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**CIBLAGE THERAPEUTIQUE DES TUMEURS
OSSEUSES PRIMITIVES PAR TRANSFERT DE GENE**

THESE DE DOCTORAT

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Biologie – Médecine – Santé
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Table des Matières

Liste des abréviations	4
Introduction	6
I. Le Tissu Osseux.....	6
A/ Organisation du tissu osseux	6
B/ L'Ostéoblaste	9
C/ L'Ostéoclaste	13
D/ Le remodelage osseux.....	15
II. La triade OPG / RANKL / RANK	18
A/ RANKL	19
B/ RANK.....	22
C/ Ostéoprotégérine (OPG).....	23
III. Les tumeurs osseuses primitives	27
A/ L'Ostéosarcome	28
B/ Le sarcome d'Ewing.....	34
C/ Limites des traitements actuels et développement de nouvelles approches thérapeutiques pour les tumeurs osseuses primitives	38
IV. Objectifs de la thèse.....	44
 Partie I: Approches thérapeutiques de l'ostéosarcome par transfert de gènes :	
Libération de principes à activité anti-résorptive, OPG 1-194 et RANK-Fc.....	47
Introduction	48
Article 1: « Pertinence thérapeutique de la libération de l'OPG par thérapie génique dans le traitement de l'ostéosarcome : blocage du cercle vicieux s'établissant entre la prolifération tumorale et la résorption osseuse ».....	51
Article 2: « Efficacité thérapeutique du récepteur activateur de NF- κ B (RANK) soluble libéré par transfert de gène non viral dans un modèle murin d'ostéosarcome ostéolytique »	52
Complément de discussion des articles 1 et 2	54
Conclusion de la partie I.....	59
 Partie II: Rôle de l'ostéoprotégérine dans les pathologies ostéolytiques malignes :	
molécule anti- ou pro-tumorale ?	61
Introduction	62

Article 3: « Les glycosaminoglycanes comme régulateurs potentiels de l'activité thérapeutique de l'OPG dans l'ostéosarcome »	63
Complément de discussion de l'article 3	64
Article 4: « Le microenvironnement tumoral osseux régule l'activité anti- ou pro-tumorale de l'OPG, dépendant de la balance locale entre le Receptor Activator of Nuclear Factor- κ B Ligand (RANKL) et le TNF Related Apoptosis Inducing Ligand (TRAIL) ainsi que de la présence des protéoglycanes »	90
Complément de discussion de l'article 4	91
Conclusion de la partie II	116
Partie III: Rôle de RANKL dans le développement tumoral	118
Introduction	119
Matériels et méthodes	122
Résultats	126
Discussions-perspectives	136
Conclusions générales et perspectives	140
Annexes	149
Bibliographie	150

Liste des abréviations

ADN: acide désoxyribonucléique
ARN: acide ribonucléique
BMP: bone morphogenetic protein
BSP: bone sialoprotein
Coll I: collagène de type I
ERK1/2: extra-cellular regulated kinase
FGF: fibroblast growth factor
GFP: green fluorescent protein
IFN γ : interféron γ
IGF : Insulin like Growth Factor
IL-: interleukine
JAK: janus kinase
MAPK: mitogen activated protein kinase
M-CSF: Macrophage-Colony Stimulating Factor
MMPs: matrix metalloproteinases
OC: ostéocalcine
OCIF: osteoclastogenesis inhibitory factor
OP: ostéopontine
OPG: ostéoprotégérine
PAL: phosphatase alcaline
PDGF: Platelet Derived Growth Factor
PI3K: phosphatidylinositol-3 kinase
PKC δ : protéine kinase C δ
PTH: parathormone
PTHrp: PTH related protein
RANK: receptor activator of nuclear factor κ B
RANKL: RANK ligand
Rb: retinoblastoma susceptibility tumor-suppressor
Shc: SH2 and collagen-homology-domain containing protein
STAT: signal transducer and activator of transcription
TGF β : transforming growth factor β
TNF α : tumor necrosis factor α

TRAIL: TNF-related apoptosis inducing ligand

TRAP: tartrate resistant acidic protein

VEGF: vascular endothelial growth factor

Introduction

I. Le Tissu Osseux

Le tissu osseux est un tissu conjonctif spécialisé composé d'une fraction organique et d'une fraction minérale, dont la substance fondamentale est calcifiée, ce qui lui confère ses propriétés de rigidité et d'élasticité. Véritable « charpente » de l'organisme, l'os minéralisé constitue la partie porteuse de l'appareil locomoteur avec trois fonctions principales : mécanique, métabolique et hématopoïétique.

En effet, il est le support mécanique essentiel du squelette intervenant dans la locomotion grâce aux articulations qui permettent aux muscles d'actionner les différents segments du squelette. De plus, il assure la protection des organes vitaux. En effet, le système nerveux central est protégé par les os du crâne et le rachis vertébral, alors que le cœur et les poumons sont entourés par la cage thoracique. Au niveau métabolique, il joue un rôle extrêmement important dans le maintien de l'homéostasie phosphocalcique, minérale et cellulaire car il est un réservoir métabolique de sels minéraux (en particulier de calcium et de phosphore) et contribue ainsi à la régulation de la composition des fluides extracellulaires. Par ailleurs, dans les espaces médullaires des os se trouve la moelle osseuse, siège de l'hématopoïèse assurant le renouvellement des cellules sanguines.

Le tissu osseux, caractérisé par sa dureté et son apparente rigidité, n'est pas pour autant figé. En effet, tout au long de la vie, le tissu osseux est en continuel remaniement, c'est-à-dire que l'os est régulièrement détruit puis reconstruit au cours des différentes phases d'un processus appelé remodelage osseux.

A/ Organisation du tissu osseux

Le tissu osseux est composé de cellules spécialisées (ostéoblastes, ostéoclastes, ostéocytes et cellules bordantes) et d'une matrice extracellulaire qui contient une phase organique et une phase minérale.

La phase organique est composée en grande majorité de fibres de collagène de type I (représentant 80% du collagène total de l'os) entourées d'une substance interfibrillaire et présentant une structure hélicoïdale. La molécule protéique de base est longue de 300 nm avec un diamètre de 1,5 nm et comprend trois chaînes élémentaires torsadées en triple hélice : deux chaînes $\alpha 1$ et une chaîne $\alpha 2$. De plus, la phase organique contient en quantité inférieure du collagène de type III (5 à 15%) et des collagènes « mineurs » de types IV et V (5%). Outre la présence de collagène dans cette matrice extracellulaire, la substance interfibrillaire composée de protéines non collagéniques représente 10% à 15% de la phase organique. Parmi celles-ci, l'ostéocalcine (10 à 20% de l'ensemble de ces protéines) contenant des résidus d'acide glutamique carboxylés jouerait un rôle dans l'attraction des ostéoclastes dans les foyers de résorption et dans la minéralisation (Swaminathan, 2001; Glowacki et al., 1991). D'autres glycoprotéines (ostéopontine, sialoprotéine osseuse) jouant un rôle dans l'adhésion des ostéoclastes sur la travée osseuse sont aussi présentes, ainsi que des protéoglycanes (détaillés dans la 2^{ème} partie), des cytokines et des facteurs de croissance tels que le Transforming Growth Factor β (TGF β), l'Insulin-like Growth Factor (IGF), le Tumor Necrosis Factor (TNF), les interleukines (IL) et les Bone Morphogenetic Proteins (BMP) (Hauschka et al., 1986). Tous ces différents facteurs jouent un rôle important dans l'activation cellulaire et dans le couplage entre la formation et la résorption osseuse (Horwood et al., 1998b).

La phase minérale de la matrice osseuse est composée de phosphate de calcium cristallisé sous forme de cristaux d'hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$, soit 70% du poids total de l'os lui conférant ainsi sa rigidité et sa solidité. De ce fait, le squelette contient environ 1 kg de calcium et constitue la principale réserve de calcium de l'organisme soit 99%. Ce calcium ainsi que 85% du phosphore et entre 40 à 60% du sodium et magnésium sont incorporés dans les cristaux d'hydroxyapatite, constituant ainsi la substance minérale osseuse.

Origine et structure des os

Il existe deux types d'os suivant l'origine d'apparition du tissu osseux :

- Les os plats ou courts (omoplate, crâne, mandibule) qui apparaissent suite à une ossification membranaire sans ébauche cartilagineuse,
- les os longs (fémur, tibia) qui ont pour origine une ossification endochondrale avec une ébauche cartilagineuse. Sur le plan anatomique, les os longs présentent une partie centrale de taille variable et creusée de la cavité médullaire et deux extrémités

renflées de forme variable, les épiphyses entrant dans la constitution des articulations avec les pièces osseuses voisines. Diaphyse et épiphyses sont jointes par des parties intermédiaires, les métaphyses.

De plus, les os sont généralement composés de deux zones distinctes : une zone dense en périphérie appelée os compact (ou cortical ou haversien) et une zone interne appelée os spongieux (Figure 1).

L'os compact représente environ 80 à 85% du tissu osseux. Il est principalement constitué d'ostéons ou du système de Havers, c'est-à-dire de petites lamelles osseuses cylindriques disposées de manière concentrique autour du canal de Havers. Dans la matrice extracellulaire se trouve des logettes appelées ostéoplastes et contenant des ostéocytes. Les canaux de Havers sont reliés entre eux ainsi qu'avec la cavité médullaire et la surface de l'os par des canaux transversaux ou obliques appelés canaux de Volkmann conférant à l'os compact une résistance optimale. Chaque canal de Havers est innervé et contient des capillaires sanguins.

L'os spongieux ou trabéculaire se trouve essentiellement au niveau des l'épiphyse des os longs, dans les os courts et les os plats. Il est appelé ainsi en raison de sa structure en éponge, faite de travées osseuses où se trouve la moelle hématopoïétique.

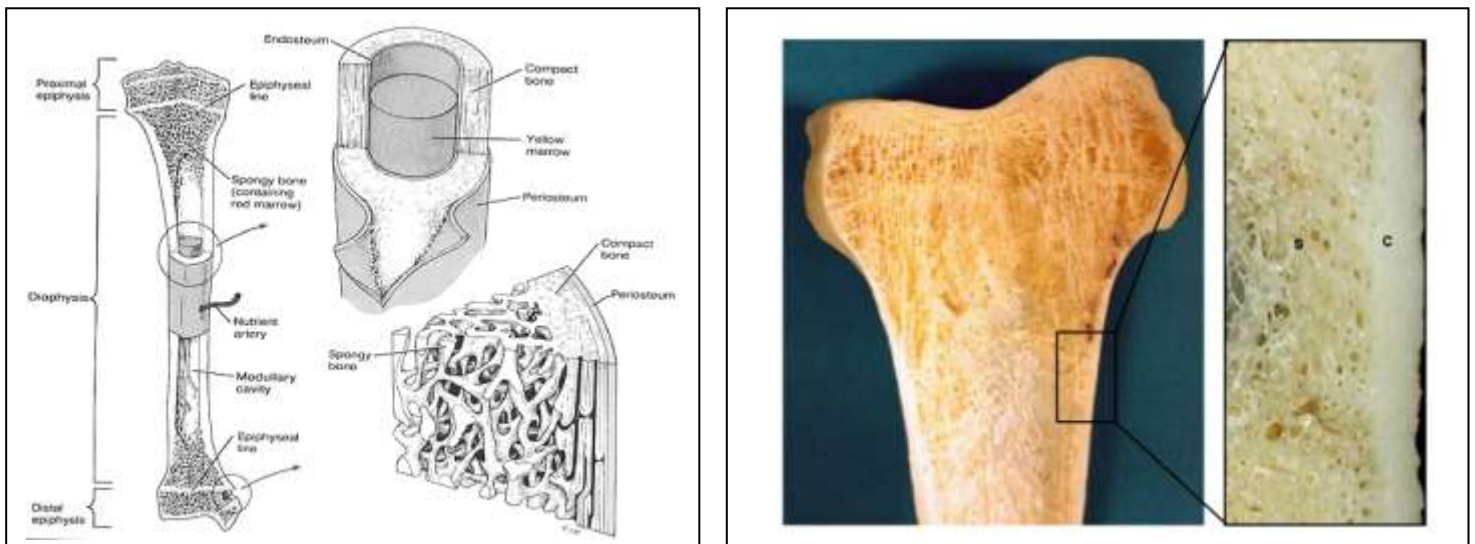


Figure 1 : Organisation structurale d'un os long.

Tout au long de la vie, le squelette subit de nombreux remaniements par un processus complexe : le remodelage osseux. Il fait intervenir deux types cellulaires : les

ostéoclastes, responsables de la dégradation ou résorption osseuse, et les ostéoblastes qui permettent la production de matrice minéralisée (apposition osseuse).

B/ L'Ostéoblaste

D'origine mésodermique dans la plus grande partie du squelette, mais issus des crêtes neurales dans les os du crâne et de la face, les progéniteurs ostéoblastiques sont des cellules mésenchymateuses. Ces cellules sont essentiellement présentes chez l'adulte dans le périoste et le stroma de la moelle osseuse, avec également l'existence de progéniteurs extrasquelettiques et/ou circulants (Bianco and Cossu, 1999). Sous le contrôle de différents facteurs de transcription, les cellules souches peuvent se différencier soit en chondrocytes, en osteoblastes, en fibroblastes, en myocytes ou en adipocytes (Owen, 1988). En effet, l'expression de Sox-9 induit la différenciation des cellules dans la voie chondroblastique, celle de Myo-D dans la voie myoblastique et celle de PPAR γ 2 dans la voie adipocytaire (Pittenger et al., 1999). Une série complexe d'étapes de prolifération et de différenciation conduit aux ostéoblastes matures depuis les cellules souches mésenchymateuses faisant intervenir l'expression du facteur de transcription Cbfa1/Runx2 (Figure 2).

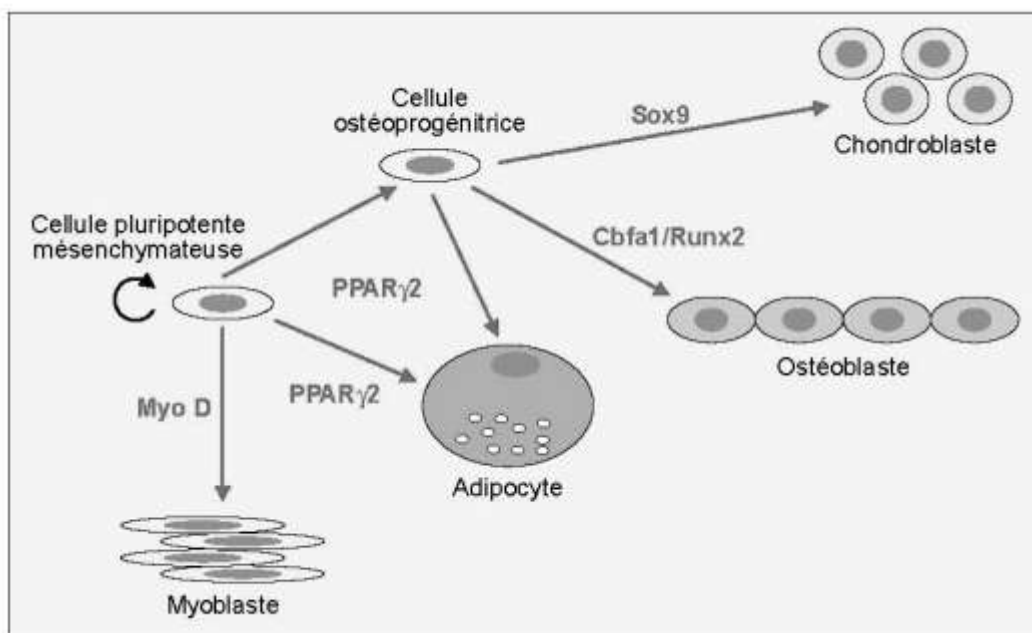


Figure 2 : Les ostéoblastes proviennent de la différenciation de cellules souches mésenchymateuses (D'après Marie P.J, Médecine/Sciences 2001).

Au niveau nucléaire, la différenciation des ostéoblastes est sous le contrôle de nombreux facteurs de transcription. En effet, des souris surexprimant c-Fos présentent une transformation des ostéoblastes et une apparition spontanée de chondrosarcome et/ou d'ostéosarcome (Grigoriadis et al., 1993; Wagner, 2002) faisant intervenir le complexe AP-1 (constitué d'homo ou hétérodimères de facteurs de transcription de la famille Jun/Fos). A l'inverse, les souris Fos $-/-$ présentent un retard de croissance, une ostéopétrose, des défauts dans le remodelage osseux et une hématopoïèse altérée (Wang et al., 1992). L'activité du complexe AP-1 est fortement diminuée lors de la différenciation terminale en ostéoblastes matures alors qu'elle est très intense dans les ostéoblastes proliférant. Le promoteur de l'ostéocalcine présente des sites de liaisons pour AP-1 et est souvent utilisé pour exprimer spécifiquement un gène dans l'ostéoblaste. La liaison d'AP-1 sur le promoteur du gène de l'ostéocalcine a donc pour effet de réprimer son expression dans les progéniteurs proliférants, inhibition qui est levée lors de la différenciation terminale (McCabe et al., 1996). Durant la phase de différenciation, les ostéoblastes expriment successivement la phosphatase alcaline (PAL ; enzyme fortement exprimée à la surface des cellules), stimulent l'activité de l'adénylate cyclase en réponse à l'hormone parathyroïdienne (PTH), synthétisent du collagène de type I (Coll I) et de l'ostéocalcine (OC) puis induisent la formation de nodules minéralisés.

Les ostéoblastes différenciés sont des cellules mononucléées de forme cuboïdale disposées en couches monocellulaires sur la surface osseuse ayant un rôle double : construire l'os et activer l'ostéoclaste. En effet, les ostéoblastes ont pour fonction principale la synthèse de protéines constituant la matrice organique, composée de collagène de type I et de substance ostéoïde (protéines non collagéniques : ostéocalcine, protéoglycanes). Du fait de leur activité de synthèse élevée, de nombreux organites se trouvent à la partie apicale du cytoplasme tels que les mitochondries, l'appareil de golgi et un ergastoplasme rugueux très développé. De plus, le cytoplasme renferme des vésicules de calcium qui permettront la minéralisation de la matrice préalablement synthétisée (chez l'homme en moyenne 10-20 jours après la déposition de la matrice). La régulation de la formation osseuse fait intervenir la PTH et les hormones sexuelles. A l'issue de la période de formation osseuse, les glucocorticoïdes en excès induisent l'apoptose de la majorité des ostéoblastes (65%), dans le cas contraire les ostéoblastes peuvent devenir des cellules bordantes ou subir une différenciation terminale en ostéocytes.

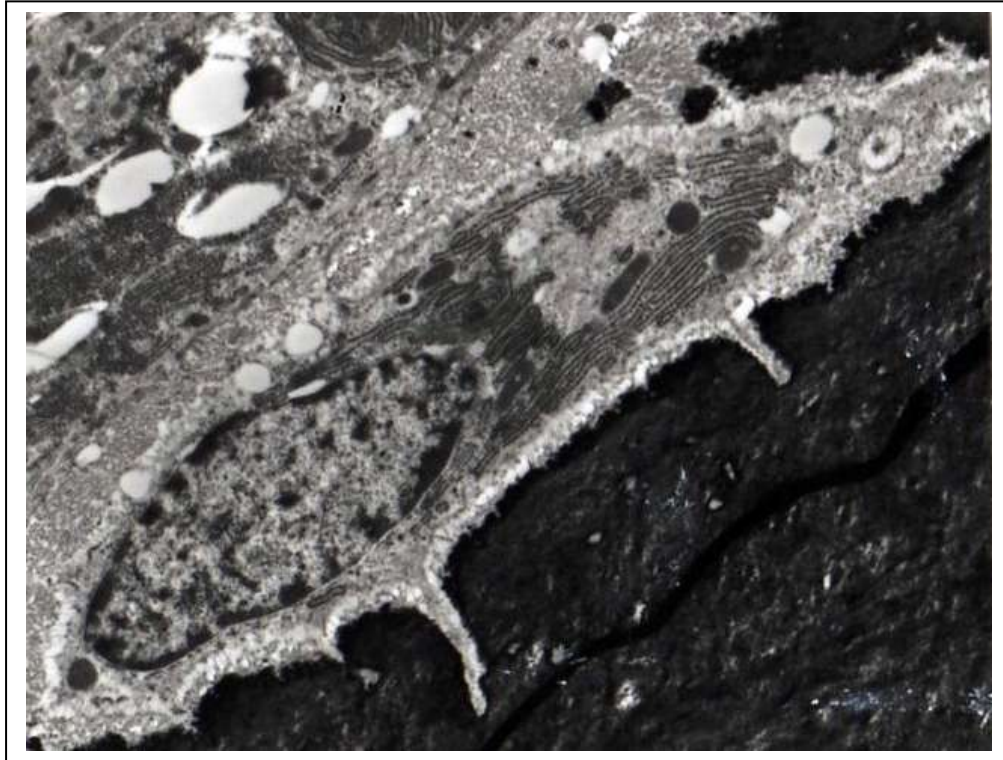


Figure 3 : Ostéoblaste emmuré dans une matrice minéralisée observé par microscopie électronique à transmission.

Les cellules bordantes ont pour origine des ostéoblastes aplatis avec une activité métabolique très réduite (cytoplasme pauvre en organites tels que les mitochondries et un réticulum endoplasmique réduit). Chez l'adulte, seules 15% des surfaces trabéculaires sont recouvertes d'ostéoblastes actifs. Le reste des surfaces osseuses est recouvert de cellules bordantes. Ces cellules n'ont aucune activité de synthèse mais ont comme principale fonction d'assurer la communication entre la surface osseuse, l'environnement cellulaire et les ostéocytes emmurés dans la matrice osseuse. De plus, les cellules bordantes sous l'influence de stimuli sont capables de se multiplier et de se redifférencier en ostéoblastes actifs et/ou de jouer également un rôle durant la phase initiale du remodelage osseux. Sous l'effet de certains signaux, elles libèrent la surface osseuse permettant ainsi l'attraction et l'ancrage des ostéoclastes (Chambers and Fuller, 1985).

Les ostéocytes proviennent de la différenciation terminale de certains ostéoblastes emmurés au sein de la matrice osseuse, à l'intérieur de lacunes périostéocytaires appelées ostéoplastes. Dix à 20% des ostéoblastes deviennent des ostéocytes soit 25000/mm³ de tissu osseux (Figure 4). Au cours de cette différenciation, les ostéoblastes perdent une grande quantité de leurs organites. Possédant de nombreux prolongements cytoplasmiques, les ostéocytes sont reliés les uns aux autres et communiquent avec les cellules bordantes recouvrant la surface osseuse. Les substances nutritives qui proviennent du sang atteignent les ostéocytes en diffusant soit dans les canalicules autour des prolongements cellulaires, soit dans les cellules elles-mêmes, passant de l'une à l'autre par des jonctions communicantes (GAP). Les ostéocytes interviennent essentiellement dans la transmission des signaux mécano-sensoriels et dans les échanges entre les cellules et le microenvironnement (Aarden et al., 1994). Ils régulent la formation osseuse *via* la sécrétion de sclérostine qui constitue un marqueur spécifique des ostéocytes (Poole et al., 2005). Il semblerait que l'apoptose de ces cellules soit le facteur déclenchant l'ancrage des ostéoclastes à la surface osseuse et par conséquent la mise en place du processus de résorption et de remodelage.

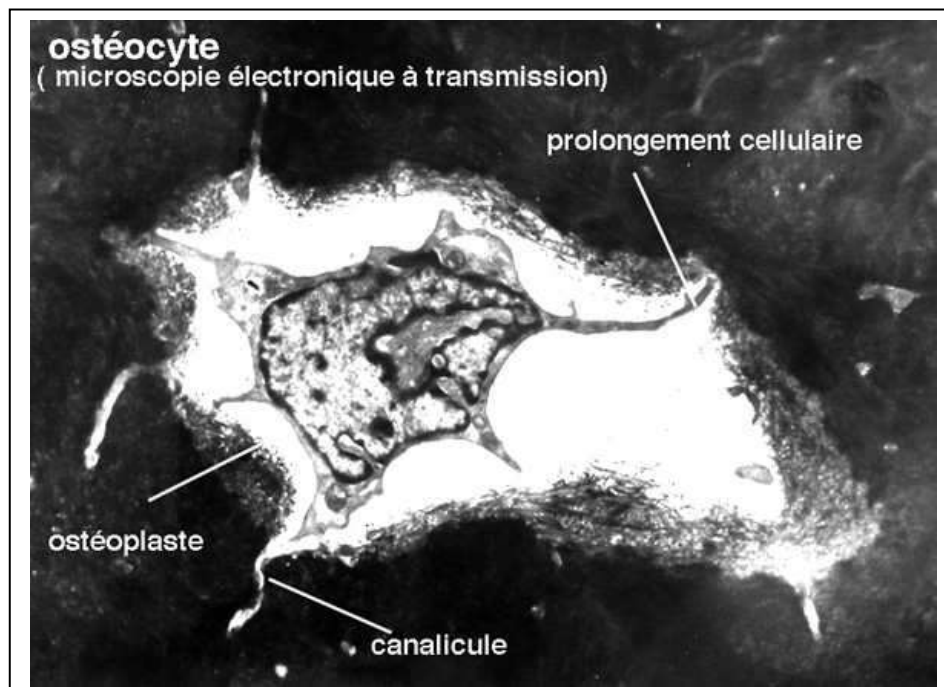


Figure 4: Ostéocyte observé par microscopie électronique à transmission (d'après le laboratoire de biologie du tissu osseux de l'université Jean Monnet, St Etienne)

C/ L'Ostéoclaste

Les ostéoclastes sont des cellules géantes multinucléées (10 à 100µm de diamètre ; entre 4 et 20 noyaux regroupés au centre de la cellule), formées par la fusion de cellules dérivées de précurseurs de la lignée myélomonocytaire communs aux cellules dendritiques, aux monocytes/macrophages et aux ostéoclastes. Ces précurseurs se différencient en ostéoclastes sous l'influence de facteurs synthétisés et sécrétés par les cellules stromales ou les ostéoblastes mais également par des contacts directs entre ces différents types cellulaires [(Udagawa et al., 1990); Figure 5]. Ce sont des cellules qui possèdent un appareil de Golgi très développé autour de chaque noyau, de nombreuses mitochondries périnucléaires ainsi que d'importantes quantités de vésicules lysosomales riches en enzymes telles que la phosphatase acide résistante au tartrate (TRAP), la cathepsine K et des collagénases. Les ostéoclastes sont des acteurs essentiels du remodelage osseux ayant comme activité principale la résorption osseuse.

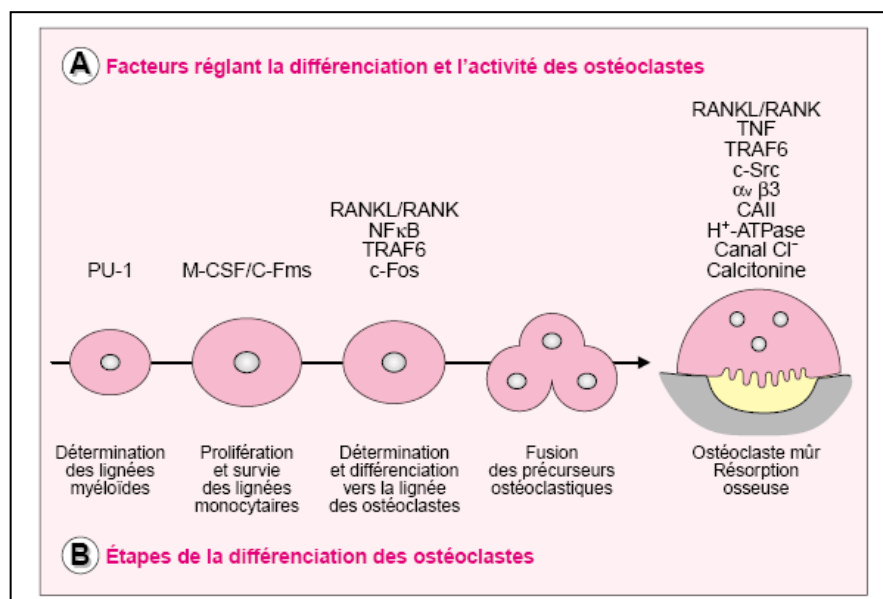


Figure 5 : Différenciation ostéoclastique à partir d'un précurseur hématopoïétique (D'après Marie P.J, Médecine/Sciences 2001).

L'ostéoclaste est une cellule étalée sans polarité membranaire particulière au repos ou en migration, mais qui acquiert une polarité verticale lorsqu'il devient actif durant le phénomène de résorption. En effet, il y a formation d'une membrane spécialisée au contact de l'os définie par une zone d'ancrage et d'une bordure plissée (ou bordure en brosse) durant la fixation de l'ostéoclaste à la matrice osseuse via les intégrines. La zone d'ancrage permet de créer un espace étroit et hermétique entre l'ostéoclaste et la matrice osseuse qui correspond au compartiment de résorption. Quant à la bordure plissée formant la zone centrale de la membrane spécialisée, elle est constituée de nombreux replis au niveau desquels s'effectueront les échanges lors de la dégradation du minéral osseux.

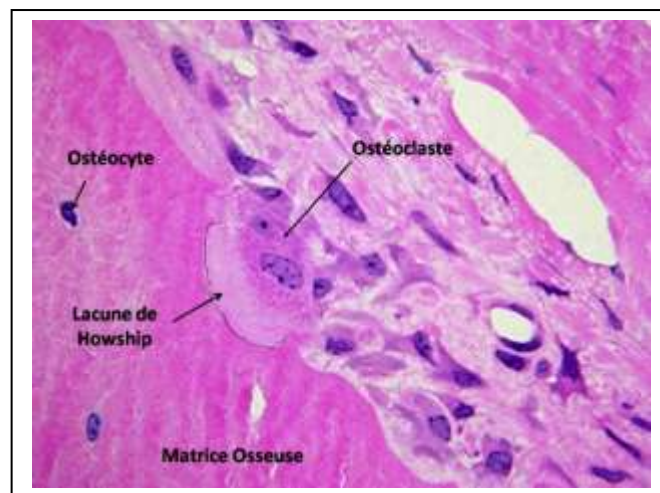


Figure 6 : Ostéoclaste résorbant une matrice osseuse après coloration hématoxyline-éosine.

L'ostéoclaste peut être observé au fond des lacunes de résorption appelées lacunes de Howship, au contact de la matrice osseuse calcifiée (figure 6 et 7). À ce niveau, l'ostéoclaste est capable d'acidifier le milieu extracellulaire (par la sécrétion de protons) grâce à des pompes à protons qui solubilisent la fraction minérale. La digestion de la trame organique s'effectue ensuite sous l'action des enzymes protéolytiques (cathepsine K, collagénases, métalloprotéases telles que la MMP-9, ...) contenues dans les lysosomes (Blair et al., 1986).

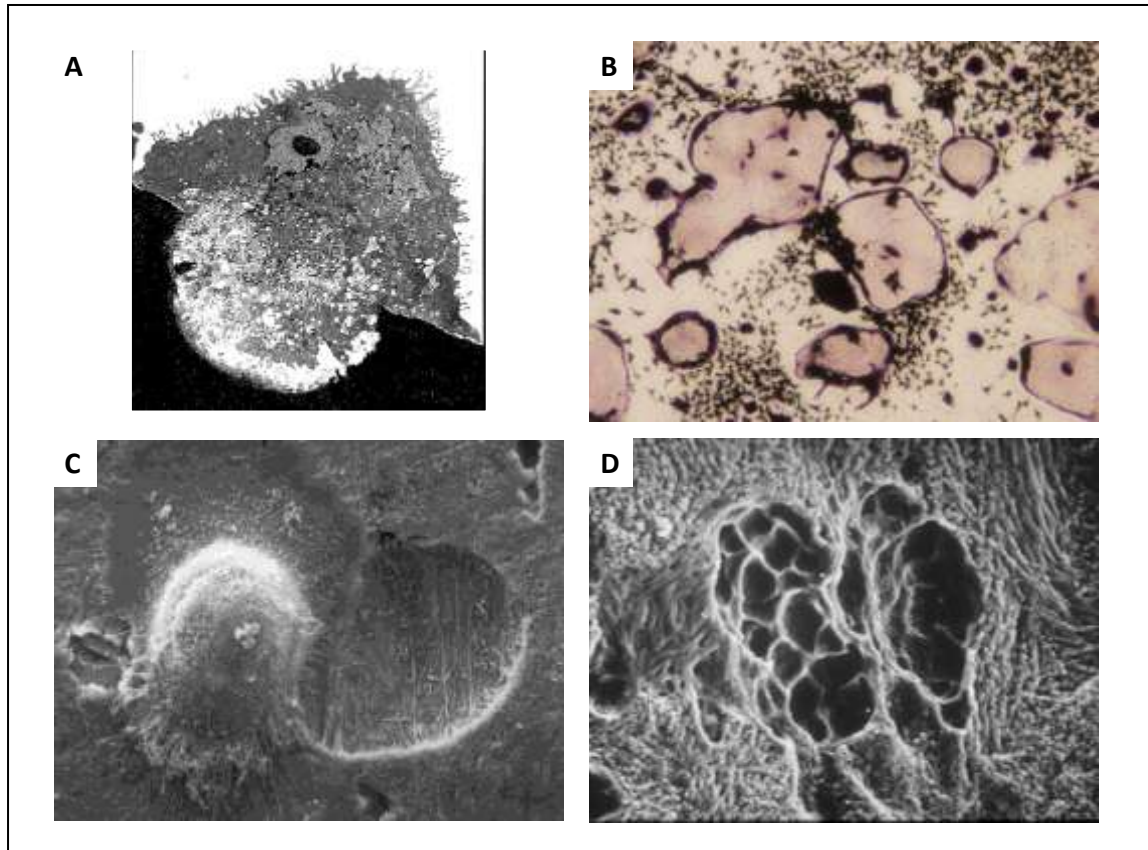


Figure 7 : Ostéoclaste résorbant issu d'une tumeur à cellules géantes et cultivé sur une pastille de dentine observé au microscope électronique à transmission (x5000; A). Ostéoclastes observés au microscope optique après coloration TRAP (x100; B). Ostéoclaste résorbant une matrice minéralisée observé en microscopie à balayage (x4500; C). Lacunes de Howship après résorption ostéoclastique en microscopie à balayage (x800; D).

D/ Le remodelage osseux

Jusqu'à l'âge de 20 ans, la masse osseuse augmente progressivement, puis reste stable pendant quelques années avant de diminuer lentement avec l'âge. Le renouvellement de l'os du squelette adulte est de l'ordre de 10% par an. Le remodelage osseux est un processus complexe qui fait intervenir deux activités opposées mais complémentaires, conduisant au

maintien de la masse osseuse : la destruction de l'os ancien par les ostéoclastes et la formation d'un tissu osseux nouveau par les ostéoblastes (Parfitt, 1994). Ces deux types cellulaires agissent de façon orchestrée dans l'espace et dans le temps. Par ces deux activités, le tissu osseux peut remplir son rôle métabolique, par la libération de sels minéraux stockés, et son rôle de soutien par des adaptations architecturales aux contraintes mécaniques.

Le remodelage osseux peut être déclenché par des stimuli de nature :

- mécanique, suite à l'altération de l'architecture locale de l'os causée entre autre par une fracture ou une prise de poids importante (Turner and Pavalko, 1998).
- hormonale, comme dans le cas de l'ostéoporose où une diminution du taux d'œstrogènes entraîne une augmentation du nombre et de l'activité des ostéoclastes (Pacifci, 1998).

Le remodelage osseux se subdivise en une succession de 4 phases parfaitement définies : activation, résorption, inversion et formation (Figure 8) (Hill, 1998).

Le cycle débute par l'activation caractérisée par la rétractation des ostéoblastes (ou cellules bordantes) à la surface du tissu osseux en réponse à des facteurs ostéorésorbants tels que la PTH, la vitamine D3, la prostaglandine E2, ... laissant place aux ostéoclastes ou aux précurseurs ostéoclastiques mononucléés qui peuvent adhérer à la matrice. Les ostéoblastes environnants vont alors favoriser la différenciation et l'activation des pré-ostéoclastes en ostéoclastes matures par la production de différentes cytokines, la plus importante étant RANKL (Receptor Activator of Nuclear factor kappa B ligand) qui va interagir avec son récepteur RANK (Receptor Activator of Nuclear factor kappa B) présent à la surface des pré-ostéoclastes.

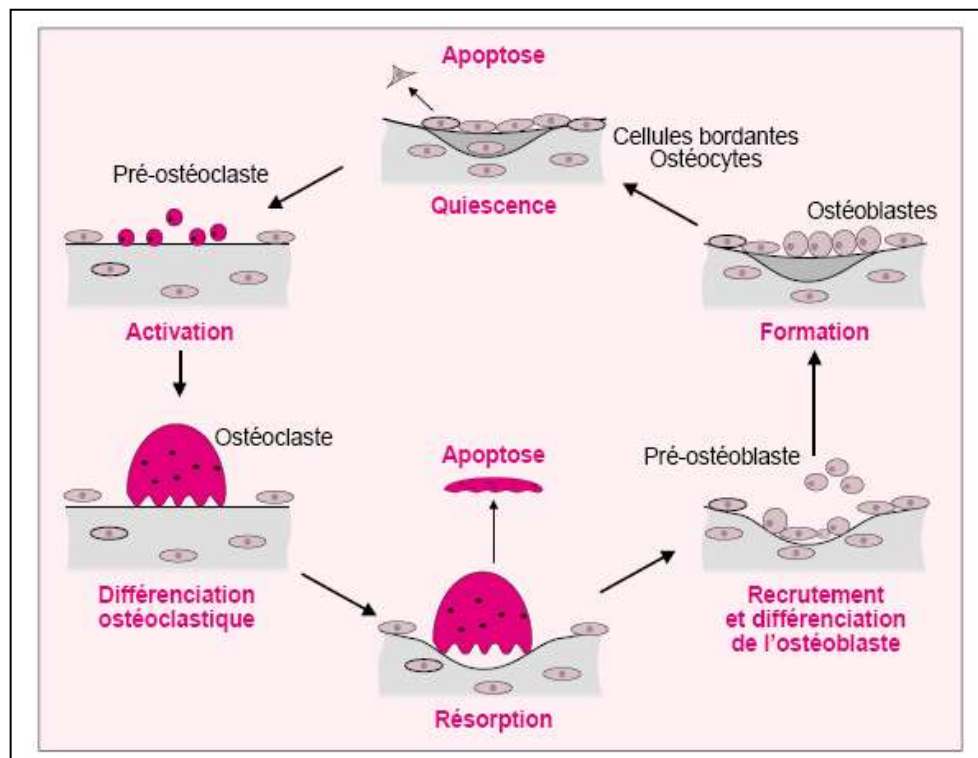
Les ostéoclastes différenciés et activés vont alors adhérer à la surface osseuse au niveau de la zone claire constituant une zone de scellement entre l'ostéoclaste et l'os. Cette adhérence assurée par l'interaction entre les protéines de la matrice organique (notamment la vitronectine) et les intégrines présentes sur la membrane ostéoclastique (notamment $\alpha_2\beta_1$ et $\alpha_v\beta_3$) signe le début de la phase de résorption et est à l'origine de la polarisation et de la formation de la bordure en brosse. Cette phase s'effectue en deux étapes successives. En effet, l'acidification du compartiment de résorption s'effectue à l'aide d'une pompe à protons qui sécrète des ions H^+ , permettant la dissolution de la phase minérale de la matrice extracellulaire. Cette acidification de la chambre de résorption est suivie par la dégradation de

la matrice organique sous l'action d'enzymes protéolytiques lysosomales telles que la phosphatase acide, la cathepsine K, les collagénases,....

La phase d'inversion suit la phase de résorption, correspondant au moment où la profondeur maximale d'érosion est atteinte, les ostéoclastes se détachent de la surface osseuse érodée et meurent par apoptose vraisemblablement médiée par le système Fas/Fas ligand (Wu et al., 2003). Le décrochement des ostéoclastes fait vraisemblablement intervenir la MMP-9 dans le déplacement des ostéoclastes. Suite à la résorption, il y a une augmentation du taux de calcium dans la lacune qui perturberait la stabilité de l'anneau du filament d'actine rendant la lacune moins hermétique avec pour conséquence une augmentation du pH au niveau de cette lacune. Ainsi la MMP-9 plus active suite à l'augmentation de pH jouerait un rôle dans le déplacement des ostéoclastes. Ils sont alors remplacés par des macrophages qui lissent le fond de la lacune.

La dernière phase du remodelage osseux est la phase de formation d'un nouveau tissu osseux qui se déroule en deux temps, au cours desquels les ostéoblastes jouent un rôle majeur. Dans un premier temps, ces cellules sont responsables de la production de la matrice extracellulaire, suivie d'une étape de minéralisation de cette matrice. En effet, durant cette phase, les ostéoblastes sont recrutés au fond de la lacune de résorption dans le but de combler cette lacune par la synthèse d'une nouvelle matrice collagénique non minéralisée (substance pré-osseuse ou tissu ostéoïde). La production de cette matrice peut être stimulée par des hormones (oestrogènes, androgènes, vitamine D,...) ou par des facteurs de croissance sécrétés par les ostéoblastes (TGF β , IGF, BMP...). A l'inverse, l'IL1 et TNF- α sont capables d'inhiber la production de matrice osseuse par les ostéoblastes.

Une fois la lacune comblée par la nouvelle matrice, la minéralisation débute à la jonction entre le tissu ostéoïde et le tissu minéralisé existant. Les ostéoblastes synthétisent alors la phosphatase alcaline qui est libérée dans de petites vésicules matricielles au niveau de la matrice non minéralisée. Cette enzyme a pour rôle d'initier la minéralisation du tissu ostéoïde par l'hydrolyse des esters phosphoriques inhibiteurs de la minéralisation. De plus l'ostéocalcine et la vitamine D3 jouent un rôle dans l'augmentation et la fixation du calcium extra-cellulaire sur le tissu ostéoïde. La carence en vitamine D3 est connue pour augmenter la sécrétion de la PTH responsable de la déminéralisation des os par l'appauvrissement en calcium et en phosphore, notamment observé dans les cas de rachitisme chez l'enfant ou d'ostéomalacie chez l'adulte.



**Figure 8 : Les différentes étapes du remodelage osseux
(D'après Marie P.J, Médecine/Sciences 2001)**

II. La triade OPG / RANKL / RANK

Le processus de remodelage osseux est étroitement régulé par l'action de nombreux facteurs locaux et systémiques (cytokines, facteurs de croissance,...) afin d'éviter toute perturbation pouvant conduire à des situations pathologiques. La découverte depuis 1997 de l'ostéoprotégérine (OPG) et l'identification consécutive de son ligand RANKL et de RANK a révolutionné les connaissances des mécanismes moléculaires à la base de la régulation du remodelage osseux (Simonet et al., 1997). Ce système a été depuis caractérisé grâce à l'utilisation de modèles animaux transgéniques qui ont permis de mieux comprendre le rôle de chaque protagoniste dans le contrôle de la biologie osseuse. L'interaction de RANKL à son récepteur RANK induit la différenciation et l'activation des précurseurs ostéoclastiques en ostéoclastes matures et joue aussi dans la survie des ces ostéoclastes.

L'OPG est un récepteur soluble capable de se lier à RANKL et de bloquer son interaction avec RANK. L'OPG est donc une molécule anti-résorption osseuse (Figure 9).

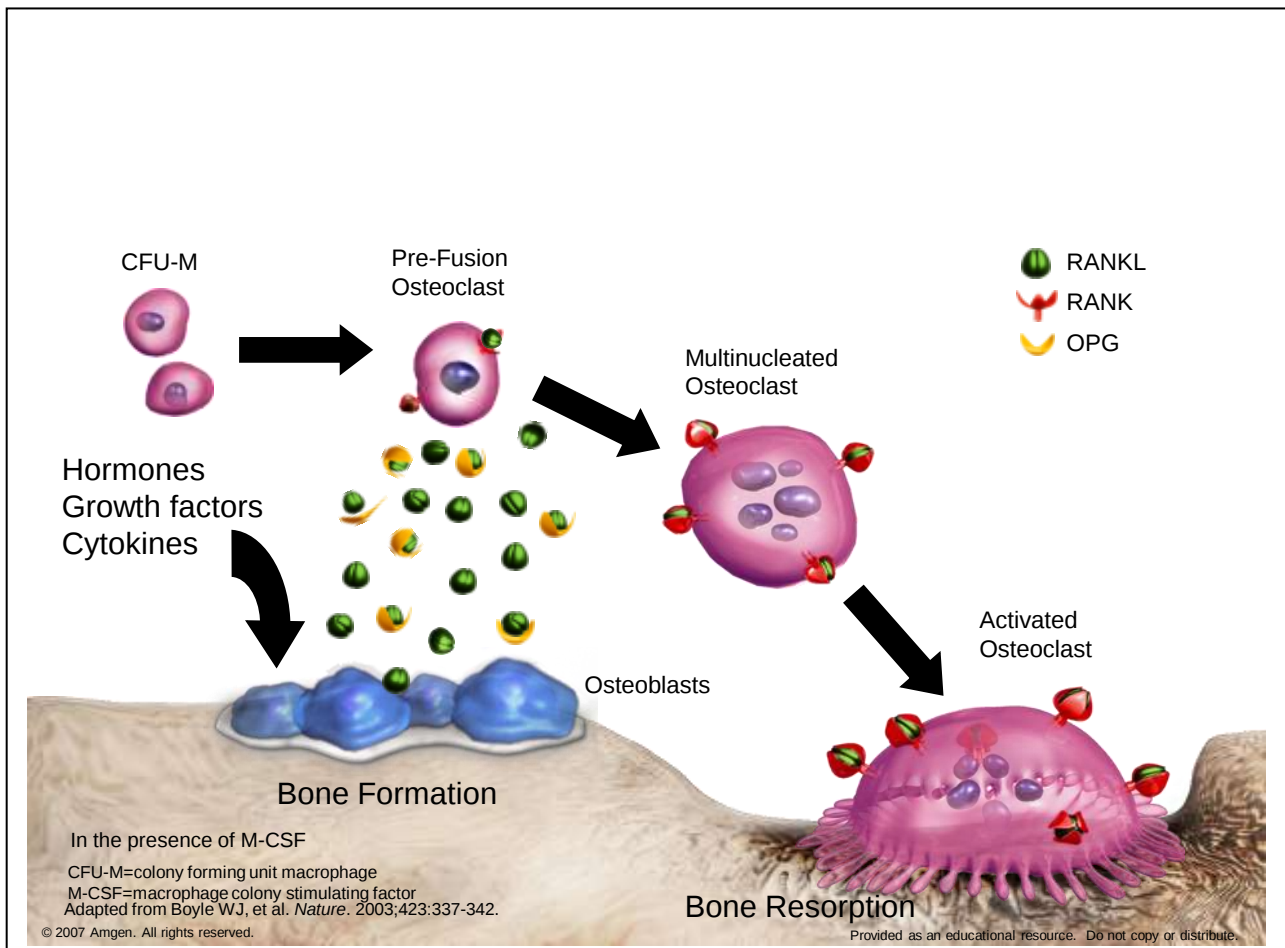


Figure 9 : Rôle de la triade OPG / RANKL / RANK dans la différenciation ostéoclastique (d'après Amgen adapté de Boyle et al., 2003)

A/ RANKL

Si RANKL, membre de la superfamille du TNF est aussi connu sous le nom d'OPGL (Osteoprotegerin Ligand), TRANCE (TNF-Related Activation-Induced Cytokine) ou ODF (Osteoclast Differentiation Factor), la nomenclature officielle est le TNFSF11 (11^{ème} membre de la superfamille du TNF). Les différentes appellations s'expliquent du fait que

RANKL a été identifié et cloné au même moment par deux groupes de recherche différents, l'un à partir d'une lignée de cellules stromales de la moelle osseuse murine ST2 (Yasuda et al., 1998b), et l'autre à partir de cellules myélomonocytaires murines 32D (Lacey et al., 1998). RANKL est une protéine transmembranaire de type II (partie N-terminale intracellulaire) sans peptide signal (Hofbauer et al., 2000) montrant une homologie d'environ 30% avec TRAIL et CD40, et d'environ 20% avec Fas-Ligand (Wong et al., 1997b).

RANKL existe sous trois isoformes (chez la souris et l'homme) résultant de l'épissage alternatif d'un même gène (Ikeda et al., 2001; Suzuki et al., 2004). L'isoforme RANKL correspond à une protéine transmembranaire de 317 acides aminés chez la souris et 316 chez l'homme, possédant un domaine intra-cytoplasmique complet lui permettant d'interagir avec des molécules intracellulaires. RANKL3 est une protéine soluble monomérique de 199 acides aminés chez la souris et 244 chez l'homme, intracytoplasmique et non sécrétée, délétée dans sa partie N-terminale pour les acides aminés correspondant aux domaines intra-cytoplasmique et transmembranaire de RANKL. Quant à l'isoforme RANKL2, elle est de structure différente suivant l'espèce : RANKL2 de souris (287 acides aminés) possède un court domaine intra-cytoplasmique alors que RANKL2 humain (244 acides aminés) en est dépourvu (Figure 10).

Dans le milieu extracellulaire, la forme soluble de RANKL peut également être libérée de la membrane plasmique après clivage par des protéases comme TACE (TNF alpha Converting Enzyme) (Lum et al., 1999), la MT1-MMP (Membrane-Type 1 Matrix Metalloproteinase) (Schlondorff et al., 2001) ou la MMP-7 (Lynch et al., 2005).

RANKL exprimé par un grand nombre de cellules et de tissus est fortement présent au niveau du squelette (os, moelle osseuse, cartilage) (Lacey et al., 1998; Yasuda et al., 1998b), dans les organes lymphoïdes (ganglions, thymus, rate, foie fœtal) et dans le système vasculaire (Collin-Osdoby et al., 2001). RANKL est exprimé dans différentes lignées de cellules stromales (ST2, MC3T3-E1) et d'ostéosarcome (UMR-106, SaOS₂), dans des ostéoblastes murins en culture primaire et dans les chondrocytes hypertrophiques murins (Kartsogiannis et al., 1999). Les ostéoblastes matures expriment peu RANKL, à l'inverse des cellules stromales qui l'expriment en quantité importante. Il est également exprimé en grande quantité dans les lymphocytes T, les cellules endothéliales et les glandes mammaires (Anderson et al., 1997; Srivastava et al., 2003). Enfin une expression faible de RANKL a été mise en évidence dans le cerveau, le cœur, les poumons, les reins et le placenta (Kartsogiannis et al., 1999).

De nombreux facteurs peuvent réguler l'expression de RANKL soit en la stimulant (vitamine D3, IL-1, IL-6, IL-11, TNF α , oncostatine M, PTH et les prostaglandines E2 (Hofbauer et al., 1998; Huang et al., 2004; Nakashima et al., 2000; Palmqvist et al., 2002), soit en l'inhibant par le TGF β et l'IL-13 (Nakashima et al., 2000).

Le principal rôle de RANKL est de stimuler la différenciation des ostéoclastes ainsi que leur maturation et d'inhiber leur apoptose. En effet, des souris invalidées pour ce gène présentent une ostéopétrose sévère associée à une absence totale d'ostéoclastes fonctionnels due à l'incapacité des ostéoblastes à supporter l'ostéoclastogenèse, induisant une augmentation de la densité minérale osseuse et une hématopoïèse compensatoire extramédullaire (Kong et al., 1999). A l'inverse, après injection de RANKL, des souris naïves ont développé une ostéoporose importante (Hofbauer et al., 2000). Ces résultats mettent en évidence le rôle clé de RANKL dans la résorption osseuse. En effet, *in vitro*, RANKL en se fixant à son récepteur RANK présent à la surface des pré-ostéoclastes (Nakagawa et al., 1998) induit en présence de M-CSF (Suda et al., 1999) leur différenciation en ostéoclastes matures (Lacey et al., 1998). RANKL est en outre un facteur de survie des ostéoclastes matures (Fuller et al., 1998; Wong et al., 1999).

De plus, les souris transgéniques invalidées pour RANKL présentent également un défaut de développement des glandes mammaires engendrant la mort des nouveaux-nés, ce processus pouvant être contrebalancé par l'injection de RANKL exogène (Fata et al., 2000).

Enfin RANKL joue un rôle majeur dans les fonctions immunitaires puisqu'il semble être très fortement impliqué dans la régulation de l'organogenèse des ganglions lymphoïdes et dans le développement du thymus (Kong et al., 1999). Le rôle de RANKL dans le système immunitaire peut également être étendu aux cellules dendritiques puisqu'il est capable de stimuler leur activation et leur survie (Anderson et al., 1997; Wong et al., 1997a).

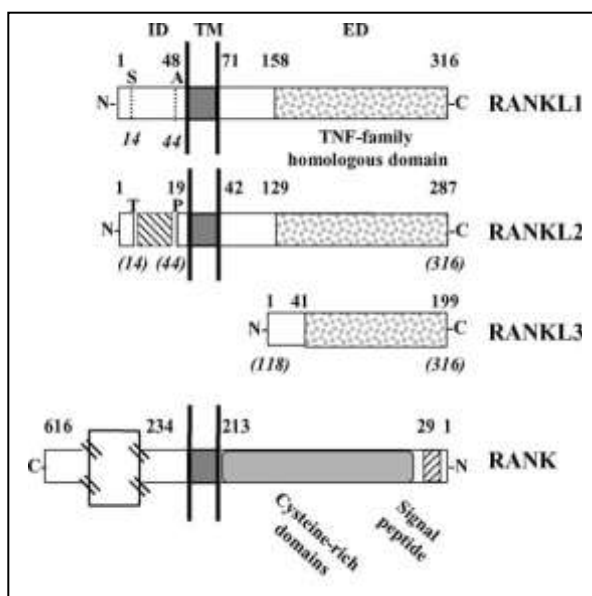


Figure 10 : Structure des isoformes de RANKL et de son récepteur RANK (Theoleyre et al., 2004).

B/ RANK

RANK ou TNFRSF11A, selon la nomenclature officielle, est une protéine transmembranaire de type I (partie N-terminale extracellulaire) appartenant à la superfamille des récepteurs au TNF et qui possède environ 40% d'homologie avec CD40. Cette protéine transmembranaire de 616 acides aminés possédant un peptide signal (Anderson et al., 1997) se présente à la membrane sous forme d'un récepteur trimérique. Le domaine extracellulaire de RANK contient 4 motifs riches en cystéine et deux sites de N-glycosylation (Hofbauer et al., 2000). RANK, récepteur de RANKL, est présent à la surface des précurseurs ostéoclastiques et des ostéoclastes matures au niveau osseux, et il est également exprimé par les lymphocytes T et les cellules dendritiques. L'expression de RANK a été détectée dans différents tissus comme le muscle squelettique, la peau, le cerveau, les poumons, le foie (Anderson et al., 1997; Min et al., 2000; Nakagawa et al., 1998). Il a aussi été mis en évidence dans de nombreux systèmes cellulaires mais la production de la protéine est restreinte aux ostéoclastes, aux cellules lymphocytaires B et T, aux cellules dendritiques, aux cellules endothéliales et aux fibroblastes.

RANK est essentiel à la différenciation et à la survie des ostéoclastes. En effet, des souris transgéniques invalidées pour ce gène (souris RANK^{-/-}) présentent une ostéopétrose sévère associée à un manque d'ostéoclastes matures, phénotype similaire à celui des souris délétées pour le gène RANKL (Li et al., 2000). Différentes études ont montré que RANKL se lie spécifiquement à RANK à la surface des pré-ostéoclastes avec une haute affinité (3 nM) et que cette liaison entraîne l'activation de RANK indispensable à l'ostéoclastogenèse (Hsu et al., 1999; Nakagawa et al., 1998). La fixation de RANKL à RANK entraîne une cascade de voies de signalisation via TRAF6 dont l'activation de kinases telles que TAB2, Src, PI3K ou AKT menant à l'activation d'effecteurs plus tardifs tels que p38, ERK, JNK ou encore NFκB, conduisant notamment à la différenciation des précurseurs ostéoclastiques et à l'activation des ostéoclastes (Figure 11).

Les souris RANK^{-/-} présentent une absence de ganglions lymphoïdes et un défaut de maturation des lymphocytes B et T alors qu'elles ont un développement thymique normal (Li et al., 2000).

Des souris transgéniques exprimant la protéine de fusion soluble RANK-Fc présentent quant à elles un phénotype squelettique similaire à celui des souris transgéniques pour l'OPG c'est-à-dire une diminution de l'ostéoclastogenèse et de la résorption osseuse, et une ostéopétrose (Hsu et al., 1999).

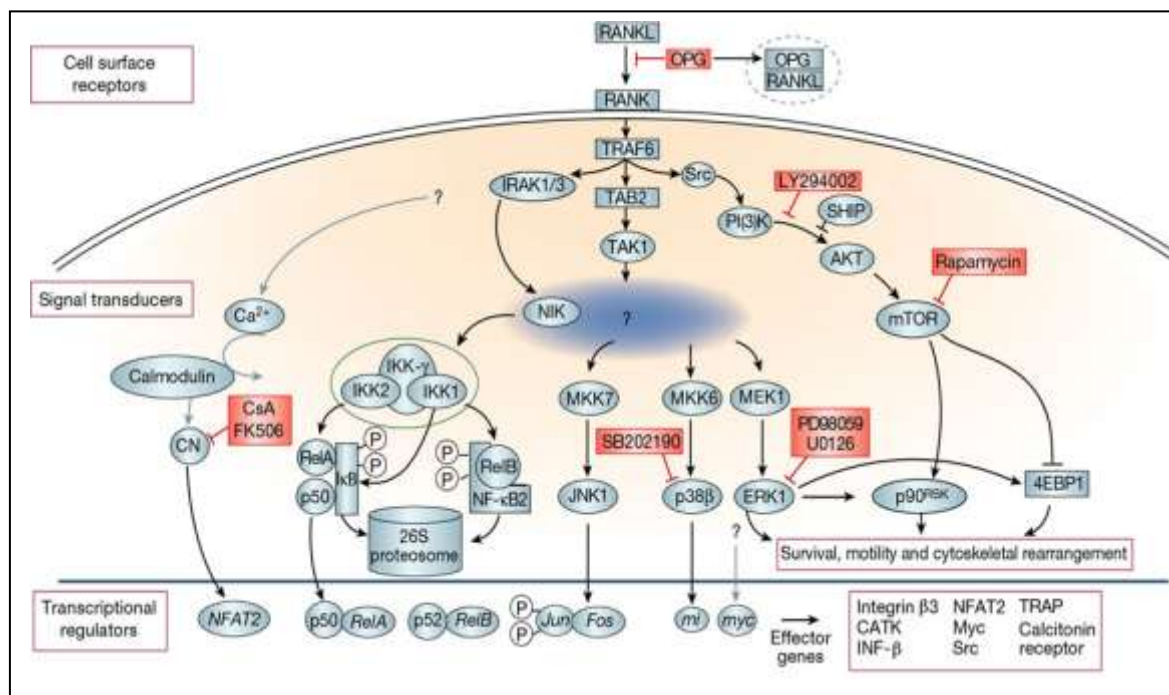


Figure 11 : Signalisation induite par RANKL via son récepteur RANK dans les ostéoclastes (Boyle et al., 2003).

C/ Ostéoprotégérine (OPG)

L'OPG initialement appelée Osteoclastogenesis Inhibitory Factor (OCIF), ou encore Follicular Dendritic Cell Receptor 1 (FDCR-1), TNF Receptor related molecule 1 (TR-1) ou selon la nomenclature officielle TNFRSF11B, a été identifiée par deux groupes indépendants comme un nouveau membre de la superfamille des récepteurs au TNF (Simonet et al., 1997; Yasuda et al., 1998a) mais diffère des autres membres par l'absence de domaine

transmembranaire. L'OPG est donc une molécule soluble ayant la capacité de se lier à différents ligands.

Le gène humain codant l'OPG est localisé sur le chromosome 8q23-24 et contient 5 exons sur un domaine de 29 kb (Yamaguchi et al., 1998). L'OPG est synthétisée sous la forme d'un propeptide de 401 acides aminés dont le peptide signal (21 acides aminés) est clivé générant une protéine de 380 acides aminés. L'OPG ne possédant pas de domaine cytoplasmique ni transmembranaire, est produite exclusivement sous forme d'une protéine soluble. Sa partie N-terminale possède 4 motifs riches en cystéine (D1-D4) impliqués dans l'inhibition de l'ostéoclastogenèse. Son extrémité C-terminale contient quant à elle deux « death domain homologous » (D5 et D6), dont la fonction est actuellement inconnue pour l'OPG mais fonctionnels (Yamaguchi et al., 1998), et un domaine de liaison à l'héparine (D7). En partie C-terminale se trouve également un résidu cystéine (Cys⁴⁰⁰) nécessaire à son homodimérisation (Figure 12). L'OPG est synthétisée sous forme de monomère (55-62 kDa) qui se dimérise par des ponts disulfures pour être sécrétée principalement en tant que glycoprotéine homodimérique (110-120 kDa) avec 4 à 5 sites potentiels de N-glycosylation (Hofbauer et al., 2000).

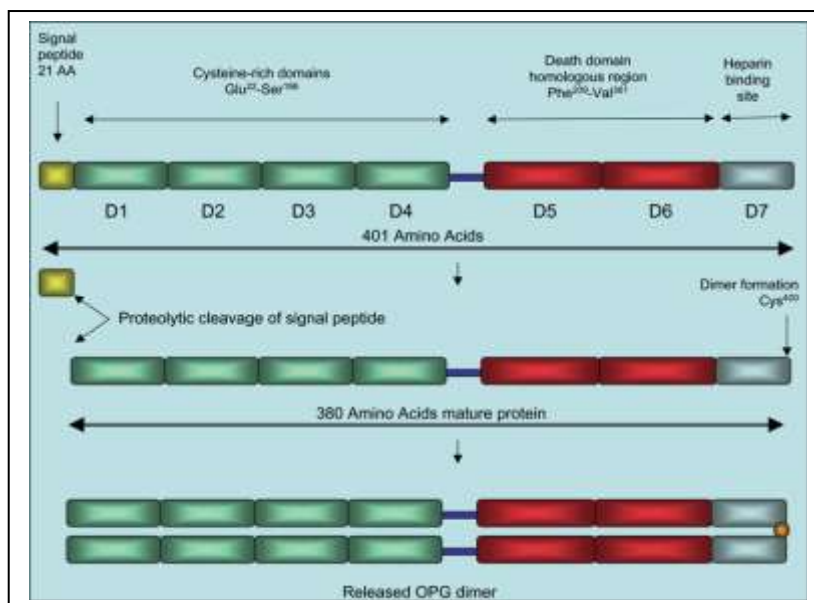


Figure 12 : Structure de l'OPG (Holen and Shipman, 2006).

L'OPG est une molécule ubiquitaire présente dans le tissu osseux, la peau, le foie, les poumons, le cœur, les reins, le placenta, les glandes mammaires et le cerveau (Simonet et al., 1997; Yasuda et al., 1998a). Elle est exprimée de façon prédominante par les cellules stromales de la moelle osseuse, mais son expression par les lymphocytes B et les cellules dendritiques peut également être induite par le CD40 (Yun et al., 1998). Enfin, les cellules

endothéliales, les fibroblastes, les monocytes, les ostéoblastes humains en culture primaire et les lignées ostéosarcomateuses telles MG-63 et SaOS₂ expriment fortement l'OPG (Hofbauer et al., 2000), ainsi que d'autres cellules tumorales des cancers du sein ou de la prostate (Holen et al., 2002; Neville-Webbe et al., 2004).

Un très grand nombre de facteurs peut réguler la synthèse et l'expression de l'OPG tels que la vitamine D₃, les oestrogènes, le TNF α , le TGF β et de l'IL-1 qui ont un effet stimulateur (Hofbauer et al., 1998; Saika et al., 2001; Thirunavukkarasu et al., 2001). La PTH et les prostaglandines E₂ exercent au contraire un effet inhibiteur sur l'expression de l'OPG (Brandstrom et al., 2001; Gao et al., 1998; Li et al., 2000), ainsi que les glucocorticoïdes qui sont de ce fait responsables de l'apparition d'ostéoporose chez les patients traités à long terme par ces molécules (Hofbauer et al., 1999).

Les fonctions principales de l'OPG ont été clairement mises en évidence par l'établissement de souris transgéniques surexprimant l'OPG. En effet, ces souris présentent une sévère ostéopétrose accompagnée d'une splénomégalie due à une hématopoïèse compensatoire extramédullaire et à un défaut de développement thymique (Simonet et al., 1997). Une augmentation de l'os trabéculaire minéralisé associée à une diminution du nombre d'ostéoclastes est également observée. Au contraire, les souris invalidées pour l'OPG présentent une ostéoporose et une diminution de la densité minérale osseuse accompagnée de multiples fractures et d'une stimulation de renouvellement osseux (Bucay et al., 1998). Ces phénomènes sont réversibles par l'injection intraveineuse d'OPG recombinante (Min et al., 2000). L'OPG joue donc un rôle primordial dans le remodelage osseux et dans le maintien de la masse osseuse.

L'OPG fonctionne en réalité comme un récepteur compétiteur de RANK puisqu'elle se fixe à RANKL empêchant ce dernier d'interagir avec RANK (figure 9). L'OPG inhibe par conséquent la différenciation et l'activation ostéoclastiques. Un autre ligand de l'OPG a été mis en évidence, il s'agit de TRAIL (TNF-Related Apoptosis-Inducing Ligand). La fixation d'OPG à TRAIL inhibe l'effet apoptotique de TRAIL qui en retour bloque l'activité anti-ostéoclastique de l'OPG (Emery et al., 1998).

L'OPG peut enfin interagir avec des molécules de la famille de l'héparine par un domaine de liaison spécifique (Yamaguchi et al., 1998). Il a notamment été montré que l'OPG est capable de se fixer à la surface des cellules de myélome *via* un protéoglycane, le syndecan-1 (Borset et al., 2000).

Le tissu osseux est en perpétuel remaniement assuré par la balance cellulaire ostéoblaste / ostéoclaste, finement régulé au niveau moléculaire par la triade OPG / RANKL / RANK, représentant les effecteurs finaux du remodelage osseux. Notamment, toute dérégulation du ratio OPG / RANKL aboutit à l'apparition de pathologies osseuses d'origine tumorale ou non.

III. Les tumeurs osseuses primitives

Les tumeurs osseuses primitives sont considérées comme des pathologies rares et peuvent être divisées en deux groupes : les tumeurs bénignes et les tumeurs malignes.

Les tumeurs osseuses bénignes sont les plus fréquentes et peuvent s'observer à tout âge avec tout de même une prédilection chez l'enfant et l'adulte jeune.

Les tumeurs osseuses primitives malignes représentent moins de 1% des cancers et environ 10% de l'ensemble des tumeurs de l'enfant et de l'adulte jeune. Cependant un deuxième pic d'incidence se situe vers 60 ans avec des tumeurs osseuses primitives qui surviennent à la suite de pathologies du remodelage osseux telles que la maladie de Paget. Aux Etats-Unis, l'incidence annuelle des tumeurs osseuses primitives chez les moins de 20 ans est estimée à 8,7 nouveaux cas par an par million d'habitants. L'ostéosarcome et le sarcome d'Ewing sont les deux principaux types de tumeurs osseuses primitives malignes chez l'enfant et l'adulte jeune, représentant environ 90% des tumeurs osseuses primitives (Desandes et al., 2004).

L'apparition d'une tumeur osseuse pose un certain nombre de problèmes de diagnostic et de traitements nécessitant une étude minutieuse incluant l'imagerie, la chirurgie et l'histologie afin d'établir avec précision le diagnostic de la tumeur de manière à entreprendre une thérapie adaptée.

Par ailleurs, le perfectionnement des techniques d'imagerie essentielles pour le diagnostic ainsi que le développement de nouvelles séquences de traitement associant poly-chimiothérapie néo-adjuvante et chirurgie conservatrice ont considérablement amélioré depuis trois décades les taux de survie des tumeurs osseuses primitives.

Techniques de diagnostic

La suspicion chez un patient d'une tumeur osseuse primitive se déroule en plusieurs étapes, bien que souvent la tuméfaction du membre associée à la douleur soit un signe probant. Tout d'abord, un examen clinique est réalisé associé à de la radiographie. La présence de la douleur dans d'autres sites pourrait suggérer la présence de métastases. Il est important, surtout chez l'enfant, de ne pas négliger la symptomatologie douloureuse, en particulier les douleurs rachidiennes qui doivent toujours être explorées. L'examen physique révèle généralement un gonflement des tissus mous au site de la tumeur primitive.

A ce jour, l'imagerie est essentielle pour le diagnostic, pour le bilan d'extension locale et à distance, l'évaluation de l'efficacité du traitement et la détection de récurrence.

L'utilisation de l'Imagerie par Résonance Magnétique (IRM) ou de la TomoDensitoMétrie (TDM) définissant l'étendue de la tumeur locale se révèle être un prédicateur exact de mesure et de définition de l'extension tumorale afin de faciliter la résection chirurgicale (Gillespy et al., 1988; Panicek et al., 1997).

En plus de l'imagerie de la tumeur primitive, diverses autres analyses radiologiques permettent de déterminer l'extension métastatique de la maladie. En effet, environ 10 à 20% des ostéosarcomes sont métastatiques au diagnostic, principalement au niveau des poumons dans 90% des cas et au niveau osseux dans 10% des cas. La fréquence des métastases est plus élevée dans le sarcome d'Ewing représentant 20 à 30% des cas. La scintigraphie osseuse au technétium-99m, permet de définir la tumeur primitive mais a surtout l'intérêt de rechercher d'autres localisations osseuses (McKillop et al., 1981), d'autant plus qu'environ 30% des patients ayant des métastases peuvent être guéris avec la chimiothérapie et de multiples résections chirurgicales (Bacci et al., 2000; Marina et al., 1992; Meyers et al., 1993).

Lors d'un diagnostic d'une tumeur osseuse primitive, la biopsie est réalisée rapidement après le bilan d'extension locale. La biopsie est donc la première intervention chirurgicale pour un patient avec une tumeur osseuse. Le type de biopsie effectué doit être soigneusement déterminé en évaluant la taille et l'emplacement de la tumeur, le diagnostic différentiel, et l'âge du patient. Il est fortement recommandé que la biopsie soit réalisée par le chirurgien qui effectuera la résection définitive. En effet une biopsie mal faite peut rendre la résection impossible (Mankin et al., 1996).

A/ L'Ostéosarcome

L'ostéosarcome représente la plus fréquente des tumeurs osseuses primitives avec 100 à 150 cas recensés par an en France, se développant majoritairement chez l'adolescent et l'adulte jeune avec un âge moyen de survenue de 18 ans (Link, 1988). Il est extrêmement rare avant l'âge de 5 ans. Une prédominance masculine est observée avec un rapport 1,4 (homme): 1 (femme). L'incidence de l'ostéosarcome des enfants afro-américains est légèrement supérieure à celle des enfants caucasiens. L'âge de survenue de la tumeur au moment de la croissance ainsi que la localisation métaphysaire suggèrent un lien entre la croissance et la survenue des ostéosarcomes.

D'origine ostéoblastique, l'ostéosarcome est une tumeur très agressive caractérisée par la production directe de substance ostéoïde par les cellules tumorales (>90%), mais cependant toujours associée à des plages d'ostéolyse. Avant l'utilisation courante de la chimiothérapie dans le traitement de l'ostéosarcome, le faible taux de survie des patients atteints de cette tumeur était de 20% à 2 ans dans les meilleures séries et après résection chirurgicale et / ou radiothérapie (Friedman and Carter, 1972; Marcove et al., 1970; Weinfeld and Dudley, 1962). Bien que l'ostéosarcome soit une maladie qui touche principalement des adolescents et des jeunes adultes, un second groupe de patients plus âgés développe cette maladie consécutivement à des pathologies qui touchent le remodelage osseux (maladie de Paget, dysplasie fibreuse) ou suite à une radiothérapie.

De plus, un certain nombre de syndromes de prédisposition génétiques peuvent être associés au développement d'ostéosarcome. La survenue de ce cancer représente la plus fréquente des tumeurs secondaires chez les sujets atteint d'un rétinoblastome héréditaire (Hawkins et al., 1996). Le développement d'ostéosarcome est également plus fréquent à la suite du syndrome de Rothmund-Thompson (Leonard et al., 1996) ou du syndrome de Li-Fraumeni (Varley et al., 1997). Il n'existe pas de lésion chromosomique spécifique reconnue comme dans le cas du sarcome d'Ewing, mais une atteinte fréquente des locus des gènes p53 et du rétinoblastome (Feugeas et al., 1996). En effet, l'inactivation de ces gènes suppresseurs de tumeurs est l'anomalie la plus fréquente, avec souvent l'inactivation combinée des deux.

Le syndrome de Li-Fraumeni qui prédispose à la survenue d'ostéosarcome présente des mutations du gène p53, ce qui suggère un rôle de p53 dans la genèse des ostéosarcomes. De plus, 75 à 80% des ostéosarcomes présentent une perte d'hétérozygotie de la région 17p qui contient le gène p53 (Yamaguchi et al., 1992) ainsi qu'une mutation ponctuelle dans 30% des cas (Miller et al., 1996). Par ailleurs, une inactivation fonctionnelle de p53 induite par une amplification du gène MDM2 (se liant à p53) a été mise en évidence dans environ 25% des tumeurs, avec une prédominance dans les tumeurs métastatiques ou en rechute, suggérant une implication dans la progression tumorale et le développement de métastases (Ladanyi et al., 1993; Miller et al., 1996). Dans environ 50 à 70% des cas, le gène Rb n'est pas exprimé dans les ostéosarcomes. En effet, cette anomalie est due à une perte d'hétérozygotie dans 60% des cas, un réarrangement dans 30% des cas ou une mutation ponctuelle dans 10% des cas (Miller et al., 1996; Yamaguchi et al., 1992). Par ailleurs, Feugeas *et al* ainsi que Wadayama *et al* suggèrent que l'altération fonctionnelle de Rb due à la perte d'hétérozygotie pourrait être un facteur pronostique de l'ostéosarcome (Feugeas et al., 1996; Fuchs and Pritchard, 2002; Wadayama et al., 1994).



Figure 13 : Radiographies d'un ostéosarcome secondaire à une maladie de Paget.

L'étiologie moléculaire de l'ostéosarcome est encore mal connue. En effet, les ostéosarcomes ne sont pas associés à une anomalie moléculaire contrairement à d'autres types de cancers tels que le sarcome d'Ewing induit par une translocation spécifique. Ce manque de données sur les mécanismes moléculaires rend plus difficile le diagnostic ainsi que le développement d'approches thérapeutiques ciblées pour cette pathologie.

Les signes déterminants pour poser un diagnostic d'ostéosarcome sont le plus souvent des douleurs osseuses, des tuméfactions osseuses, ou encore des fractures spontanées sans contraintes, et au niveau radiologique la présence de plages d'ostéolyse, de zones d'ostéocondensation ou bien des formes mixtes (Meyers and Gorlick, 1997). Les patients ont généralement des symptômes pendant plusieurs mois (en moyenne, 3-4 mois, mais souvent supérieur à 6 mois) avant qu'un diagnostic soit établi.

Bien que l'ostéosarcome puisse se développer dans tous les os, le site le plus fréquent est la métaphyse des os longs. Les plus courants sont le fémur, le tibia proximal et l'humérus (Dahlin and Coventry, 1967; Dahlin and Unni, 1977; Weinfeld and Dudley, 1962), avec environ 50% des cas se développant au niveau du genou (Dahlin and Coventry, 1967; Weinfeld and Dudley, 1962).

Au diagnostic, environ 15-20% des patients présentent un développement métastatique détectable par radiographie (Kaste et al., 1999; Meyers and Gorlick, 1997) avec l'apparition de métastases qui augmentent souvent à la suite de la résection tumorale (Dahlin and Coventry, 1967; Friedman and Carter, 1972; Marcove et al., 1970; Weinfeld and Dudley,

1962). L'ostéosarcome métastase majoritairement au niveau des poumons (Fitzgerald et al., 1973), induisant des détresses respiratoires, principale cause de décès des patients (Meyers and Gorlick, 1997). Cependant, le développement de métastases peut aussi survenir au niveau osseux à des sites différents de la tumeur primitive, ainsi qu'au niveau des tissus mous (Jeffree et al., 1975; Kaste et al., 1999).

Au niveau histologique, l'ostéosarcome montre des plages ostéoformatrices et la présence d'ostéoblastes tumoraux ainsi que de nombreuses mitoses indispensables au diagnostic, parfois minoritaires (Fig. 14A). L'aspect hétérogène de l'ostéosarcome permet de visualiser sur une même tumeur des zones présentant de nombreuses travées osseuses de tailles et formes variables mettant en évidence la composante lytique de la tumeur (Fig. 14B). La résection après une chimiothérapie d'induction chez un bon répondeur montre une disparition des cellules tumorales, remplacées par un tissu fibreux hyalin et des travées osseuses irrégulières (Fig 14C)

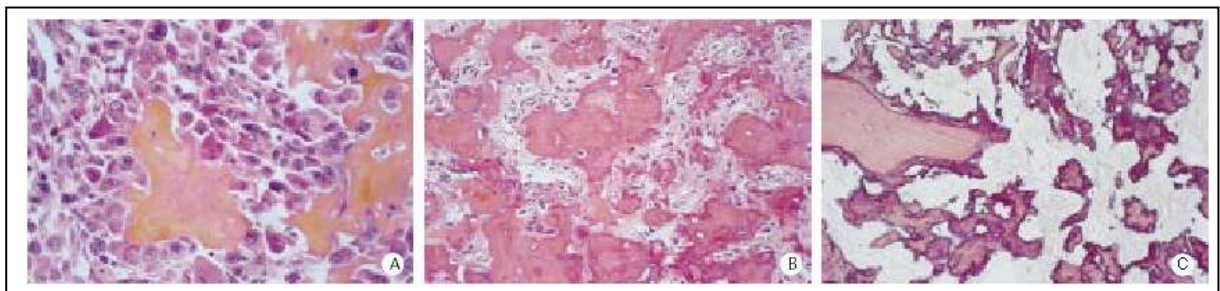


Figure 14 : Aspect histologique d'un ostéosarcome (A : x400, B : x200, C : x200 ; JM Guinebretière 2001).

Traitements

Les approches thérapeutiques des ostéosarcomes sont l'objet d'une récente revue [(Lamoureux et al., 2007c), en annexe].

Jusque dans les années 70, le traitement de l'ostéosarcome était purement local avec comme seul traitement l'acte chirurgical. L'amputation du membre représentait le traitement radical de la tumeur primitive, mais augmentait la survenue de métastases majoritairement pulmonaires chez environ 80% des patients dans les semaines ou les mois suivant l'amputation et conduisant au décès du patient. En effet, avec la chirurgie seule, la survie à 5 ans était de 12% et 3 patients sur 4 décédaient dans les deux ans suivant le diagnostic. Depuis 20 ans, les progrès de la chimiothérapie utilisée de manière néo-adjuvante associée à l'amélioration des techniques chirurgicales conservatrices ont considérablement

augmenté les taux de survie qui atteignent 70% à 5 ans dans les meilleures séries. Le concept de chimiothérapie « néo-adjuvante » a été introduit par Rosen en 1976, où la chimiothérapie préopératoire avait diminué le volume de la tumeur chez 17 patients sur 20 et ainsi facilité l'ablation de la tumeur et la conservation du membre (Rosen et al., 1976). Deux études randomisées ont comparé un groupe de patients traités avec chimiothérapie adjuvante et un groupe de patients sans chimiothérapie. Les résultats mettaient en évidence une augmentation significative de la survie dans le groupe de patients traités par chimiothérapie (Eilber et al., 1987; Link et al., 1986).

Même si aucune preuve formelle ne démontre son avantage (Goorin et al., 2003), la chimiothérapie initiale administrée en pré-opératoire offre de multiples avantages, notamment un traitement prophylactique précoce des métastases, une diminution de la taille de la tumeur primitive et la possibilité d'évaluer la réponse histologique à la chimiothérapie préopératoire sur la pièce de résection. En effet, l'étude du taux de nécrose des cellules tumorales au moment de l'exérèse chirurgicale après chimiothérapie préopératoire se révèle être un facteur pronostique en corrélation avec la survie globale du patient. La nécrose tumorale est évaluée par la classification universellement reconnue et établie en 1977 par Huvos (Huvos et al., 1977) qui détermine le pourcentage moyen de cellules viables sur la pièce opératoire. Si moins de 5% de cellules viables (correspondant au grade III ; Grade IV, aucune cellule viable) sont présentes sur la pièce opératoire, le patient est considéré comme bon répondeur. Les séquences de chimiothérapie adjuvante sont adaptées en fonction de la qualité de la réponse histologique à la chimiothérapie préopératoire. La chimiothérapie adjuvante est différente de celle d'induction et est définie suivant la réponse histologique du patient.

Plusieurs drogues sont actives dans le traitement de l'ostéosarcome et ont montré leur efficacité dans des études de phase II en monothérapie (plus de 20% de réponse dans l'ostéosarcome): le méthotrexate à hautes doses (HDMTX) (Jaffe et al., 1973) et l'adriamycine (Doxorubicine) (Cores et al., 1972) ont été les premières drogues utilisées. Le cis-platinum (Gasparini et al., 1985), l'ifosfamide (Marti et al., 1985) et plus récemment le thiotepa à hautes doses (Lucidarme et al., 1998) ont successivement été joints au traitement de l'ostéosarcome améliorant ainsi la survie des patients. Depuis les années 70, les protocoles les plus utilisés comportent tous du HDMTX qui demeure la drogue de référence dans le traitement des ostéosarcomes localisés. Cependant, le HDMTX doit être utilisé en association avec une des drogues citées ci-dessus, telle que l'adriamycine (Meyers et al., 1992; Winkler et al., 1988). Deux études ont montré qu'il fallait privilégier des fortes doses de MTX plus

efficaces que des doses modérées (Bacci et al., 1990; Meyers et al., 1992). Néanmoins, du fait de sa toxicité, les doses de MTX varient de l'enfant ($<12\text{g/m}^2$) à l'adulte (dose maximale de 8g/m^2).

Toutefois, la faible tolérance au MTX chez les jeunes adultes, ainsi qu'un certain nombre de contraintes (administration hebdomadaire, hospitalisations répétées,...) sont en faveur de développement de protocoles excluant le MTX (Kalifa et al., 1988). Une large étude multicentrique de l'European Osteosarcoma Intergroup (EOI) incluant environ 400 patients utilisant un protocole associant l'adriamycine et le cisplatine montrent des résultats équivalents à une chimiothérapie multi-drogues dérivée du protocole T10 de Rosen (Rosen, 1986) basée sur le MTX à haute dose, en terme de réponse histologique, survie sans récurrence et survie globale (Souhami et al., 1997).

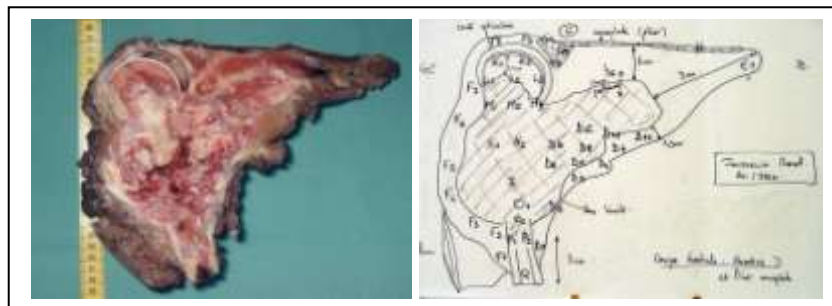


Figure 15: Résection d'un ostéosarcome au niveau de la hanche et analyse anatomopathologique pour l'estimation du nombre de cellules tumorales résiduelles.

La combinaison chimiothérapie d'induction, chirurgie et chimiothérapie adjuvante a transformé le pronostic de cette lésion auparavant gravissime. En effet, le taux de survie est maintenant de 70% à 5 ans mais reste cependant faible (20%) lorsque des métastases pulmonaires sont détectées au moment du diagnostic.

B/ Le sarcome d'Ewing

Le sarcome d'Ewing, décrit par James Ewing en 1921, est considéré comme d'origine neuro-ectodermique et appartient à l'entité des « tumeurs de la famille PNET (Peripheral Neuro Ectodermal Tumors) ».

La tumeur d'Ewing touche principalement l'enfant et l'adulte jeune, rare avant 5 ans et après 30 ans. Le pic de survenue se situe essentiellement entre 10 et 30 ans et l'âge pédiatrique médian est de 12 ans. C'est une tumeur rare avec 2,9 nouveaux cas par million d'enfants de moins de 20 ans aux USA (Ries et al., 1999) et 3,2 nouveaux cas par million d'enfants de moins de 15 ans représentant environ 80 nouveaux cas par an en France (Desandes et al., 2004). De plus, cette tumeur osseuse de l'adolescent est plus fréquente chez les garçons avec un rapport de 1,2 à 1,5. Par ailleurs, elle est exceptionnelle dans les populations noires, africaines ou américaines ainsi que chez les sujets d'origine asiatique.

La tumeur osseuse est généralement localisée au niveau de la diaphyse des os longs (fémur, tibia, péroné, humérus,...) mais également au niveau des os plats avec une fréquence variable (tableau I).

Tableau I - Répartition des sarcomes d'Ewing

Siège de la tumeur primitive	Fréquence
Ceinture pelvienne	22
Côtes	18
Vertèbre	10
Fémur	10
Tibia	10
Péroné	10
Humérus	8,5
Autres	11,5

Source : étude SFOP EW 88 "Memphis" sur 142 patients porteurs de tumeur localisée.

Au niveau étiologique, le mécanisme physiopathologique du sarcome d'Ewing découvert en 1984 et caractérisé en 1992 par l'équipe dirigée actuellement par Olivier Delattre est maintenant bien connu (May et al., 1993). Il s'agit le plus souvent d'une translocation chromosomique fusionnant de façon constante le gène EWS porté par le chromosome 22 avec d'autres gènes tels que le gène FLI-1 situé sur le chromosome 11 (dans environ 85% des cas), ou plus rarement le gène ERG porté par le chromosome 21 (dans 10 à 15% des cas) ou le gène E1AF situé sur le chromosome 17. Ces différentes translocations sont responsables de l'apparition de protéines de fusion comme la protéine EWS/FLI-1 qui exerce une action oncogénique par une activation transcriptionnelle anormale de gènes cibles, telle que l'inhibition de l'expression d'IGFBP-3 (Insulin-like Growth Factor Binding Protein 3). En effet, au moment de la croissance chez les adolescents, les cellules normales sont soumises à une intense signalisation par l'hormone de croissance et notamment par l'IGF-1, ce dernier pouvant être inhibé par IGFBP-3. Dans le sarcome d'Ewing, la protéine altérée (EWS/FLI1) empêche l'expression d'IGFBP-3. Par conséquent, le facteur de croissance IGF-1 n'est plus inhibé pouvant alors transmettre en continu un signal ordonnant aux cellules de proliférer de manière anarchique (Prieur et al., 2004). IGFBP-3, impliqué en amont dans cette voie de signalisation, pourrait servir de cible thérapeutique pour bloquer la prolifération cellulaire anormale induite par IGF-1.

Au niveau histologique, le sarcome d'Ewing est une tumeur à petites cellules rondes basophiles, d'architecture plus ou moins lobulée, dense, d'aspect uniforme (Figure 16). Parfois l'arrangement est circulaire autour d'une formation vasculaire en pseudo rosettes. Les cellules sont en effet relativement monomorphes, comportant des noyaux arrondis à ovalaires, vésiculeux à membrane nucléaire cernée, à chromatine finement granitée homogène renfermant un petit nucléole. Les cytoplasmes sont pâles, mal limités, peu abondants. Si le nombre des mitoses est réduit, il peut exister des formes avec un plus grand pléomorphisme et une activité mitotique augmentée. Il n'y a pas de réseau réticulinique intercellulaire. La vascularisation est riche, parfois masquée par la densité cellulaire. Il peut exister des remaniements hémorragiques, des aspects pseudo-angiomateux. Le glycogène est d'aspect variable, mieux vu sur des prélèvements congelés, présent dans 70 % des cas.

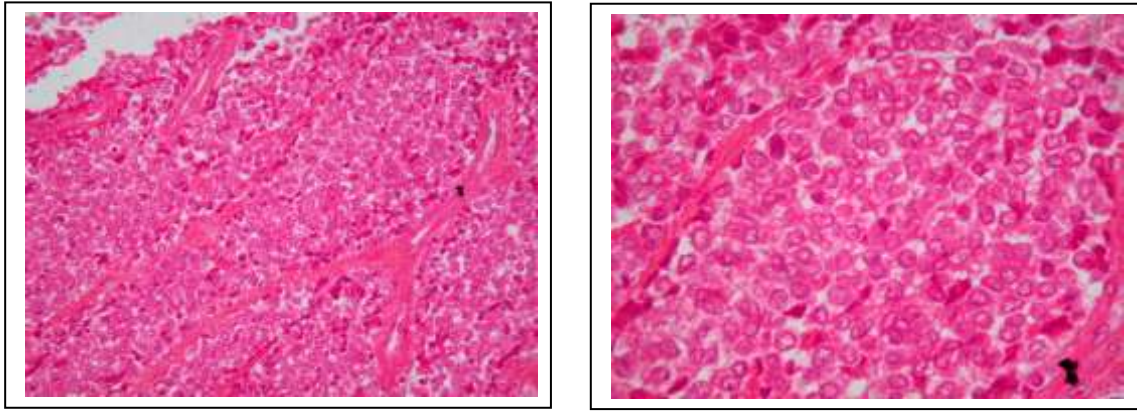


Figure 16 : Aspect histologique d'un sarcome d'Ewing extra-squelettique chez une jeune fille de 13 ans. Cette tumeur est caractérisée par de petites cellules rondes qui groupent de nombreuses entités, au noyau pourvu de chromatine et aux limites du cytoplasme imprécises (gauche : x200, droite : x400).

Comme l'ostéosarcome, les signes révélateurs de la tumeur d'Ewing sont généralement des douleurs osseuses, parfois capricieuses pouvant expliquer un retard fréquent de plusieurs mois au diagnostic. Il apparaît ensuite un gonflement de la zone touchée, parfois secondaire aux douleurs et pouvant être d'aspect inflammatoire. Dans 15 à 20% des cas, d'autres symptômes plus généraux peuvent apparaître tels que de la fièvre, une asthénie, un amaigrissement... Signes généraux souvent associés à une maladie métastatique ou à une localisation pelvienne.

Traitement

Le traitement du sarcome d'Ewing a beaucoup évolué ces 50 dernières années avec la mise en place de traitements systémiques. Auparavant, l'amputation était systématique, et 90% des patients atteints de tumeurs d'Ewing décédaient de métastases osseuses ou pulmonaires dans les trois ans suivant le diagnostic. L'utilisation de la chimiothérapie adjuvante associée à un traitement local a permis de révolutionner le pronostic, ciblant ainsi la tumeur et les micrométastases. La radiothérapie, en tant que traitement local, a progressivement laissé place à la chirurgie, mais continue d'être utilisée dans les formes inopérables et plus fréquemment complémentaires à la chirurgie, assurant ainsi un contrôle loco-régional.

Comme dans le traitement de l'ostéosarcome, la chimiothérapie d'induction a été introduite comme thérapie dans la tumeur d'Ewing dans les années 80 avec un double

objectif : prévenir l'apparition de métastases et réduire la masse tumorale afin de faciliter le traitement local (chirurgie ou radiothérapie).

Les protocoles de chimiothérapie les plus anciens combinaient 4 médicaments avec une efficacité certaine démontrée mais transitoire en phase II de monochimiothérapie : vincristine, l'actinomycine D, l'adriamycine et le cyclophosphamide. L'association des 4 (VACA) a montré une efficacité supérieure à d'autres associations, notamment de 3 médicaments, VAC (vincristine, actinomycine D et cyclophosphamide) (Nesbit et al., 1990). Ce protocole VACA, faisant partie des plus anciens protocoles de chimiothérapie néo-adjuvante, était administré de façon variable, sous forme cyclique, c'est-à-dire 2 à 3 jours toutes les 3 semaines ou à faibles doses de manière continue 1 semaine sur 2.

L'apparition de l'ifosfamide en supplément dans ce type de protocole a permis d'améliorer le pronostic des grosses tumeurs (Paulussen et al., 2001) et d'augmenter la survie sans récurrence après association à l'étoposide (Grier et al., 2003), mais son association au protocole VACA reste encore discutée

Actuellement, le traitement du sarcome d'Ewing en Europe suit le protocole Euro-EWING 99 qui consiste en 6 cures de polychimiothérapie néo-adjuvantes associant vincristine, ifosfamide, adriamycine (= doxorubicine) et étoposide (VIDE) suivies d'une résection chirurgicale si la tumeur est opérable. Une randomisation est ensuite effectuée en fonction du volume tumoral, de la réponse histologique et de la présence de métastases. Si la tumeur a bien répondu à la chimiothérapie inductive, le traitement adjuvant sera randomisé entre une chimiothérapie comportant de l'ifosfamide (VAI) versus une chimiothérapie comportant du cyclophosphamide (VAC). Si le pourcentage de cellules résiduelles est supérieur à 10%, le traitement sera randomisé entre une chimiothérapie conventionnelle (VAI) et une chimiothérapie à haute dose comportant du busulfan et du melphalan (figure 17).

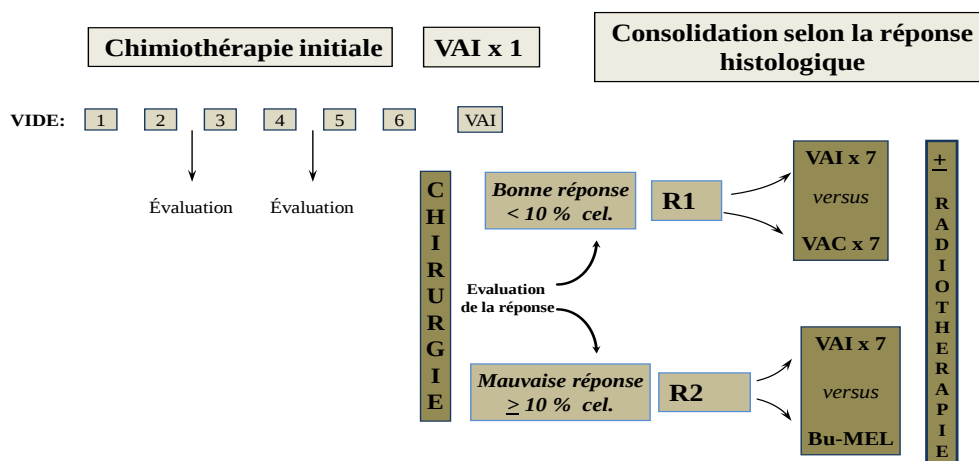


Figure 17 : Protocole Euro-EWING 99

Malgré des progrès récents, le sarcome d'Ewing reste une des tumeurs osseuses primitives avec le plus sombre pronostic : 60% de survie à 5 ans tous stades confondus, moins de 20% en cas de métastases et 5% en cas de métastases ostéo-médullaires (Paulussen et al., 2001). Des biopsies ostéo-médullaires à plusieurs endroits permettent de rechercher des micrométastases ostéo-médullaires lors du bilan d'extension initial. Même si le traitement chirurgical a beaucoup progressé avec une optique de conservation de membre, elle aboutit cependant à 15% d'amputation. Les effets secondaires liés à la chimiothérapie ne sont pas négligeables non plus, notamment ceux liés à l'Ifosfamide avec un risque à long terme de tubulopathie, en fonction de la dose cumulée, ainsi que de cancers secondaires liés principalement à l'Etoposide (Paulussen et al., 2001).

C/ Limites des traitements actuels et développement de nouvelles approches thérapeutiques pour les tumeurs osseuses primitives

Comme nous venons de le voir, les tumeurs osseuses primitives sont responsables d'une morbidité importante. Le traitement actuel, basé sur une polychimiothérapie néo-adjuvante souvent mal tolérée associée à une chirurgie conservatrice, a largement contribué à l'amélioration du taux de survie des patients dans ces dernières années. Cependant, une absence de réponse aux drogues anti-tumorales est souvent observée et demeure un problème majeur conduisant au développement de métastases puis au décès du patient. De plus, les séquences de traitement, les hospitalisations répétées font de la chimiothérapie un traitement difficile à supporter dégradant la qualité de vie du patient. Il devient donc nécessaire de développer de nouvelles approches thérapeutiques pour ces pathologies.

La problématique biologique du développement tumoral en site osseux repose sur l'existence d'un cercle vicieux entre cellules tumorales et résorption osseuse (Figure 18) (Chirgwin and Guise, 2000; Guise, 2002). En effet, il est maintenant bien établi que le tropisme de certaines tumeurs (primitive ou secondaire) pour le site osseux est maintenu par l'environnement osseux lui-même, contenant un ensemble de facteurs de croissance (Insulin-like Growth Factor-1, Transforming Growth Factor- β , Fibroblast Growth Factor...) favorables à la prolifération tumorale. A leur tour, les cellules tumorales produisent directement ou suite à l'interaction avec les cellules stromales, des facteurs solubles qui

activent la différenciation et la maturation des ostéoclastes, favorisant ainsi la résorption osseuse. Comme exemple, la sécrétion de PTHrP par les cellules tumorales est le principal facteur stimulateur de la production de RANKL par les ostéoblastes. RANKL joue un rôle pivot dans l'activation des ostéoclastes et entraîne une augmentation de la formation, de l'activation et de la survie des ostéoclastes provoquant une ostéolyse accrue, qui par la libération des facteurs stockés dans la matrice osseuse, entretient ce cercle vicieux. Outre ces processus, l'ostéolyse libère et augmente au niveau local la concentration de calcium extracellulaire (Ca^{2+}), également montré comme favorisant la croissance tumorale et la production de PTHrP. Ces mécanismes décrits dans le cas des métastases osseuses peuvent être également envisagés pour les tumeurs osseuses primitives.

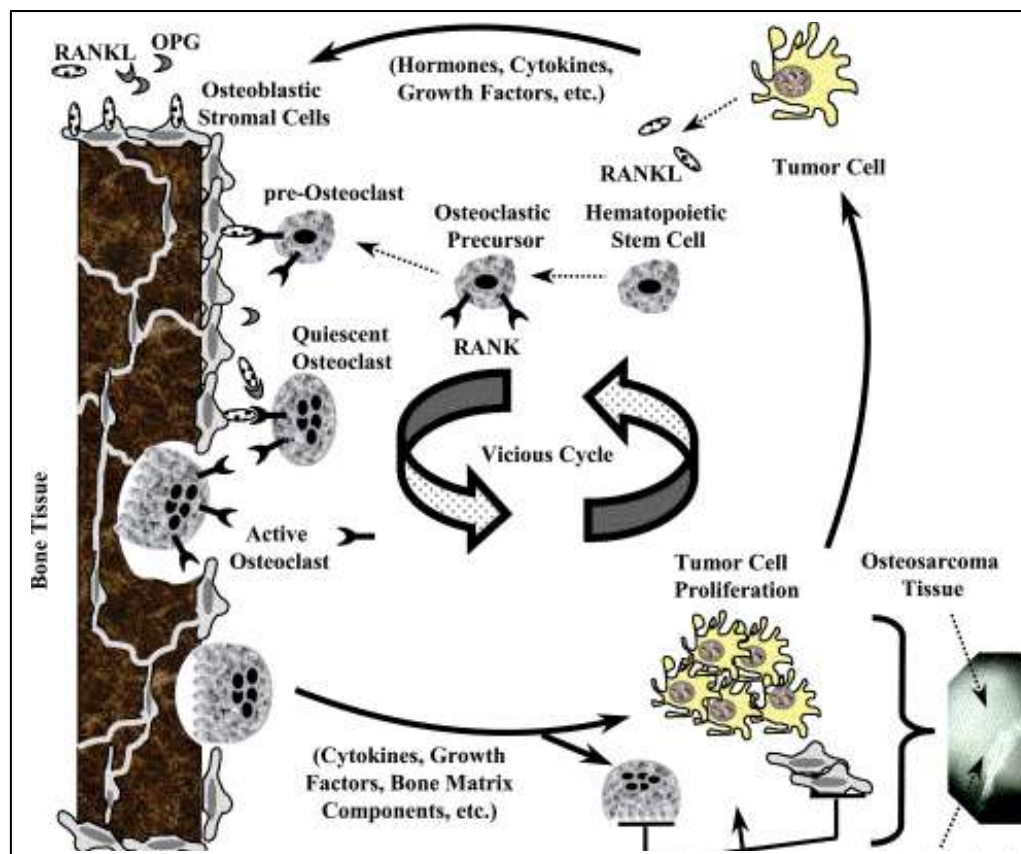


Figure 18 : Cercle vicieux entre prolifération tumorale et hyper-résorption osseuse d'origine maligne. Entre résorption et prolifération tumorale s'instaure un cercle vicieux dans lequel des facteurs de croissance libérés de la matrice lors de l'hyper-résorption osseuse stimulent l'activité et la prolifération des cellules tumorales, qui à leur tour produisent des facteurs qui vont activer la résorption osseuse (RANKL), directement ou via les ostéoblastes (Wittrant et al., 2004).

Afin de rompre ce cercle vicieux deux hypothèses thérapeutiques peuvent être envisagées : - inhiber la prolifération des cellules tumorales notamment par des agents conventionnels de chimiothérapie, bloquant ainsi la libération de facteurs pro-ostéoclastiques.

- inhiber la résorption osseuse, et donc la libération de facteurs de croissance piégés dans la matrice osseuse favorables et à la prolifération des cellules tumorales. Cette hypothèse thérapeutique est au centre des recherches menées dans notre laboratoire.

D'un point de vue général, les nouvelles approches envisagées dans le traitement des tumeurs osseuses primitives ciblent plus particulièrement la tumeur:

- le développement d'anticorps dirigés contre IGF-1R puisque cette voie est impliquée dans la croissance et la survie des cellules tumorales (Beech et al., 2003; Benini et al., 2006; MacEwen et al., 2004; Toretsky et al., 2001). Manara *et al* ont démontré dans un modèle de sarcome d'Ewing chez des souris athymiques que l'inhibition d'IGF-1R par un inhibiteur spécifique, le NVP-AEW541 () induisait une inhibition de la migration du développement de métastases et de l'angiogenèse (Manara et al., 2007). Le « Pediatric Preclinical Testing Program » (PPTP) montre *in vivo* une activité anti-tumorale du SCH717454, un anticorps humain anti-IGF-1R, aussi bien dans des modèles d'ostéosarcome que de sarcome d'Ewing (Kolb et al., 2008). Deux autres études montrent que des anticorps anti-IGF-1R peuvent être utilisés seuls dans le traitement du sarcome d'Ewing (Atzori et al., 2008; Olmos et al., 2008).

- le Muramyl tripeptide phosphatidyl-ethanolamine (MTP-PE) est un analogue lipophile synthétique du muramyl dipeptide. Le MTP-PE a été encapsulé dans des liposomes qui vont aider à délivrer le principe actif dans les monocytes/macrophages, les rendant ainsi activés et tumoricides. L'addition du MTP-PE à la chimiothérapie a montré une amélioration significative de la survie globale dans une étude incluant 662 patients atteints d'ostéosarcome sans métastases détectables (Meyers et al., 2008).

- le développement de nouvelles drogues au stade expérimental dans le traitement de l'ostéosarcome, telles que le Topotecan utilisé dans une thérapie de phase II incluant 26 patients avec un ostéosarcome métastatique qui ne montrait aucune amélioration (Seibel et al., 2001). Le Gemcitabine a été utilisé seul dans une étude de phase II, montrant une action limitée chez des patients avec un stade avancé de sarcomes (Okuno et al., 2003). Cependant, en association avec du Docetaxel, un effet bénéfique était observé dans différents sarcomes dont l'ostéosarcome (Leu et al., 2004). L'Ecteinascidin 743, employé seul dans une étude de phase II dans le prétraitement de patients atteints d'ostéosarcome ne présentait pas de résultats

encourageants (Laverdiere et al., 2003) mais peut être envisagé en association avec le cisplatine ou la doxorubicine. L'interféron a été utilisé seul comme thérapie adjuvante dans un essai de 1971 à 1990 incluant 89 patients traités de 18 mois à 5ans. La survie sans métastases à 10 ans sur un suivi de 12 ans est de 39% pour le traitement le plus court et 43% pour le traitement sur 5 ans (Muller et al., 2005). Cependant l'absence de groupe contrôle limite les conclusions.

- l'IL2 a été utilisée dans l'immunothérapie en alternance avec de la polychimiothérapie en pré-et postopératoire sur 18 patients atteints d'ostéosarcome. Les résultats ont montré une réponse immunitaire en réponse à l'IL2 (Luksch et al., 2003).

- l'utilisation d'allogreffes de cellules souches a été testée après conditionnement non-myéloablatif (« minigreffes ») afin de s'affranchir de la toxicité des conditionnements préparatoires, et de conserver l'effet anti-tumoral du système immunitaire du donneur. Cette forme d'immunothérapie s'est avérée efficace dans différentes tumeurs solides (Fagioli et al., 2002; Resnick et al., 2005) dont un cas d'ostéosarcome avec une rémission complète des métastases après résection de la tumeur primitive malgré une récurrence après environ 100 jours (Fagioli et al., 2003).

- l'immunothérapie utilisant des lymphocytes T cytotoxiques (CTL) peut être une approche innovante dans le traitement des tumeurs osseuses primitives. En effet, les sarcomes comme la plupart des tumeurs expriment à leur surface des antigènes spécifiques servant de cibles aux CTL qui vont ensuite tuer sélectivement les cellules tumorales. Récemment, l'expression de MAGE, SSX et SART-3 ont été identifiés comme antigènes spécifiques de l'ostéosarcome et ont permis d'envisager l'immunothérapie dans l'ostéosarcome utilisant des CTL stimulés par des cellules dendritiques chargées en corps apoptotiques de cellules tumorales (Fagioli et al., 2008).

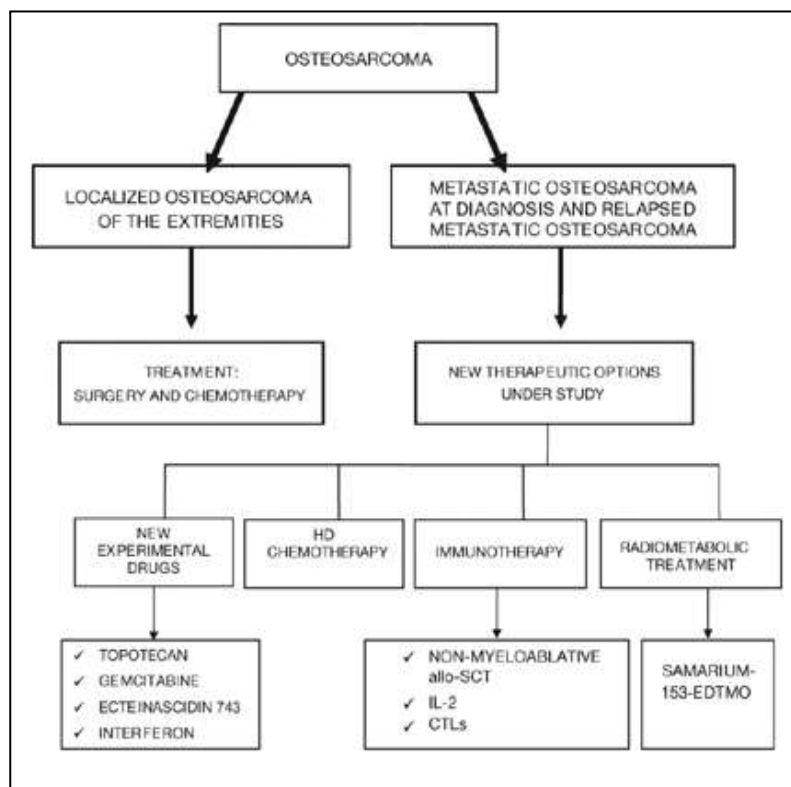


Figure 19 : Approches thérapeutiques des ostéosarcomes (Fagioli et al., 2008).

Malgré des avancées dans la recherche de différentes approches innovantes (Figure 19), il persiste un manque de réponse de la tumeur à ces thérapies ou une toxicité trop importante pour le patient. La deuxième hypothèse thérapeutique pour rompre le cercle vicieux est de bloquer la résorption osseuse.

Pour cela, l'utilisation des bisphosphonates connus pour leur forte inhibition de la résorption osseuse est, à l'heure actuelle, une des approches les plus utilisées dans le traitement palliatif des métastases osseuses mais également envisager dans le traitement des tumeurs osseuses primitives telles que l'ostéosarcome. Les bisphosphonates sont des analogues synthétiques du pyrophosphate endogène (PPi), constitués par une structure de base phosphate-carbone-phosphate (P-C-P) et de deux chaînes R1 et R2. La structure P-C-P, ainsi que la chaîne R1 leur confèrent la capacité à se fixer aux ions divalents tels que le Ca^{2+} ainsi qu'une très haute affinité pour le minéral osseux (Rogers et al., 2000). La chaîne R2 est responsable des propriétés biologiques de ces composés. Les bisphosphonates attirés vers les zones de remodelage osseux atténuent les douleurs osseuses et améliorent la qualité de vie des patients. Ils préviennent les pertes osseuses par inhibition de la résorption osseuse médiée par les ostéoclastes et éventuellement induisent l'apoptose des ostéoclastes (Green, 2003). Plusieurs études démontrent qu'ils sont aussi capables d'induire directement l'apoptose des

cellules tumorales et de réduire l'adhésion cellulaire et l'invasion dans des modèles pré-cliniques de tumeurs osseuses (Fromigue et al., 2003; Santini et al., 2003), cependant la pertinence clinique de l'effet direct des bisphosphonates sur la tumeur reste à éclaircir. Utilisés dans le traitement des métastases osseuses des cancers du sein, de la prostate, des poumons et dans le myélome, les bisphosphonates ont montré leur efficacité à réduire les événements osseux (douleurs, fractures, pertes osseuses, ...) liés aux métastases dans plusieurs essais cliniques (Body et al., 1995; Hillner et al., 2000; Kanis et al., 1996; Lipton et al., 2003; Paterson et al., 1993; Rosen et al., 2001; Rosen et al., 2003a; Rosen et al., 2003b).

Concernant les tumeurs osseuses primitives, nous avons montré, *in vitro* que l'acide zolédronique était capable d'agir directement sur les cellules d'ostéosarcome, de chondrosarcome et de sarcome d'Ewing, justifiant son utilisation *in vivo*. Nous avons montré pour la première fois les effets anti-résorption et anti-tumoral de l'acide zolédronique dans un modèle d'ostéosarcome de rat, ainsi que son association bénéfique avec un agent anti-tumoral, l'ifosfamide, dans la prévention des récurrences tumorales (Heymann et al., 2005). De plus, l'acide zolédronique est capable de réduire l'apparition de nodules pulmonaires dans un modèle tumoral pulmonaire suite à l'injection de cellules d'ostéosarcome en site intraveineux en s'affranchissant totalement du microenvironnement osseux (Ory et al., 2005). L'efficacité anti-tumorale de l'acide zolédronique a été confirmée par l'inhibition de la prolifération tumorale aussi bien *in vitro* qu'*in vivo* dans un modèle de chondrosarcome chez le rat (Gouin et al., 2006).

Au niveau clinique, l'acide zolédronique a été utilisé dans le traitement des tumeurs osseuses primitives dans un premier essai clinique multicentrique mis en place en 2005, ayant comme investigateur principal le Pr Gouin (CHU Nantes) : « Etude de phase II des effets de l'acide zolédronique sur la récurrence locale des tumeurs osseuses à cellules géantes après intervention chirurgicale », tumeurs ostéolytiques épiphysaires, vascularisées et récidivantes. Cette étude incluant 23 patients sur un suivi de 3 ans a pour objectif d'étudier le taux de récurrence des tumeurs à cellules géantes après résection puis dans un deuxième temps d'évaluer la tolérance générale et locale du traitement à l'acide zolédronique.

Une deuxième étude multicentrique est actuellement en cours, en collaboration avec l'institut Gustave Roussy, avec pour objectif l'association de l'acide zolédronique à des agents conventionnels de chimiothérapie utilisés dans le traitement de l'ostéosarcome chez l'enfant et l'adulte afin d'évaluer les bénéfices de cette association. Ce programme envisage l'inclusion de 470 patients sur une période de 6 ans avec un suivi de 3 ans après la dernière inclusion. L'investigateur principal est le Dr Brugières (IGR), le service d'orthopédie de

Nantes ainsi que notre laboratoire sont associés à ce protocole respectivement comme centre recruteur et centre d'analyse des paramètres du remodelage osseux.

L'utilisation accrue des bisphosphonates induit certes des effets bénéfiques dans le traitement de pathologies osseuses malignes, mais n'est pas sans effets secondaires. L'hypocalcémie aiguë est l'effet indésirable commun à tous les bisphosphonates, qui peut être contrôlé par un supplément en calcium et en vitamine D. Les effets secondaires sont variables suivant le mode d'administration. L'administration orale de bisphosphonates induit des effets sévères tels que des brûlures d'estomac, des nausées, des fièvres, des vomissements, des douleurs osseuses et dans quelques cas des œsophagites sévères. L'injection intraveineuse peut produire une irritation locale au site d'injection et une légère thrombophlébite. Une insuffisance rénale peut se produire en cas de haute dose intraveineuse ou lors de perfusion de courte durée. Des anémies et thrombopénies peuvent apparaître en cas de traitement à long terme. Des cas de nécrose de la mâchoire, qui restent rares ont été rapporté sous traitement au pamidronate ou à l'acide zolédronique (Ruggiero et al., 2004). L'utilisation des bisphosphonates a nettement amélioré le traitement des pathologies osseuses, mais demeure un traitement « lourd » à endurer dû à des séquences d'injection répétées ainsi qu'à des effets secondaires plus ou moins douloureux, n'améliorant pas pour autant la survie du patient. La découverte de nouvelles approches thérapeutiques dans l'optique de toujours améliorer l'efficacité, la survie, la qualité de vie du patient et de développer un traitement simple, non douloureux, en réduisant au maximum les effets indésirables est toujours d'actualité.

IV. Objectifs de la thèse

Le développement d'une tumeur osseuse a pour conséquence d'affecter rapidement la balance entre l'apposition et la résorption osseuse. En effet, dans la plupart des tumeurs osseuses primitives (ostéosarcome et sarcome d'Ewing), la malignité de la tumeur se caractérise le plus souvent par l'ostéolyse associée au développement tumoral. Or, la balance apposition/résorption est sous le contrôle de la triade moléculaire OPG / RANKL / RANK. Dans la majorité des pathologies ostéolytiques d'origine tumorale, cette triade est dérégulée en faveur d'une augmentation du rapport RANKL/OPG.

Parmi les facteurs d'activation des ostéoclastes, RANKL est le plus important et se situe au centre de l'hypothèse du cercle vicieux. En effet RANKL est impliqué dans le

développement de métastases osseuses résultant d'un large spectre de types de tumeurs tels que les cancers du sein, de la prostate, le neuroblastome, le myélome multiple, la thyroïde, rénale et pulmonaire (Roodman, 2004).

Dans le contexte de l'ostéolyse tumorale, l'équilibre apposition/résorption, sous le contrôle de la triade moléculaire OPG/RANK/RANKL, est dérégulé. Les objectifs de cette thèse ont été de développer de nouvelles approches thérapeutiques innovantes des tumeurs osseuses primitives ciblant cette triade en utilisant des modèles expérimentaux spécifiques (ostéosarcome et sarcome d'Ewing) permettant ainsi de mieux comprendre le rôle de chaque acteur de cette triade ainsi que leurs régulations potentielles par divers protagonistes du microenvironnement tumoral osseux.

RANKL étant une des cibles privilégiées pour rompre le cercle vicieux, l'utilisation de l'OPG ou toute autre molécule (récepteur soluble RANK-Fc) capable de bloquer la liaison RANKL-RANK représente un intérêt thérapeutique majeur pour le traitement des tumeurs osseuses primitives. Les molécules thérapeutiques délivrées par transfert de gène ont été utilisées *in vivo* afin de bloquer la fixation de RANKL à son récepteur RANK. Les stratégies développées ont permis d'utiliser des outils novateurs : des modèles animaux originaux d'ostéosarcome chez la souris et le rat, ainsi que le transfert de gène à l'aide de vecteurs synthétiques en comparaison aux vecteurs viraux.

Dans un premier temps, la libération de l'OPG a été induite par transfert de gène à l'aide de vecteurs synthétiques, afin de diminuer ou de bloquer la résorption osseuse dans le traitement de l'ostéosarcome, ce blocage permettant de démontrer l'existence du cercle vicieux entre résorption osseuse et prolifération tumorale dans les tumeurs osseuses primitives. L'effet thérapeutique potentiel de l'OPG libérée par transfert de gène a été évalué dans un modèle d'ostéosarcome ostéolytique chez la souris et dans un modèle d'ostéosarcome ostéocondensant chez le rat, présentant toujours une phase d'ostéolyse précédant celle de formation osseuse tumorale, d'un point de vue clinique, biologique et histologique. Le choix de l'utilisation de la forme tronquée OPG 1-194, dépourvue de son domaine de liaison à l'héparine et du domaine potentiel de liaison à TRAIL (TNF-Related Apoptosis Inducing Ligand) a permis de s'affranchir des problèmes de liaison potentielle à TRAIL ou d'autres molécules, sujet controversé dans la littérature actuelle sur l'activité anti-tumorale de l'OPG.

L'effet de molécule anti-résorption osseuse sur le développement tumoral et l'implication du cercle vicieux étendue aux tumeurs osseuses primitives telles que l'ostéosarcome sera confirmé par l'utilisation de RANK-Fc soluble. Cette molécule libérée

également par transfert de gène agit comme récepteur leurre de RANKL bloquant ainsi la résorption osseuse.

Les données actuelles sont assez confuses sur l'activité pro- ou anti-tumorale de l'OPG et sur le domaine impliqué dans la liaison à TRAIL. En effet, l'OPG complète pourrait jouer le rôle de facteur de survie pour les cellules tumorales par l'inhibition de l'activité pro-apoptotique de TRAIL. C'est pourquoi, les objectifs suivants ont été de comparer la forme tronquée (1-194) à la forme complète 1-401 de l'OPG, dans le but de déterminer la relation structure-fonction de la partie délétée et de déterminer le rôle thérapeutique de l'OPG ainsi que sa régulation par d'autres protagonistes tels que les protéoglycanes.

Puis, le rôle de RANKL dans le développement de tumeurs osseuses sera approfondi dans une troisième partie. En effet, RANKL n'est pas seulement impliqué en tant que molécule clé dans le développement de pathologies malignes ostéolytiques, mais il l'est également dans les pathologies tumorales ostéocondensantes et, plus récemment, dans la migration des cellules tumorales exprimant son récepteur RANK. Pour tenter de montrer le rôle central joué par RANKL dans le développement tumoral en site osseux (ou non), nous avons utilisé un modèle tumoral développé au laboratoire, induit par l'injection de cellules surexprimant ou non RANKL permettant d'observer et de comprendre la contribution de RANKL dans le développement tumoral en site osseux et d'envisager de nouvelles approches thérapeutiques.

Partie I

**Approches thérapeutiques de
l'ostéosarcome par transfert de gènes :
Libération de principes à activité anti-
résorptive, OPG 1-194 et RANK-Fc.**

Introduction

Le développement tumoral primaire ou secondaire est dans la plupart des cas associé à l'apparition de lésions osseuses, conséquence d'une perturbation de la balance entre l'apposition et la résorption osseuse suite à l'invasion de la tumeur. Il s'instaure alors un cercle vicieux entre prolifération tumorale et résorption osseuse, qui se traduit par la mise en place d'un environnement propice à la prolifération tumorale par la libération de facteurs de croissance (IGF-1, TGF β ,...) libérés de la matrice osseuse lors de sa dégradation par les ostéoclastes. A leur tour, les cellules tumorales vont délivrer des facteurs favorables à l'hyper-résorption osseuse en agissant directement sur l'activation des ostéoclastes ou indirectement sur les ostéoblastes. Le développement d'une tumeur primaire ou secondaire entraîne également, au niveau moléculaire, une dérégulation du système OPG/RANK/RANKL (Grimaud et al., 2003) en faveur d'une augmentation du rapport RANKL/OPG. En effet, RANKL a été détecté dans plusieurs tumeurs et peut être considéré comme un facteur clé impliqué dans les lésions osseuses associés au développement tumoral (Brown et al., 2001; Huang et al., 2002; Michigami et al., 2001; Zhang et al., 2001). Cependant, l'expression de RANKL n'est pas ou peu détectée *in vitro* dans les cellules tumorales du cancer du sein ou du mélanome (Chikatsu et al., 2000; Thomas et al., 1999), bien qu'il soit présent *in situ* au niveau de la tumeur, ce qui souligne son implication dans la mise en place du cercle vicieux.

De nouvelles stratégies thérapeutiques peuvent être envisagées afin de rompre ce cercle vicieux. En effet, il est possible de briser le cercle vicieux en bloquant directement la prolifération tumorale par des agents anti-tumoraux tels que les drogues de chimiothérapie, ou bien de bloquer la résorption osseuse et donc indirectement la prolifération tumorale. Cette première partie sera consacrée au développement de nouvelles approches thérapeutiques ciblant la résorption osseuse et plus précisément l'inhibition de l'expression ou de l'activité de RANKL, cytokine clé dans ce développement tumoral.

L'OPG, molécule anti-résorptive, semble représenter un candidat idéal dans le développement de nouvelles voies thérapeutiques des tumeurs ostéolytiques. En effet, l'OPG, déjà utilisée avec succès dans le traitement de l'ostéoporose et du myélome multiple (Body et al., 2003), pourrait être employée pour briser le cercle vicieux établi au cours de la progression tumorale et de la dégradation osseuse.

Cependant, les travaux de Fisher et collaborateurs montrent des effets biologiques différents suivant la structure d'OPG utilisée. En effet, l'OPG complète aurait un effet pro-

tumoral par sa fixation à TRAIL (TNF-Related Apoptosis-Inducing Ligand) empêchant ainsi l'apoptose des cellules tumorales induite par la cytokine. A l'inverse, l'OPG 1-194, dépourvue du domaine de liaison à l'héparine, exercerait un effet anti-tumoral (Fisher et al., 2006). Ainsi, la forme 1-194 de l'OPG retient notre intérêt pour cette étude. Plusieurs données bibliographiques valident le concept de libération de l'OPG par thérapie génique utilisant des vecteurs viraux, tels que les Adeno-Associated Virus (AAV), les lentivirus et les adénovirus (Bolon et al., 2001; Doran et al., 2004; Kostenuik et al., 2004; Ulrich-Vinther et al., 2002; Yang et al., 2002). En effet, l'utilisation de vecteurs viraux dans le traitement de diverses pathologies osseuses (myélomes multiples, ostéoporose,...) entraîne une production de protéines pouvant atteindre jusqu'à plusieurs µg/ml (Bolon et al., 2001). Encore peu utilisés et malgré une plus faible efficacité de transfection par rapport aux vecteurs viraux, les vecteurs synthétiques semblent très prometteurs car ils ne présentent pas ou peu d'immunogénicité. De plus, ils sont plus simples à produire que les vecteurs viraux et permettent la transfection de plusieurs types cellulaires, aussi bien *in vitro* qu'*in vivo*. En effet, différentes études montrent l'efficacité de l'utilisation de vecteurs synthétiques dans la libération de protéines au niveau musculaire (Pitard et al., 2002; Richard et al., 2005a; Richard et al., 2005b).

Utilisant la même stratégie de transfert de gène, nous étudierons ensuite une construction codant RANK-Fc murin afin d'évaluer la capacité de ce récepteur leurre soluble de RANKL à prévenir les lésions ostéolytiques et potentiellement inhiber la progression tumorale. L'étude de Zhang *et al.* a montré que RANK-Fc était capable de prévenir les lésions ostéoblastiques associées au développement tumoral dans le cancer de la prostate, de réduire le volume tumoral et de diminuer les l'expression sériques d'antigènes tumoraux spécifiques du cancer de la prostate. Cependant, RANK-Fc n'avait aucun effet sur une tumeur prostatique implantée en sous cutanée, c'est-à-dire sans environnement osseux (Zhang et al., 2003). Ces données suggèrent que RANK-Fc diminue la progression tumorale indirectement par l'inhibition de la résorption osseuse. Le principal avantage de l'utilisation de RANK-Fc en thérapie anti-ostéolytique d'origine tumorale est qu'il ne se fixe pas à TRAIL ne bloquant donc pas l'apoptose des cellules tumorales induite par TRAIL.

Dans cette première partie, deux études utilisant le transfert de gène de molécule anti-résorptive montrent les effets bénéfiques sur la prévention de la résorption osseuse et l'inhibition de la progression tumorale dans deux modèles d'ostéosarcome chez les rongeurs. Le modèle d'ostéosarcome de rat est issu d'un ostéosarcome initialement radio-induit chez le rat Sprague-Dawley de souche OFA par du Ce¹⁴⁴ et dont l'évolution correspond aux données cliniques recueillies chez l'homme (Allouche et al., 1980; Heymann et al., 2005). Dans ce

modèle, le développement tumoral s'accompagne d'un remodelage osseux intense débutant par des lésions ostéolytiques suivies d'une formation directe d'os par les cellules tumorales associées au développement de métastases pulmonaires. Le modèle tumoral murin POS-1 est issu d'un ostéosarcome qui se développe spontanément dans la souche murine C3H/HeN. Suite à l'injection de cellules tumorales POS-1, les souris développent une tumeur primitive au site d'injection et des métastases pulmonaires. Un fragment de la tumeur primitive est ensuite transplanté au contact du fémur de souris de même souche, induisant le développement d'un ostéosarcome ostéolytique (confirmé par microscanner) et de métastases pulmonaires, reproduisant le tableau clinique de la pathologie humaine. La première étude utilise une construction codant l'OPG 1-194 murine, tandis que la deuxième utilise une construction codant RANK-Fc murin. Deux méthodes de transfert de gènes différentes ont été utilisées : une approche adénovirale et une approche novatrice utilisant un vecteur synthétique, le F68, poly(ethyleneoxide)₇₅-poly(propyleneoxide)₃₀-poly(ethyleneoxide)₇₅-block copolymer (POE : hydrophile ; POP : hydrophobe) (famille des LUTROL[®] ; en collaboration avec le Dr. Bruno Pitard). C'est un polymère amphiphile non ionique dont la formulation permet d'optimiser la transfection de cellules *in vivo*.

Article 1

« Pertinence thérapeutique de la libération de l'OPG par thérapie génique dans le traitement de l'ostéosarcome : blocage du cercle vicieux s'établissant entre la prolifération tumorale et la résorption osseuse »

Cancer Res, Vol 67 :7308-18, 2007, F. Lamoureux, P. Richard, Y. Wittrant, S. Battaglia, P. Pilet, V. Trichet, F. Blanchard, F. Gouin, B. Pitard, D. Heymann, F. Rédini.

L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives qui touche principalement une population jeune avec un âge median à 18 ans. Malgré les améliorations thérapeutiques dans le traitement de l'ostéosarcome, le taux de survie est seulement de 30% à 5 ans pour des patients présentant des métastases pulmonaires au moment du diagnostic. L'ensemble de ces données justifie le développement de nouvelles approches thérapeutiques, parmi lesquelles l'utilisation de l'ostéoprotégérine, molécule anti-résorption osseuse, semble être prometteur pour bloquer le cercle vicieux qui s'établit en site osseux entre la résorption osseuse et la prolifération tumorale. L'OPG étant aussi connue pour se lier et inhiber l'activité du Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), la forme tronquée de l'OPG 1-194 murine sera utilisée. L'ADNc codant l'OPG 1-194 a été administré par deux techniques de transfert de gène : soit en utilisant un adénovirus ou en étant associé à des polymères amphiphiles dans deux modèles d'ostéosarcome chez les rongeurs. Dans ces deux modèles, l'OPG libérée par transfert de gène prévient la formation de lésions ostéolytiques associées au développement de l'ostéosarcome, réduit l'incidence et la croissance tumorale, ce qui entraîne une augmentation des taux de survie 28 jours après l'implantation tumorale. Néanmoins, l'OPG n'a pas prévenu le développement de lésions tumorales pulmonaires dans un modèle qui s'affranchit du micro-environnement osseux, suggérant que celui-ci est nécessaire pour l'efficacité thérapeutique de l'OPG. En effet, *in vitro*, l'OPG n'exerce pas d'activité directe sur les cellules d'ostéosarcome (liaison à la cellule, prolifération cellulaire, apoptose, ou effet sur le cycle cellulaire). Nous avons montré que l'OPG exerce un effet inhibiteur indirect sur la progression tumorale vraisemblablement par l'inhibition de RANKL dont sa production est augmentée dans l'environnement tumoral osseux. Ces résultats confirment l'inhibition de l'ostéolyse induite par l'OPG mise en évidence par la diminution du nombre d'ostéoclastes.

Therapeutic Relevance of Osteoprotegerin Gene Therapy in Osteosarcoma: Blockade of the Vicious Cycle between Tumor Cell Proliferation and Bone Resorption

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Abstract

Osteosarcoma is the most frequent primary bone tumor that develops mainly in the young, the median age of diagnosis being 18 years. Despite improvement in osteosarcoma treatment, survival rate is only 30% at 5 years for patients with pulmonary metastases at diagnosis. This warrants exploration of new therapeutic options, and among them, osteoprotegerin (OPG), a naturally occurring protein that inhibits bone resorption, is very promising in blocking the vicious cycle between bone resorption and tumor proliferation that takes place during tumor development in bone site. As OPG binds and inhibits the activity of tumor necrosis factor-related apoptosis-inducing ligand, the truncated form of murine OPG 1-194 was used. The cDNA encoding OPG was administered by gene transfer using replication-defective adenoviral vector or was associated with an amphiphilic polymer in two models of rodent osteosarcoma. In both models, OPG gene transfer was effective in preventing the formation of osteolytic lesions associated with osteosarcoma development, in reducing the tumor incidence and the local tumor growth, leading to a 4-fold augmentation of mice survival 28 days postimplantation. On the contrary, OPG did not prevent the development of pulmonary metastasis alone, suggesting that bone environment is necessary for OPG therapeutic efficacy. Because OPG has no direct activity on osteosarcoma cells *in vitro* (cell binding, cell proliferation, apoptosis, or cell cycle distribution), we show that OPG exerts indirect inhibitory effect on tumor progression through the inhibition of RANKL whose production is enhanced in bone tumor environment, leading to osteolysis inhibition as reflected by osteoclast number decrease. [Cancer Res 2007; 67(15):7308–18]

Introduction

Osteosarcoma is defined as a malignant tumor of mesenchymal cells, characterized by the direct formation of malignant osteoid and/or woven bone by the tumor. Some are composed largely of

fibroblastic cells, others have abundant bone formation, some show chondroid differentiation, and still others are highly vascular. Osteosarcoma lesion is also characterized by bone destruction with high biological virulence, seeming to be caused by active osteoclasts. Although osteosarcoma is a rare malignant disease, it is the most common primary malignant bone tumor in both children and adults (1). After initial diagnosis is made with a biopsy, treatment consists of preoperative chemotherapy, followed by definitive surgery and postoperative chemotherapy. Survival has improved over the past several decades. Indeed, nonmetastatic disease has an ~70% chance of long-term survival. Unfortunately, patients with metastatic disease at diagnosis or those who have recurrent disease have a poor prognosis, with ~20% surviving at 5 years, indicating that new therapeutic options should be actively explored.

As evidenced for bone metastases, a vicious cycle between osteoclasts, bone stromal cells/osteoblasts, and cancer cells has been hypothesized during the progression of primary bone tumors (2). Accordingly, suppression of osteoclasts or inhibition of osteoclast activity would be a promising approach to inhibit local cancer growth. Osteoprotegerin (OPG), a soluble protein of the tumor necrosis factor (TNF) receptor superfamily, offers considerable promise as a new modality for treating osteolysis associated to bone tumors. OPG acts as a decoy receptor and inhibits osteoclast formation, function, and survival by preventing the binding of receptor activator of nuclear factor- κ B ligand (RANKL) to RANK (3). Transgenic mice overexpressing OPG exhibit an osteopetrotic phenotype, whereas OPG knock-out mice have severe osteoporosis (4, 5). The OPG/RANKL/RANK system is also involved in various pathologies associated with tumors in the bone (6, 7). For example, increased expression of RANKL has been observed in osteolytic malignancies, such as breast cancer and multiple myeloma (8–10). A recent study showed that the RANKL/OPG ratio was significantly increased in patients suffering from severe osteolysis from tumor origin or not (7). As a result, the inhibition of osteoclastogenesis has been considered as an intervention strategy in the malignant pathologies that develop in bone: treatment of osteolytic bone metastases with OPG inhibits osteolysis and decreases skeletal tumor burden in myeloma and breast cancer (11, 12). Moreover, a recent publication reports that OPG is able to inhibit cancer cell migration and bone metastasis through the inhibition of the RANKL-induced effects in RANK-expressing cells from tumor origin (13). The relevance of using OPG as a therapeutic approach in bone pathologies including osteolytic tumors has been well documented. Indeed, OPG was shown to inhibit tumor-induced

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osteoclastogenesis and bone tumor growth in osteopetrotic mice (14), to reduce bone cancer pain by the blockade of the ongoing osteoclast activity (15), to decrease the number and area of radiographically evident lytic bone lesions in a model of mouse colon adenocarcinoma (12), to exhibit beneficial effects in experimental models of myeloma (11, 16), and to inhibit osteolytic lesions associated with prostate cancer (17). However, no studies were done on OPG effects in primary bone tumors.

Previous data showed that it is feasible to provide long-lasting expression of OPG at bone-protective levels using a gene therapy approach (18). However, the toxicity associated with the use of adenoviral vectors is extremely complex, involving both the innate and adoptive immune responses. Recently, a new class of synthetic vectors has been reported for *in vivo* gene transfer in various organs, including skeletal and cardiac muscles (19, 20) and in the lung (21). These new synthetic vectors result from the association of plasmid DNA with amphiphilic polymers consisting of blocks of poly(ethylene oxide) and of poly(propylene oxide). I.m. injections of these synthetic vectors led to the synthesis of proteins for local benefit such as dystrophin or of systemic erythropoietin (22).

The aim of this study was first to determine the therapeutic relevance of OPG in osteosarcoma by using viral and nonviral gene transfer approaches in two models of osteosarcoma in rodents.

Materials and Methods

Cell Lines

The murine osteosarcoma cell line POS-1, derived from mouse spontaneous osteosarcoma (23), was cultured in RPMI 1640 (Bio Whittaker) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 2 mmol/L L-glutamine.

The cell line OSRGa, rat osteosarcoma, was cultured in RPMI supplemented with 10% FBS and antibiotics (penicillin, streptomycin; ref. 24).

The C2C12 cell line has been subcloned from mouse myoblasts previously established (25). They are cultured in DMEM (Bio Whittaker) supplemented by 10% FBS and 2 mmol/L L-glutamine.

OPGΔ Delivered by Synthetic Vectors in a Mouse Model of Osteosarcoma

In vitro experimentations

Plasmids. The cDNA encoding the truncated form (1-194) of murine OPG (named mOPGΔ) kindly provided by Amgen Inc. was transferred from the mOPGΔ pCEP4 to the pcDNA3 plasmid (Invitrogen) between the *Hind*III and *Xho*I sites using standard cloning procedures. The pcDNA3-enhanced green fluorescent protein (EGFP) was used as a control.

Cell transfections. For POS-1 and C2C12 transfections, polyethyleneimine/DNA complexes were prepared by equivolumetric mixing polyethyleneimine (charge ratio, ± 4) in water with plasmid DNA solution at the desired concentration (4 μ g per well) in 150 mmol/L NaCl. Transfections were done at 70% to 80% of confluence in each well, by adding 200 μ L of complex formulations in 0.5 mL of culture DMEM deprived of FBS. After 6 h at 37°C, the transfection medium was replaced by 1 mL of DMEM containing 10% FBS and 1% penicillin/streptomycin (complete medium). The transfection efficacy is controlled by the quantification of pcDNA3-EGFP-transfected cells.

In vivo experimentations

Mouse osteosarcoma model. Four-week-old male C3H/He mice (Elevages Janvier) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. The mice were anesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with an i.m. injection of buprenorphine (0.05 mg/kg; Temgésic, Schering-Plough) before s.c. inoculation of POS-1 cell suspension (containing 2×10^6 cells in 50 μ L PBS) in the hind footpad of the mice.

Under these conditions, mice develop a primary tumor at the site of injection in 3 weeks that can be transplanted to mice of the same strain as a small fragment ($2 \times 2 \times 2$ mm³) in close contact with the tibia. For this purpose, the periosteum of the diaphysis was opened and resected along a length of 5 mm, and the underlying bone was intact. The osteosarcoma fragment was placed contiguous to the exposed bone surface without the periosteum, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site approximately 8 days later associated with the development of pulmonary metastases in a 3-week period. The tumor that develops in contact to the femora leads to osteolytic lesions that reproduce the osteolytic form of human osteosarcoma (26). The tumor volume (*V*) was calculated from the measurement of two perpendicular diameters using a caliper according to the following formula: $V = 0.5 \times L \times S^2$, where *L* and *S* are, respectively, the largest and smallest perpendicular tumor diameters. Relative Tumor Volumes (RTV) were calculated from the formula $RTV = (V_{21}/V_{15})$, where *V*₂₁ is the tumor volume on day 21 and *V*₁₅ is the tumor volume at day 15. A model of pulmonary metastases was used in a separate experiment, induced in 3 weeks by the i.v. injection of 150,000 POS-1 cells in C3H/He mice.

Formulation preparations. For i.m. injections, mice were anesthetized with hypnomidate (40 mg/kg, i.p. injection). About 50 μ L of block copolymers/DNA formulations were injected into shaved tibial anterior muscles at one site using a microfine syringe (U100, Becton Dickinson). Lutrol, a block copolymer consisting of poly(ethyleneoxide)₇₅-poly(propyleneoxide)₃₀-poly(ethyleneoxide)₇₅, was generously provided by BASF (Mount Olive, NJ, USA). Stock solutions were prepared at 20% (w/v) in water and stored at 4°C. Formulations of DNA with block copolymers were prepared by equivolumetric mixing block copolymers in water and DNA solution at the desired concentration (50 μ g per muscle) as already reported (22).

Experimental protocols. To determine the effect of mOPGΔ delivered by synthetic vectors on osteosarcoma development, the mice were transplanted with POS-1 osteosarcoma fragments as described above. Groups of six to eight mice were assigned, respectively, as controls (no injection), control vectors (Lutrol/pcDNA3 alone), and p-mOPGΔ (Lutrol/pcDNA3-mOPGΔ). A preventive treatment was applied, where the Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning at 7 days before osteosarcoma implantation up to 28 days postimplantation. A separate experiment was conducted in which the Lutrol/DNA formulation was only injected in the contralateral muscle, compared with the muscle implanted with the tumor fragment. The tumor volume was calculated as described above. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation. Lung tumor dissemination was assessed at necropsy. Three independent experiments were done.

OPGΔ Delivered by Adenoviral Vectors in a Rat Osteosarcoma Model

In vitro experimentation

Construction of replication-defective adenoviral vectors. The OPGΔ cDNA was transferred from the mOPGΔ pCEP4 construct into the shuttle vector pTrackCMV using the pAdEasy-1 system (27). Recombinant adenoviral plasmids recovered by homologous recombination in *Escherichia coli* were transfected into 911 cells. Appropriate recombinant plaques containing mOPG were isolated, propagated and titrated. Adenovirus containing GFP was used as a control (Ad-EGFP).

In vivo experimentations

Rat osteosarcoma model. The osteosarcoma was initially induced by a local injection of colloidal radioactive ¹⁴⁴cerium in rats (28). The evolution of the tumor is comparable at the temporal (ratio 1:100 between rats and humans) and physiologic levels to the development of human osteosarcoma. The tumor can be regrafted as described above and maintained *in vivo* for many months, or fragments can be frozen until reuse. Lung metastases are observed in 75% to 90% of rats bearing advanced malignant bone tumors. Four-week-old male Sprague-Dawley rats (IFFA-CREDO) were housed under pathogen-free conditions at the Experimental Therapy Unit as described above. For the implantation, the rats were anesthetized by

inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with an i.m. injection of buprenorphine (0.05 mg/kg). A 10-mm³ fragment of osteosarcoma was placed contiguous to the exposed bone surface as previously described, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site approximately 7 to 10 days later.

Experimental protocol. Rats bearing growing tumors with a volume >1,200 mm³, which were considered as progressive tumors (10–12 days postimplantation), were individually identified and assigned to the control groups (control tumor, CT and Ad-EGFP) or treatment group (Ad-mOPGΔ, 6–8 animals per group, depending on the series). Ad-EGFP and Ad-mOPGΔ [5 × 10⁹ particle-forming units (pfu)] were administered once by i.v. or i.m. injection to the animals 14 days posttumor implantation. The animals were weighed twice a week, and the tumor volume was calculated as described above.

RANKL expression. RANKL expression quantified by ELISA (TRANCE/TNFSF11, R&D) was compared in the serum of C3H/He mice bearing POS-1 osteosarcoma or not. RANKL expression was also compared at the tissue level between control mice (bone, muscle) and osteosarcoma-bearing mice (tumor) by immunohistochemistry using a polyclonal rabbit anti-RANKL antibody (RANKL-N19; 1/100, Santa Cruz). The slides were incubated with anti-rabbit biotinylated antibody (Sigma) and then with extravidin-peroxidase at 1/100 (Sigma) and revealed with the AEC chromogen staining kit (Sigma). Preparations were counterstained with hematoxylin. Negative controls were realized without primary antibody.

Terminal nucleotidyl transferase-mediated nick end labeling assay for apoptosis. Apoptotic cells in the osteosarcoma were detected with an *in situ* cell death detection kit (Roche Diagnostics) based on the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method (TUNEL). Four to six sections per animal were prepared for staining of apoptotic cells. The number of TUNEL-positive cells were counted by microscopic examination with a 40× objective lens, and indices were determined as the mean percentages of positive cells among total cells.

Tartrate-resistant acid phosphate 5b activity. Tartrate-resistant acid phosphate (TRACP)-5b activity was measured using the Mouse TRAP Assay (Immunodiagnostic Systems Ltd.) according to the manufacturer's instructions. Assay specificity is determined by the color intensity of the kinetic activity of TRACP 5b. TRACP 5b is immunocaptured using a polyclonal antibody and then assayed using a chromogenic substrate to develop a color reaction. The reaction is stopped, and the absorbance of the samples is determined by reading in a multiwell plate reader.

Microscanner and radiological analyses. Analysis of architectural parameters was done using the high-resolution X-ray micro-computed tomography (CT) system for small animal imaging SkyScan-1072 (SkyScan). Relative volume (BV/TV) of the femora [total bone (cortical + trabecular) or trabecular bone] was quantified at necropsy in the osteosarcoma groups that received mOPGΔ (associated with adenovirus or synthetic vectors), compared with that of control mice. Radiographs on anesthetized animals (Nesdonal, 50 mg/kg) were taken every week and at the time of necropsy with a mammography PLANMED Sophie apparatus (SN RAH 40710, Helsinki, Finland).

Gene and protein expression analysis

RNA extraction and semiquantitative reverse transcription RT-PCR analysis. At confluence, pcDNA3- and -mOPGΔ-transfected C2C12 cells were used for total RNA extraction using the TRIzol reagent (Invitrogen). First, RNA was reversed transcribed (RT) using 400 U MMLV-RT from Invitrogen. To determine the expression of mOPGΔ, 2 μL of the RT reaction mixture were subjected to PCR using upstream and downstream primers (30 pmol each) and Taq polymerase (Eurobio). The band densities were measured using the ImageQANT computer software program (Molecular Dynamics). The relative expression of each gene was calculated as the ratio to 18S signal.

Osteoprotegerin ELISA

Cell culture. pcDNA3- and -mOPGΔ-transfected C2C12 cells were grown in six-well plates under standard conditions (cell density, 150,000 cells per well). Media were collected after 48 h, and OPG levels were determined using a human OPG ELISA kit (R&D Systems) following the manufacturer's recommendations.

Serum and muscle. Blood was drawn intermittently from the retro-orbital vein to monitor serum mOPGΔ level. At necropsy, the tumor and muscle tissues were lysed in reporter lysis buffer 1× (Promega) supplemented with protease inhibitor cocktail (Roche), broyed during 30 s using Ultraturax, centrifuged at 10,000 rpm during 5 min at 4°C, and were processed for OPG detection using the ELISA test previously described.

OPG immunohistochemistry. Sections of 6 μm of decalcified, paraffin-embedded tumors, bone, or muscle tissues were used for the determination of OPG immunoreactivity using a biotinylated anti-mouse OPG/TNFRSF11B antibody (R&D Systems, 30 μg/mL), then revealed as described above.

In vitro analyses

Cell proliferation. Replicate subconfluent cell cultures of POS-1 or OSRGa cells in 96-well plates were treated for 12 to 72 h with increasing concentrations of OPG-Fc (10, 50, and 100 ng/mL). Cell viability was determined by the sodium 3′[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche Molecular Biomedicals).

Caspase activity. POS-1 or OSRGa cells (2 × 10⁴) grown in 24-well plates were treated with 10 ng/mL OPG-Fc for the indicated times, washed, and lysed with 50 μL of radioimmunoprecipitation assay buffer for 30 min in the presence of protease inhibitors. The cells were then scraped off, and the protein amount was quantified using the BCA [bicinchoninic acid + copper(II) sulfate] test (Pierce Chemical Co.). Caspase-3 activity was assessed on 10 μL of cell lysate with the CaspACE assay kit (Promega) following the manufacturer's recommendations. Cells treated with UV light for 30 s 24 h before harvesting were used as a positive control for caspase activity.

Cell cycle analysis. Confluent POS-1 or OSRGa cells (treated with increasing concentrations of OPG-Fc for 24, 48, and 72 h) were removed from culture dishes by trypsinization, washed twice in PBS, and incubated in PBS containing 0.12% Triton X-100, 0.12 mmol/L EDTA, and 100 μg/mL DNase-free RNase A (Sigma Chemical Co.). Then, 50 μg/mL propidium iodide (Sigma) were added for each sample for 20 min at 4°C in the dark. The stained nuclei were analyzed by flow cytometry (FACScan, BD Biosciences) using the CellQuest software. Cell cycle distribution was based on 2N and 4N DNA content.

OPG binding on osteosarcoma cells. A coupling of the FITC on the mouse OPG-Fc was achieved by using a FluoroTag kit (Sigma). About 1 mg of OPG-Fc was incubated in the presence of 0.3 mg of FITC protected from light during 2 h at room temperature. The purification of the OPG-Fc-FITC was done using a Sephadex G-25M column. The OPG-Fc-FITC was then quantified by the ELISA technique (R&D Systems). POS-1, OSRGa, and 293RL 2.0 (2.93 cells transfected with the cDNA encoding the full-length form of hRANKL were used as positive controls) cells were seeded in a 96-well plate (1 × 10⁶ cells per well) in the presence of 2 μg of OPG-Fc-FITC in PBS/bovine serum albumin 1% during 1 h at 4°C. At the end of the incubation period, the cells were washed with PBS and analyzed by flow cytometry (FACScalibur, data base Biosciences) using the CellQuest software to analyze the fixation of OPG.

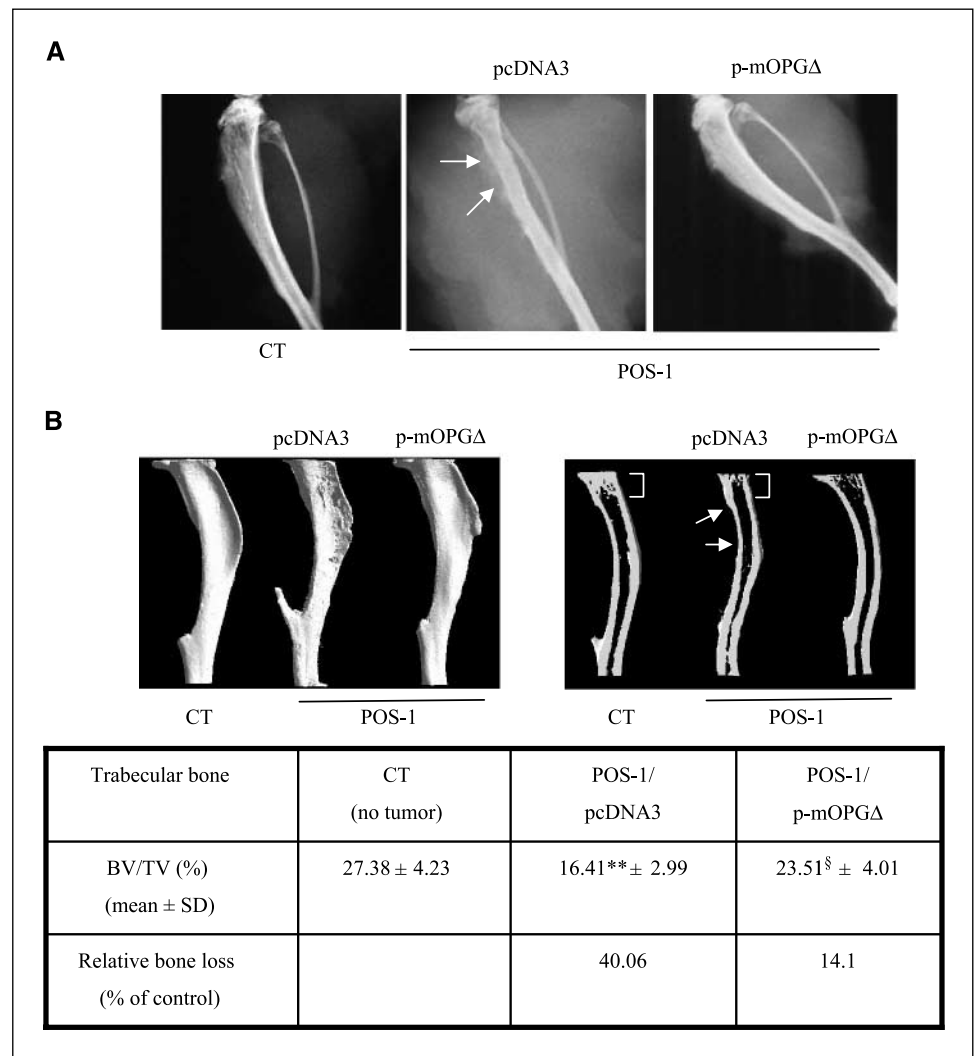
Data analyses. For *in vivo* experimentations, the nonparametric Wilcoxon test was used. The differences of actuarial survival were determined by the *K*² test. Statistical evaluation of the *in vitro* proliferation data was done by Student's *t* test. Results are given as mean ± SD, and results with *P* < 0.05 were considered significant.

Results

Effect of Murine OPGΔ Associated with Synthetic Vectors on Mouse POS-1 Osteosarcoma Tumor Development

Transfection efficiency of osteosarcoma cells. A pilot experiment was designed *in vitro* using a pcDNA3-EGFP construct to compare the transfection efficiency between murine myoblasts C2C12 and osteosarcoma cells POS-1 to determine which cell type

Figure 1. mOPGΔ transgene expression prevents osteosarcoma-associated osteolysis. Mice were transplanted with POS-1 osteosarcoma fragments as described in Materials and Methods. Groups of six and eight mice were assigned, respectively, as CT (controls: no tumor), POS-1/pcDNA3 (control vectors: Lutrol/pcDNA3 alone in POS-1 osteosarcoma-bearing mice), and POS-1/p-mOPGΔ (Lutrol/pcDNA3-mOPGΔ in POS-1-bearing mice). A preventive treatment was applied, where the Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning at 7 d before osteosarcoma implantation up to 28 d postimplantation (time of sacrifice). At that time, the tumor-associated osteolysis was analyzed by radiography (A) and the microarchitectural parameters quantified at the trabecular bone level by microscanner analysis (B). BV, bone volume; TV, total volume ($n = 8$). **, $P < 0.001$, POS-1/pcDNA3 versus CT; §, $P < 0.01$, POS-1/mOPGΔ versus POS-1/pcDNA3. Relative bone loss was calculated as $[(BV/TV)_{CT} - (BV/TV)_{POS \text{ or } mOPG}]/(BV/TV)_{CT}$.



shows the best transfection efficiency to direct the injections in the further *in vivo* experimentations. POS-1 cells showed poor transfection efficiency whatever the method used (synthetic vectors: cationic or amphiphilic biopolymers, lipoplexes, and calcium phosphate). Therefore, the i.m. site was chosen for the further *in vivo* protocol.

Validation of transgene expression *in vitro* and *in vivo*. The aim of this study was to show the induction of mOPGΔ expression using the polyethyleneimine *in vitro* and the amphiphile block copolymer Lutrol *in vivo*, which showed the best efficiency, respectively, *in vitro* and *in vivo* (21, 29). Preliminary experiments validated the induction of mOPGΔ expression *in vitro* in C2C12 cells transfected with p-mOPGΔ associated to polyethyleneimine at the mRNA and protein levels, whereas the osteosarcoma POS-1 cells showed a poor transfection efficiency. According to these *in vitro* results, mice were treated with Lutrol associated with p-mOPGΔ or pcDNA3 alone by i.m. injections (in the tibial anterior muscle). Induction of mOPGΔ transgene expression was evidenced in the muscle (site of injection) by ELISA and immunohistochemistry (data not shown).

Protective effect of OPG transgene expression on bone resorption associated with osteosarcoma development. The mouse osteosarcoma POS-1 model used in this study has been

previously associated with osteolytic lesions by microscanner analysis (26). Because control tumors and pcDNA3-treated mice exhibit similar bone alterations, only radiographs and microscanner analysis of the tibiae of mice treated with pcDNA3 alone are shown. Radiographs of the POS-1 implanted mouse tibiae revealed a high bone remodeling, resulting in cortical destruction and intensive interactions between altered bone tissue and tumor cells, as compared with control mice (Fig. 1A). Osteolytic lesions were no more observed in osteosarcoma-bearing mice treated with p-mOPGΔ associated with Lutrol, and the long bones exhibited high bone density as observed on radiographies, reflecting decreased bone resorption. These results were confirmed and quantified by bone microarchitecture analysis, showing bone osteolysis both at the total and trabecular bone levels in the POS-1 osteosarcoma group that was abrogated in mOPGΔ-treated animals (Fig. 1B). The specific trabecular bone volume was significantly decreased in osteosarcoma-bearing mice as compared with control animals (respectively, 16.41% versus 27.38%, $P < 0.001$). OPG treatment induced a partial recovery of the bone microarchitecture (23.51% versus 16.41% in osteosarcoma-bearing mice; $P < 0.01$). The relative bone loss has been deduced from these values and estimated as 14.1% and 40%, respectively, in POS-1-bearing mice treated or not with mOPGΔ

(Fig. 1B). Overall, these results showed the antitumor resorption effect of mOPGΔ delivered by gene transfer using synthetic vectors in a preventive protocol developed in an osteolytic mouse model of osteosarcoma.

OPG transgene expression limits osteosarcoma progression and augments animal survival. A preventive protocol was designed to determine not only a potential protective effect of mOPGΔ on bone resorption associated with osteosarcoma development, but also on the tumor progression itself. The results of tumor development of a representative series out of three (six mice per group in the series presented in Results) show both a decrease of the tumor incidence and a diminution of the tumor volume itself (Fig. 2A and B). Indeed, when each animal is considered individually, only 30% of mice developed a primary tumor in the p-mOPGΔ group versus more than 80% in the pcDNA3 group (Fig. 2A and B). Moreover, in the mice that develop primary tumors, the mean tumor volume is smaller in the mOPGΔ-treated mice than in control mice (for example, mean volume of 984 mm³ at day 21, versus 3,166 in the controls; $P < 0.005$). The tumor progression calculated between days 15 and 21 is also strongly inhibited by the mOPGΔ-transgene expression: 1,394 mm³ versus 2,603 mm³ in pcDNA3 mice ($P < 0.05$, Fig. 2C). These results show that mOPGΔ expression diminished the tumor incidence and progression in an osteolytic model of osteosarcoma. As a consequence, overall survival was significantly increased in the mOPGΔ-treated mice: 100% survival 28 days posttumor implantation versus 33% in pcDNA3 (Fig. 2D, $P < 0.001$). The control and pcDNA3 mice died or were sacrificed because of the huge tumor

development or respiratory distress caused by pulmonary metastasis dissemination. In this experiment, OPG-treated mice were sacrificed at the same time as control/pcDNA3 mice (28 days posttumor implantation). No pulmonary metastases could be evidenced in the mice that received the mOPGΔ transgene (0/6 at the time of necropsy versus 5/6 in controls).

The OPG therapeutic potential was further tested as a curative protocol. The same formulation Lutrol associated with pcDNA3-mOPGΔ was given when tumor was detectable (14 days postimplantation) and then once a week until the sacrifice. Unfortunately, no therapeutic beneficial effect could be shown with this treatment sequence on osteosarcoma progression (data not shown). One hypothesis could be the relative low transgene expression to compensate for aggressive osteosarcoma development. Therefore, a protocol using adenoviral construction was developed to get a higher OPG production that can be effective in curing established osteosarcoma.

Therapeutic Relevance of OPGΔ Delivered by Adenoviral Vectors in a Rat Osteosarcoma Model

OPGΔ expression is evidenced in tumor tissue. A pilot experiment was done to assess the OPG expression profile mediated by adenoviruses carrying DNA encoding the truncated form of murine OPG (Ad-mOPGΔ). Induction of murine OPGΔ production at the tumor site was evidenced by immunostaining on histologic tumor slices of animals that were i.m. injected, 7 days after injection as compared with animals that received Ad-EGFP (Fig. 3A).

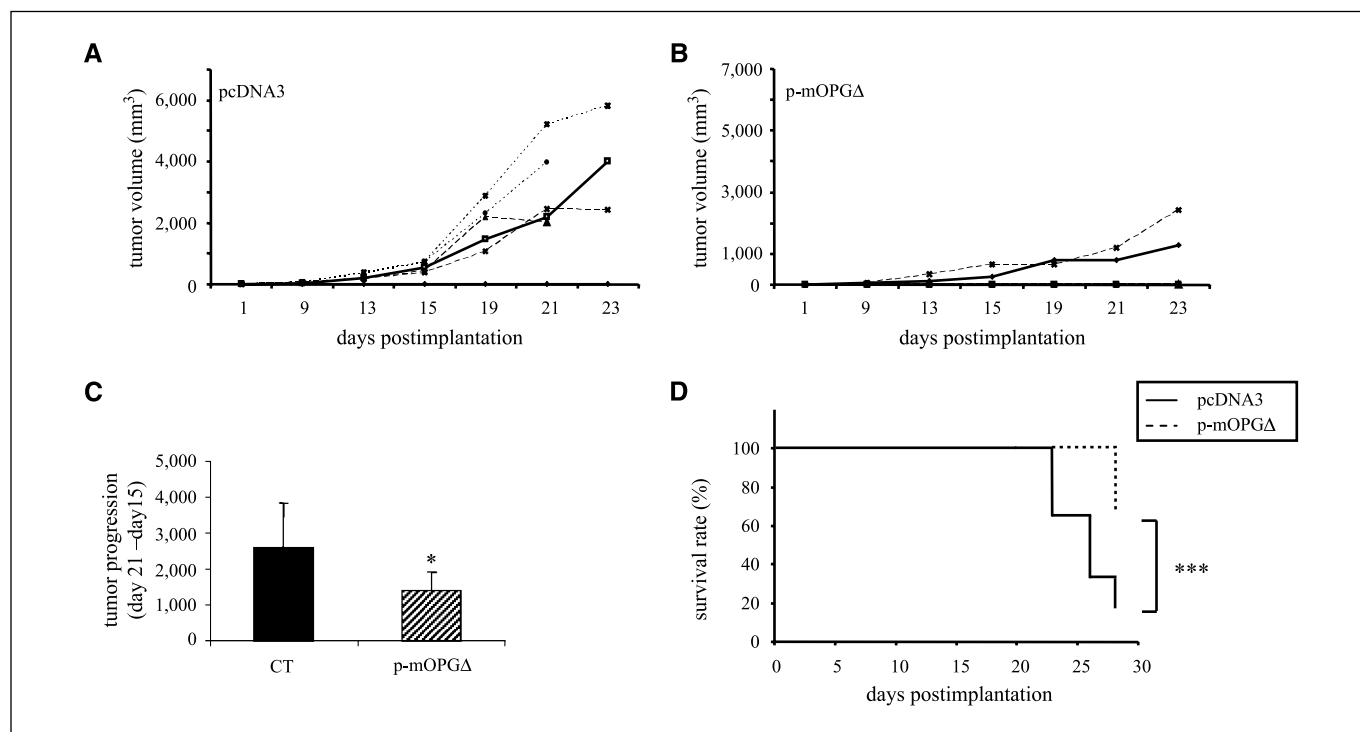
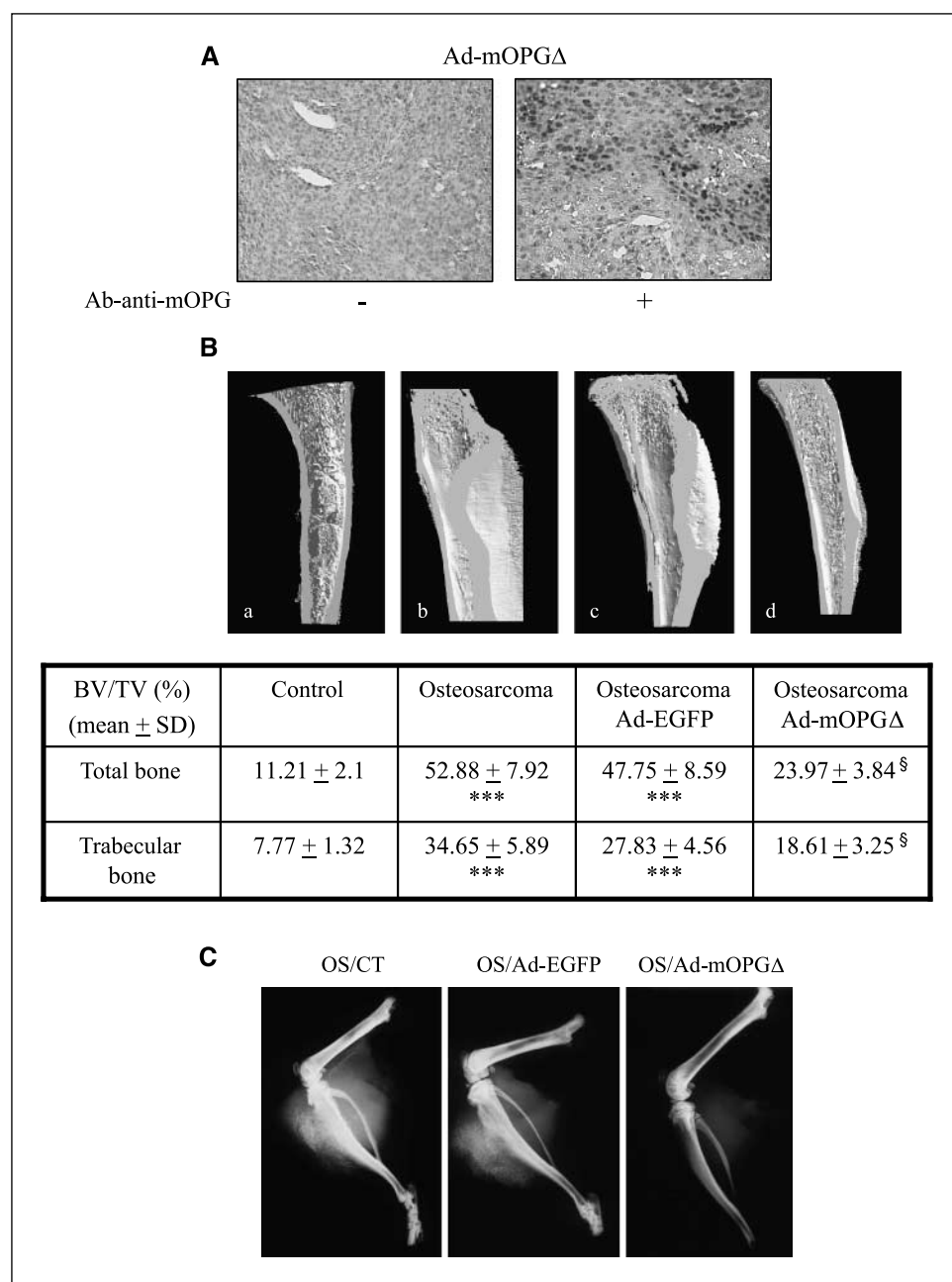


Figure 2. mOPGΔ transgene expression diminishes tumor incidence, tumor volume, and progression and augments survival rate in an osteolytic model of mouse osteosarcoma. The same protocol was applied as in Fig. 1. The tumor volume (V) was calculated from the measurement of two perpendicular diameters, according to the following formula: $V = 0.5 \times L \times S^2$, where L and S are, respectively, the largest and smallest perpendicular tumor diameters. The tumor volumes of individuals that received pcDNA3/Lutrol formulation (*pcDNA3*) are compared with that of mice treated with p-mOPGΔ/Lutrol (*p-mOPGΔ*), 50 μg in both tibial anterior muscles (respectively, A and B). The tumor progression was estimated as the relative tumor volumes (RTV) calculated from the formula: $RTV = (V_{21}/V_{15})$, where V_{21} is the tumor volume on day 21, and V_{15} is the tumor volume at day 15 (C). *, $P < 0.01$. D, overall survival rate was compared between mice that received pcDNA3/Lutrol formulation (*pcDNA3*) and p-mOPGΔ/Lutrol (*p-mOPGΔ*) over a 28-d period.

Figure 3. *In vivo* validation of mOPG transgene tissue expression and biological activity in rats. Immunologic analysis of OPG expression in osteosarcoma bearing rats injected i.m. with Ad-mOPG (5×10^9 pfu) as compared with Ad-EGFP (5×10^9 pfu) revealed a positive staining 7 d postinjection (original magnification, $\times 20$; A). Validation of Ad-mOPG biological activity on the tumor-associated bone remodeling was shown in a curative protocol developed in a rat model of osteosarcoma by the quantification of the microarchitectural parameters assessed by microscanner analysis (B). *Control*, naive rats; *Osteosarcoma*, rats that were implanted with osteosarcoma fragments 52 days before as described in Materials and Methods; *Osteosarcoma/Ad-EGFP*, osteosarcoma-bearing rats injected i.v. with Ad-EGFP 16 d posttumor implantation; *Osteosarcoma/Ad-mOPGΔ*, osteosarcoma-bearing rats injected with Ad-mOPG in the same conditions. BV, bone volume; TV, total volume ($n = 6$). ***, $P < 0.001$, osteosarcoma or osteosarcoma/Ad-EGFP versus control. §, $P < 0.01$, osteosarcoma/Ad-mOPGΔ versus osteosarcoma/Ad-EGFP. C, bone lesions associated with osteosarcoma were also analyzed by radiography in control rats bearing osteosarcoma (OS/CT) and osteosarcoma-bearing rats inoculated with the Ad-EGFP construct (OS/Ad-EGFP) as compared with osteosarcoma-bearing rats that received the adenoviral construct encoding mOPG (OS/Ad-mOPGΔ).



OPGΔ expression protects from bone remodeling associated with tumor development. Ad-mOPGΔ (5×10^9 pfu) was injected i.m. and i.v. to rats bearing osteosarcoma following a curative protocol, when the tumor volume is superior to 1,200 mm³ (14–16 days posttumor implantation), corresponding to progressive tumors. Microscanner analyses of hemisagittal sections of tibias taken at the time of necropsy revealed that osteosarcoma-bearing rat controls or receiving Ad-EGFP showed intense bone remodeling with a huge increase of specific total bone volume (respectively, 52.88% and 47.75% compared with 11.21% in normal rats, $P < 0.001$, Fig. 3B). However, because the bone formation process is always preceded by bone resorption (osteolytic lesions have been previously shown in this model by our group ref. 30), a therapeutic strategy using an antibone resorption agent is relevant in this model. Rats that received an i.v. injection of Ad-mOPGΔ displayed

less bone remodeling, as revealed by a significant decrease of the specific bone volume when compared with that of osteosarcoma-bearing control rats (23.97% as compared with 47.75% and 52.88%, respectively, for Ad-EGFP and control rats bearing osteosarcoma, $P < 0.01$). OPG treatment contrasts to the increased bone formation associated with tumor development, but cannot totally inhibit it. Both osteolytic lesions and bone formation, characteristic of osteosarcoma development, were clearly evidenced on radiographs of osteosarcoma-bearing rats (Fig. 3C). These results validated the biological activity of the OPGΔ transgene at the bone level.

OPGΔ expression diminishes osteosarcoma tumor progression. All animals that received i.v. injection of Ad-mOPGΔ exhibit a rapid and total tumor regression within 15 days after adenovirus inoculation, as compared with animals that received Ad-EGFP in the same conditions or control osteosarcoma-bearing rats, which

developed progressive tumor (Fig. 4A, mean volume of 3,410 mm³ 43 days postinoculation, range 1,372–6,534 mm³, versus 0 for Ad-mOPGΔ; $P < 0.001$; Fig. 4B). When the adenovirus construct was injected i.m., intermediary results were observed: 50% of tumors were progressive (mean tumor volume of 3,137 mm³, 43 days postinoculation), 25% stopped their progression (936 and 850 mm³ at day 43), and 25% totally regressed (Fig. 4C). As a result, animals that received an i.v. injection of Ad-mOPGΔ showed a high increase in survival (100% versus 50% in the control group, 65 days posttumor implantation, $P < 0.001$, Fig. 4D). However, overall survival was not significantly modified between the Ad-OPGΔ i.m. and control groups (62.5% and 50%, respectively, 65 days posttumor implantation).

In vitro experimentations. Because OPGΔ exerts antitumoral activities in both osteosarcoma models, several experiments were done *in vitro* to determine the mechanisms involved in these effects by studying whether OPG directly affects tumor cells or not.

No direct binding of murine OPG-Fc on osteosarcoma cells. To see whether OPG has a potential direct effect on osteosarcoma cells, binding of FITC-labeled OPG-Fc (with the same sequence as the OPG-Δ used in the preclinical studies) was studied by fluorescence-activated cell sorting (FACS) analysis on both mouse POS-1 and rat OSRGa cells. Human 2.93 cells overexpressing full-length murine RANKL were used as positive controls. Results presented in Fig. 5A show no binding of FITC-Fc-OPG on murine

POS-1 cells. The same result was obtained in the presence of rat OSRGa cells.

Effect of OPGΔ on tumor cell proliferation, apoptosis, and gene expression

Effect of OPGΔ on osteosarcoma cell proliferation and apoptosis. The influence of OPG overexpression was studied *in vitro* on mouse POS-1 and rat OSRGa cell proliferation using a XTT-based method and cell viability by trypan blue exclusion. The studies were done in the presence of recombinant OPG-Fc, corresponding to the same sequence of the transgene (OPG 1-194) to dispose of a great amount of corresponding protein for dose- and time-dependent analyses. Figure 5B shows that OPG-Fc does not affect POS-1 cell proliferation over a 72-h period, whatever the concentration used (10–100 ng/mL). Cell viability was not affected either as assessed by cell counting after trypan blue staining (data not shown). Similarly, OPG-Fc treatment affects neither the caspase activity nor the cell repartition between the different cell cycle phases (Fig. 5C and D, respectively). The same results were obtained with OSRGa cells (data not shown). These overall results confirmed the absence of OPG effect observed on cell proliferation and apoptosis because no modification could be evidenced when POS-1 and OSRGa osteosarcoma cells were treated with OPG 1-194.

Transcript analysis. The effect of OPG-Fc studied on the phenotype of osteosarcoma cells by reverse transcription-PCR

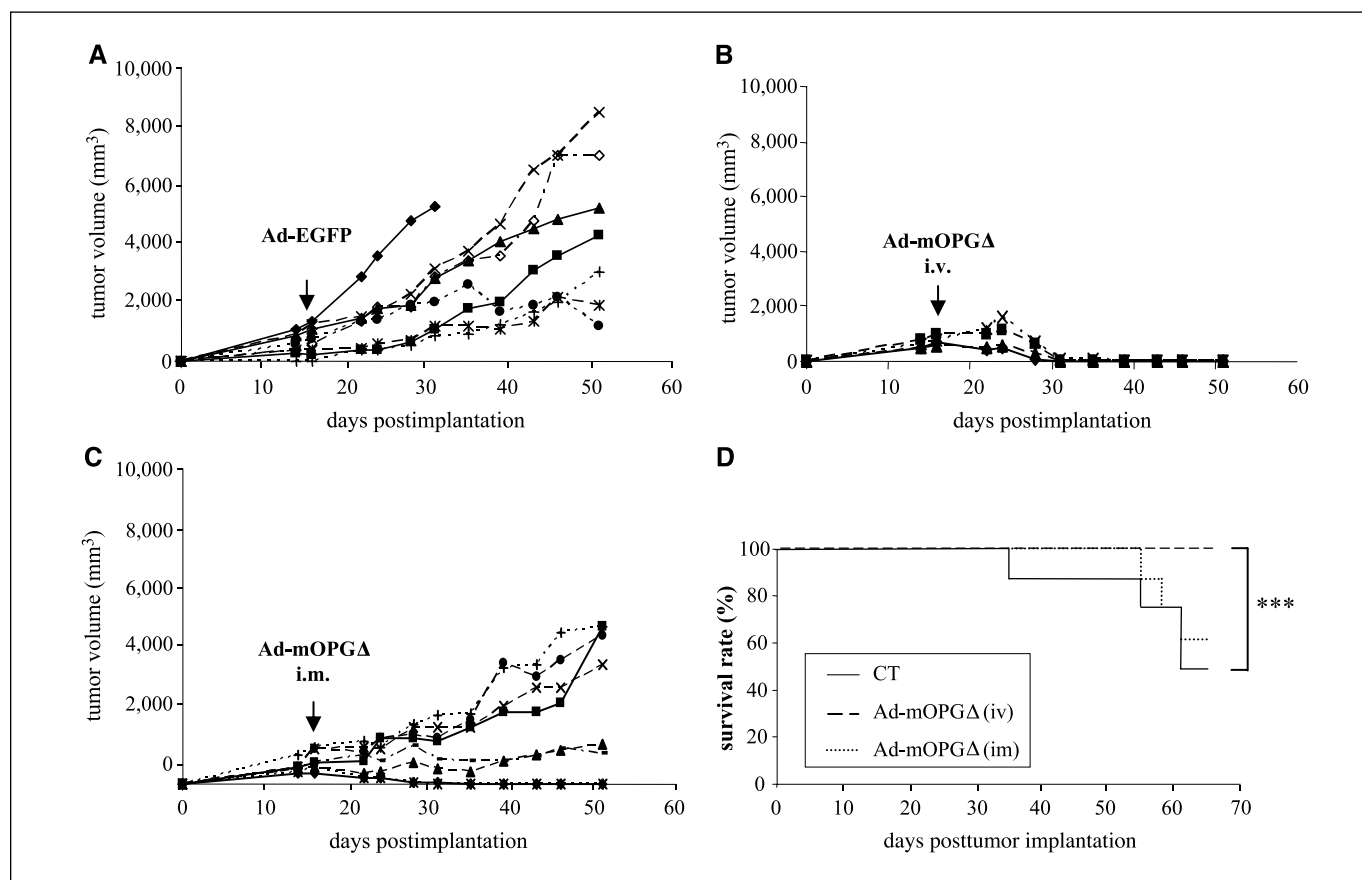


Figure 4. Ad-mOPGΔ administered as a curative protocol by i.v. injection induces a total regression of tumor development in a rat model of osteosarcoma. Rats implanted with osteosarcoma fragment (as described in Fig. 3) were treated with Ad-EGFP (5×10^9 pfu, $n = 8$; A) or with 5×10^9 pfu Ad-mOPGΔ i.v. ($n = 4$; B) or i.m. ($n = 8$; C) 16 d posttumor implantation, i.e., when tumors were progressive. The tumor volume was calculated as described in Fig. 3. D, overall survival rate was compared between control rats that received Ad-EGFP (CT) and rats that received Ad-mOPGΔ i.v. or i.m. over a 65-d period. ***, $P < 0.001$.

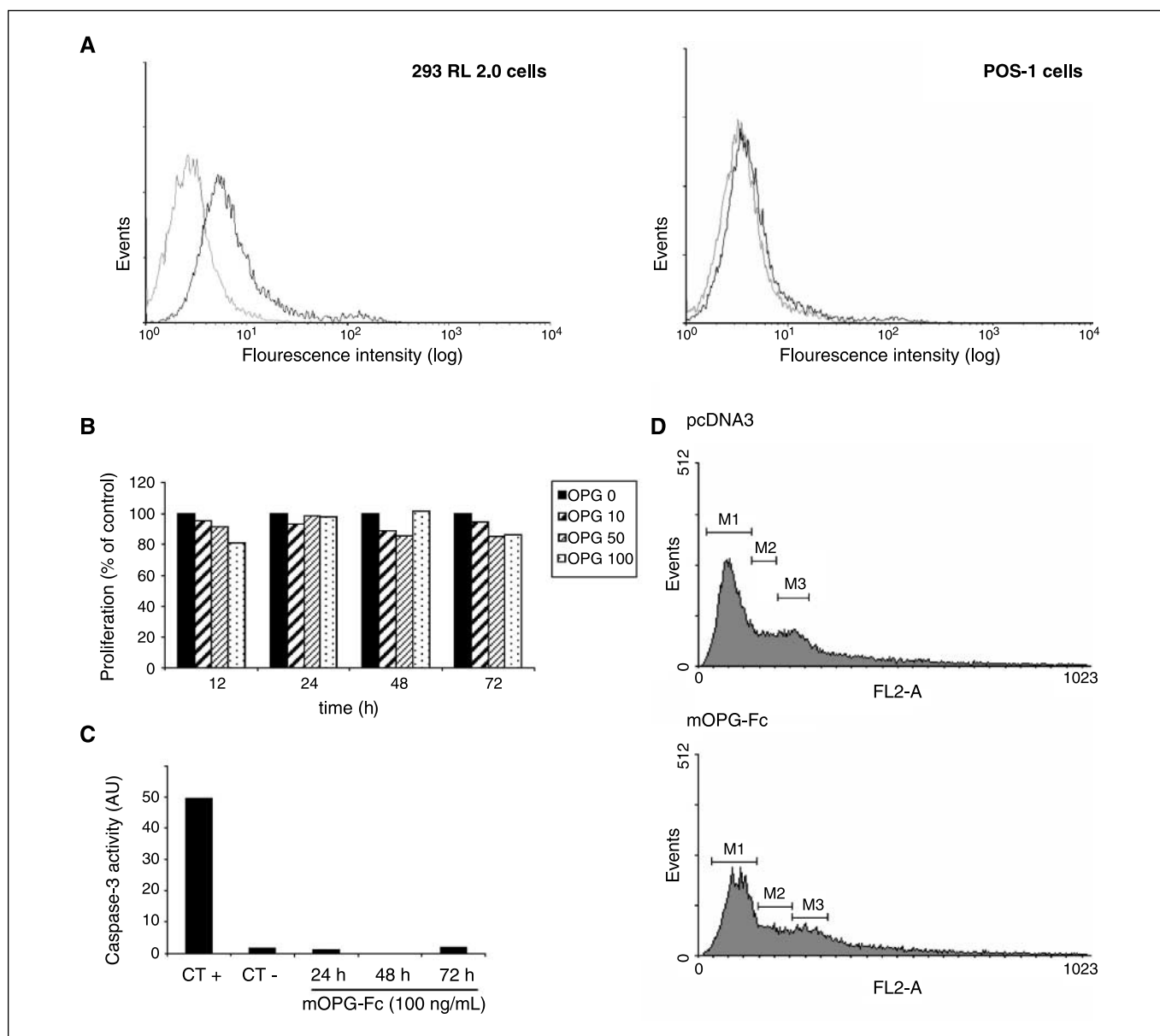


Figure 5. OPG-Fc does not bind to osteosarcoma cells nor affect osteosarcoma cell proliferation and apoptosis. Murine osteosarcoma POS-1 cells were incubated with FITC-labeled murine OPG-Fc and analyzed by FACS (A). Human fetal 293 cells overexpressing the murine form of full-length RANKL (293 RL 2.0) were used as positive controls. The effects were assessed on POS-1 cell proliferation using an XTT assay as described in Materials and Methods. POS-1 cells were treated for 12 to 72 h with increasing concentrations of murine OPG-Fc (10, 50, and 100 ng/mL) that exhibits the same sequence as the mOPG Δ transgene used for *in vivo* experiments (B). To determine the OPG influence on tumor cell apoptosis, the caspase-3 activity was analyzed in the cell lysate of POS-1 cells treated with 100 ng/mL mOPG-Fc for 24, 48, and 72 h (C). CT+, staurosporin (1 μ M/L, 6 h) was used as positive control; CT-, POS-1 cells alone. Parallel experiments were done on cell cycle distribution in the absence or the presence of 100 ng/mL mOPG-Fc during 24, 48, and 72 h (D; because no effect could be detected, only the results obtained after 72 h of incubation are shown).

showed no modulation of gene transcription in POS-1 and OSRGa cells in the presence of 100 ng/mL OPG-Fc (data not shown).

It can be therefore concluded that the inhibitory effect of OPG on tumor progression is not due to a direct effect on osteosarcoma POS-1 and OSRGa cells.

Indirect Inhibitory Antitumor Effect of OPG via RANKL Inhibition

Apoptosis induced by OPG *in vivo*. TUNEL staining on biopsies of POS-1 tumors revealed the presence of few apoptotic

cells in the tumor itself, corresponding to the necrosis areas. An increase of the number of apoptotic cells was evidenced in OPG-treated mice, which may explain the inhibition of tumor development previously observed (Fig. 6A).

Osteoclast number as measured by TRACP 5b activity. Two forms of TRACP circulate in blood, known as TRACP 5a and TRACP 5b, TRACP 5b being derived from osteoclasts and TRACP 5a from macrophages. Osteoclasts secrete TRACP 5b into the blood circulation. Recent studies have shown that TRAP 5b indicates the number of osteoclasts rather than their activity (31, 32). Figure 6B shows that OPG-treated mice evidenced a 51.6% reduction in

osteoclast number as compared with osteosarcoma-bearing mice ($P < 0.001$).

Elevation of RANKL expression in osteosarcoma-bearing mice is due to direct production by tumor cells. Because the inhibitory effects of OPG on osteosarcoma development are not caused by direct antitumoral effects on osteosarcoma cells, we may suggest that OPG indirectly influence tumor progression by inhibiting bone resorption through RANKL interaction, therefore blocking the vicious cycle between bone resorption and tumor proliferation. To assess this hypothesis, RANKL expression was searched for in serum of tumor-bearing mice treated or not with OPG and by immunohistochemistry in tissues of the corresponding tumors. Results presented in Fig. 6C showed an increase in RANKL production in the serum of osteosarcoma-bearing animals as compared with controls, which was abrogated in OPG-treated mice. Immunohistochemical analyses revealed a direct RANKL production by the tumor cells themselves, which may contribute to the elevated RANKL detection in the serum of osteosarcoma-bearing mice (Fig. 6D). The immunostaining for RANKL totally disappeared in the residual tumor tissues of OPG-treated mice (Fig. 6D).

Discussion

Osteosarcomas represent the most frequent primary malignant bone tumor, with a 5-year survival rate of about 60% to 70%,

decreasing to only 20% when pulmonary metastases are detected at the time of diagnosis. The vicious cycle that has been described in osteolytic metastases consists of release of osteolytic mediators by tumor cells, bone degradation, release of growth factors from degraded bone, enhanced tumor cell growth, and further release of osteolytic mediators (2). Inhibitors of bone resorption thus appear one of the more promising tools to manage skeletal metastases. One can speculate that this vicious cycle may also apply in the case of the primary bone tumors, and that inhibitors of bone resorption such as OPG may interfere with primary tumor development at a skeletal site. Delivery of Fc-OPG as a recombinant protein has shown promise as a potential therapy through experiments in animal models in that OPG limits hypercalcemia and osteolysis induced by myeloma, breast, lung, or prostate cancer and reduces tumor establishment in bone (11, 12, 33, 34).

In the present study, we describe for the first time the therapeutic interest of the truncated form of OPG in the treatment of osteosarcoma not only by the prevention of osteosarcoma-induced osteolysis, but also by the inhibition of associated tumor development, leading to increased survival rate in OPG-treated animals.

Two models of osteosarcoma were used in the present study: a mouse model that exhibits osteolytic lesions as confirmed by microscanner analysis (26) and a rat model that reproduces the clinical conditions of osteosarcoma in patients: osteolytic lesions

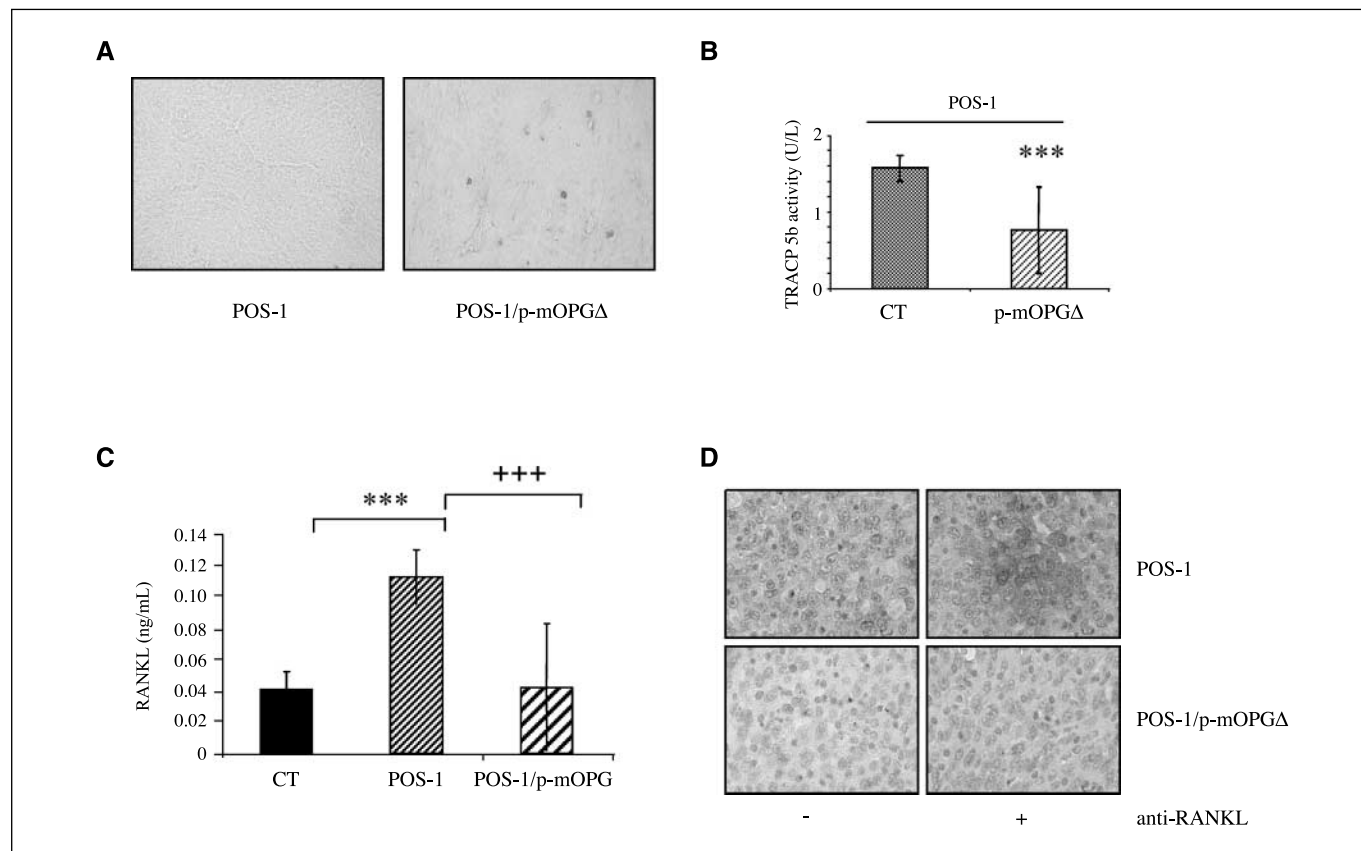


Figure 6. Comparison of tumor cell apoptosis, osteoclast number, and RANKL expression between POS-1 osteosarcoma bearing mice treated or not with p-mOPGΔ. **A**, tumor cell apoptosis was investigated *in vivo* by TUNEL staining and compared between POS-1 mice treated or not with OPG (POS-1/p-mOPGΔ). Original magnification, $\times 400$. **B**, the effect of p-mOPGΔ on osteoclast number was studied by TRACP 5b activity in the serum of POS-1 osteosarcoma bearing mice. **C**, RANKL production was measured by ELISA test in the serum of POS-1 osteosarcoma-bearing mice treated or not with OPG (POS-1/p-mOPGΔ) as compared with control mice (CT). **D**, localization of RANKL expression was investigated by immunohistochemistry in the tumor microenvironment of POS-1 osteosarcoma-bearing mice treated or not with OPG (POS-1 and POS-1/p-mOPGΔ). Original magnification, $\times 640$.

together with direct bone formation by tumor cells (30). In this model, even if the modifications of bone remodeling result in an overall bone formation as quantified by microarchitectural parameters (BV/TV), the initial process is always osteolysis. In these conditions, the use of OPG as an antbone resorption molecule is justified. Because OPG is a decoy receptor for TRAIL, blocking TRAIL-induced apoptosis of several tumor cell lines, it could confer a survival advantage on cancer cells (35, 36). Indeed, recent data from the literature highlighted the differences of biological activity between full-length OPG produced by tumor cells and therapeutic administered Fc-OPG (37). Therefore, to avoid a potential survival role of OPG on osteosarcoma cells, the DNA construct encoding the truncated form of OPG (1-194) was used in this study.

The preventing effect of OPG on osteosarcoma-associated osteolysis is the first report of its efficacy as an antbone resorption molecule *in vivo* in a model of primary bone tumor. OPG has been reported to exert antbone resorption activities through RANKL inhibition in several pathologic osteolyses. For example, OPG was shown to augment bone volume with reduced osteoclast number (18), reverse established osteopenia (38) in ovariectomized mice, or prevent osteoclastic resorption in wear debris-induced osteolysis (39). OPG inhibitory activity (inhibition of osteoclast formation and bone-resorbing activity) has also been reported in osteolysis associated with tumor pathologies as in giant cell tumor of bone, in which both the osteoclast formation and activation processes are promoted by RANKL (40). In multiple myeloma, tumor cells express RANKL, and treatment of mice with recombinant OPG protein prevented the development of osteolytic lesions (11). OPG also inhibits osteoclastogenesis in prostate cancer (17, 34) and mouse models of experimental bone metastasis (12). In most of the osteolysis models associated with tumor development, OPG also shows antitumor activity. Because the RANKL system may be abnormally regulated in bone tumor, targeting this system may represent a novel therapeutic approach. For example, besides murine models of myeloma (11, 16) and prostate cancer (17, 34), targeting this system with OPG has been shown to inhibit the development of bone disease *in vivo* in established osteoblastic prostate cancer bone metastases (41). A recent study of Corey et al. (42) revealed that OPG overexpression in prostate cancer cells does not directly affect proliferation of tumor cells, but indirectly decreases growth of prostate tumors in the bone environment. The experiments described in the present study revealed similar results in two models of primary bone tumor (osteosarcoma), with no direct effect of OPG on tumor cells as revealed by the absence of OPG binding on osteosarcoma cells and confirmed by the absence of OPG effect on cell proliferation, apoptosis, cell cycle distribution, or gene expression, but an indirect preventing effect on tumor development via the inhibition of associated osteolysis. The hypothesis relies on the fact that OPG, by inhibiting bone resorption, causes not only a reduction in the extent of osteolytic lesions, but also deprives tumor cells of bone-derived growth factors that are required for tumor-cell proliferation (2). This OPG

inhibitory effect on tumor progression may be the consequence of the RANK-RANKL axis blockade because RANKL concentration is increased in the serum of osteosarcoma-bearing mice, reflecting an increase of RANKL production in the tumor microenvironment caused by a direct RANKL expression by osteosarcoma cells. This hypothesis was indeed confirmed in our osteosarcoma models, as an inhibition of RANKL expression was evidenced in OPG-treated animals. In other bone tumor models, RANKL can be directly produced by tumor cells (11, 34) or is present in the tumor environment, produced by neighboring cells (8, 43). We previously reported that POS-1 osteosarcoma cells express RANK but not RANKL *in vitro* (26), and the present results suggest that *in vivo*, some factors present in the bone tumor environment induce RANKL expression by tumor cells themselves, such as growth factors released by bone degradation. The therapeutic hypothesis that is emerging for bone tumors focuses on targeting bone microenvironment rather than tumor cells themselves. A recent study from Jones et al. (13) shows that *in vivo* neutralization of RANKL by OPG results in complete protection from paralysis and a marked reduction in tumor burden in bones in a mouse model of melanoma metastasis, but not in other organs. In the 5T2MM murine model of myeloma, the OPG-like peptidomimetic OP3-4 that selectively inhibits RANKL but not TRAIL prevented the tumor-induced decrease in the cancerous bone area and the development of osteolytic lesions, but also reduced tumor burden when compared with the controls (44). The importance of the microenvironment of the local host tissue that actively participates in the propensity of certain cancers to metastasize to the bone has been studied, confirming the "seed-and-soil" hypothesis proposed by Paget (45): metastasis depends on cross-talks between selected cancer cells (the seeds) and specific organ microenvironment (the soil). Among the homeostatic factors that promote tumor cell growth in the bone, RANKL is a good candidate influencing the local bone resorption process that is necessary for tumor development in bone site (46).

In conclusion, using two methodologic approaches of gene transfer in two models of osteosarcoma, we can suggest that by targeting RANKL, the main cytokine that mediates bone resorption, it may be possible to modify the osteosarcoma itself and inhibit its development.

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Article 2

« Efficacité thérapeutique du récepteur activateur de NF- κ B (RANK) soluble libéré par transfert de gène non viral dans un modèle murin d'ostéosarcome ostéolytique »

Molecular Cancer Therapeutics, 2008 Oct ; 7 (10) : 3389-98,

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L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives qui touche principalement une population jeune avec un âge median se situant à 18 ans. Malgré les améliorations thérapeutiques dans le traitement de l'ostéosarcome, le taux de survie est seulement de 30% à 5 ans pour des patients avec la présence de métastases pulmonaires au moment du diagnostic. C'est pourquoi il est essentiel de développer de nouvelles approches thérapeutiques. L'utilisation de RANK soluble en tant que molécule anti-résorption osseuse est vraiment une approche prometteuse pour bloquer le cercle vicieux qui s'instaure en site osseux entre la résorption osseuse et la prolifération tumorale.

L'ADNc codant RANK-Fc murin a été administré par transfert de gène utilisant des polymères amphiphiles dans un modèle d'ostéosarcome ostéolytique murin. Les paramètres cliniques et la microarchitecture osseuse ont été étudiés par radiographie et analyses au micro-scanner. Des expériences *in vitro* ont été réalisées afin d'étudier le mécanisme d'action de mRANK-Fc sur la prolifération tumorale (analyse XTT), l'apoptose (test de l'activité caspase), le cycle cellulaire (analyse par cytométrie en flux), ou sur l'expression de gènes (RT-PCR).

Le traitement par mRANK-Fc a prévenu la formation de lésions ostéolytiques associées au développement de l'ostéosarcome, réduit l'incidence et la croissance tumorale ainsi que la dissémination métastatique induisant une augmentation de 3,9 fois de la survie des souris 28 jours après l'implantation. Cependant, mRANK-Fc ne prévient pas le développement de nodules tumoraux en site non osseux, suggérant que l'environnement osseux est nécessaire pour l'efficacité thérapeutique de mRANK-Fc. De plus, *in vitro*, mRANK-Fc n'a pas d'activité directe sur les cellules d'ostéosarcome.

mRANK-Fc exerce donc un effet inhibiteur indirect sur la progression de l'ostéosarcome par l'inhibition de la résorption osseuse.

Therapeutic efficacy of soluble receptor activator of nuclear factor- κ B-Fc delivered by nonviral gene transfer in a mouse model of osteolytic osteosarcoma

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Abstract

Osteosarcoma is the most frequent primary bone tumor that develops mainly during youth, the median age of diagnosis being 18 years. Despite improvement in osteosarcoma treatment, survival rate is only 30% after 5 years for patients with pulmonary metastases at diagnosis. This warrants exploration of new therapeutic options. The anti-bone resorption molecule receptor activator of NF- κ B (RANK) is very promising, as it may block the vicious cycle between bone resorption and tumor proliferation that takes place during tumor development in bone site. The cDNA encoding murine RANK-Fc (mRANK-Fc) was administered by gene transfer using an amphiphilic polymer in a mouse model of osteolytic osteosarcoma. Clinical and bone microarchitecture variables were assessed by radiography and micro-CT analyses. *In vitro* experiments were designed to determine the mechanism of action of RANK-Fc on tumor cell proliferation (XTT assays), apoptosis (caspase activation), cell cycle distribution (fluorescence-activated cell sorting analysis), or gene expression (reverse transcription-PCR). RANK-Fc was effective in preventing the formation of

osteolytic lesions associated with osteosarcoma development and in reducing the tumor incidence, the local tumor growth, and the lung metastases dissemination leading to a 3.9-fold augmentation of mice survival 28 days after implantation. On the contrary, mRANK-Fc did not prevent the development of nonosseous tumor nodules, suggesting that bone environment is necessary for mRANK-Fc therapeutic efficacy. Furthermore, mRANK-Fc has no direct activity on osteosarcoma cells *in vitro*. mRANK-Fc exerts an indirect inhibitory effect on osteosarcoma progression through inhibition of bone resorption. [Mol Cancer Ther 2008;7(10):1–10]

Introduction

With an estimated incidence of 2 cases per million people per year, osteosarcoma is the most frequent primary bone malignant tumor excluding hematopoietic intraosseous tumors. Osteosarcoma generally affects young patients with a peak incidence at age 18 years. The unifying histologic feature found in all types and subtypes of osteosarcomas is the presence of osteoid tissue produced by the neoplastic cells (1). As these tumors frequently penetrate and destroy the cortical substance of the bone and extend into the surrounding tissues, a strong osteolytic activity is often associated with osteosarcoma development. In spite of newly devised chemotherapy regimes combined with wide-margin, limb-sparing surgery, osteosarcoma continues to confer a generally poor prognosis in patients with lung metastasis (<30%; ref. 2). As this survival rate has not evolved since two decades, there is an urgent need for new therapeutic strategies, adjuvant to surgery and chemotherapy.

The final effector molecules that ultimately control osteolysis associated with tumor development in bone site are receptor activator of NF- κ B ligand (RANKL) and osteoprotegerin (OPG; ref. 3). RANKL directly stimulates osteoclastic differentiation and osteoclast activation and survival by signaling through its membrane receptor RANK, expressed on the osteoclast precursor surface. Physiologically, RANKL signaling is negatively regulated by the soluble antagonist receptor protein OPG, which induces osteoclast apoptosis. Targeting RANKL signaling with OPG inhibits tumor-associated osteolysis in several experimental bone tumor models including mouse colon adenocarcinoma, myeloma, breast, lung, prostate cancer, and osteosarcoma (4–9). However, OPG has been shown to bind the tumor necrosis factor receptor [TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)] and to block TRAIL-mediated apoptosis in cancer cells (10–13). Thus, alternative agents, such as soluble RANK-Fc that block RANKL-mediated bone resorption but that do not

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interact with TRAIL, may provide useful therapies to prevent tumor development in bone. RANK-Fc is a chimeric protein formed by fusing the four cysteine-rich pseudorepeats of RANK that are responsible for RANK-L binding with the Fc portion of human IgG1. Moreover, RANK-Fc has the potential advantage over OPG of greater specificity for RANKL. Fusion of the four cysteine-rich domains of RANK to the constant region of hIgG1 dictates homodimerization, which probably increases its avidity for RANKL. The sRANK differs from OPG because, in addition to containing four cysteine-rich domains, OPG also contains two death domains and a heparin-binding domain (14). Accordingly, RANK-Fc has been recently shown to cause a marked reduction in tumor burden in two SCID-hu multiple myeloma models (15) and also an inhibition of prostate cancer progression in bone (16). However, no study was done on RANK-Fc effects in primary bone tumors.

Previous data showed that it is feasible to provide long-lasting expression of RANK-Fc at bone-protective levels using a retroviral gene therapy approach (17). However, the toxicity associated with the use of viral vectors is extremely complex involving both innate and adaptative immune responses. Recently, a new class of synthetic vectors has been reported for *in vivo* gene transfer in various organs including skeletal and cardiac muscles (18, 19) and in lungs (20). These new synthetic vectors result from the association of plasmid DNA with amphiphilic polymers consisting in blocks of poly(ethylene oxide) and poly(propylene oxide). Intramuscular injections of these synthetic vectors lead to the synthesis of proteins for local benefit such as dystrophin or for systemic use such as erythropoietin (21).

The aim of this study was to determine the therapeutic relevance of RANK-Fc in a murine osteolytic osteosarcoma model by using a nonviral gene transfer approach.

Materials and Methods

Cell Lines

The osteosarcoma cell line POS-1, derived from mouse spontaneous osteosarcoma (22), was cultured in RPMI 1640 (Bio Whittaker) supplemented with 10% fetal bovine serum (Hyclone) and 2 mmol/L L-glutamine.

The osteoclast precursor RAW 264.7 cells from the monocyte macrophage lineage were obtained from the American Tissue and Cell Collection and grown in α MEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 1% nonessential amino acids (Invitrogen).

The human osteosarcoma MG63 cell line was purchased from the American Tissue and Cell Collection and used for *in vitro* assay of TRAIL biological activity and cultured in DMEM (BioWhittaker) supplemented by 10% fetal bovine serum (Hyclone) and 2 mmol/L L-glutamine.

In vitro Experimentations

Plasmid Constructions. The pcDNA3.1-RANK-Fc construction (kindly provided by Dr. Y. Choi) contains a DNA sequence encoding the extracellular domain of murine

RANK (mRANK; 0.7 kb) fused to the coding sequences of the constant portion of human IgG1 (0.5 kb; ref. 23). The soluble RANK-Fc cDNA is inserted in the pcDNA3.1 plasmid between *Xba*I and *Xho*I under the control of the cytomegalovirus promoter. For the *in vivo* and *in vitro* studies, the empty pcDNA3.1 was used as a control.

Cell Transfection. To assess the cellular expression of RANK-Fc, 2 μ g pcDNA3.1 and pcDNA3.1-RANK-Fc were transfected by nucleofection into RAW 264.7 cells using the Cell Line Nucleofector Kit V program D-032 (AMAXA Biosystems) following the manufacturer's recommendations. The transfection efficacy is controlled by quantification of 2 μ g pmaxGFP-transfected cells by fluorescence microscopy (AMAXA Biosystems).

Osteoclast Differentiation. The biological activity of the transgene was compared between pcDNA3.1-RANK-Fc- and pcDNA3.1-nucleofected RAW 264.7 cells plated in 96-well plates (3,000 per well) during 5 days. Medium was replaced twice (after 2 and 48 h) with α MEM 10% fetal bovine serum, 1% nonessential amino acids, and increasing concentrations of recombinant human soluble RANKL (50, 75, and 100 ng/mL; kindly provided by Amgen). After 5 days of culture, multinucleated cells (>3 nuclei) were counted after a May-Grünwald Giemsa staining.

Cell Proliferation. Replicate subconfluent cell cultures of POS-1 cells in 96-well plates were treated for 24 to 72 h with increasing concentrations of mRANK-Fc (R&D Systems; 0, 25, 50, and 100 ng/mL). Cell viability was determined by the XTT cell proliferation reagent assay kit (Roche Molecular Biomedicals).

Caspase Activity. POS-1 cells (2×10^4 per well) cultured in 24-well plates were treated with 100 ng/mL RANK-Fc for 24 to 72 h, washed and lysed with 50 μ L radio-immunoprecipitation buffer for 30 min in the presence of protease inhibitors. The cells were then scraped off and the protein amount was quantified using the BCA (bicinchonic acid + copper II sulfate) test (Pierce Chemical). Caspase-3 activity was assessed on 10 μ L cell lysate with the CaspACE assay kit (Promega) following the manufacturer's recommendations. Cells treated with 1 μ mol/L staurosporin 6 h before harvesting were used as a positive control for caspase activity.

Cell Cycle Analysis. Confluent POS-1 cells treated with increasing concentrations of RANK-Fc (0, 50, and 100 ng/mL) for 24, 48, and 72 h were removed from culture dishes by trypsinization, washed twice in PBS, and incubated in PBS containing 0.12% Triton X-100, 0.12 mmol/L EDTA, and 1 μ g/mL DNase-free RNase A (Sigma). Each sample was incubated 20 min at 4°C in the dark with 50 μ g/mL propidium iodide (Sigma). The stained nuclei were analyzed by flow cytometry (FACScan; BD Biosciences) using the CellQuest software. Cell cycle distribution was based on 2N and 4N DNA content.

TRAIL Biological Activity. Replicate subconfluent cell cultures of human osteosarcoma MG63 cells in 96-well plates were treated for 72 h with increasing concentrations of human TRAIL (R&D Systems; 0, 50, and 100 ng/mL) in the presence or absence of 100 ng/mL RANK-Fc.

Cell viability was determined by the XTT cell proliferation reagent assay kit as described above.

In vivo Experimentations

Mouse Osteosarcoma Models. Male C3H/He mice (4 weeks old; Elevages Janvier) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. The mice were anesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with an intramuscular injection of buprenorphine (0.05 mg/kg; Temgesic, Schering-Plough) before s.c. inoculation of POS-1 cell suspension containing 2×10^6 cells in 50 μ L PBS in the hind footpad of the mice. Under these conditions, mice develop a primary tumor at the site of injection in 3 weeks that can be transplanted to mice of the same strain as a small fragment ($2 \times 2 \times 2$ mm³) in close contact with the tibia. For this purpose, the periosteum of the diaphysis was opened and resected along a length of 5 mm; underlying bone was intact. The osteosarcoma fragment was placed contiguous to the exposed bone surface without periosteum, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site approximately 8 days later associated with the development of pulmonary metastases in a 3-week period. The tumor that develops in contact to the tibia induces osteolytic lesions that reproduce the osteolytic form of human osteosarcoma (22). The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a caliper according to the following formula: $V = 0.5 \times L \times S^2$, S^2 , where L and S are the largest and smallest perpendicular tumor diameters, respectively. A model of pulmonary metastases was developed to study RANK-Fc effect independent of bone environment, where mice were anesthetized by inhalation of a mixture of isoflurane/air combined with an intramuscular injection of buprenorphine as described above before i.v. injection of 50 μ L POS-1 cell suspension containing 1.5×10^5 cells. In these conditions, pulmonary metastases developed rapidly, leading to the death of the animals in 3 weeks after POS-1 cell injection.

Formulation Preparations. For intramuscular injections, mice were anesthetized with a combination isoflurane/air. Fifty microliters of block copolymers/DNA formulations were injected into shaved tibial anterior muscles at one site using a microfine syringe (U100; Becton Dickinson). Lutrol, a block copolymer consisting of poly(ethyleneoxide)₇₅-poly(propyleneoxide)₃₀-poly(ethyleneoxide)₇₅, was generously provided by BASF. Stock solutions were prepared at 6% (w/v) in water and stored at 4°C. Formulations of DNA with block copolymers were prepared by equivolumetric mixing block copolymers in water and DNA solution at the desired concentration (50 μ g/muscle) as already reported (21).

Experimental Protocols. To determine the effect of RANK-Fc delivered by synthetic vectors on osteosarcoma development, the mice were transplanted with POS-1

osteosarcoma fragments as described above. Groups of 8 mice were assigned as controls (no injection), control vectors (Lutrol/pcDNA3.1 alone), and RANK-Fc (Lutrol/pcDNA3.1-RANK-Fc), respectively. Preliminary results from the laboratory have already shown that the vector Lutrol alone does not affect tumor development in the same mouse osteosarcoma model (9). A preventive treatment was applied, where the Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning 7 days before osteosarcoma implantation up to 21 days post-implantation. The tumor volume was calculated as described above. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation. Lung tumor dissemination was assessed at necropsy.

Radiographs on animals anesthetized with 50 mg/kg Nesdonal (Merial) were taken every week and at the time of necropsy with a mammography PLANMED Sophie apparatus (SN RAH 40710). Analysis of architectural variables was done using the high-resolution X-ray micro-CT system for small animal imaging SkyScan-1072 (SkyScan). Relative volume (BV/TV) of the tibia [total bone (cortical + trabecular) or trabecular bone] was quantified at necropsy in the osteosarcoma groups that received RANK-Fc compared with that of control mice. The results of one of three representative experiments are shown.

Gene and Protein Expression Analysis

To assess RANK-Fc transgene expression *in vitro* at the mRNA and protein levels, pcDNA3.1- and pcDNA3.1-RANK-Fc-nucleofected RAW 264.7 cells were cultured in six-well plates (2×10^6 per well) under standard conditions during 48 h.

In vivo, blood was drawn intermittently from the retro-orbital vein to monitor serum RANK-Fc level (days 7, 10, or 15 post-injection). At necropsy, the tumor and muscle tissues were lysed in 1 $\times$ reporter lysis buffer (Promega) supplemented with protease inhibitor cocktail (Roche Molecular Biomedicals), buoyed during 30 s using Ultraturax, centrifuged at 10,000 rpm during 5 min at 4°C, and processed for RANK-Fc detection using the ELISA test described below.

RNA Extraction and Semiquantitative Reverse Transcription-PCR Analysis. RANK-Fc mRNA expression was determined by reverse transcription-PCR (RT-PCR) after total RNA extraction using Trizol reagent (Invitrogen). First, RNA was reverse transcribed using 400 units MMLV-RT (Invitrogen). Then, 2 μ L RT reaction mixture was subjected to PCR using upstream (5'-GCTGGCTACCACTGGAAGCTC-3') and downstream (5'-GTGGGCATGTGTGAGTTTTG-3') primers (30 pmol each) of sRANK-Fc and Taq polymerase (1.25 units; Eurobio). The effect of RANK-Fc was assessed on tumor cell phenotype by studying the expression of several bone markers by RT-PCR.

RANK-Fc ELISA. RANK-Fc protein levels were determined by ELISA test: 96-well plates were coated with goat polyclonal anti-mouse RANK extracellular

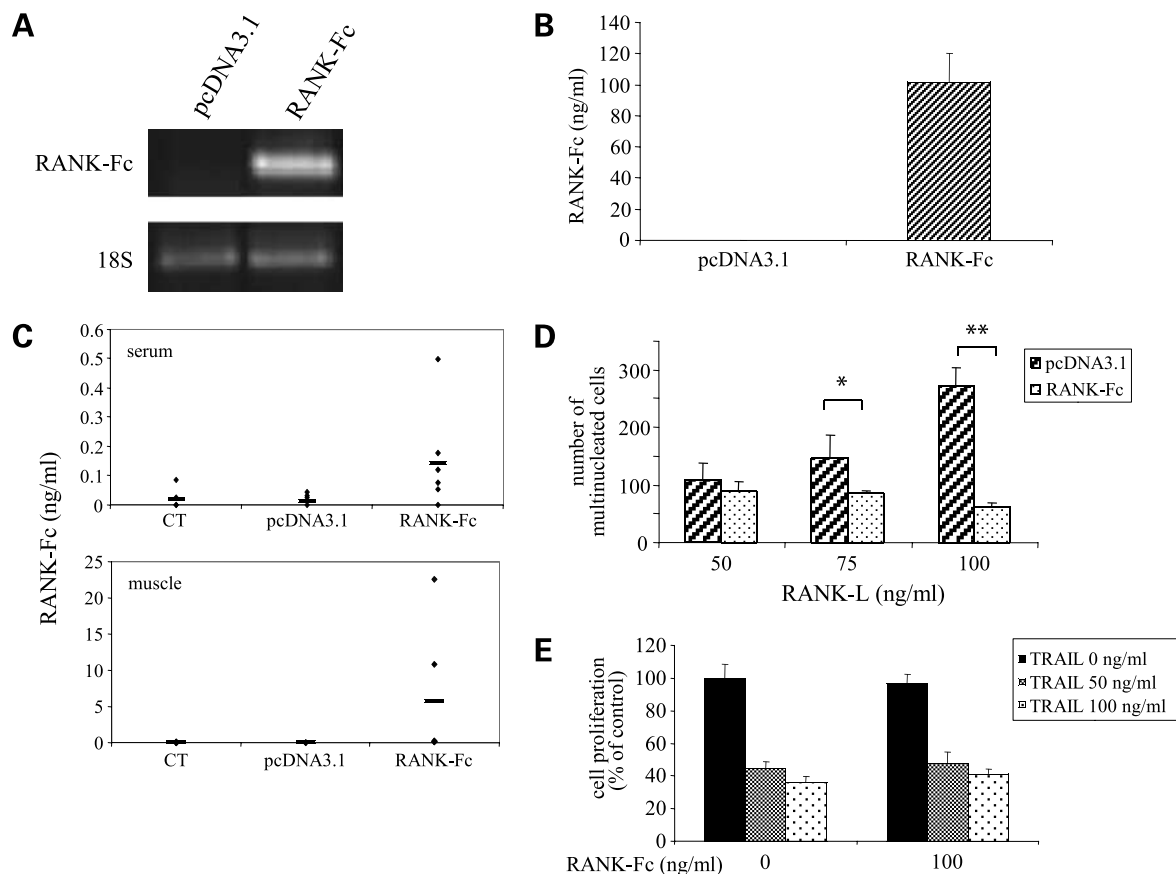


Figure 1. *In vitro* and *in vivo* validation of RANK-Fc transgene overexpression and biological activity. mRANK-Fc transgene expression was assessed 48 h after transfection in murine RAW 264.7 cells using a AMAXA nucleofector /pcDNA3-mRANK-Fc formulation at the transcript level by RT-PCR (**A**) and at the protein level by ELISA in the culture medium (**B**). *In vivo*, formulations of pcDNA3-mRANK-Fc with Lutrol block copolymers were assessed. The formulations were prepared by equivolumetric mixing block copolymers in water and DNA solution at the desired concentration (50 μ g/muscle). The DNA-Lutrol formulation was administered in the tibial anterior muscle of both legs at weekly intervals. mRANK-Fc expression was analyzed 7 d post-injection by ELISA test in the muscle and in the serum (**C**). The biological activity of the mRANK-Fc transgene was assessed by its ability to block the RANKL-induced multinucleated cell formation in RAW 264.7 cells (**D**). RAW 264.7 cells transfected by nucleofection with pcDNA3.1 or pcDNA3.1-mRANK-Fc (RANK-Fc) were cultured in the presence of 50, 75, and 100 ng/mL RANKL during 5 d to assess multinucleated cell formation. *, $P = 0.048$, pcDNA3.1/RANK-Fc-versus pcDNA3.1-transfected RAW cells cultured with 75 ng/mL RANKL; **, $P = 0.003$, pcDNA3.1-mRANK-Fc- versus pcDNA3.1-transfected RAW cells cultured with 100 ng/mL RANKL. Viability assay using XTT test of MG63 cells cultured in the presence of 50 or 100 ng/mL human TRAIL were realized with recombinant human RANK-Fc (100 ng/mL), which exhibits the same sequence than the RANK-Fc transgene produced *in vivo* (**E**).

domain (Jackson ImmunoResearch Laboratories; 0.2 μ g/well) overnight at room temperature and then washed and incubated for 1 h with a blocking solution (1% bovine serum albumin, 5% sucrose PBS). After washing, wells were incubated at room temperature with dilute serum or medium from nucleofected RAW 264.7 cells for 2 h followed by a 2 h incubation with a biotin-SP-conjugate donkey anti-human IgG (1:20,000; R&D Systems). Detection was done with conjugate streptavidin horseradish peroxidase (1:200; R&D Systems) incubation for 30 min. Reaction was stopped by 50 μ L of 1 mol/L H_2SO_4 , and 410 nm absorbance was determined (VICTOR² Multilabel counter; Perkin-Elmer). The ELISA detection limit was 0.02 ng/mL.

Histology

After sacrifice, tibia were conserved and fixed in 10% neutral-buffered formalin at 4°C and embedded in

glycol methyl methacrylate for TRAP staining, and 5 μ m sections were cut. The sections were stained for TRAP to identify osteoclasts by 1 h incubation in a 2 mg/mL naphthyl phosphate (Sigma) and 5 mg/mL fast violet salt (Sigma) solution. The counterstain was done with toluidine blue after a 1 h inactivation of the first solution in sodium fluoride. The number of osteoclasts was evaluated on the cortical bone by manually counting by light microscopy.

Data Analyses

For *in vivo* experimentations, the Tukey/Bonferroni test was used for microscanner and nonparametrical test by Kruskal-Wallis analysis for the tumoral progression evaluation. The differences of actuarial survival were determined by the log-rank test on a Kaplan-Meier survival curve. Statistical evaluation of the *in vitro* proliferation data was done by Student's *t* test. Results

are given as mean $\pm$ SD and results with $P < 0.05$ were considered significant.

Results

In vitro Analysis of Transgene Expression

mRANK-Fc overexpression was investigated at the mRNA and protein levels by RT-PCR and ELISA analyses done on RAW 264.7 cells, which were transfected with pcDNA3.1 or pcDNA3.1-RANK-Fc using the AMAXA nucleofactor system. RT-PCR analysis showed a major expression of RANK-Fc mRNA in pcDNA3.1-RANK-Fc-transfected cells, whereas no expression was observed in the corresponding pcDNA3.1-transfected cells (Fig. 1A). This result was confirmed at the protein level by ELISA analysis, as supernatant from pcDNA3.1-RANK-Fc-transfected cells showed a high level of mRANK-Fc protein (101.6 ± 15.88 ng/mL) after 48 h when compared with pcDNA3.1-transfected cells, which showed no protein expression (Fig. 1B).

In vivo Validation of Transgene Expression

After injection of the DNA-Lutrol complexes into the tibial anterior muscles, an overexpression of the RANK-Fc transgene was measured at both systemic (serum) and local (muscle) levels in mice treated with pcDNA3.1-RANK-Fc. The production varied from 0.053 to 0.499 ng/mL in the serum and from 0.09 to 22.57 ng/mL in the muscle, being significantly higher to the pcDNA3.1 or control groups where no RANK-Fc expression could be detected, either

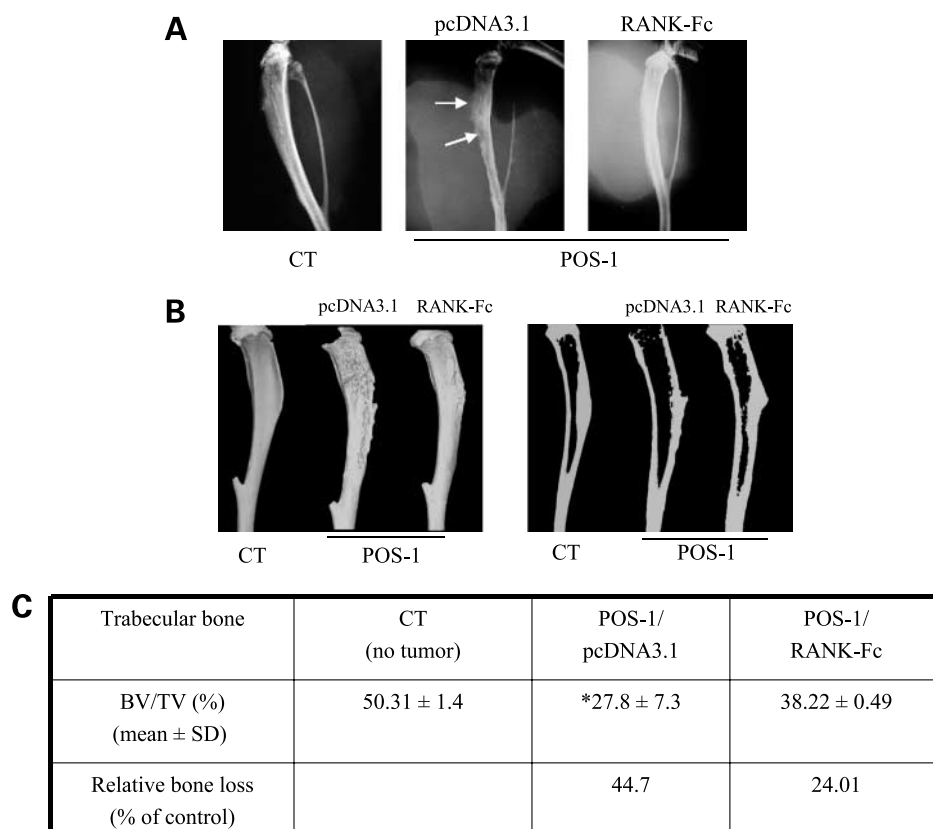
in serum or in muscle (Fig. 1C). No correlation could be established between RANK-Fc concentration (either systemic or local) and its therapeutic efficacy. The immunogenicity of the human Fc part of RANK-Fc was checked previously in a immunocompetent rat model of osteosarcoma, and no antibodies were detected up to 3 weeks (not shown).

In vitro Validation of Transgene Biological Activity

To confirm that RANK-Fc expressed by nonviral gene transfer was biologically active, we investigated its ability to block RANKL-induced multinucleated cell formation in the osteoclast precursor RAW 264.7 cells. Inhibition of osteoclast formation was analyzed by multinucleated cell counting after May-Grünwald Giemsa staining of pcDNA3.1- and pcDNA3.1-RANK-Fc-transfected RAW 264.7 cells cultured in the presence of soluble RANKL. The results presented in Fig. 1D show that RAW 264.7 cells transfected with pcDNA3.1-RANK-Fc induced a significant inhibition of multinucleated cell formation in the presence of RANKL compared with cells transfected with pcDNA3.1 [84.7 ± 4 and 148.3 ± 39.1 multinucleated cells ($P = 0.048$) in the presence of 75 ng/mL RANKL and 62.7 ± 7.4 and 272.7 ± 30.3 ($P = 0.003$) for 100 ng/mL RANKL, respectively].

To ensure that RANK-Fc does not block TRAIL-mediated apoptosis, the effect of RANK-Fc was assessed on the human osteosarcoma cell line MG63 treated with increasing concentrations of TRAIL. To dispose of great amount of

Figure 2. mRANK-Fc transgene expression prevents osteosarcoma-associated osteolysis. Mice were transplanted with POS-1 osteosarcoma fragments as described in Materials and Methods. Groups of six to eight mice were assigned as CT (controls: no tumor), POS-1/pcDNA3.1 (control vectors: Lutrol/pcDNA3.1 alone in POS-1 osteosarcoma-bearing mice), and POS-1/RANK-Fc (Lutrol/pcDNA3.1-mRANK-Fc in POS-1-bearing mice), respectively. A preventive treatment was applied, where the Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning at 7 d before osteosarcoma implantation up to 21 d post-implantation (time of sacrifice). At that time, the tumor-associated osteolysis was analyzed by radiography (A) and the microarchitectural variables by micro-CT (B and C). The trabecular specific bone volume was quantified in percentage by the following formula: BV / TV (C), where BV is bone volume and TV is total volume. Relative bone loss was calculated as percentage of control following the formula: $[(BV / TV)_{CT} - (BV / TV)_{POS \text{ or mRANK-Fc}}] / (BV / TV)_{CT}$. *, $P < 0.05$, POS-1/pcDNA3.1 versus CT.



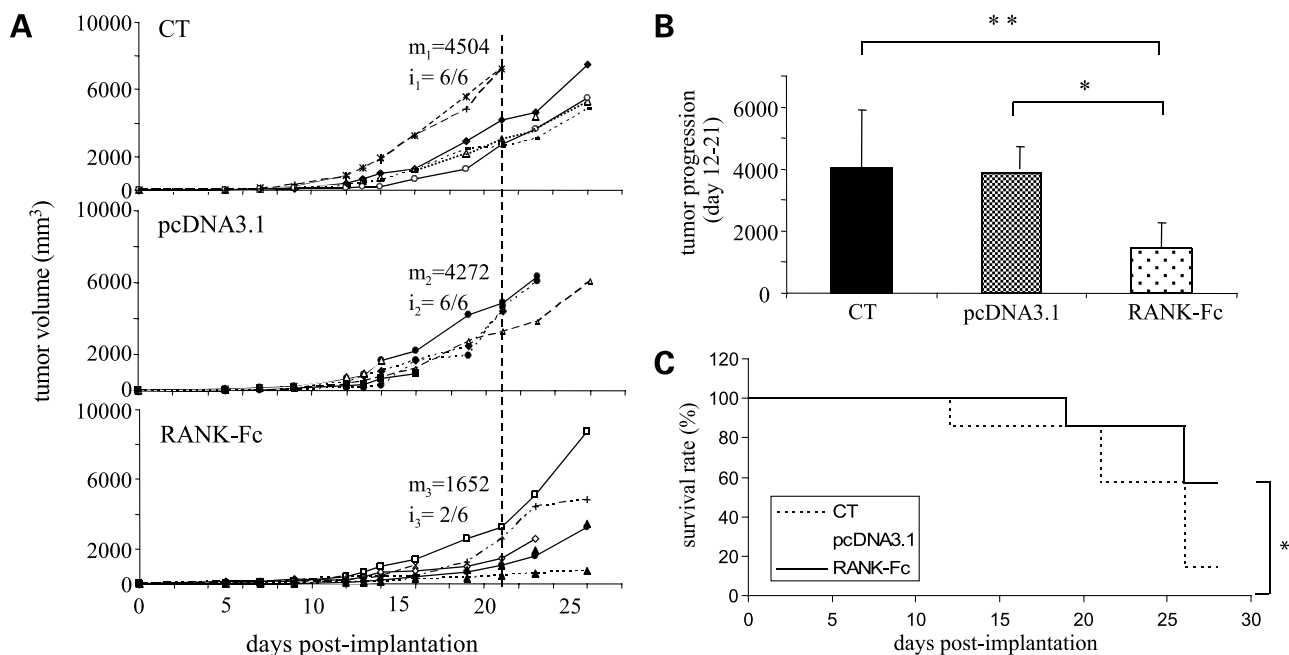


Figure 3. mRANK-Fc decreases tumor volume, tumor progression, and increases mouse survival. The same protocol was applied as in Fig. 2. The tumor volume (V) was calculated from the measurement of two perpendicular diameters according to the following formula: $V = 0.5 \times L \times S^2$, where L and S are the largest and smallest perpendicular tumor diameters, respectively. The tumor volumes of individuals untreated or treated by pcDNA3.1/Lutrol formulation (named pcDNA3.1) are compared with that of mice treated with pcDNA3.1-mRANK-Fc/Lutrol (RANK-Fc), 50 μ g in both tibial anterior muscles (A). The mean tumor is indicated at day 21 in each group as m_{1-3} . The incidence of animals bearing progressive tumors at day 21 is also indicated as i_{1-3} . The mean tumor progression volume between days 12 and 21 (B). *, $P < 0.05$, pcDNA3.1-mRANK-Fc-treated versus pcDNA3.1 mice; **, $P < 0.01$, pcDNA3.1-mRANK-Fc-treated versus untreated mice (CT). The overall survival rate was compared between control mice and mice that received pcDNA3/Lutrol formulation (pcDNA3) and pcDNA3.1-mRANK-Fc/Lutrol (RANK-Fc) over a 28-d period (C). *, $P < 0.02$.

protein for dose and time-dependant analysis *in vitro*, a recombinant RANK-Fc corresponding to the same sequence as the transgene used in the *in vivo* study was chosen. No effect of RANK-Fc treatment could be shown on TRAIL-induced apoptosis of MG63 cells (Fig. 1E). Similar results were obtained with other TRAIL-sensitive cell lines, such as the A673 human Ewing sarcoma cell line.

These results show that RANK-Fc protein is expressed after nonviral gene transfer both *in vitro* and *in vivo* and that it exerts an antiresorption activity *in vivo* (against RANKL) and not a protumoral one (unable to block TRAIL-induced apoptosis).

Protective Effect of mRANK-Fc Transgene Expression on Bone Resorption Associated to Osteosarcoma Development

The mouse osteosarcoma POS-1 model used in this study has been associated previously with osteolytic lesions by micro-CT analysis (22). To show the inhibitory effect of RANK-Fc on the development of osteolytic lesions, mice transplanted with osteosarcoma were treated with pcDNA3.1-RANK-Fc or pcDNA3.1 alone (control vector) using the amphiphile block copolymer Lutrol, which have shown previously the best transfection efficiency *in vivo* (20, 21). Because control tumors and pcDNA3.1 (control vector)-treated mice exhibit similar bone alterations (9), only pcDNA3.1-treated mice tibiae radiographs and micro-CT analyses were shown. Important osteolytic lesions were

observed on the tibia of POS-1 osteosarcoma-bearing mice treated with pcDNA3.1 alone compared with the pcDNA3.1-RANK-Fc group that exhibit minor lesions (Fig. 2A). These results were confirmed by the bone microarchitecture analysis, as the quantification of the specific bone volume revealed a reduced trabecular bone loss in RANK-Fc-treated mice compared with the pcDNA3.1 group: 24.01% versus 44.7%, respectively (Fig. 2B and C). The anti-bone resorption activity of the RANK-Fc transgene was thus confirmed *in vivo*, as RANK-Fc treatment induces a decrease of osteolytic lesions in the POS-1 model of osteosarcoma compared with the control group (CT: without tumor).

mRANK-Fc Transgene Expression Limits Osteosarcoma Progression and Increases Animal Survival

Mice received a preventive treatment with DNA-Lutrol complexes, the first injections being realized 7 days before osteosarcoma implantation. This strategy was chosen because *in vivo* transgene production is optimal 7 days after the construct injection, this time point corresponding to the day of tumor transplantation. The tumor volume was calculated twice a week following the measure of the two perpendicular diameters. The results show that the tumor volume of each animal was smaller in the RANK-Fc-treated group compared with the pcDNA3.1 or the control groups (Fig. 3A). As a result, the mean tumor volume 21 days after tumor implantation was significantly lower in the group

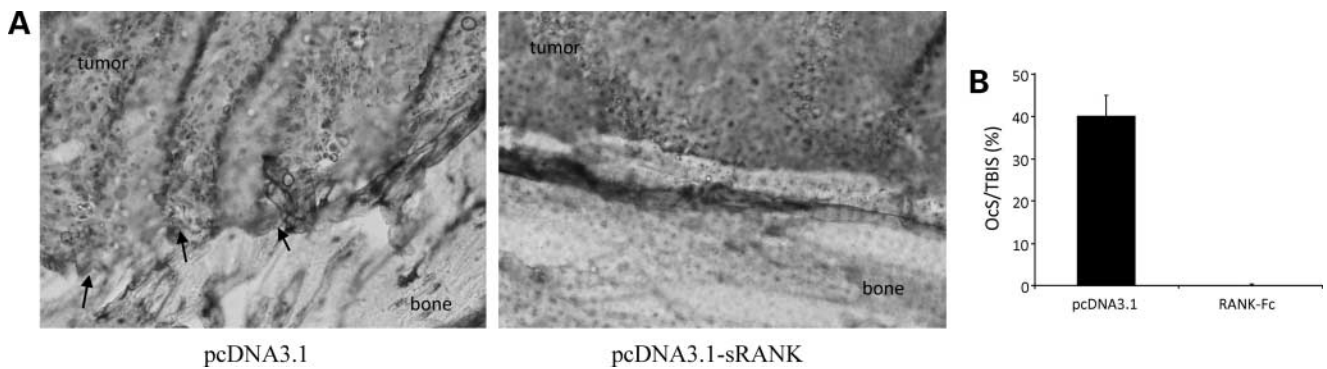


Figure 4. mRANK-Fc gene transfer inhibits osteosarcoma progression by preventing bone resorption. TRAP staining was realized on 6 μ m sections of tibias and tumors from pcDNA3.1- and RANK-Fc-treated mice (**A**). Numerous TRAP-positive osteoclasts were observed in POS-1 osteosarcoma-bearing mice treated by pcDNA3.1 (arrows). Magnification, $\times 320$. Osteoclast numbers at the tumor-bone interface surface (OcS/TBIS; **B**).

receiving RANK-Fc compared with the pcDNA3.1 group or the control group ($P < 0.05$), reaching $1,652.7 \pm 1,055$, $4,272.7 \pm 712.8$, and $4,504.1 \pm 2,162.2$ mm³, respectively (Fig. 3A). In addition, the incidence of animals bearing progressive tumors (tumor volume $> 2,000$ mm³) is significantly lower at day 21 in the RANK-Fc-treated animals (2/6) compared with controls or pcDNA3.1-treated mice (6 of 6 for both; Fig. 3A). These results enabled us

to calculate the relative tumor progression between days 12 and 21, which was significantly decreased by 62.7% ($P < 0.05$) and 64.3% ($P < 0.01$) in the RANK-Fc-treated group compared with the pcDNA3.1-treated and untreated groups, respectively (Fig. 3B). As a consequence, a significant increase ($P < 0.02$) of animal survival was observed with 57.1% of survival at day 28 in the RANK-Fc-treated group versus 0% in the pcDNA3.1-treated group

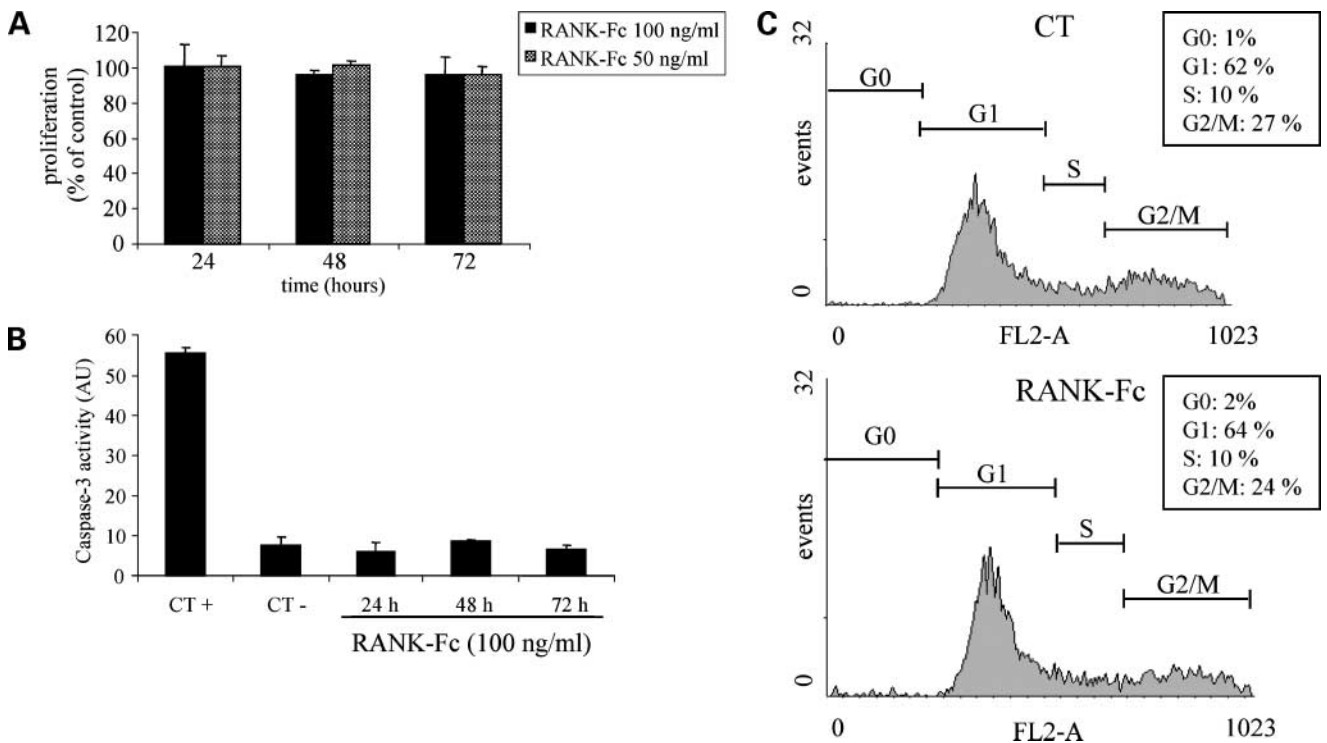


Figure 5. mRANK-Fc exerts no direct effect on osteosarcoma cells *in vitro*. Effects were assessed on POS-1 cell proliferation using a XTT assay as described in Materials and Methods. POS-1 cells were treated for 24 to 72 h with increasing concentrations of recombinant mRANK-Fc (50 and 100 ng/mL) that exhibits the same sequence as the mRANK-Fc transgene used for *in vivo* experiments (**A**). To determine the RANK influence on tumor cell apoptosis, the caspase-3 activity was analyzed in the cell lysate of POS-1 cells treated with 100 ng/mL mRANK-Fc for 24, 48, and 72 h (**B**). CT+, staurosporin (1 μ mol/L, 6 h) was used as positive control; CT-, POS-1 cells alone. Paralleled experiments were done on cell cycle distribution by fluorescence-activated cell sorting analysis in the absence or the presence of 100 ng/mL mRANK-Fc during 24, 48, and 72 h (**C**). As no effect could be detected, only the results obtained after 72 h of incubation are shown.

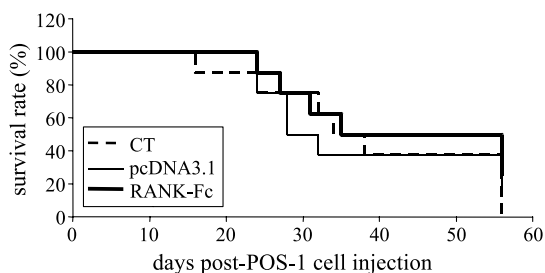


Figure 6. mRANK-Fc exerts no therapeutic effect in a model of tumor lesions that develop in lungs. A model of pulmonary tumor lesions was induced in C3H/HeN mice by the i.v. injection of 200 000 POS-1 cells in the retro-orbital vein. The Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning at 7 d before POS-1 cells injection up to 21 d post-implantation. The mice were separated into three groups ($n = 8$) treated as follows: control mice received vehicle (PBS) alone (CT), pcDNA3.1/Lutrol (pcDNA3.1), or pcDNA3.1-mRANK-Fc/Lutrol (RANK-Fc). The overall survival rate was compared with the three groups over a 56-d period.

and 14.3% in the untreated group (Fig. 3C). Animals died of respiratory distress because of lung metastasis or were sacrificed due to huge tumor development. Animals treated with RANK-Fc showed no pulmonary metastases at day 26.

All these results show that RANK-Fc not only exerts a protective effect on osteosarcoma associated bone lesions but also diminishes the tumor progression in bone, increasing the animal survival in an osteolytic model of osteosarcoma.

Expression of mRANK-Fc Inhibits Osteosarcoma Progression by Preventing Bone Resorption

Complementary to radiographic and micro-CT analyses, TRAP staining was done on osteosarcoma sections from mice treated or not with RANK-Fc to determine whether this cytokine could inhibit osteosarcoma-induced osteoclast formation. The development of large osteosarcoma tumors in this model is accompanied by increased osteoclastic bone resorption and increased recruitment of osteoclasts to the tumor-bone interface (Fig. 4A, arrows). The presence of TRAP-positive osteoclasts was abrogated in the sRANK-Fc group (Fig. 4A and B), confirming at the cellular level the inhibitory effect of RANK-Fc on osteosarcoma associated osteolysis.

In vitro, RANK-Fc Has No Effect on Tumor Cell Proliferation, Apoptosis, Migration, and Gene Expression

To determine whether *in vivo* RANK-Fc-inhibitory effect on tumor progression is direct on tumor cells, several *in vitro* experiments were done on osteosarcoma cell proliferation, apoptosis, cell migration, gene expression, and cell cycle using the POS-1 mouse osteosarcoma cell line corresponding to the *in vivo* model.

The viability of POS-1 cells treated with increasing concentrations of RANK-Fc was analyzed from 24 to 72 h using a XTT-based method. No significant effect was noticed even after 72 h of treatment with 100 ng/mL RANK-Fc (Fig. 5A) nor on POS-1 cell apoptosis as analyzed

by caspase activity assay (Fig. 5B). Similarly, RANK-Fc treatment had no effect on POS-1 cells migration (data not shown) and on the cell cycle phase distribution (Fig. 5C). RT-PCR analysis shows that a 100 ng/mL treatment during 72 h with RANK-Fc does not induce gene expression modulation of POS-1 cells (data not shown).

These data show that the inhibitory effects observed on tumor progression *in vivo* for the RANK-Fc-treated group are not due to a direct effect on POS-1 tumor cells.

RANK-Fc Treatment Does Not Affect Tumor Nodule Development in Lungs

As RANK-Fc exerts no direct effect on tumor cells, we hypothesize that the inhibitory effect observed in osteosarcoma development is the indirect consequence of bone resorption inhibition, thus depending on bone microenvironment. To show this hypothesis, another set of experiments was done in mice developing tumor lesions in lungs. This model was induced after POS-1 cell injection in the retro-orbital vein. The results presented in Fig. 6 show no significant differences between the different groups in the overall mouse survival rate, indicating that RANK-Fc exerts no inhibitory effect on tumor development outside the bone microenvironment.

Discussion

The mouse model of osteosarcoma used in this study is characterized by the presence of osteolytic lesions (24). The release of latent growth factors (such as insulin growth factor, transforming growth factor- β , bone morphogenetic protein, platelet-derived growth factor, and vascular endothelial growth factor) stored in the bone matrix during osteolysis stimulates the tumor growth in a vicious cycle that leads to tumor cell proliferation and tumor progression in bone sites (25, 26). Bone resorption inhibitors thus appear as one of the most promising tools to manage osteolytic lesions associated to primary or secondary bone tumors. The OPG/RANKL/RANK pathway offers multiple molecular checkpoints for therapeutic targeting in osteolytic tumors. Inhibition of this axis has shown therapeutic efficacy in restricting tumor-mediated osteolysis *in vitro* as well as in animal models of both bone metastasis (16, 27–29) and osteosarcoma (9). Indeed, previous results obtained in our laboratory in the same mouse osteosarcoma model with OPG gene transfer showed the relevance of using such antiresorptive factors as promising therapeutic approaches for primitive osteolytic tumors. However, the ability of OPG to block the TRAIL apoptosis pathway in cancer cells was noticed and there were concerns that this could lead to a burst in tumor growth (30). Denosumab is a fully human monoclonal antibody directed against RANKL and is currently under investigation in phase III clinical trials (31, 32). Denosumab was generated by immunizing the XenoMouse (33) with full-length human RANKL protein, producing a fully human IgG1 monoclonal antibody, which recognizes an epitope with a single amino acid difference between the mouse and the human sequences. Because human IgG1 can induce complement-dependent

cytotoxicity or antibody-dependent cell cytotoxicity to target cells (34), it was converted to a noncytotoxic IgG2 monoclonal antibody, known as Denosumab, which has an extremely high affinity (K_d , $\sim 10^{-12}$ mol/L) for human RANKL. The fact that Denosumab does not recognize rodent RANKL has complicated preclinical development, with only one relevant animal study conducted in cynomolgus monkeys (35). Therefore, as these anti-RANKL antibodies cannot be used in our mouse model of osteosarcoma, RANK-Fc, another member of the tumor necrosis factor receptor family was used in the present study. Indeed, RANK-Fc, the fusion of the extracellular domain of RANK (amino acids 22-209) with the constant region of human IgG1 has the potential advantage over OPG of greater specificity for RANKL (15). Delivery of RANK-Fc as a recombinant protein has shown promising results as a potential therapy through experiments in animal models, in that RANK-Fc limits hypercalcemia and osteolysis induced by myeloma or prostate cancer and reduces bone tumor establishment in these models (15, 16, 29, 36). Moreover, a recent study reported that RANK-Fc inhibition of RANKL has an antiosteoclast activity at doses that have no detectable immunoregulatory activity (37). Even if long-lasting expression of RANK-Fc could be provided at bone-protective levels using a retrovirus-mediated gene transfer approach by the use of genetically modified mesenchymal stem cells (17), the toxicity associated with the use of such viral vectors is extremely complex.

The results from the present study validate the nonviral gene transfer method and the therapeutic interest of RANK-Fc in osteosarcoma. This methodology has proven previously its efficacy to deliver and express therapeutic gene in the same mouse model of osteosarcoma (9). In the present study, we show that when injected every week, the DNA-Lutrol complexes can induce RANK-Fc overexpression at both local (muscle) and systemic levels in sufficient quantities to induce anti-bone resorption activity. The overproduction reached its maximum 7 days after injection and lasted for 15 days at the systemic level. The intramuscular injection of the RANK-Fc/Lutrol complexes induces a local RANK-Fc transgene production that blocks bone resorption as confirmed by radiography and histology and quantified by micro-CT analysis in an osteolytic model of osteosarcoma. Moreover, the same inhibitory effect on bone osteolytic lesions has been obtained by adenoviral delivery of RANK-Fc in a rat osteocondensant osteosarcoma (data already published; refs. 3, 27).

Given its role in osteoclastogenesis, it is likely that inhibition of osteosarcoma progression by RANK-Fc reflects, at least in part, its ability to inhibit bone resorption. Indeed, RANK-Fc has no direct activity on osteosarcoma cells as shown by *in vitro* studies on proliferation, migration, apoptosis, cell cycle, or phenotype analysis. Furthermore, the observation that RANK-Fc did not diminish development of nonosseous tumors (pulmonary tumor lesions induced by the same osteosar-

coma POS-1 cells injected i.v.) suggests that the ability of RANK-Fc to inhibit osteosarcoma development was not caused by a direct effect on tumor but rather specific to factors in the bone microenvironment. Given that RANKL expression has been shown previously in the POS-1 tumor by immunohistochemical analyses (9), the present data suggest that inhibition of RANKL activity diminishes the osteosarcoma progression. Moreover, other experiments done in our laboratory have shown that osteosarcoma cells express RANK and that RANKL is able to induce modulation of gene expression in these cells (24, 38, 39). Targeting RANKL is therefore a promising approach in bone tumor therapy. It has been shown that RANKL is implicated in the pathogenesis of bone metastasis at several levels: increased bone resorption as a result of excess RANKL results in the release of growth factors that facilitate tumor cell division and survival, and a recent study suggested a role for RANKL as a chemoattractant for certain cancer cells metastatic to bone (40). Furthermore, RANKL can stimulate both angiogenesis (41) and endothelial cell survival (42), suggesting a role for RANKL in supporting vascularization of bone metastases. This role could also be extended to osteosarcoma, which is a naturally highly vascularized tumor. As RANKL participates to the regulation of Treg function, it could also interfere in tumor response at this level (43, 44).

To inhibit RANKL activity, three candidates are potentially promising: the decoy receptor OPG, the soluble receptor RANK-Fc constructed as the fusion of RANK extracellular domain with the constant region of the human IgG1, and antibodies directed against human RANKL. The advantage of using anti-RANKL antibodies is that they do not inhibit the TRAIL apoptosis pathway. In a recent randomized, double-blind, double-dummy, active-controlled multicenter phase I clinical study, Body et al. (45) showed the efficacy of denosumab. A single s.c. dose of denosumab given to patients with multiple myeloma or bone metastasis from breast cancer yielded a dose-dependent and sustained reduction in bone resorption. However, as this therapeutic agent cannot be used in immunocompetent models of bone tumors in rodents, RANK-Fc, which is more specific of RANKL binding than OPG, seems therefore a better therapeutic tool for these pathologies.

In conclusion, these data show that RANK-Fc delivered by nonviral gene transfer is a promising therapeutic approach for osteolytic bone tumors such as osteosarcoma by targeting RANKL in the bone microenvironment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Complément de discussion des articles 1 et 2

L'ostéosarcome représente la plus fréquente des tumeurs osseuses primitives, avec un taux de survie de 65% à 5 ans justifiant le développement de nouvelles approches thérapeutiques pour cette pathologie. Comme cela a été décrit dans le cadre des tumeurs osseuses secondaires (Guisse, 2002), la problématique biologique de l'ostéosarcome repose sur l'existence d'un cercle vicieux qui s'établit entre la prolifération tumorale et la résorption osseuse. Les facteurs de croissance (tels que IGF, TGF- β , BMP, PDGF et VEGF) stockés dans la matrice osseuse et libérés au cours de l'ostéolyse tumorale stimulent en retour la croissance de la tumeur en site osseux. Ainsi, l'inhibition de la résorption osseuse est une des stratégies envisageables dans le traitement des tumeurs osseuses primitives pour rompre ce cercle vicieux.

RANKL, responsable de la différenciation, de l'activation et de la survie des ostéoclastes par son interaction avec son récepteur RANK est ainsi largement impliqué dans la régulation de la résorption osseuse. Dans l'optique de bloquer RANKL qui semble être une cytokine clé dans le développement de l'ostéosarcome ou d'autres pathologies malignes osseuses, d'autres approches thérapeutiques sont en cours d'étude. Ainsi, le Denosumab (AMG162), anticorps monoclonal entièrement humanisé et dirigé contre RANKL est actuellement l'objet d'une étude clinique de phase III (Lonberg, 2005; Weiner, 2006). Cependant, le Denosumab a été généré par l'immunisation de souris avec la forme complète de la protéine humaine RANKL, produisant un anticorps ne reconnaissant pas le RANKL murin, ce qui a limité le développement de protocoles précliniques, avec seulement une étude chez l'animal réalisée chez le singe cynomolgus (Kostenuik, 2005). Etant donné que l'anticorps anti-RANKL ne peut être utilisé dans notre modèle murin d'ostéosarcome, l'utilisation d'agents anti-résorption et potentiellement anti-tumoraux, tels que OPG ou RANK-Fc, libérés par transfert de gènes, semble représenter des candidats idéaux dans le développement d'une nouvelle approche thérapeutique de l'ostéosarcome.

En effet, plusieurs études démontrent que l'hypercalcémie et l'ostéolyse induites par les cellules tumorales dans le cas du myélome, du cancer du sein, des poumons ou de la prostate peuvent être prévenues par l'utilisation de l'OPG (Capparelli et al., 2000; Croucher et al., 2001; Morony et al., 2001; Zhang et al., 2001) ou de RANK-Fc administré en tant que protéines recombinantes (Feeley et al., 2006; Oyajobi et al., 2001; Sordillo and Pearce, 2003;

Zhang et al., 2003) et même associées à une réduction de la progression tumorale en site osseux dans les modèles animaux correspondants. De plus, une étude récente a rapporté que l'inhibition de l'activité des ostéoclastes en bloquant RANKL par RANK-Fc se fait à des concentrations n'induisant pas de réponses immunitaires (Miller et al., 2007).

Dans la première étude (article 1), nous décrivons pour la première fois l'intérêt thérapeutique de la forme OPG 1-194 dans le traitement de l'ostéosarcome, non seulement par la prévention des lésions osseuses associées à la tumeur, mais également par l'inhibition de la progression tumorale elle-même et l'apparition de métastases pulmonaires, conduisant ainsi à l'augmentation de la survie des animaux. Ces résultats ont été obtenus aussi bien dans un modèle d'ostéosarcome ostéolytique murin par transfert de gène utilisant des vecteurs synthétiques que dans un modèle d'ostéosarcome mixte (ostéocondensant et ostéolytique, reproduisant le tableau clinique humain) chez le rat par une approche adénovirale.

Précédent notre étude, d'autres données avaient déjà rapporté les effets anti-résorption osseuse de l'OPG sous forme de protéine recombinante dans plusieurs pathologies ostéolytiques par l'inhibition de l'activité de RANKL. En effet, l'OPG est capable de réduire le nombre d'ostéoclastes, d'augmenter le volume osseux (Bolon et al., 2001) et de réverser l'ostéopénie (Kostenuik et al., 2004) chez des souris ovariectomisées. Dans des pathologies malignes osseuses, l'OPG est également capable d'inhiber la perte osseuse associée au développement tumoral et d'augmenter la survie des animaux (Chanda et al., 2008). De plus, l'OPG est capable d'inhiber l'activité de RANKL exprimé par les cellules tumorales dans le myélome multiple et ainsi de prévenir les lésions ostéolytiques (Croucher et al., 2001). De même, OPG inhibe l'ostéoclastogénèse (Yamaguchi et al., 1998; Yonou et al., 2007; Zhang et al., 2001) et l'apparition de lésions ostéoblastiques (Kiefer et al., 2004) dans le cancer de la prostate.

Une étude récente de Corey et collaborateurs a révélé que la surexpression de l'OPG dans les cellules tumorales prostatiques n'affectait pas directement la prolifération de ces cellules, mais diminuait indirectement la croissance de la tumeur dans le microenvironnement osseux (Corey et al., 2005).

Les données de notre étude montrent des résultats similaires dans les deux modèles d'ostéosarcomes utilisés (rat et souris). Or l'OPG n'exerce pas d'effets directs sur les cellules tumorales en terme de fixation aux cellules tumorales, de prolifération, de distribution dans le cycle cellulaire ou encore d'apoptose, mais exerce un effet indirect sur la progression tumorale par l'inhibition de l'ostéolyse associée. Ces résultats peuvent s'expliquer par le fait

que l'OPG, par l'inhibition de l'ostéolyse médiée par RANKL, bloque la libération de facteurs de croissance piégés dans la matrice osseuse et favorables à la prolifération tumorale.

L'ensemble de ces résultats a été confirmé par la seconde étude (article 2) utilisant un autre agent anti-résorption, RANK-Fc qui correspond à la fusion du domaine extracellulaire de RANK murin (acides aminés 22-201) avec la région constante de l'immunoglobuline G1 humaine (IgG1). L'utilisation de RANK-Fc a un double avantage : il possède une plus grande spécificité pour RANKL par rapport à l'OPG (Sordillo and Pearce, 2003), et n'a pas la capacité de se lier à TRAIL contrairement à l'OPG. En effet, l'aptitude de l'OPG à bloquer l'apoptose des cellules tumorales potentiellement induite par TRAIL, et par conséquent le fait que l'OPG pourrait représenter un agent potentiellement pro-tumoral, limite son utilisation en tant qu'agent thérapeutique dans les pathologies ostéolytiques d'origine tumorale. Les résultats de cette étude valident pour la deuxième fois, l'utilisation d'une approche non virale de transfert de gènes et l'intérêt thérapeutique de molécules à activité anti-résorption osseuse comme RANK-Fc dans le traitement de l'ostéosarcome. Dans la présente étude, nous avons montré que la surexpression local (muscle) et systémique du transgène était suffisante pour induire une inhibition de la résorption osseuse. En effet, lorsque l'ADN codant RANK-Fc murin associé au vecteur synthétique F68 de la famille du Lutrol[®] est injecté chaque semaine en site intra-musculaire, une surexpression de RANK-Fc est produite au niveau locale (muscle) et systémique. Nous avons ainsi mis en évidence une prévention des lésions ostéolytiques par radiographie, quantifiée par analyse au microscanner, ainsi qu'une diminution du nombre d'ostéoclastes recrutés sur des coupes de tibia/tumeur après marquage TRAP. De plus, nous avons obtenu des résultats similaires sur l'inhibition de lésions ostéolytiques avec l'utilisation d'adénovirus codant RANK-Fc dans un modèle d'ostéosarcome de rat ostéocondensant [données publiées (Baud'huin et al., 2007; Wittrant et al., 2004)].

Compte tenu de son rôle dans l'ostéoclastogenèse, il est probable que l'inhibition de la progression de l'ostéosarcome par RANK-Fc peut être le résultat de l'inhibition de la résorption osseuse. Comme l'OPG, RANK-Fc n'exerce pas d'activité directe sur les cellules d'ostéosarcome POS-1. De plus, la libération de RANK-Fc n'a pas d'effet thérapeutique sur le développement de nodules tumoraux pulmonaires induits par l'injection de cellules d'ostéosarcomes POS-1 en site intraveineux. Ces résultats suggèrent que la capacité de RANK-Fc à inhiber le développement de l'ostéosarcome n'a pas été causée par un effet direct sur la tumeur, mais plutôt indirectement par l'inhibition de la résorption osseuse.

De plus, nous avons mesuré une augmentation de l'expression de RANKL sérique chez les souris développant un ostéosarcome [article 1, (Lamoureux et al., 2007b)] qui peut être le reflet de l'augmentation de la concentration de RANKL observée dans le microenvironnement tumoral osseux. Une question reste cependant en suspens : quelles sont les cellules responsables de l'augmentation de la concentration de RANKL dans le microenvironnement tumoral osseux ? Dans d'autres modèles de tumeurs osseuses, RANKL est produit directement par les cellules tumorales (Croucher et al., 2001; Zhang et al., 2001) ou plus généralement par des cellules présentes dans l'environnement tumoral osseux (Chikatsu et al., 2000; Thomas et al., 1999). Nous avons précédemment démontré et publié que les cellules d'ostéosarcome murin POS-1 expriment le récepteur RANK mais pas RANKL *in vitro* [(Wittrant et al., 2006); en annexe]. Par conséquent, les résultats de la présente étude suggèrent qu'*in situ* plusieurs facteurs présents dans le microenvironnement tumoral osseux, probablement libérés lors de l'hyper-résorption osseuse associée à la tumeur, pourraient induire l'expression de RANKL par les cellules tumorales elles-mêmes. A l'heure actuelle, on ne sait pas si ce sont vraiment les cellules tumorales qui synthétisent RANKL, car l'immunohistochimie n'est pas assez précise, mais révèle la présence de l'expression de RANKL sous forme de petits îlots sur des coupes de tumeurs.

De ce fait, l'effet inhibiteur de l'OPG ou de RANK-Fc sur la progression tumorale peut être la conséquence du blocage de l'interaction RANK/RANKL. Cette hypothèse a été confirmée par l'observation de l'inhibition de l'expression sérique de RANKL chez les souris traitées avec l'OPG comparativement aux souris non traitées. D'autres expérimentations réalisées dans notre laboratoire ont montré que les cellules d'ostéosarcome exprimaient RANK membranaire et qu'il était fonctionnel, car RANKL était capable d'induire la modulation de l'expression des gènes dans ces cellules (Mori et al., 2007a; Mori et al., 2007b; Wittrant et al., 2006). RANKL est donc une cible thérapeutique privilégiée dans le traitement des tumeurs osseuses.

Il a été démontré que RANKL était impliqué dans le développement de métastases osseuses à plusieurs niveaux: par l'augmentation de la résorption osseuse car un excès de RANKL induit la libération de facteurs de croissance qui facilitent la division des cellules tumorales et leur survie. De plus, une étude récente a suggéré que RANKL pouvait jouer un rôle important dans le tropisme de cellules cancéreuses à métastaser à l'os (Jones et al., 2006). Il a été également rapporté que RANKL peut stimuler l'angiogenèse (Min et al., 2003) et la survie des cellules endothéliales (Kim et al., 2002), suggérant un rôle important de RANKL

dans la vascularisation des métastases osseuses. Ce rôle pourrait aussi être étendu à l'ostéosarcome, qui est une tumeur naturellement très vascularisée. De plus, nous avons montré que l'OPG était capable d'inhiber le développement de métastases pulmonaires dans le modèle d'ostéosarcome murin (article 1).

En conclusion de ces deux études, nous avons démontré par l'utilisation de deux approches méthodologiques différentes de transfert de gène le potentiel thérapeutique de l'OPG 1-194 et de RANK-Fc dans le traitement de l'ostéosarcome, montrant pour la première fois que RANKL est une cytokine clé dans le développement de cette pathologie par sa capacité à induire la résorption osseuse. Ainsi RANKL est une cible thérapeutique privilégiée dans le traitement de l'ostéosarcome.

Conclusion de la partie I

Les résultats obtenus dans ces deux études par la présence de la surexpression d'OPG ou de RANK-Fc dans des modèles d'ostéosarcome ostéolytique chez la souris ou ostéocondensant chez le rat sont en accord avec ceux de la littérature, notamment en terme de prévention des lésions osseuses associées à la progression tumorale primitive (résultats présents) ou secondaire (bibliographie) en site osseux.

Par ces deux études, nous avons validé la mise en place de protocoles thérapeutiques utilisant le transfert de gènes par une approche non virale utilisant les vecteurs synthétiques dans un modèle d'ostéosarcome de souris.

Il est maintenant bien admis que RANKL, par l'augmentation de sa concentration dans le microenvironnement tumoral osseux est fortement impliqué dans le développement tumoral et la mise en place du cercle vicieux qui s'instaure entre la prolifération tumorale et la résorption osseuse. Ces données font de cette cytokine une cible thérapeutique privilégiée. Pour inhiber l'activité de RANKL, trois stratégies sont potentiellement prometteuses:

- l'utilisation de l'OPG, facteur anti-résorption, qui a montré son potentiel thérapeutique par sa capacité à prévenir les lésions ostéolytiques et à inhiber indirectement la progression de la tumeur en site osseux et ainsi d'augmenter la survie des animaux.

- l'utilisation de l'anticorps anti-RANKL (Denosumab) qui est actuellement testé dans une étude clinique de phase III. Dans une étude multicentrique récente randomisée en double aveugle de phase I, Body *et al.* (Body, 2006) avait montré l'efficacité du Denosumab (AMG 162). En effet, une seule dose de Denosumab administrée en sous-cutané à des patients présentant un myélome multiple ou des métastases osseuses suite à un cancer du sein diminue de manière dose-dépendante et durable la résorption osseuse. Toutefois, le Denosumab représentant une forme humanisée ne peut pas être utilisé dans des modèles de tumeurs osseuses syngéniques chez les rongeurs, limitant l'utilisation de cet anticorps dans des études pré-cliniques.

- RANK-Fc, qui est plus spécifique contre RANKL que l'OPG, semble être un meilleur outil thérapeutique pour les pathologies osseuses malignes. L'avantage principal de l'utilisation de RANK-Fc, tout comme le Denosumab, est qu'il n'entrave pas la voie de TRAIL contrairement à l'OPG. En effet, l'OPG possède la capacité de se lier à TRAIL et d'inhiber l'apoptose des cellules tumorales induite par cette cytokine pouvant ainsi

représenter un facteur de survie pour les cellules tumorales. Les preuves cliniques de l'importance de l'apoptose des cellules tumorales médiée par TRAIL sont confirmées par le fait que de nombreuses cellules tumorales y compris les cellules tumorales osseuses expriment les récepteurs de TRAIL (Bouralexis et al., 2004; Debatin and Krammer, 2004; Wang and El-Deiry, 2003). De plus, TRAIL est considéré comme un agent anticancéreux prometteur (Kapoor et al., 2008; Shipman and Croucher, 2003).

Pour conclure, l'ensemble de ces données pré-cliniques montrent l'intérêt thérapeutique de l'utilisation de l'OPG et de RANK-Fc par la prévention des lésions ostéolytiques associée à une inhibition indirecte du développement tumoral dans le traitement de l'ostéosarcome.

Partie II

**Rôle de l'ostéoprotégérine dans les
pathologies ostéolytiques malignes :
molécule anti- ou pro-tumorale ?**

Introduction

L'inhibition de la résorption osseuse dans le traitement des tumeurs touchant l'os est une des voies thérapeutiques possible pour rompre le cercle vicieux entre la prolifération tumorale et la résorption osseuse au regard des résultats obtenus dans la partie I. La triade moléculaire OPG/RANK/RANKL constitue l'axe thérapeutique privilégié dans le développement de ces nouvelles stratégies. Comme nous l'avons vu précédemment, l'inhibition de l'interaction de RANKL à son récepteur RANK par l'utilisation de molécules anti-résorptives telles que RANK-Fc ou l'OPG a montré des résultats prometteurs.

Cependant le rôle de l'OPG comme agent thérapeutique dans les cancers est remis en question du fait de sa capacité à lier TRAIL pouvant par conséquent devenir un facteur de survie pour les cellules tumorales. Cette deuxième partie est consacrée à mieux définir le rôle de l'OPG dans le traitement de pathologies malignes osseuses et plus particulièrement de l'ostéosarcome.

L'article 3 présentera une comparaison de l'activité thérapeutique de deux formes d'OPG délivrées par transfert de gènes utilisant des vecteurs synthétiques : la première représente la forme complète ou native de l'OPG (1-401) alors que la deuxième est la forme tronquée OPG 1-194, dépourvue de ses deux domaines de mort, du domaine de liaison à l'héparine et de la cystéine 400 responsable de la dimérisation de l'OPG. L'objectif de cette étude sera d'essayer de comprendre la relation structure-fonction de la partie 195-401 de l'OPG, vis-à-vis de son activité anti-tumorale et anti-résorption osseuse dans un modèle de tumeur osseuse ostéolytique et d'étudier les mécanismes moléculaires associés : liaison à TRAIL, implication des protéoglycanes (PGs) dans la biodisponibilité et l'activité biologique de l'OPG.

L'article 4 est une revue qui fait état de nombreuses études débattant sur le rôle de l'OPG dans le cancer comme molécule anti- ou pro-tumorale. La compréhension du rôle du microenvironnement tumoral osseux est complexe et dépend des différents protagonistes présents (OPG, RANKL, TRAIL, PGs,...) dont les fonctions sont déterminées par leur relative concentration, expression spatiale à un temps donné. Cette étude résume les connaissances actuelles *in vitro* et *in vivo* sur la place qu'occupe l'OPG dans l'établissement de ce microenvironnement tumoral osseux et si elle peut être encore considérer comme molécule thérapeutique dans le traitement de cancers osseux.

Article 3

« Les glycosaminoglycanes comme régulateurs potentiels de l'activité thérapeutique de l'OPG dans l'ostéosarcome »

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L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives maligne qui se développe principalement chez les enfants et les adolescents. Malgré les avancées récentes de la chimiothérapie et de la chirurgie, le taux de survie est d'environ 50% à 5 ans. L'ostéoprotégérine (OPG) est un inhibiteur de la différenciation et de l'activation ostéoclastique, mais son utilisation comme agent thérapeutique dans le traitement de cancer associé à une ostéolyse demeure controversée, du fait de sa capacité à se lier et inhiber l'effet apoptotique du TNF-Related Apoptosis Ligand (TRAIL) sur les cellules tumorales. Les effets thérapeutiques de la forme native de l'OPG (1-401) et de l'OPG 1-194 dépourvu de son domaine de liaison à l'héparine, libérées par transfert de gène non viral, ont été comparées dans un modèle d'ostéosarcome ostéolytique murin. L'incidence tumorale, la progression et les lésions osseuses associées ont été significativement diminuées dans le groupe traité avec l'OPG 1-194, mais pas dans le groupe traité avec l'OPG 1-401 en comparaison aux groupes contrôles. Comme nous avons montré que l'expression de RANKL, TRAIL et des glycosaminoglycanes était augmentée dans le microenvironnement tumoral en comparaison au tissu contrôle, ceci suggère que l'activité biologique de l'OPG 1-401 peut être modulée par un de ces protagonistes. Les analyses par résonance plasmonique de surface réalisées avec l'OPG, TRAIL et les GAGs ont révélé que TRAIL était capable de se lier aux deux formes d'OPG avec la même affinité. De plus, comme l'OPG 1-194 et l'OPG 1-401 inhibent de façon similaire l'apoptose des cellules tumorales médiée par TRAIL, il est suggéré que TRAIL n'est pas impliqué dans la modulation de l'activité biologique de l'OPG dans l'ostéosarcome. Cependant, les GAGs étant capables d'inhiber la liaison de l'OPG 1-401 mais pas de l'OPG 1-194 avec TRAIL ou RANKL, ils peuvent représenter des régulateurs potentiels de la biodisponibilité et donc de la modulation de l'activité anti-tumorale de l'OPG dans le microenvironnement tumoral osseux.

Glycosaminoglycans as potential regulators of osteoprotegerin therapeutic activity in osteosarcoma

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Running title: glycosaminoglycans regulate OPG antitumoral activity *in vivo*

Key words: osteoprotegerin, glycosaminoglycan, TRAIL, RANKL, osteosarcoma

Abstract

Osteosarcoma is the most frequent primary bone malignant tumor that develops mainly in children and adolescents. Despite recent improvements in chemotherapy and surgery, survival rate is around 50% after 5 years. Osteoprotegerin (OPG) is a potent inhibitor of osteoclast differentiation and activation but its use as therapeutic agent in cancer associated osteolysis remains controversial due to its ability to bind and inhibit the apoptotic effect of TNF-Related Apoptosis Inducing-Ligand (TRAIL) on tumor cells. The therapeutic effects of full length OPG (1-401) and OPG 1-194 lacking its heparin-binding domain delivered by non-viral gene therapy were compared in a murine model of osteolytic osteosarcoma. Tumor incidence, progression and associated bone lesions were significantly diminished in the OPG 1-194 group, but not in the OPG 1-401 group as compared to controls. As RANKL, TRAIL and glycosaminoglycans (GAGs) were shown to be over-expressed in osteosarcoma environment as compared to control tissue, OPG 1-401 bioactivity may be modulated by one of these protagonists. Surface plasmon resonance analyses performed with OPG, TRAIL and GAGs revealed that TRAIL binds both forms of OPG with the same affinity. In addition as OPG 1-194 and 1-401 similarly inhibit TRAIL-induced apoptosis, it suggests that TRAIL is not involved in the modulation of OPG bioactivity. However, as GAGs inhibit OPG 1-401 but not OPG 1-194 binding to TRAIL or to RANKL, they may represent potent regulators of OPG availability and anti-tumor activity in bone tumor microenvironment.

INTRODUCTION

Osteosarcoma, the most common primary malignant bone tumor in both children and adults is characterized by the development of bone or osteoid substance by the tumor cells (1). The disease develops mainly in young patients between 10 and 25 years old with a peak of incidence at 18 years (2). The tumor generally develops on the average part of the long bones (femur, tibia), at the vicinity of an articulation, mainly the knee or the shoulder. Current therapeutic protocols consist in neo-adjuvant poly-chemotherapy associated to definitive surgery with limb salvage. The rate of long-term survival is 50-70% at 5 years for patients with localized tumor but only 15 to 20% when pulmonary metastases are detected at diagnosis or not responding to therapy, or with disease relapse, indicating the need to develop new adjuvant options for this pathology.

Primary tumor development in bone site is related to the existence of a vicious cycle between bone resorption and tumor proliferation, as reported for secondary bone tumors (3). Thus, it is suggested that osteosarcoma is accompanied by a disorder in the expression of osteoprotegerin (OPG), receptor activator of nuclear factor κ B (RANK) and its ligand RANKL (4), the main molecular protagonists involved in the regulation of bone resorption mechanisms. During bone pathology from tumor origin, the activated osteoclasts degrade bone tissue, leading to the release of factors favourable to tumor cell proliferation (Transforming Growth Factor- β , Insulin Growth Factor). In turn, tumor cells secrete factors [RANKL, Tumor Necrosis Factor (TNF) α and β , Interleukin-1 and 6, PTH-rP] allowing the differentiation and activation of osteoclasts (5, 6). Therefore, blocking this vicious cycle by an anti-bone resorption strategy is a promising therapeutic approach. Among these anti-bone resorption molecules, the member of the TNF receptor superfamily OPG is a promising candidate (7) as it acts as a decoy receptor for RANKL, thus inhibiting the differentiation and the activation of osteoclasts and their osteolytic activity (6). The results obtained with OPG in the treatment of different tumor associated bone pathologies such as multiple myeloma, bone metastases or osteosarcoma are encouraging (8-10).

However, the role of OPG in cancer is controversial (11), as for example, a recent study reported that OPG produced by tumor cells increase tumor growth whereas OPG-Fc exerts opposite effects (12). Indeed, OPG may play a role in tumor cell survival due to its ability to bind another member of the TNF super family, the TNF Related Apoptosis Inducing Ligand (TRAIL) which induces apoptosis of tumor cells without affecting normal cells (13). To this regard, several *in vitro* studies have demonstrated that OPG is a survival factor for

TRAIL expressing tumor cells such as prostate cancer (14), breast cancer (15) or multiple myeloma (16) cells.

As OPG contains an heparin-binding domain, the glycosaminoglycan (GAG) part of proteoglycans may also modulate OPG biological activity. GAGs are unbranched polysaccharides composed of repeated units of alternating uronic acids and hexamine or galactose (17). Most GAGs are covalently bound to core proteins to form proteoglycans (PGs). Posttranslational modifications result in specific motifs that bind to a large variety of ligands, regulating growth factor signalling, cellular behaviour, inflammation, angiogenesis and the proteolytic environment (18). The role of the heparin-binding domain in OPG has been reported by Standal *et al.* who demonstrated that myeloma cells internalize and degrade OPG through its binding to syndecan-1 (19). This observation has been strengthened by the fact that syndecan-1 is involved in OPG-induced monocyte chemotaxis (20). More recently, our group demonstrated a key function of heparin and heparan sulfate in the activity of the molecular triad RANK-RANKL-OPG (21). However, no study on GAG modulation of OPG bioactivity has been conducted so far *in vivo*.

To get further insight into the regulation of OPG bioactivity in bone tumors, *in vivo* experiments were performed comparing the biological activity of two forms of OPG [OPG full length (1-401) and OPG (1-194) lacking its heparin binding domain, the two death domains, and the C-400 responsible for OPG dimerization] administered by non viral gene transfer in a model of osteolytic osteosarcoma. Moreover, plasmon resonance surface analyses allow us to determine the relative involvement of TRAIL and GAGs in the regulation of OPG binding properties, to further hypothesize on the structure-function relation of the OPG 195-401 domain towards its anti-tumor and anti-resorption activities in osteosarcoma.

MATERIALS & METHODS

Cell lines

- The murine osteosarcoma cell line POS-1 derived from mouse spontaneous osteosarcoma (22), was cultured in RPMI 1640 medium (Bio Whittaker) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 2 mmol/L L-glutamine.
- The human osteosarcoma cell line MG63 purchased from American Type Culture Collection (Manassas) was cultured in Dulbecco's modified Eagle's medium

(DMEM, Bio Whittaker) supplemented with 5% fetal bovine serum and 2 mM L-glutamine.

- Human embryonic kidney cells (2.93) stably transfected with the cDNA encoding the full-length form of murine RANKL (2.93RL) were a kind gift of Dr. R. Josien (INSERM U643, Nantes, France). The pcDNA3 – full-length form of murine RANKL – was stably transfected into the 2.93 cells cultured in DMEM medium that contained 10% FBS by using polyethylenimine. After 4 days, the cells were transferred to media containing G418 (Invitrogen) for continuous selection of the surviving cells. Two weeks later, the surviving clones were analyzed by Western blot analysis to detect the expression of RANKL protein.

In vivo experimentations

- *Plasmids*: cDNAs encoding the murine OPG truncated form (1-194) or full length sequence (1-401) were kindly provided by Amgen Inc and were cloned in the pcDNA3 plasmid (Invitrogen) respectively between the *HindIII* / *XhoI* and *Not I* / *Xba I* sites using standard cloning procedures. The empty pcDNA3 was used as a control.

- *Mouse osteosarcoma model*: four-week-old male C3H/HeJ mice (Elevages Janvier) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. The mice were anaesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with buprenorphine (0.05 mg/kg; Temgésic®, Schering-Plough) prior to subcutaneous inoculation of POS-1 cell suspension (2×10^6 cells in 50 μ l PBS) in the hind footpad of the mice. Mice develop a primary tumor at the site of injection in three weeks that can be transplanted to mice of the same strain as a small fragment ($2 \times 2 \times 2$ mm³) in close contact with the tibia, as previously reported (23). Tumors which appeared at the graft site approximately 8 days later lead to osteolytic lesions that reproduce those observed in human osteosarcoma and are associated with the development of pulmonary metastases over a 3 week-period (23). The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a calliper, according to the following formula: $V = 0.5 \times L \times (S)^2$, where L and S are respectively the largest and smallest perpendicular tumor diameters.

- *Formulation preparations*: ®Lutrol, a block copolymer consisting of poly(ethyleneoxide)₇₅ - poly(propyleneoxide)₃₀ - poly(ethyleneoxide)₇₅ was generously provided by BASF (Mount Olive). Stock solutions were prepared at 20% (w/v) in water and stored at 4°C. Formulations of DNA with block copolymers were prepared by equivolumetric mixing block copolymers in

water and DNA solution at the desired concentration (50 µg/muscle), as already reported (24). For intramuscular injections, mice were anesthetized with hypnomidate (Etomidate®, 40 mg/kg) and fifty microliters of block copolymers/DNA formulations were injected into shaved tibial anterior muscles at one site using a microfine syringe.

- Experimental protocols: Groups of 6 mice transplanted with POS-1 osteosarcoma fragments were assigned respectively as controls (no injection), control vectors (®Lutrol/empty pcDNA3 plasmid), p-mOPG 1-401 (®Lutrol/pcDNA3-mOPG full length) and p-mOPG 1-194 (®Lutrol/pcDNA3-mOPG 1-194). The ®Lutrol/DNA formulations were injected into both tibial anterior muscles once a week, beginning 7 days before osteosarcoma implantation up to 21 days post-implantation. The tumor volume was calculated as described above. Treatment continued until each animal showed signs of morbidity, which included cachexia or respiratory distress, at which point they were sacrificed by cervical dislocation. Lung tumor dissemination was assessed at necropsy. Three independent experiments were performed.

- Micro-scanner and radiological analyses: Analysis of architectural parameters was performed using the high resolution X-ray micro-CT system for small animal imaging SkyScan-1072 (SkyScan). Relative volume (BV/TV) of the femora [total bone (cortical + trabecular) or trabecular bone] was quantified at necropsy in the osteosarcoma groups that received p-mOPG 1-194 or p-mOPG 1-401, compared to that of osteosarcoma control mice (no injection or control vector). Radiographs on anesthetized animals [xylazine (Rompun®) – ketamine (Imalgène 500®) combination at respectively 8% and 13% in PBS; 100µl/10g] were realized every week and at the time of necropsy with a mammography PLANMED Sophie apparatus (SN RAH 40710).

Histology Analysis

After sacrifice, tibia were conserved and fixed in 10% neutral buffered formalin at 4°C, decalcified (PBS-EDTA) and embedded in paraffin for Tartrate Resistant Acid

Phosphatase (TRAP) staining. Five-µm sections were cut and stained for TRAP to identify osteoclasts by one hour incubation in a 1 mg/ml naphthol-AS-TR-phosphate, 60mM N-N-Dimethylformamid, 100mM Sodium tartrate and 1 mg/ml Fast red TR salt solution (all from Sigma Chemical Co). The counterstain was performed with hematoxyline. The number of osteoclasts was evaluated on the cortical bone by manually counting by light microscopy.

RNA isolation and real-time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen). First strand cDNA was synthesized at 37°C for 1 hour from 5 µg of total RNA in a 50 µl mixture containing RT buffer, 0.5 µg

Random Primers, 0.5 mM dNTP mix, 20 U RNAsout and 400 U Murine Moloney Leukemia Virus-Reverse Transcriptase (MMLV-RT) (all from Invitrogen). Sequences of primers used for real-time PCR are listed in table 1. The real-time PCR contained, in a final volume of 10 μ l, 10 ng of reverse transcribed total RNA, 300 nM of the forward and reverse primers and 5 μ l of 2x SYBR green buffer (Biorad, Marnes-la-Coquette, France). PCRs were carried out in triplicate in 96-well plates, using the Chromo4TM System (Biorad). *Mus musculus* hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), Beta-actin (*B-actin*), cyclophilin B (*cycloB*) and cytochrome c-1 (*cyc1*) were used as invariant controls. Analysis was performed using the Vandesompele method (25).

Glycosaminoglycan content in tumor tissue

- Small animal imaging: *GAG imaging was performed using a γ camera especially devised for small animal imaging (γ IMAGER, Biospace Mesures). The gamma camera consists of a R 3292 Hamamatsu position-sensitive photomultiplier having a continuous 4 mm thick x 120 mm diameter CsI(Na) crystal leading to a 10 cm field of view. The energy resolution and intrinsic planar resolution of the basic system are given as 11% at 140 keV and < 2mm full width at half maximum (FWHM) respectively. For mouse planar imaging, the camera was equipped with a 1.3/0.2/20 collimator (hole diameter/septum thickness/height in mm) leading to a sensitivity given as 330 cpm/ μ Ci. For imaging procedures, mice were anaesthetised by intraperitoneal (i.p.) injection of 40 μ l of a solution of ketamine and xylazine solution (4:1). Anaesthetized animals iv injected with the tracer of glycosaminoglycan ^{99m}Tc-N-[triethylammonium]-3-propyl-[15]ane-N5 (NTP 15-5) (25 MBq) were placed supine over the collimator of the gamma camera, and a posterior whole body image started 15 min after tracer administration. Acquisition was performed in List Mode for a 10 minute-duration, with a 15% window centered on the 140-keV photopeak of ^{99m}Tc.*

- Radiolabelled tracers: *the NTP 15-5 was prepared and radiolabelled with ^{99m}Tc by the stannous chloride method as previously described, with a specific radioactivity of 25 MBq/ μ mol (26). The quality control was performed with Whatman Partisil® KCF18F strip thin layer chromatography, using methanol/acetonitrile /tetrahydrofuran /ammonium acetate (1N) (3:3:2:2) as eluent.*

- *alcian blue staining: the tumor was fixed in 10% neutral buffered formalin and embedded in paraffin. Four- μ m sections were cut using microtome, dehydrated and stained with alcian blue (0.1%), and then counterstained with Groat's haematoxylin and Phosphomolybdic acid/Orange-G. The sections were examined by light microscopy.*

Cell proliferation analysis

Replicate subconfluent cultures of MG63 cells seeded in 96-well plates were treated for 24 to 72 hours with 50 ng/ml of recombinant human TRAIL (Promokine) in the absence or the

presence of OPG. In this assay, two forms of recombinant murine OPG were used: a truncated 22-194 form (generous gift from Amgen Inc) and a complete form (22-401) purchased from R & D. Cell viability was determined by the sodium 3' [1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche Molecular Biomedicals). The effects of heparin (50nM; Sigma) were studied in the same experimental conditions.

ELISA assays

RANKL levels were measured in supernatant of 2.93 RANKL cells using a DUOset ELISA, with a sensitivity of 0.05 ng/ml (TRANCE/TNFSF11, R&D System). The procedure was carried out according to the manufacturer's recommendations and the absorbance at 450 nm was measured using a microplate reader (Victor, Hewlett Packard). Detection of mOPG was also realized *in situ* at the injection site: the tibial anterior muscle, as previously described (10).

Surface Plasmon Resonance analysis

The experiments were carried out on a BIAcore 3000 instrument (BIAcore). All experiments were performed at 25°C. For kinetic analysis, 260 resonance units (RU) of TRAIL were covalently coupled to a research grade CM5 chip (BIAcore) as recommended by the manufacturer. Increasing concentrations of OPG 1-401 or OPG 1-194 ranging from 1 to 520 nM in Hepes-buffered saline were injected at a flow rate of 30 µl/min for 5 min and dissociation was monitored for 15 min. Regeneration was achieved with NaOH (4.5mM) after each cycle. For binding analysis, RANKL was immobilized at 1630 RU on a CM5 chip. Binding of OPG 1-401 alone or preincubated for 60 min with oligosaccharide 16 (16 sugar units, kindly provided by Pr Gallagher, University of Manchester) was assayed for 3 min at a flow rate of 20 µl/min, followed by dissociation for 3.5 min. The resulting sensorgrams were fitted using BiaEval 4.1 software (BIAcore).

Data analyses:

For *in vivo* experimentations, the ANOVA test was used. The differences of actuarial survival were determined by the log-rank test on a Kaplan Meier survival curve. Statistical evaluation

of the *in vitro* proliferation data was performed using the Student's *t*-test. Results are given as mean $\pm$ SD and results with $p < 0.05$ were considered significant.

RESULTS

OPG 1-194 but not OPG 1-401 transgene expression decreases osteosarcoma development

Mice received an intramuscular injection of DNA formulation encoding murine OPG 1-194 or OPG 1-401 as a preventing treatment 7 days before osteosarcoma implantation, followed by one injection per week during 3 weeks. This time schedule has been chosen as the OPG transgene production is optimal 7 days after the injection of the DNA formulation, leading to a high OPG production at the time of osteosarcoma induction by tumor transplantation. Over-expression of mOPG (1-194 and 1-401) was confirmed by ELISA in muscle of the corresponding mice versus mice injected with pcDNA3 alone or control osteosarcoma-bearing mice (data not shown). All animals injected i.m. with the plasmid construct encoding mOPG 1-194 exhibit a significant decrease of the tumor volume at day 20 (633.1 mm^3) as compared to the animals that received pcDNA3 alone or OPG 1-401 in the same conditions (respectively 3048.8 mm^3 , $p < 0.01$ and 2674 mm^3 , $p < 0.05$), or to control osteosarcoma-bearing mice which developed progressive tumor (3848.4 mm^3 , $p < 0.001$; Fig 1A). In addition, when each animal is considered individually, the tumor incidence of mice developing a progressive primary tumor (tumor volume $\geq 1000 \text{ mm}^3$) is significantly diminished at day 18 in OPG 1-194 treated animals (0/5) as compared to controls, pcDNA3 or OPG 1-401 treated mice (respectively 6/6, 5/5 and 5/5; Fig 1B). These results reveal that the OPG anti-tumor activity is structure-dependent: the anti-tumor effect of OPG 1-194 was confirmed as previously reported in osteosarcoma (10) whereas OPG 1-401 over-expression did not significantly affect tumor growth (tumor volume was similar to pcDNA3-injected mice). A significant increase of survival rate was observed in the OPG 1-194 group (Fig 1C, $p < 0.01$) as a consequence of decreased pulmonary metastases dissemination as compared to the other groups. Indeed, at day 20, all mice died or were euthanized due to a high tumor burden in the group treated with OPG 1-401, 80% in the pcDNA3 group, 100% in the control group as compared to the OPG 1-194 treated group where all mice were still alive (Fig 1C).

Effects of different forms of over-expressed OPG on bone resorption associated with osteosarcoma development

Because control tumors and pcDNA3-injected mice exhibit similar bone lesions, only radiographs and microscanner analysis of the tibiae of mice treated with pcDNA3 alone are shown. Radiographs of the tibiae of pcDNA3-treated mice confirmed important osteolytic lesions with cortical and fibular destruction as a consequence of an intense bone remodelling associated to tumor growth, as compared to naïve mice (without osteosarcoma, CT: Fig 2A), as previously reported (23). Murine OPG treatment differentially modulated bone lesions associated to osteosarcoma development depending on OPG structure: mOPG 1-194 markedly inhibited tumor induced bone alteration as compared to pcDNA3-treated mice, whereas mOPG 1-401 treatment induced intermediary results with less aggressive osteolytic lesions at the cortical and fibular levels, than those observed in pcDNA3-treated mice (Fig 2A).

Quantification of the osteolytic lesions by microcomputed tomography analysis confirmed radiographic data (Fig 2B). POS-1 tumors are associated with bone lesions with cortical degradation in pcDNA3-treated mice as compared to naïve animals, whereas OPG 1-194-treated mice showed a protective effect on bone resorption associated with osteosarcoma development (Fig 2B). Indeed, the specific trabecular bone volume is strongly decreased in pcDNA3-treated mice as compared to control naïve mice (respectively, 27.3% versus 50.28%, $p < 0.001$), while BV/TV of OPG 1-194-treated mice are closer to those of control mice (respectively, 42.2% versus 50.28%, ns). However, animals treated with OPG 1-401 show intermediary results as compared to OPG 1-194- and pcDNA3-treated mice (32.1% versus 42.2% and 27.3% respectively). As a consequence, the relative bone loss calculated as percent of controls reached 45.7% in the pcDNA 3.1 group, and 36.5 or 16.1% respectively in OPG 1-401 and OPG 1-194 treated mice.

In addition, TRAP staining performed on osteosarcoma sections revealed an increase of osteoclasts recruitment to the tumor-bone interface associated with osteosarcoma development (pcDNA3 mice, Fig 2C), whereas the presence of TRAP positive osteoclasts was totally absent in OPG 1-194-treated mice and only partially in OPG 1-401-treated mice (Fig 2C). Osteoclast counting confirm these analyses, with respectively 2 and 15 osteoclasts / tumor-bone interface surface (TBIS) observed respectively in the OPG 1-194 and 1-401 groups as compared to 38 Ocs/TBIS in controls (Fig. 2C), revealing a higher protective effect of OPG 1-194 on tumor induced bone lesions as compared to OPG 1-401-treated mice. Comparing the different effects exerted by OPG1-194 and OPG 1-401 towards prevention of bone degradation and tumor development, it may be suggested that OPG 1-401 exerts diminished effects due to loss of bioactivity. Two molecules are potential candidates to

interfere with OPG 1-401 bioactivity: TRAIL and Glycosaminoglycans, which binding domain to OPG has been described (GAG) or hypothesized (TRAIL) in the 195-401 domain.

The presence of RANKL and TRAIL is higher in the tumor than in control muscle

We next investigated by real-time PCR analysis the respective expression profile of TRAIL and RANKL at the transcript level in tumor tissue as compared to contralateral muscle as control tissue (Fig. 3). RANKL and TRAIL expression were evidenced in the tumor as compared to the control tissue (muscle; Fig. 3A and B). As these cytokines were not expressed in cultured POS-1 cells, it could be suggested that infiltrated cells such as lymphocytes are involved in this phenomenon.

TRAIL binds both forms of OPG with the same affinity

To determine whether TRAIL is the protagonist involved in the modulation of OPG bioactivity *in vivo*, two sets of experiments were performed: (i) TRAIL binding to both forms of mOPG was compared by BIAcore analysis, (ii) OPG ability to inhibit TRAIL pro-apoptotic activity was compared between both forms of mOPG.

Quantitative measurement of TRAIL interactions with both forms of mOPG was performed by BIAcore analysis. TRAIL was captured on the CM5 chip and increasing concentrations of mOPG 1-194 or 1-401 were injected over the chip surface. The response expressed in response units (RU) was recorded for each analyte concentration in the form of sensorgram (Fig 4A). The data were then fitted using BiaEval 4.1 software in order to calculate the dissociation constant (Kd). TRAIL binding affinity for mOPG 1-194 or mOPG 1-401 are similar with respective Kd of 21nM and 24nM (Fig. 4A), suggesting that TRAIL binds OPG through its 22-194 domain.

In addition, at the biological level, both forms of OPG abolished the TRAIL induced decrease of MG63 cell proliferation (-12% and -4% of controls respectively for OPG 1-401 and OPG 1-194; Fig. 4B).

Therefore, as the results revealed that both forms of OPG exert a quantitative similar effect on TRAIL-induced apoptosis, it suggests that TRAIL binds OPG through its 1-194 domain and that another protagonist may be involved in the difference of OPG biological activity observed between OPG 1-194 and OPG 1-401 *in vivo*. To this regard, the other candidate may be GAGs.

PGs content in the tumor tissue

PG or GAG content was therefore analysed in POS-1 tumor tissue using complementary methodological approaches. The overall presence of glycosaminoglycans was first investigated by Alcian blue staining, as compared to chondrosarcoma as positive control (Fig. 5A). Few stained areas were observed in POS-1 tumor, revealing the presence of GAGs in this tissue.

Then, the presence of GAGs was confirmed and quantified in POS-1 tumor, by specific imaging using ^{99}mTc -NTP 15-5 as *in vivo* radiotracer of PGs (26). The results showed an intense radioactivity uptake associated with the osteosarcoma development (Fig. 5B) and a significant higher fixation of the tracer is evidenced at the tumor level as compared to the controlateral muscle as control tissue: the Tumor to Background Ratio (TBR) was increased 2.2 times between day 9 and day 23 (Fig 5C).

In addition, the mRNA expression profile of Aggrecan, Decorin, Biglycan, Syndecan-1, -2 and -4 was investigated in the tumor tissue by real-time PCR (Fig. 5D). Results showed a significant increase of GAG mRNA expression in the tumor tissue compared to the control (muscle): 34 times for biglycan, 146 times for syndecan-1, 26 times for syndecan-2, 26 times for syndecan-4.

These results highlighted that GAG expression is significantly higher in osteosarcoma tumor as compared to controlateral muscle suggesting that their presence in the tumor microenvironment may interfere with OPG bioactivity.

GAGs can modulate OPG bioactivity

To demonstrate the hypothesis of GAG modulation of OPG bioactivity *in vivo*, surface plasmon resonance-binding assays were performed with both forms of OPG pre-incubated or not for 45 min with oligosaccharides (16 sugar units) as GAGs, and further binding to RANKL or TRAIL. The results showed that OPG 1-401 pre-incubated with GAGs did not further bind to immobilized RANK-L or TRAIL (Fig. 6A), whereas OPG 1-194 lacking heparin-binding domain was still able (Fig. 6B). These results confirm the involvement of the OPG 195-401 domain as a target for GAGs to modulate OPG binding to RANKL or TRAIL.

GAGs were then tested for their ability to modulate OPG 1-401 bioactivity as assessed in a model of 2.93 cells stably transfected to over-express RANKL. OPG 1-401 binds RANKL present in the supernatant of 2.93-RANKL cells, decreasing its detection by 75% (Fig. 6C). When heparin or oligo16 were co-incubated with OPG 1-401, RANKL detection decrease was only of 24 and 60% respectively, suggesting that GAGs inhibit OPG-RANKL interaction.

DISCUSSION

Despite recent progress in surgery and chemotherapy, the outcome of patients with osteosarcoma is poor, especially for metastatic localizations with a third of patients not surviving past the 10-year mark. The OPG/RANKL/RANK pathway is a therapeutic target in osteolytic tumors with significant effects demonstrated in restricting tumor-mediated osteolysis *in vitro* as well as in animal models of both secondary (6, 9, 27, 28) and primary bone tumors (10). However, the role of OPG in tumor-induced osteolytic lesions remains controversial (11) as in addition to inhibit osteoclastic differentiation and activation (29), OPG is able to bind TRAIL, and therefore inhibits its apoptotic effect (14, 16). Moreover, OPG can interact with molecules of the GAG family through its heparin binding domain (30).

OPG is composed of four cysteine-rich domains (1-194), two death-domains and one heparin-binding domain in the 195-401 region. In the present study, the biological activity of OPG full length (1-401) and OPG (1-194) administered by non viral gene transfer were compared *in vivo* in a model of osteolytic osteosarcoma in terms of osteolysis prevention and inhibition of associated tumor development. Our results clearly demonstrated that OPG 1-194 is more effective in both anti-tumor and anti-bone resorption activities than OPG 1-401, suggesting that protagonists present in the tumor environment bind to the 195-401 domain of the full length OPG further decreasing its biological activity or bioavailability. Excepting RANKL, two other potential candidates could bind to OPG and modulate its biological activity: TRAIL or the GAG part of PGs.

The first hypothesis is the implication of TRAIL in the modulation of OPG activity, but the precise OPG region for TRAIL binding is debated in the literature. It has been demonstrated that OPG 1-401 inhibits TRAIL-induced apoptosis of tumor cells, and conversely that TRAIL reverses the ability of OPG to inhibit osteoclastogenesis (13, 31). Others studies confirmed the protective effect of OPG on TRAIL-induced apoptosis of tumor cells using various forms of OPG: OPG 1-401, recombinant OPG-Fc and/or OPG produced directly by tumor cells or Bone Marrow Stromal Cells (BMSCs) in myeloma, breast and prostate cancer (12, 14-16, 32). However, contradictory data were reported on the OPG domain involved in TRAIL binding: Cheng *et al* have shown that the osteoprotegerin-like peptidomimetic (OP3-4) designed to bind RANKL and to inhibit osteoclastic bone resorption is also able to bind TRAIL with a low affinity (33). On the opposite, Heath *et al* have reported that OP3-4 had no effect on TRAIL-induced apoptosis *in vitro* suggesting that there is no interaction between TRAIL and OP3-4 (34). However, these results were all obtained from *in*

vitro studies, and no data demonstrated that OPG could protect tumor cells from TRAIL-induced apoptotic effects *in vivo*.

Several studies investigated the therapeutic potential of recombinant OPG-Fc to prevent the development of tumor bone disease such as myeloma, breast and prostate cancer, to reduce the tumor burden and increase survival (9, 27, 35-37). The main question remaining is: what would be the OPG action (anti-bone resorption by binding to RANKL or anti-apoptotic by binding TRAIL) in a given bone tumor environment in which both RANKL and TRAIL are present? Indeed, depending on the relative concentrations of TRAIL, RANKL and OPG in the tumor environment, the consequence of OPG binding between TRAIL and RANKL will be quite opposite. Holen and co-workers have demonstrated that the protective effect of OPG on TRAIL-induced apoptosis could be eliminated by addition of an excess of RANKL, which suggest that RANKL has a higher affinity for OPG than TRAIL (32). Shipman and Croucher have highlighted that the role of OPG is dependant of the relative concentrations, timing and location of OPG, TRAIL and RANKL expressions in the bone tumor microenvironment (16). In our model, we have demonstrated by real-time PCR that RANKL and TRAIL mRNA expression were increased in the tumor compared to the control tissue (muscle). Vitovski *et al* have demonstrated that OPG binds RANKL or TRAIL with a similar affinity, and that OPG plays a pivotal role in regulating the biological activity of these two ligands (31). However, the TRAIL/OPG affinity was demonstrated to be lower than that of OPG/RANKL in other studies (13, 38). Our present *in vivo* data do not reveal any protective effect of OPG 1-401 on tumor development as compared to the control group and we showed, by plasmon resonance analysis, that both forms of OPG are able to bind TRAIL with the same affinity. These results clearly demonstrate that TRAIL is not implicated in the *in vivo* difference of tumor proliferation and bone lesions observed between the two forms of OPG in our osteosarcoma model and evidence for the first time that TRAIL binds OPG in its 1-194 region (cysteine-rich domain), which is also recognized by RANKL (39).

Furthermore, because OPG is also composed of a heparin-binding domain at its C terminal portion, the cytokine is able to bind the GAG part of PGs which may regulate its bioavailability and biological activity in the bone environment. Indeed, PGs or GAGs are associated to the cell membrane or resident in the extracellular matrix and one of their main property is their ability to store growth factors or cytokines at both levels (17). In the present study, a higher GAGs expression and production were evidenced in osteosarcoma environment as compared to control tissue (muscle), analysed by alcian blue staining, specific GAG imaging and real time PCR suggesting that the PGs present in tumor environment may

modulate OPG 1-401 bioavailability and consequently its biological activity. Using RT-qPCR, we demonstrated that syndecan-1 is the main proteoglycan overexpressed in osteosarcoma tumor with a 340 fold increase as compared with controlateral muscle, whereas decorin was not expressed at all and the three other PGs studied were less increased. It is quite surprising that biglycan is not the most overexpressed PG, because osteosarcoma is characterized by the direct synthesis of osteoid substance by tumor cells and that biglycan is the most PG present in the bone matrix (17). However, overexpression of syndecan-1 has been already reported in tumors, especially in myeloma (19). Among several examples of OPG bioactivity regulation by PGs reported in the literature, Irie *et al* have reported that heparin was able to bind OPG thereby preventing OPG-mediated inhibition of osteoclastic bone resorption *in vitro* (40). However, another study revealed that dermatan sulfate is able to inhibit osteoclastogenesis like OPG by binding to RANKL (41). In pathological situations, it has been shown that OPG is able to bind to syndecan-1 expressed at the surface of myeloma cells (42) and that these tumor cells then internalize and degrade OPG through its binding to syndecan-1 (19). A similar process could be proposed to explain the decreased biological activity of full length OPG in osteosarcoma expressing syndecan-1. Our group recently demonstrated by kinetic studies of molecular interactions that OPG binds to heparin with a high affinity (K_d : 0.28 mM) and that the pre-incubation of OPG with heparin inhibited in a dose-dependent manner the further OPG binding to the RANK-RANKL complex (21). In addition, plasmon resonance analyses reveal in the present report that GAGs strongly interfere with full length OPG binding to RANKL or TRAIL, suggesting that GAGs may modulate OPG bioavailability and consequently block the inhibitory effects of OPG 1-401 on RANKL and TRAIL biological activities. The present results associated to bibliographic data suggest that OPG 1-401, contrary to OPG 1-194, can be stored in the extracellular matrix of bone tumor by binding to PGs, thereby diminishing its biological activity.

However, other hypotheses cannot be excluded. We don't rule out the possibility that the two death domains present in the 195-401 OPG domain are involved in this phenomenon. Even if the exact role of these domains is not elucidated in OPG, one study demonstrated that they are active with a high potential for mediating a cytotoxic signal when there are fused with the transmembrane region of Fas (30). Another hypothesis is that OPG 1-401 is able to dimerize and Schneeweis *et al.* have reported that OPG dimerization was required for the inhibition of the RANK/RANKL interaction *in vitro* (43). However in the present study, we demonstrated that OPG 1-194 which is not able to form a dimer is more active *in vivo* than

OPG 1-401, so the preferential hypothesis is that the GAG part of PGs present in the tumor environment inhibit OPG bioactivity by storing this cytokine in the tumor matrix.

Therefore, in the light of the present results, the use of truncated OPG as a recombinant protein should be preferred for clinical application, but the use of denosumab, the human antibody against RANKL is more promising as it does not interact with TRAIL. In conclusion, we demonstrated that the difference in OPG structure regulates its biological activities in a mouse model of primary bone tumor, and confirmed that OPG 1-194 is the best candidate for the therapeutic approach of bone tumors (10).

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Pour voir les figures, consulter la thèse sous sa forme papier

Legends to the figures:

Figure 1: mOPG 1-194 transgene expression significantly diminishes tumor volume and progression, and augments survival rate in an osteolytic model of mouse osteosarcoma.

Mice were transplanted with POS-1 osteosarcoma fragments as described in Materials and Methods. ®Lutrol/DNA formulations were injected as a curative protocol into both tibial anterior muscles once a week. The tumor volume (V) was calculated from the measurement of two perpendicular diameters, according to the following formula: $V = 0.5 \times L \times S^2$. The mean (A) or the individual (B) tumor volume of mice treated with p-mOPG 1-194/®Lutrol formulation is compared with that of mice that received p-mOPG 1-401/®Lutrol, pcDNA3/®Lutrol or control mice without tumors. ***, $p < 0.005$; **, $p < 0.01$; *, $p < 0.05$; i_x , tumor incidence. (C) Overall survival rate was compared between mice that received p-mOPG 1-194/®Lutrol, p-mOPG 1-401/®Lutrol, pcDNA3/®Lutrol and control mice over a 20-d period. One representative experiment out of three is shown. *: $p < 0.01$.

Figure 2: Higher prevention of bone lesions associated to tumor development by OPG 1-194 than OPG 1-401.

(A) Representative radiographs of mice legs bearing osteosarcoma from each group (control, POS-pcDNA3, POS-OPG 1-401 and POS-OPG 1-194) at day 20. Arrows indicate tumor-induced osteolytic lesions. (B) Micro-computed tomography analysis and microarchitectural parameters quantified at the trabecular level. BV, bone volume; TV, total volume. **: $p < 0.001$ (POS-pcDNA3 versus CT and POS-OPG 1-401 versus CT); *: $p < 0.01$ (POS-OPG 1-194 versus POS-OPG 1-401). (C) TRAP-stained sections of each tibia were evaluated. Arrows indicate osteoclasts stained in red for TRAP activity. In the control tumor and control pcDNA3 groups, numerous osteoclasts are observed on the remaining trabeculae and within the tumor (only control pcDNA3 is shown). OPG 1-401 treatment preserved bone trabeculae more than control groups but bone lesions are still observed by the presence of TRAP positive osteoclasts. However, OPG 1-194-treated mice showed a preserved cortical bone and complete osteoclast suppression. Osteoclast numbers at the tumor-bone interface surface (OcS/TBIS); **, $p < 0.001$; *, $p < 0.01$.

Figure 3: Comparison of RANKL, TRAIL expression in tumor tissue

RANKL and TRAIL mRNA expression were analyzed by real-time PCR in the tumor tissue as compared to the controlateral muscle as control tissue. Results are expressed as fold increase compared to the POS-1 cells. Experiments were performed at least 3 times in triplicate.

Figure 4: TRAIL binds OPG through its 1-194 domain.

The binding of OPG 1-401 and OPG 1-194 (1, 2, 4, 8, 16, 32, 65 and 130 nM) to TRAIL immobilized on a research grade CM5 chip was visualized as the change in surface plasmon resonance in the form of sensorgrams (A). The regions used in determining equilibrium dissociation constants are only shown. The resulting sensorgrams were fitted using BiaEval 4.1 software (BIAcore). (B) The effects of both forms of OPG were assessed on TRAIL-induced apoptosis on MG63 osteosarcoma cells using an XTT assay as described in Materials and Methods. MG63 cells were seeded and treated with 50 ng/ml TRAIL in the presence or not of OPG 1-401 or OPG 1-194 (100 ng/ml).

Figure 5: PGs are highly expressed in osteosarcoma tissue:

(A) The presence of GAGs was evaluated by alcian blue staining on the osteosarcoma tumor tissue as compared to chondrosarcoma as a positive control, unstained chondrosarcoma is given as a negative control. (B) Whole-body planar scintigraphy of a representative POS-osteosarcoma bearing mouse at the stage Day-23. Planar static image (10-min-duration) was acquired 15 min after i.v. injection of ^{99m}Tc -NTP 15-5 radiotracer (20 MBq/animal). A clear uptake of the radiotracer was observed within the tumour-bearing paw (arrow) respectively to the controlateral paw. (C) Quantitative analysis of ^{99m}Tc -NTP 15-5 tumoural uptake at early (day-9) and late stage of study (Day-23); Mean TBR (Tumor to Background Ratio tumor/muscle) values $\pm$ standard deviation are presented at each time-point. * Statistical significance ($p < 0.05$). (D) GAGs expression (biglycan, syndecan-1, -2 and -4 were analyzed at the transcript level by real-time PCR in the tumor tissue as compared to control tissue (muscle without tumor). Results are expressed as fold increase compared to the POS-1 cells. Experiments were performed at least 3 times in triplicate.

Figure 6: GAGs inhibit OPG binding to TRAIL (A) or RANKL (B) and modulate OPG bioactivity *in vitro*

Surface Plasmon resonance was used to study interactions between OPG (1-401 and 1-194), RANK-L and GAGs (oligosaccharides containing 16 sugar units) or OPG (1-401 and 1-194), TRAIL and GAGs. Recombinant OPG 1-401 or OPG 1-194 previously pre-incubated or not at room temperature for 45 min with 400 nM GAGs were injected over immobilized RANKL (A) or TRAIL (B); flow rate, 20 μ l/min; injected volume, 200 μ l. (C) RANKL production in the supernatant of 2.93 cells stably transfected with full length murine RANKL was detected by ELISA in the presence or absence of OPG 1-401 (50 ng/ml) alone or preincubated with heparin (50 nM) or oligo 16 (200 nM) during 45 min at room temperature. **, $p < 0.001$; *, $p < 0.05$ when compared with control; \$: $p < 0.05$ when OPG+oligo16 is compared to OPG alone; §: $p < 0.001$ when OPG + heparin is compared to OPG alone.

Complément de discussion de l'article 3

Malgré les progrès récents en chirurgie et en chimiothérapie, la survie des patients atteints d'ostéosarcome reste faible de l'ordre de 50% à 5 ans. Les tumeurs osseuses sont associées à un remodelage osseux intense souvent caractérisé par des plages d'ostéolyse, faisant de la triade moléculaire OPG/RANK/RANKL la cible privilégiée dans le développement de nouvelles approches thérapeutiques. En effet, dans le cadre du cercle vicieux entre la résorption osseuse et la prolifération tumorale, l'inhibition de la résorption osseuse a pour conséquence une diminution du volume tumoral associée à une augmentation de la survie.

Cependant, l'utilisation thérapeutique de l'OPG comme agent anti-résorption et anti-tumoral dans les tumeurs osseuses est actuellement très controversée, du fait qu'un grand nombre d'études rapportent que l'OPG est capable de se lier à TRAIL et d'inhiber l'apoptose des cellules tumorales médiée par TRAIL (Fisher et al., 2006; Holen et al., 2002; Neville-Webbe et al., 2004; Nyambo et al., 2004; Shipman and Croucher, 2003).

L'OPG, récepteur compétiteur de RANKL, est une protéine soluble de 401 acides aminés et de 380 après clivage du peptide signal. Elle est composée de différents domaines dont quatre domaines riches en cystéines responsables de son activité anti-résorptive, ainsi que deux domaines de mort actifs (Yamaguchi et al., 1998) mais dont la fonction est encore inconnue pour l'OPG et un domaine de liaison à l'héparine se situant dans la partie 195-401. Les objectifs de cette étude étaient de comparer l'activité biologique de deux formes d'OPG délivrées par thérapie génique non virale dans un modèle d'ostéosarcome ostéolytique murin: la forme complète OPG 1-401 et une forme tronquée OPG 1-194 afin d'essayer de déterminer la relation structure-fonction de la partie 195-401 de l'OPG dans son activité anti-résorption osseuse et anti-tumorale. Les résultats démontrent clairement que l'OPG 1-194 est plus efficace dans la prévention des lésions ostéolytiques associées à la tumeur et dans l'inhibition de la progression tumorale comparée à l'OPG 1-401. Ces données suggèrent que d'autres protagonistes présents dans l'environnement tumoral osseux peuvent réguler la biodisponibilité et/ou l'activité biologique de l'OPG 1-401 par la capacité à se lier à la partie 195-401. Outre la fixation avec RANKL, les candidats potentiels capables de se fixer à l'OPG et de moduler son activité biologique pourraient être TRAIL ou les protéoglycanes.

La première hypothèse est l'implication de TRAIL dans la régulation de l'activité biologique de l'OPG car le domaine de liaison entre l'OPG et TRAIL n'est pas clairement

défini dans la littérature. Des études démontrent que l'OPG 1-401 est capable d'inhiber l'apoptose des cellules tumorales induite par TRAIL, et inversement TRAIL peut inhiber l'activité anti-ostéoclastique de l'OPG (Emery et al., 1998; Vitovski et al., 2007). D'autres études confirment la capacité de l'OPG à inhiber l'activité apoptotique de TRAIL sur les cellules tumorales de myélome, de cancer du sein ou de prostate (Fisher et al., 2006; Holen et al., 2002; Neville-Webbe et al., 2004; Nyambo et al., 2004; Shipman and Croucher, 2003). Cependant, deux études sont en désaccord sur le domaine impliqué dans la liaison de l'OPG à TRAIL. D'une part, Cheng *et al* ont montré que le peptide OP3-4 établi à partir de l'OPG et correspondant au site de liaison de RANKL permettant d'inhiber spécifiquement l'ostéoclastogénèse est aussi capable de lier TRAIL avec une faible affinité (Cheng et al., 2004) suggérant que les sites impliqués dans la liaison avec TRAIL et RANKL sont quasiment similaires. D'autre part, Heath *et al* ont montré que ce même peptide OP3-4 n'exerçait aucun effet sur l'apoptose des cellules tumorales médiée par TRAIL suggérant que TRAIL n'interagit pas avec OP3-4 (Heath et al., 2007). Toutefois, tous les résultats mettant en évidence le rôle de facteur de survie de l'OPG pour les cellules tumorales ont été obtenus par des études réalisées *in vitro* en s'affranchissant des conditions pathologiques et de l'environnement tumoral osseux et restent donc à être confirmés *in vivo*.

A l'heure actuelle, il est clairement défini que l'OPG empêche la résorption osseuse par sa liaison à RANKL, inhibant ainsi l'activité ostéoclastique de RANKL, mais réduit également le volume tumoral et augmente la survie des animaux par le blocage du cercle vicieux dans différents modèles tumoraux (Corey et al., 2005; Croucher et al., 2001; MacEwen et al., 2004; Morony et al., 2001; Vanderkerken et al., 2003; Zhang et al., 2001).

L'ensemble de ces données suggèrent que l'OPG peut être non seulement un facteur de survie pour les cellules tumorales de par sa fixation à TRAIL mais également peut être un facteur anti-tumoral indirect de par sa fixation à RANKL. Par conséquent la présence de chaque cytokine dans l'environnement tumoral et l'affinité de chacune pour l'OPG détermineront l'activité biologique de l'OPG. Des études rapportent que RANKL est plus affiné pour l'OPG que TRAIL. Notamment, Holen *et al* qui démontrent qu'un excès de RANKL peut réverser l'effet protecteur de l'OPG vis-à-vis de l'apoptose des cellules tumorales médié par TRAIL (Holen et al., 2002). A l'inverse, Vitovski *et al* rapportent que l'OPG lie RANKL et TRAIL avec la même affinité, jouant un rôle pivot dans l'activité biologique de ces deux cytokines (Vitovski et al., 2007). Ces auteurs concluent que finalement le rôle de l'OPG est dicté par les concentrations relatives, le moment et la

localisation de ces cytokines dans le microenvironnement tumoral osseux (Shipman and Croucher, 2003).

Par cette étude nous avons essayé de répondre à toutes ces controverses. Nous avons tout d'abord démontré une augmentation de l'expression de RANKL et de TRAIL dans le tissu tumoral en comparaison à du tissu sain (muscle). Nous avons, certes, observé une différence de progression tumorale entre les groupes traités avec les deux formes d'OPG, mais l'OPG 1-401 n'induisait pas d'effet pro-tumoral (protecteur des cellules tumorales) en comparaison au groupe contrôle. De plus, nous avons montré *in vitro* par des expériences d'inetractions moléculaires par la technique BIAcore que TRAIL est capable de se fixer aux deux formes d'OPG avec la même affinité. L'ensemble de ces résultats démontre clairement que TRAIL n'est pas impliqué dans la différence de progression tumorale et de prévention osseuse observée *in vivo* entre les deux formes d'OPG dans notre modèle d'ostéosarcome. De plus, nous montrons pour la première fois que TRAIL lie l'OPG sur la partie 1-194 (domaine riche en cystéine), qui reconnaît également RANKL.

Comment peut s'expliquer cette différence d'activité biologique observée *in vivo* si TRAIL n'est pas impliqué ? L'OPG possède un domaine de liaison à l'héparine par son côté C-terminal, domaine délété dans la forme OPG 1-194. Par conséquent, l'OPG est capable de se lier à la partie oligosaccharidique (GAGs) des protéoglycanes, qui peuvent ainsi réguler sa biodisponibilité et son activité biologique. Les PGs ou GAGs sont associés à la membrane cellulaire ou présents dans la matrice extracellulaire avec comme rôle principal de stocker des facteurs de croissance ou des cytokines dans la matrice osseuse [(Lamoureux et al., 2007a); en annexe]. Dans cette étude, une augmentation de l'expression des protéoglycanes est mise en évidence dans la tumeur en comparaison au tissu contrôle (muscle) par coloration au bleu alcian, imagerie spécifique des GAGs et PCR quantitative, suggérant que les PGs présents dans l'environnement tumoral pourraient réguler la biodisponibilité et donc l'activité biologique de l'OPG 1-401. Quelques études montrent que les GAGs ou les PGs peuvent effectivement réguler l'activité biologique de l'OPG notamment en inhibant son activité anti-ostéoclastique, en l'internalisant et la dégradant (Borset et al., 2000; Irie et al., 2007; Mosheimer et al., 2005; Shinmyouzu et al., 2007; Standal et al., 2002). Une étude réalisée récemment dans notre laboratoire a démontré que les GAGs pouvaient moduler l'activité de la triade moléculaire OPG/RANK/RANKL (Theoleyre et al., 2006). En effet, les GAGs peuvent inhiber les interactions OPG 1-401/RANKL et OPG 1-401/TRAIL mais pas celles de l'OPG 1-194 avec RANKL ou TRAIL, suggérant fortement que la présence de GAGs (dans l'environnement tumoral osseux) peut moduler l'activité biologique de l'OPG 1-401 par

rapport à TRAIL et RANKL, contrairement à l'OPG 1-194, probablement par le fait qu'elle soit stockée dans la matrice extracellulaire osseuse.

Cependant nous ne pouvons exclure d'autres hypothèses, comme par exemple l'implication des deux domaines de mort au rôle toujours inconnu au sein de l'OPG, mais dont l'effet cytotoxique a été déjà rapporté lorsque qu'ils sont fusionnés avec la région transmembranaire de Fas (Yamaguchi et al., 1998). Une autre hypothèse est liée à l'implication de la dimérisation dans l'activité de l'OPG. En effet, Schneeweis *et al* rapportent que l'OPG sous sa forme dimérique est requise pour inhiber l'interaction RANK/RANKL (Schneeweis et al., 2005). Or, dans notre étude nous rapportons que la forme OPG 1-194 qui n'est donc pas capable de se dimériser, possède une meilleure efficacité thérapeutique que l'OPG 1-401. Par conséquent cette hypothèse ne semble pas déterminante dans notre système et par conséquent nous supportons l'hypothèse selon laquelle les protéoglycanes régulent l'activité biologique de l'OPG.

En conclusion, nous démontrons dans un modèle murin de tumeur osseuse primitive que l'activité biologique de l'OPG dépend de sa structure et nous confirmons que l'OPG 1-194 reste un bon candidat dans le traitement des ostéolyses d'origine tumorale comme l'ostéosarcome.

Article 4

« Le microenvironnement tumoral osseux régule l'activité anti- ou pro-tumorale de l'OPG, dépendant de la balance locale entre le Receptor Activator of Nuclear Factor-KB Ligand (RANKL) et le TNF Related Apoptosis Inducing Ligand (TRAIL) ainsi que de la présence des protéoglycanes »

Cytokine Growth Fact. Rev., *soumis*, F. Lamoureux, G. Picarda, G. Moriceau, J. Rousseau, V. Trichet, F. Rédini.

Le développement tumoral en site osseux, souvent associé à des fractures, des pertes osseuses et des douleurs nécessite le développement de nouvelles approches thérapeutiques. Les tumeurs osseuses sont associées à l'existence d'un cercle vicieux entre la résorption osseuse et la prolifération tumorale dans lequel la triade moléculaire OPG/RANK/RANKL joue un rôle majeur. L'OPG, molécule anti-résorptive a montré des résultats prometteurs en termes de prévention osseuse et d'inhibition de la croissance tumorale associée. Cependant, son utilisation thérapeutique dans le traitement des tumeurs osseuses demeure controversée due à sa capacité de se lier et d'inhiber TRAIL, inducteur d'apoptose des cellules tumorales. Cette revue fait le point sur l'implication du microenvironnement tumoral osseux, principalement de la balance RANKL/TRAIL et de la présence de protéoglycanes dans la régulation de l'activité biologique de l'OPG dans les tumeurs osseuses.

Tumor bone microenvironment regulates Osteoprotegerin anti- or pro-tumoral activity depending on the local Receptor Activator of Nuclear Factor- κ B Ligand/TNF Related Apoptosis Inducing Ligand balance and the presence of proteoglycans

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Key words: bone resorption, bone tumor, vicious cycle, OPG, RANKL, TRAIL, proteoglycan

Short running title: OPG bioactivity is regulated by bone tumor environment

Abstract

Tumor development in bone site, often associated with fractures, bone loss and bone pain still needs improvement in therapeutic approaches. Bone tumors are related to the existence of a vicious cycle between bone resorption and tumor proliferation in which the molecular triad OPG/RANK/RANKL plays a pivotal role. The anti-resorptive molecule OPG showed promising results in bone prevention and inhibition of associated tumor growth. However, its therapeutic use in bone tumors remains controversial due to its ability to bind and inhibit TRAIL, a potent inducer of tumor cell apoptosis. This is an overview on the involvement of the micro-environment, mainly the balance RANKL/TRAIL and the presence of proteoglycans in the regulation of OPG biological activity in bone tumors.

Introduction

Bone lesions associated to primary and secondary malignant tumor development remain a major clinical problem and need therapeutic improvement. Indeed, these bone lesions are often associated to pathological fractures, palpable mass, bone loss, spinal cord compression and severe bone pain (1, 2). Tumor development in bone site relies on the existence of a vicious cycle between bone resorption and tumor proliferation (3, 4). Thus, it is suggested that bone pathology from tumor origin is associated with a disorder in the expression of the main cytokines involved in the regulation of bone remodeling: OPG (osteoprotegerin), RANK (Receptor Activator of Nuclear Factor κ B) and RANKL (RANK Ligand), members of the TNF (Tumor Necrosis Factor)/TNF Receptor superfamilies.

Bone is a dynamic tissue continuously remodeled dependently on osteoclast and osteoblast activities. The discovery by two different groups in 1997 of RANKL (5, 6) and its activating (RANK) (7) and decoy (OPG) (8, 9) receptors have allowed a better knowledge of the molecular mechanisms involved in the regulation of bone remodeling. In bone site, OPG and RANKL are expressed by osteoblasts and bone marrow stromal cells whereas RANK is found at the surface of mature osteoclasts and osteoclast precursors. The binding of RANKL to RANK on pre-osteoclasts induces their differentiation, maturation and activation leading to bone resorption. Therefore, RANKL is an essential mediator of osteoclast formation, function and survival, whereas OPG, a decoy receptor for RANKL operates as a real competitor of RANK/RANKL interaction and prevents this latter to interact with RANK. Consequently, OPG inhibits the osteoclastic differentiation and activation, leading to protection against bone loss.

The discovery in the past few years of this molecular triad OPG/RANK/RANKL and its implication in the pathophysiology of primary bone tumors and skeletal metastasis has allowed to define promising targets for therapeutic applications (10-13). For example, as OPG blocks the RANK/RANKL interaction, it represents a potent anti-resorptive molecule to use in degradative bone pathologies from tumor origin or not (4, 8, 14-16).

However, the role of OPG in cancer remains controversial (17) due to its ability to bind TRAIL (TNF Related Apoptosis Inducing Ligand), another member of the TNF superfamily and therefore to inhibit the apoptosis of tumor cells induced by this factor (18). To this regard, OPG could represent a survival factor for tumor cells.

This review discusses the results on the dual role of OPG in bone tumor microenvironment in correlation with the affinity and local concentrations of RANKL and

TRAIL, together with the involvement of the matrix proteoglycans (PGs)/glycosaminoglycans (GAGs) as regulators of OPG bioactivity.

The molecular triad OPG/RANK/RANKL plays a pivotal role in the vicious cycle

OPG also called Osteoclastogenesis Inhibitory Factor (OCIF), Follicular Dendritic Cell Receptor 1 (FDCR-1), TNF Receptor related molecule 1 (TR1) or, as the official nomenclature, TNFRSF11B, has been identified in the late 90's as a new member of the TNF receptor superfamily (8, 9). OPG is synthesized as a propeptide of 401 amino acids which generates a protein of 380 amino acids after cleavage of the signal peptide. As OPG does not possess cytoplasmic or transmembrane domain, it is only secreted as a soluble protein. Its N-terminus possesses 4 cysteine-rich domains involved in osteoclastogenesis inhibition. Its C-terminus domain in turn contains two homologous death domains, which function is currently unknown, and a heparin binding domain. In C-terminus is also a cysteine residue (Cys400) necessary for its homodimerization (Fig.1). OPG is synthesized as a 55 kDa monomer which dimerizes by disulfide-binding to be secreted as a homodimeric glycoprotein of 110 kDa with 4 to 5 potential sites of N-glycosylation (19). OPG plays direct roles in the formation, function and survival of osteoclasts, as it decreases the number of mature osteoclasts and finally increases bone density (8). Indeed, transgenic mice for OPG showed a severe osteopetrosis caused by a defect in the later stages of osteoclast differentiation (8). In addition, Bucay *et al.* have reported that OPG knockout mice have a decreased bone density associated to a high fracture incidence (20), these phenomena being reversed by the intravenous injection of recombinant OPG (21) showing that the presence of OPG is absolutely essential to maintain bone mass. *In vitro*, many studies have shown that OPG is able to block the terminal stages of osteoclastic precursor differentiation, to suppress the activation of mature osteoclasts and indirectly to induce their apoptosis (5, 8, 9, 22).

To maintain constant bone mass, the balance between bone resorption and bone formation is tightly regulated by osteoblast and osteoclast functions and at the molecular level by the RANKL / OPG equilibrium. Any alteration in the expression of one of these protagonists leading to an imbalanced OPG/RANKL ratio is associated to bone pathologies, and among them cancer induced bone diseases. An increased RANKL level was demonstrated in several solid tumors developing in bone site which seems to be a critical mechanism for these pathologies (23-27). Furthermore in primary and metastatic bone cancers, RANKL

plays a key role in the vicious cycle between bone destruction and tumor growth in which tumor cells increase RANKL-mediated osteoclast activity (3) (Fig. 2). Indeed, tumors in bone environment produce factors such as parathyroid hormone-related peptide (PTH-rP) which increases RANKL production by osteoblasts or by bone marrow stromal cells promoting differentiation, activation and maturation of pre-osteoclasts in mature osteoclasts. Tumor cells secrete other factors such as interleukin (IL)-6, IL-11, TNF- α , IL-1 α , Colony Stimulating Factor-1 or Macrophage Inflammatory Protein-1 α which are described as osteoclast activating factors. When activated, osteoclasts degrade the bone matrix, allowing the release of growth factors stored in the bone matrix, such as Transforming Growth Factor β and Insulin like Growth Factor-1 which in turn stimulate tumor cells and osteoblast proliferation (28, 29). Even if the majority of bibliographic data report that RANKL is produced by stromal cells or by osteoblasts in response to tumor cell stimuli, RANKL production by tumor cells themselves increasing osteoclast activity and leading to excess bone loss has also been described (24, 25, 30, 31).

OPG inhibits bone resorption by blocking RANK/RANKL interaction

Several studies report that RANK expression has been evidenced on several tumor cell types in bone, suggesting an additional role for RANKL on tumor cell migration and homing in bone (32-34). To this regard in the case of tumor-associated bone loss, main therapeutic approaches used to reverse bone lesions rely on the inhibition of RANKL-RANK interaction not only to block osteoclast-mediated bone resorption but also to reduce tumor growth in bone. Several preclinical studies have shown that OPG represents a promising therapeutic candidate in animal models of bone loss from various pathological origins. The role of OPG as a regulator of bone remodeling has been first evidenced by the injection of OPG-Fc in OPG knockout mice, restoring bone equilibrium in the osteoporotic mice (20, 21) and preventing vascular calcifications (21, 35). Using an osteolytic tumor model, Clohisy *et al.* showed that OPG treatment reduces the number of osteoclast precursors, bone tumor growth and the expression of RANKL at the tumor site (36). Similar results were observed on the decrease of osteoclast number in nude mice bearing tumor associated with humoral hypercalcemia of malignancy (37, 38). OPG could also exert a beneficial effect on bone pain by inhibiting bone resorption which indirectly induced diminution of the neurochemical changes in spinal cord in nude mice with bone cancer (39, 40). These data highlight the

potential therapeutic role of OPG in the treatment and prevention of bone lesions in various tumoral pathologies.

Besides its role as anti-resorptive agent, a number of studies report that recombinant OPG-Fc is also able to inhibit tumor progression associated with bone prevention, and to increase the survival rate of tumor-bearing mice in breast cancer, multiple myeloma and prostate cancer models (14, 41-46). Moreover, when compared or associated with bisphosphonates (Zoledronic acid or Ibandronate) OPG exerts similar effects on the inhibition of bone resorption and tumor volume decrease respectively in human lung cancer cells injected in the tibiae of nude mice and in prostate and breast cancer models (47-49). Zheng *et al.* reported a similar anti-resorptive and anti-tumor effect of OPG-Fc (1 mg/kg/day) and Ibandronate (160 microg/kg/day) in the MDA-MB-231 breast cancer model without additive and/or synergistic effects when both agents were combined (49), emphasizing the potential role of OPG in the treatment of bone resorption.

It was also reported that OPG delivered by gene transfer reverses osteopenia by an increase of bone volume (50), inhibits bone resorption in several non malignant models (51-53), and that the tumor burden decrease is the consequence of the osteolysis inhibition by blocking the RANKL/RANK interaction (4). We have demonstrated for the first time in primary bone tumor (osteosarcoma) that OPG delivered by gene transfer using synthetic vectors inhibited bone resorption and consequently decreased tumor growth and increased animal survival without exerting direct effect on tumor cells (4). More recently, Chanda *et al.* reported that OPG over-expression (110.75 ng/ml) using a recombinant adeno-associated viral vector in a mouse model of osteolytic breast cancer restored bone integrity and reduced tumor volume in bone site (54). In these studies, despite its effects on bone resorption and tumor growth, OPG treatment failed to provide long-term survival. Indeed, OPG did not reduce the tumor volume in non osseous tissues such as lungs, heart, and adrenal glands in which the presence of visible metastasis induced by MDA-MB-435 cells was observed. In the light of these results, the authors highlighted the importance of the bone microenvironment in OPG therapy (54).

In this context, two clinical studies were realized showing the beneficial effect of OPG-Fc in osteolytic pathologies from tumor origin or not. Bekker *et al.* described the effect of a single subcutaneous dose of recombinant OPG-Fc in a randomized, double-blind, placebo-controlled, sequential dose escalation study (0-3 mg/kg) which was conducted in 52 postmenopausal women. OPG-Fc was well tolerated, and rapidly and profoundly reduced bone turnover for a sustained period (55). In 2003, a phase I study (randomized, double-blind,

double-dummy, active-controlled, single-dose, dose escalation study) using a recombinant OPG construct (AMGN-007, 1.0 mg/kg) was conducted in 28 patients suffering from multiple myeloma and 26 patients suffering from breast carcinoma, both diseases being associated to osteolytic lesions. Results showed that a single subcutaneous dose of OPG-Fc was well tolerated and suppressed bone resorption as indicated by a rapid and sustained decrease of urinary markers of bone resorption (NTX/creatinin levels) comparable to the results obtained with the bisphosphonate pamidronate (56) .

OPG inhibits tumor cells from TRAIL-induced apoptosis

However, despite the positive effect of OPG to prevent bone destruction and subsequently to inhibit tumor growth in bone, the ability of OPG to bind TRAIL and to protect tumor cells from TRAIL-induced apoptosis renders its use more questionable in osteolysis associated with bone tumor. Therefore, OPG would exert anti- or pro-tumoral effect depending on its binding to RANKL or TRAIL, and mainly depending on the relative concentration of both cytokines in the bone tumor environment. The dual role of OPG in cancer is discussed in various studies. In the literature, Van Poznak *et al.* reported the spatial expression of OPG, RANKL, and TRAIL in breast tissue and breast cancer (57). The hypothesis of a simultaneous production of RANKL, OPG and TRAIL could be paradoxical, as it would suggest that tumor cells produce at the same time factors favorable to its growth and survival (OPG) together with factors inducing its apoptosis (TRAIL). However, the presence and the concentrations of OPG, RANKL and TRAIL in the bone tumor microenvironment may direct tumor cells to produce molecules known to avoid their destruction by host defense systems and to adapt to bone micro-environment, as it has been already shown in other cancer models.

Results of *in vitro* studies suggest that the presence of OPG in tumor bone microenvironment may induce tumor cell survival in myeloma (58, 59), prostate (60, 61) and breast (62, 63) cancers. For example, Shipman *et al.* demonstrated *in vitro* that OPG produced by MG63 osteoblast-like and primary bone marrow stromal cells (BMSC) may function as a paracrine survival factor in the bone marrow microenvironment in multiple myeloma by inhibiting TRAIL-induced apoptosis (59). Similar results were reported by Neville-Webbe *et al.* in breast cancer as the levels of OPG present in BMSC conditioned medium increased survival of breast cancer cells (63). Holen *et al.* reported that OPG is directly expressed by

breast cancer cells and by breast tumors and that the OPG levels produced *in vitro* by tumor cells were sufficient to inhibit TRAIL-induced apoptosis. Consequently, OPG can act as an endocrine survival factor for breast cancer, but the authors gave no clear evidence for these findings *in vivo* (62). Similar results were obtained in prostate cancer in which the role of OPG on tumor cell survival was reversed by co-administration of 100-fold molar excess of RANKL (60), highlighting that RANKL alters the binding of OPG to TRAIL. As both TRAIL and RANKL are members of the TNF superfamily, a competition between RANKL and TRAIL binding to OPG could be envisaged. However, the significance of this finding for the *in vivo* situation depends on the local concentration of TRAIL and to its ability to induce apoptosis in the bone microenvironment where RANKL is present.

Besides its pro-tumoral effect as inhibitor of TRAIL-induced apoptosis of cancer cells, OPG can also prevent TRAIL-induced osteoclast apoptosis by binding to endogenous TRAIL produced in human osteoclast cultures (64). Indeed, even if TRAIL is known to induce mainly the apoptosis of tumor cells, an effect on cells from non tumor origin such as osteoclasts has been already reported (64-66). In a paradoxical way, OPG could represent in this case a pro-resorptive molecule by inhibiting TRAIL-induced osteoclast apoptosis. Brunetti and co-workers report that the OPG/TRAIL complex could be involved in the formation and survival of osteoclasts in an *in vitro* osteoclastogenesis system derived from human multiple myeloma-bone disease (67). Moreover, after the addition of anti-TRAIL antibodies, the OPG/TRAIL complex was dissociated which induced a decrease of osteoclast formation (68), suggesting the important role played by TRAIL in bone cell regulation. Moreover, myeloma cells sensitized osteoblasts from TRAIL-induced apoptosis, whereas OPG produced by primary osteoblasts protected myeloma cells from TRAIL-mediated apoptosis. In this context, the suppression of osteoblast-mediated bone formation by myeloma cells may participate to the increased bone loss observed in multiple myeloma patients (69).

However, the direct role of TRAIL on osteoclastogenesis is unclear and discussed in the literature. The majority of the studies report that TRAIL negatively regulates osteoclastogenesis by direct inhibition of preosteoclast differentiation and by inducing apoptosis of mature osteoclasts (64, 68, 70, 71) without affecting the OPG anti-osteoclastic activity (72). In contrast, Labrinidis and co-workers support the idea that TRAIL exerts a direct effect on tumor cells within the tumor bone microenvironment and consequently an indirect effect on osteoclast differentiation and survival, resulting in the abrogation of the vicious cycle (73, 74). One recent study evidenced a new role for TRAIL in the direct induction of osteoclast formation in human monocytes and murine RAW 264.7 macrophages

via a direct engagement with TRAIL death receptor through a signaling pathway distinct from apoptosis (75).

OPG, RANKL and TRAIL in the tumor microenvironment

The tumor bone microenvironment represents a very complex cellular and molecular network and the knowledge of its regulation is under current investigation. The functions of several cytokines and their receptors present in the microenvironment are determined by their relative concentrations at a given time. If the cytokines are present simultaneously at similar concentrations in the bone tumor environment, their respective affinity for one ligand will be determinant. In this regard, data of the literature are controversial on OPG affinity for TRAIL or RANKL whose extracellular domains share 30% homology. Several studies reported the protective effect of OPG on TRAIL-mediated apoptosis of tumor cells that could be reversed by addition of an excess of RANKL, suggesting that RANKL has a higher affinity for OPG than TRAIL (59, 60). Other studies have shown that OPG/TRAIL affinity was lower than OPG/RANKL affinity (18, 76). More recently, Vitovsky and co-workers have demonstrated that OPG/TRAIL affinity is comparable to that of OPG/RANKL but is temperature dependant (76, 77). However a much higher concentration of TRAIL than RANKL was used in this study (77).

Besides the difference of OPG affinity for its ligands, the structure of OPG (full length or truncated) is determinant for its biological activity. Schneeweis *et al* reported that OPG dimerization is required for an optimal inhibition of RANK/RANKL interaction (78). The knowledge of the precise domains implicated in the OPG binding to RANKL, TRAIL or other protagonists is crucial for the understanding of the molecular mechanisms involved in the regulation of OPG bioactivity in tumor micro-environment. In the literature, the OPG domain involved in TRAIL binding is discussed. Indeed, Cheng and co-workers have demonstrated that the OPG-like peptidomimetic (OP3-4) designed to bind RANKL and to restore bone loss *in vivo* is also able to bind TRAIL with a low affinity (79). In contrast, Heath *et al* have shown that OP3-4 had no effect on TRAIL-mediated apoptosis of myeloma cells *in vitro* suggesting that there is no interaction between TRAIL and OP3-4 (80).

Moreover, our team has observed a difference of biological activity between OPG 1-401 and OPG 1-194 overexpressed using the same delivery system (synthetic vectors) in a murine model of osteolytic osteosarcoma. Indeed, whereas OPG 1-194 significantly prevented bone lesions, tumor growth and increased survival, OPG 1-401 has little or no effect. As TRAIL

and RANKL are both produced in the osteosarcoma microenvironment, these cytokines may compete for OPG binding. *In fine*, their relative concentration will determine preferential OPG binding to TRAIL or to RANKL, and will direct OPG anti-bone resorption/anti-tumoral (RANKL binding) or pro-tumoral (TRAIL binding) activity. In this study, the difference of OPG bioactivity could not be explained by a difference in blocking TRAIL-induced apoptosis as both forms of OPG were able to bind TRAIL with the same affinity, demonstrating clearly for the first time that TRAIL binds OPG in its 22-194 domain (cysteine-rich domain), which is also recognized by RANKL (*unpublished data* and (81)).

A study recently reported the pro-tumoral effect of full length OPG *in vivo*. In this study, Fisher *et al.* demonstrated that full length OPG (native OPG) overexpressed by modified MCF-7 breast cancer cells which didn't express OPG initially, enhanced tumor growth *in vivo* associated to increased osteolysis (82). Conversely in the same study, treatment with OPG-Fc (22-194) inhibited bone lesions and consequently tumor proliferation in bone. Moreover, the effects of native OPG seemed to be TRAIL-independent because the authors observed no difference in apoptotic cell number between tumors from OPG over-expressing cells and those from parental derivatives, and no difference of TRAIL-induced apoptosis between MCF-7 and MCF-7 + PTHrP cells with or without OPG expression (82). The authors supported the idea that native OPG promotes tumor cell growth but not OPG-Fc, and explained this discrepancy by the difference of OPG molecular structure as OPG-Fc lacks its heparin binding domain, the death domains and the C-400 responsible for OPG dimerization which consequently cannot participate in intracellular actions (82).

Majority of the studies thus evidenced a beneficial effect of OPG on tumor growth in bone site indirectly through inhibition of bone degradation. However, other studies also demonstrate a potential pro-tumoral role of the OPG in its complete form, but these results were mainly obtained *in vitro*, except for two *in vivo* studies in which TRAIL was demonstrated not to be involved in this pro-tumoral effect. However, the OPG structure could be involved in this process as the beneficial effect on bone resorption and tumor growth were only observed with the truncated OPG form (22-194) which is not able to dimerize as compared to the native OPG (81, 82). Corresponding to the lacking domain 195-401, other protagonists such as proteoglycan (PG)/glycosaminoglycan (GAG) may also interfere in the regulation of OPG bioactivity by binding to the heparin binding domain located in the 361-400 region.

PGs regulate OPG bioavailability and bioactivity

The GAG moiety of PG associated to the tumor cell membrane or resident in the extracellular matrix plays a key role in various tumors in term of cancer cell proliferation, invasion, metastasis and angiogenesis (83-85). Besides its interaction with RANKL or TRAIL, OPG is also able to bind GAG present in tumor bone environment by its heparin binding domain. The involvement of GAG/PG in the regulation of OPG bioactivity has been first demonstrated by Standal *et al.* in myeloma cells which internalize and degrade OPG through its binding to syndecan-1 expressed at the myeloma cell membrane (86). Syndecan-1 is also involved in OPG induced monocyte chemotaxis as reported by Mosheimer *et al* (87). More recently, Theoleyre *et al* demonstrated the potential role of GAGs in regulating OPG binding activity to the RANK/RANKL complex by molecular interaction analyses (88). Indeed, OPG binds heparin with a high affinity and pre-incubation of OPG with heparin inhibited in a dose-dependent manner OPG binding to the RANK-RANKL complex. Moreover, pre-incubation of OPG with GAGs strongly inhibited the OPG induced decrease of membrane RANKL half-life in 293 cells overexpressing RANKL (88). Irie and co-workers recently reported that heparin enhances osteoclastic bone resorption in coculture of bone marrow cells and osteoblasts by inhibiting OPG activity without interacting with RANK or RANKL (89).

For the first time *in vivo*, we have recently shown that PGs/GAGs highly expressed in osteosarcoma microenvironment could regulate OPG bioactivity. Indeed, the difference of OPG biological activity observed between OPG 1-194 and OPG 1-401 on bone lesions prevention and tumor growth inhibition may be explained by the modulation of OPG 1-401 bioavailability but not OPG 1-194 which lack its heparin binding domain (*unpublished data and* (81)). In addition, surface plasmon resonance studies revealed that GAGs were able to disturb OPG 1-401/RANKL as well as OPG 1-401/TRAIL interaction. In this model, we suggest that PGs/GAGs could play a role as inhibitors of OPG 1-401 activity through its sequestration in tumor bone matrix.

These overall results suggest different levels of bone resorption regulation by PGs/GAGs: directly through the regulation of OPG bioavailability and biological activity, and indirectly on RANKL activity in bone tumor environment (90). PGs/GAGs may also directly regulate RANKL activity through a strong binding to RANKL and inhibition of signal transduction in osteoclast progenitor cells (91). In this study, the authors demonstrated that dermatan sulphate (DS) present in bone and in the cartilage matrix was able to inhibit the

interaction RANK/RANKL and to suppress osteoclast formation induced by RANKL and M-CSF in mouse bone marrow cultures, suggesting that DS plays an important regulating role in bone remodelling (91).

Conclusion

The role of OPG in cancer remains unclear and conflicting between anti-resorptive activity associated to indirect anti-tumoral effect and the potential survival effects mediated through TRAIL inhibition that could never be demonstrated *in vivo*. Indeed, the pro-tumoral effects induced by the inhibition of TRAIL activity were only observed *in vitro*. These results remain to be confirmed *in vivo* in the presence of all protagonists which intervene in the establishment and maintenance of tumor bone microenvironment. The timing, spacial expression and relative concentration of each cytokine and their receptors will determine their respective roles in this particular microenvironment.

In a tumor context, the role of OPG may be regulated by the RANKL/TRAIL balance since these two cytokines seem to have the same affinity for OPG. If the concentration of RANKL is higher than that of TRAIL in the tumor bone microenvironment, OPG could exert an anti-resorptive effect and indirectly antitumor activity by preferential binding to RANKL rather than TRAIL (Fig. 3). In contrast, if TRAIL concentration is higher than that of RANKL, OPG could bind preferentially TRAIL and would represent a survival factor for tumor cells by inhibition of TRAIL-mediated apoptosis, provided that TRAIL concentration is lower than OPG (Fig.3). Indeed, when TRAIL is over-expressed (30 mg/kg/dose) in murine breast cancer cells injected in bone, it prevented bone destruction and reduced tumor burden (74). These data indicate that TRAIL exerts a direct effect on tumor cells and indirectly on bone resorption by blocking the vicious cycle, and suggests that OPG present within the tumor bone microenvironment is not sufficient to bind TRAIL in excess and to induce pro-tumoral effect.

In addition besides TRAIL and RANKL, the bio-availability and bioactivity of OPG may be regulated by GAG/PG present at the surface of tumor cells or in the tumor bone matrix. GAGs/PGs are able to bind OPG by its heparin binding domain and probably to store OPG in the tumor bone matrix thereby blocking its activity. However, all studies evidenced that the use of OPG 22-194 without the heparin binding domain shows a best therapeutic efficiency as compared to the native OPG, highlighting the importance of OPG structure in the regulation of its activity.

This review confirms the complexity of the molecular mechanisms involved in the tumor bone microenvironment and the precise role of each protagonist. The spatial and time-dependent expression, and the relative concentration of TRAIL, RANKL, OPG, PGs and probably other protagonists seem to be determinant to define their respective role in the tumor bone microenvironment. Despite of the advances in the knowledge of these molecules, many questions remain unresolved on the conflicting role of OPG in bone cancer and call into question the possibility to use OPG as a therapeutic approach in bone tumors.

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Pour voir les figures, consulter la thèse sous sa forme papier

Legends

Figure 1

Comparison of the OPG 1-401 and OPG 1-194 functional domains

Molecular structures of OPG allow the binding with different ligands such as RANKL and TRAIL on the cysteine-rich domains, and GAG/PG on the heparin binding domain.

Figure 2

Vicious cycle between tumor proliferation and bone resorption

Osteoclast activating factors (PTH-rP, IL, TNF- α ,...) expressed by tumor cells induce RANKL mediated bone resorption which allows the release of growth factors (TGF- β , BMP, IGF-1) which in turn stimulate tumor growth.

Figure 3

Regulation of OPG activity by the bone tumor micro-environment

The relative concentration of each OPG ligand (RANKL, TRAIL or GAG/PG) within the tumor bone micro-environment will determine its anti- or pro-tumoral activity.

Table I Regulation of OPG bioactivities depends on the nature of its ligand

OPG ligands	Biological effects	References
RANKL	Inhibition of osteoclast formation Decrease tumor growth in bone and increase survival rate	[2, 12, 14, 19-21, 34-54, 74-77]
TRAIL	Survival of tumor cells Bone cell regulation	[55-63]
GAG/PG	Regulation of OPG bioavailability by internalization, degradation and storage in the tumor bone matrix Inhibition of OPG/RANKL interaction	[76, 81-85]

Complément de discussion de l'article 4

Le rôle de l'OPG dans les cancers demeure confus et conflictuel entre son activité anti-résorptive associée à son effet anti-tumoral indirect et son rôle potentiel de facteur de survie des cellules tumorales médié par l'inhibition de TRAIL, mais qui n'a jamais été démontré *in vivo*. En effet, les travaux rapportant que l'OPG serait un facteur de survie par l'inhibition de l'apoptose des cellules tumorales induite par TRAIL ont été réalisés *in vitro* ne reflétant pas les réelles conditions du microenvironnement tumoral osseux. Les résultats publiés sont donc à confirmer *in vivo* en présence de tous les protagonistes intervenant dans la mise en place et le maintien du microenvironnement tumoral. L'expression spatiale et temporelle ainsi que la concentration relative de chaque cytokine et de leurs récepteurs sont déterminants dans le rôle que chacun jouera à l'intérieur de cet environnement tumoral.

Dans ce contexte, le rôle de l'OPG peut être régulé par la balance RANKL/TRAIL puisque ces deux cytokines sont capables de se lier à l'OPG sur un site voisin voire commun et avec une affinité similaire. Si la concentration de RANKL est supérieure à celle de TRAIL dans le microenvironnement tumoral osseux, alors l'OPG exercera préférentiellement du fait de sa fixation à RANKL un effet anti-résorptif qui entraînera un effet anti-tumoral. A l'inverse, si TRAIL est en concentration supérieure par rapport à RANKL dans l'environnement tumoral, alors l'OPG jouera plutôt le rôle de facteur de survie pour les cellules tumorales par sa fixation et l'inhibition de l'activité apoptotique de TRAIL, mais à la condition que TRAIL soit moins exprimé que l'OPG. Dans le cas inverse, le rôle de TRAIL comme inducteur d'apoptose des cellules tumorales sera supérieur à celui de l'OPG. En effet, une étude rapporte qu'une surexpression de TRAIL (30mg/kg/dose) dans un modèle murin de cellules de cancer du sein injectées en site intra-osseux a pour effet de réduire le volume tumoral et de prévenir les lésions osseuses associées (Thai le et al., 2006). Ces données indiquent que TRAIL exerce un effet direct sur les cellules tumorales et indirect sur la résorption osseuse via le blocage du cercle vicieux, mais de plus suggère que l'OPG n'est pas présent en quantité suffisante pour contrecarrer l'effet de TRAIL, présent en excès, et induire un effet pro-tumoral.

D'autres protagonistes ont été rapportés pour réguler la biodisponibilité et l'activité biologique de l'OPG, ce sont les PGS/GAGs présents à la surface des cellules ou dans la matrice tumorale osseuse. Les PGS/GAGs sont capable de lier l'OPG par son domaine de liaison à l'héparine et ainsi de moduler son activité biologique par différents mécanismes

tels que l'internalisation, la dégradation ou le stockage. L'article précédent souligne l'intérêt thérapeutique de l'OPG 1-194 dépourvu du domaine de liaison à l'héparine et l'importance de la structure de l'OPG dans la détermination de sa fonction.

Par cette revue nous soulignons la complexité des mécanismes moléculaires impliqués dans la mise en place du microenvironnement tumoral osseux et le rôle de chaque protagoniste déterminé par leur localisation, leur concentration à un temps donné. Malgré les avancées des connaissances sur ces molécules, le rôle de l'OPG comme agent thérapeutique dans le traitement des cancers osseux demeure un sujet à controverse.

Conclusion de la partie II

Ces deux études démontrent que le rôle de l'OPG dans les tumeurs osseuses n'est encore pas clairement défini et dépend du microenvironnement tumoral osseux et des protagonistes présents, notamment de la balance RANKL/TRAIL capable de faire basculer la fonction de l'OPG d'une molécule anti-tumorale à une molécule pro-tumorale. De plus, sa structure semble jouer un rôle très important dans l'orientation de sa fonction biologique. Néanmoins, le rôle de l'OPG comme facteur de survie des cellules tumorales n'a été rapporté jusqu'à présent que dans des études *in vitro*. Paradoxalement, les études réalisées à partir de modèles *in vivo* rapportent plutôt un effet bénéfique de l'OPG sur la prévention des lésions osseuses et sur l'inhibition de la croissance tumorale. Les contradictions observées entre les études *in vitro* et *in vivo* peuvent être discutées sur plusieurs points :

- L'absence du microenvironnement tumoral osseux et des différents facteurs et cytokines associés est un élément essentiel non reproduit dans les études *in vitro*.
- La mise en place de modèles animaux est souvent limitée à l'implantation ou l'injection de cellules tumorales au niveau de sites uniques osseux et non d'un développement tumoral osseux à partir de cellules circulantes.
- De plus, il est possible que l'effet inhibiteur de l'OPG sur l'apoptose des cellules tumorales induite par TRAIL ne peut s'effectuer qu'à un stade précoce du développement tumoral, lorsque le nombre de cellules tumorales est faible. En effet, l'activité biologique de l'OPG peut être modulable dans le temps, suivant la concentration de chaque protagoniste présent dans le microenvironnement tumoral osseux et donc favorable à la croissance tumorale. Aucune donnée bibliographique démontre cette hypothèse, mais l'importance du facteur temps dans le développement tumoral est fortement suggéré (Holen and Shipman, 2006). Nos résultats obtenus par transfert de gène en traitement curatif et non préventif montraient qu'il y avait un effet seuil au niveau du volume tumoral. En effet, à partir d'un volume trop important, l'OPG n'exerçait plus d'effet thérapeutique. Ces observations soutiennent l'hypothèse que le rôle de l'OPG peut être modulé dans le temps suggérant aussi une possible variation des concentrations de TRAIL et RANKL dans l'environnement osseux, déterminants dans l'activité biologique de l'OPG.

En conclusion de cette partie, seule l'OPG 1-194 montre des effets bénéfiques sur la prévention des lésions osseuses et l'inhibition indirecte de la progression tumorale. Bien que sa structure semble jouer un rôle majeur dans ces effets, il n'en demeure pas moins que le rôle de l'OPG dans le développement tumoral dépend également de l'expression des différents protagonistes moléculaires présents dans l'environnement tumoral osseux pouvant moduler son activité biologique.

Partie III

Rôle de RANKL dans le développement tumoral

Introduction

Les recherches sur la triade moléculaire OPG/RANK/RANKL ont révélé le rôle essentiel de ces molécules dans la différenciation et l'activité des ostéoclastes en conditions normales physiologiques, et leur implication dans le développement des lésions osseuses d'origine tumorale.

L'interaction RANK/RANKL induit directement une cascade de signalisation menant à la différenciation, l'activation et la survie des ostéoclastes et par voie de conséquence à la résorption osseuse. L'OPG agit comme un récepteur leurre soluble de RANKL et bloque son interaction avec RANK, limitant ainsi l'ostéoclastogénèse. Par conséquent, la balance RANKL/OPG est déterminante dans la régulation de l'ostéoclastogénèse et donc de la résorption osseuse. De plus, RANKL se trouve anormalement régulé dans les conditions pathologiques malignes. En effet, l'ostéoclaste, en résorbant l'os, libère plusieurs facteurs actifs dans le microenvironnement osseux, tels que le TGF- β , les FGFs, les BMPs, l'IGF-1. Certains facteurs sont favorables à la croissance tumorale, alors que d'autres comme le TGF- β ne semblent pas intervenir dans la croissance des cellules tumorales mais conduisent à la production de PTHrP par ces cellules (Guisse, 2000). Cette production de PTHrP est responsable d'une augmentation de RANKL et donc d'une accélération de la résorption osseuse, avec la libération de facteurs favorables à la croissance tumorale. Ces processus font de RANKL un élément critique dans l'établissement du cercle vicieux qui s'instaure entre la résorption osseuse et la prolifération tumorale.

Ainsi la compréhension du rôle de RANKL et de sa signalisation dans le développement de lésions osseuses d'origine tumorale est une des étapes-clés dans le développement de stratégies thérapeutiques, puisqu'il est maintenant très clair que l'inhibition de RANKL dans des pathologies osseuses malignes est favorable à la prévention osseuse et à l'inhibition de la progression tumorale (Corey et al., 2005; Croucher et al., 2001; Kiefer et al., 2004; Lamoureux et al., 2007b; Morony et al., 2001; Pearse et al., 2001; Zhang et al., 2001; Zhang et al., 2003).

Il a été rapporté que la présence de RANKL est augmentée dans le microenvironnement tumoral osseux aussi bien dans les tumeurs osseuses secondaires (sein, poumons, prostate) que primitives (ostéosarcome). Dans les pathologies osseuses malignes, il est rapporté que différents facteurs (PTHrP, IL-1, IL-6, IL-11 et TNF- α) peuvent augmenter l'expression de RANKL par les ostéoblastes et les cellules stromales (Haaber et al., 2008;

Horwood et al., 1998a; Lee and Lorenzo, 1999; Palmqvist et al., 2002; Thomas et al., 1999). Dans le myélome multiple, RANKL est augmenté mais il a été démontré que RANKL pouvait également être exprimé par les lymphocytes T (Giuliani et al., 2002) et à des faibles concentrations par les cellules tumorales elles-mêmes (Farrugia et al., 2003; Heider et al., 2003; Lai et al., 2004). De plus, l'expression de l'OPG se trouve diminuée (Giuliani et al., 2001; Seidel et al., 2001; Standal et al., 2002) dans le myélome multiple accentuant l'augmentation de la balance RANKL/OPG. L'expression de RANKL est également augmentée dans le cancer du sein (Huang et al., 2002; Kitazawa and Kitazawa, 2002).

L'ensemble de ces données est concordant avec l'implication importante de RANKL dans les pathologies osseuses malignes et est renforcé par des expérimentations *in vivo* dans lesquelles l'inhibition de RANKL prévient les lésions osseuses et la progression tumorale. Outre son implication dans le développement de lésions osseuses associées à la tumeur, RANKL peut stimuler la production de facteurs ostéotropiques (IL-1, IL-6, CSF-1), angiogéniques (IL-8, CXCL-2) et chimio-attractants (CXCL-1, 2, 3, 5 et 6) par les cellules tumorales exprimant RANK (Tometsko et al., 2004). De plus, RANKL est capable d'augmenter directement la migration de cellules tumorales exprimant RANK, médiant un tropisme osseux pour les cellules tumorales (Jones et al., 2006). RANKL semble donc jouer un rôle critique dans l'invasion des cellules tumorales en site osseux.

Actuellement, l'activité ostéoclastique et plus précisément l'inhibition de RANKL est la cible majeure dans le développement de nouvelles stratégies thérapeutiques pour les pathologies osseuses d'origines tumorales (bisphosphonates, denosumab, peptidomimétiques).

C'est pourquoi, après des approches utilisant OPG et RANK-Fc, il nous a paru nécessaire de mettre en place une stratégie utilisant l'ARN interférence pour directement bloquer RANKL. La première étape a alors été d'établir un modèle animal dans lequel nous pouvions aisément tester nos siRNA anti-RANKL, et une des approches envisagée a été d'induire un modèle avec une forte concentration de RANKL facilement dosable au niveau sérique pour pouvoir tester *in vivo* l'efficacité des siRNA anti-RANKL. Pour ce faire, des cellules rénales fœtales humaines 2.93 ont été transfectées pour exprimer RANKL puis injectées à des souris immuno-déprimées.

Le modèle obtenu a non seulement permis d'induire une augmentation significative de RANKL au niveau sérique mais également montré de façon inattendue un développement tumoral et des lésions osseuses associées proportionnelles à la concentration

de RANKL mesurée au niveau sérique. Ce modèle met en évidence le rôle majeur de RANKL dans le développement de lésions osseuses et de la tumeur en site osseux.

Matériels et méthodes

2.1 Cultures cellulaires

2.1.1 Milieux, Solutions et Matériels utilisés

- Milieu de culture DMEM à 4.5 g/l de glucose, 2mM L-glutamine (Biowhittaker, Verviers, Belgique)
- Sérum de veau fœtal (SVF ; Hyclone Perbio, Logan, USA)
- Antibiotiques : Penicilline 100U/ml et Streptomycine 100µg/ml (Invitrogen, Cergy-Pontoise, France)
- DPBS (Dulbecco's Phosphate Buffered Saline sans Ca ni Mg ; Biowhittaker)
- Versene:Trypsine 0.05% / EDTA 0.02% (Biowhittaker)
- Supports plastiques stériles (Corning Costar, Dominique Dutscher, Brumath, France)

2.1.2 Lignée cellulaire 2.93

Il s'agit de cellules humaines de rein fœtal cultivées en milieu DMEM supplémenté avec 10% de SVF. De base, ces cellules n'expriment pas RANKL. Elles ont ensuite été transfectées par les différentes isoformes de RANKL murin utilisant le PEI (polyéthylénimine; fourni par le Dr. Bruno Pitard) puis les cellules transfectées ont ensuite été sélectionnées à la blasticidine pour obtenir une transfection stable. Des clones cellulaires ont ensuite été sélectionnés en fonction de leur niveau d'expression des différentes isoformes de RANKL. Les cellules 2.93RL correspondent à la transfection de l'isoformes mRANKL 1, qui est la forme complète de RANKL pouvant être membranaire ou soluble après clivage. Les cellules 2.93RL 2.0 correspondent à la transfection de l'isoforme mRANKL 2, dépourvue de la partie centrale de sa partie intracellulaire. De ce fait, il est moins exprimé à la membrane que l'isoforme mRANKL 1. Puis les cellules 2.93RL 2.1 correspondent à la transfection de l'isoforme mRANKL sans domaine intracellulaire mimant hRANKL2. De ce fait, après transfection, cette isoforme reste intracytoplasmique car elle n'est pas adressée à la membrane.

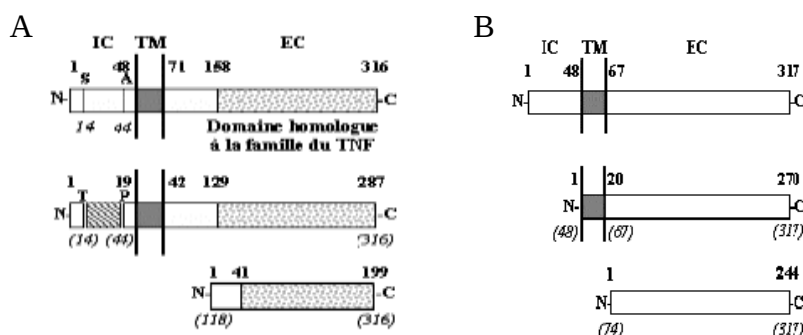


Schéma représentatif des différentes isoformes de RANKL murin (A) et humain (B).

La culture s'effectue dans une atmosphère saturée en humidité avec 5% de CO₂ à 37°C. Une fois à confluence, ces cellules adhérentes sont décollées dans une solution de Trypsine–EDTA. Après quelques minutes à 37°C, elles sont rincées par le DPBS, et la viabilité est estimée par la méthode d'exclusion au Bleu Trypan. Le comptage s'effectue sur une cellule de Malassez.

2.2 Experimentations *in vivo*

2.2.1 Modèle tumoral murin

Des souris mâles Nudes Athymiques (Elevage Harlan) de 4 semaines sont hébergées dans des conditions exemptes de pathogènes à l'Unité de Thérapeutique Expérimentale de la faculté de Médecine de Nantes en accord avec la réglementation du Comité d'Ethique Français. Après anesthésie par inhalation d'une combinaison isoflurane / air (1,5%, 1L/min), les souris reçoivent une injection de 2 millions de cellules 2.93, 2.93RL, 2.93RL 2.0 ou 2.93RL 2.1 en site intramusculaire dans le muscle tibial antérieur. Dans ces conditions, les souris développent une tumeur au contact du tibia en environ 30 jours associée à une surexpression de RANKL sérique et de sévères lésions ostéolytiques.

2.2.2 Approches thérapeutiques par OPG

- Expérimentations de transfert de gène *in vivo* :

La solution ADN/F68 est préparée à la concentration de 50µg d'ADN et de F68 à 3% dans un volume final de 50µl NaCl 150 mM. Les souris anesthésiées à l'éthomidate (40 mg/kg, voie intra péritonéale) reçoivent deux injections intra-musculaires de 50µl chacune, du mélange plasmide (ADN)/ vecteur (F68) ou de plasmide seul (ADN nu), au niveau du muscle tibial antérieur de chaque patte. Différents groupes (n=6) sont définis suivant l'ADN d'intérêt étudié : contrôle pcDNA3.1 seul/F68, OPG humain/F68.

La première injection du complexe ADN/vecteur est réalisée au moment où la tumeur est palpable, suivie de 4 autres injections à 7 jours d'intervalle.

- Injection de protéines recombinantes :

L'OPG-Fc de rat, protéine recombinante dépourvue du domaine de liaison à l'héparine, des domaines de mort et de la cystéine 400 (OPG 22-194), a été gracieusement fournie par les laboratoires Amgen Inc. (Thousand Oaks, USA). Les groupes sont traités avec du PBS ou de l'OPG-Fc (2,5 mg/kg) à raison de 3 injections en site sous-cutané par semaine durant le temps de l'expérimentation.

2.2.3 Suivi clinique

Le volume tumoral est mesuré tous les deux jours et calculé suivant la formule $[(L \times l^2)/2]$ où L correspond au plus grand axe et l au plus petit axe. Des prélèvements sanguins au niveau du sinus veineux sont réalisés afin de déterminer l'expression sérique de RANKL par dosage ELISA (§ 2.3). Les souris sont euthanasiées 50 jours post-implantation, et les pattes où se sont développées les tumeurs sont conservées pour analyses immunohistochimiques, radiographiques et au microscanner.

2.3 Dosage sérique de RANKL par ELISA

Le dosage de RANKL murin est effectué à l'aide d'un kit ELISA (R&D Systems, Abingdon, Grande-Bretagne). Une plaque 96 puits est recouverte par 100µL d'anticorps de capture anti-RANKL de souris à 2µg/mL pendant une nuit à température ambiante. Après trois rinçages avec une solution détergente de PBS / 0,05% Tween 20, 100µL d'une solution PBS/BSA 1% avec 5% de sucrose sont déposés dans chaque puits afin de saturer les sites non fixés par les anticorps. Après une incubation d'une heure, trois rinçages sont effectués avec la solution détergente. Après une dilution adéquate, chaque échantillon à doser est déposé en duplicat à raison de 100µL par puits. En parallèle, une gamme étalon de RANKL-Fc murin à différentes concentrations (0 ; 0,015 ; 0,03 ; 0,062 ; 0,125 ; 0,25 ; 0,5 et 1ng/ml) est réalisée. Après une incubation à température ambiante de deux heures et rinçage, 100µL d'anticorps anti-RANKL biotinylé (200ng/mL dilués dans du PBS/BSA 1%) sont ajoutés par puits. Suite à une nouvelle incubation de deux heures et rinçages, 100µL de Streptavidine Conjugase diluée au 200^{ème} sont ajoutés, puis incubés 20 minutes à température ambiante. Après rinçages, 100µL de substrat TMB (Tetramethylbenzidine) sont ajoutés, puis la plaque est incubée 20 minutes à température ambiante à l'obscurité. La réaction enzymatique est ensuite stoppée par l'ajout de 50µL d'une solution d'acide sulfurique 1M. La densité optique est alors mesurée à 450 nm à l'aide d'un lecteur de plaque ELISA (Victor, Hewlett Packard).

2.4 Immunohistochimie

Dans un premier temps, les prélèvements (tumeur et muscle) sont fixés dans une solution de formol 4%, puis inclus en paraffine et des coupes de 3µm des blocs sont réalisées à l'aide d'un microtome Reichert-Jung®. Les coupes sont ensuite recueillies et déposées sur lame de verre. Après déparaffinage (bains de toluène) et réhydratation, l'activité des enzymes endogènes par de l'H₂O₂ (3%) est bloquée pendant 5 minutes et rincé par le PBS pendant 2 minutes. Les liaisons non spécifiques des protéines sont bloquées par une solution de BSA (5%) pendant 10 minutes. Les différents anticorps (anti-KL1, 1/100^{ème}, Immunotech ; anti-CKAE1/AE3, 1/60^{ème}, DAKO ; anti-PS100, 1/1000^{ème}, DAKO ; anti-CD31, 1/20^{ème}, DAKO ; anti-CD34, 1/20^{ème}, Tebu-Bio) dilués dans du PBS/BSA 1% sont ajoutés sur les coupes qui sont incubées durant une heure, puis un rinçage au PBS de 5 minutes est réalisé. Les lames sont ensuite déposées dans un bain contenant le complexe enzymatique extravidine-peroxydase (Sigma-Aldrich) dilué au 100^{ème} pendant 20 minutes à température ambiante. Les lames sont rincées dans du PBS pendant 5 minutes, puis l'activité peroxydase est révélée par un kit AEC chromogène (AEC staining kit, Sigma-Aldrich). La contre-coloration est réalisée par un bain d'hématoxyline Mayer. Les lames sont montées en milieu aqueux grâce à un système Immunomount (ThermoShandon, Pittsburgh, USA). Les photographies des lames sont réalisées au microscope (NIKON Eclipse E600) grâce à une caméra (NIKON Digital Camera DXM1200).

2.5 Statistiques

Pour les expérimentations *in vivo*, le test ANOVA a été utilisé. Les différences de survie sont déterminées par le test log-RANK sur la courbe de survie Kaplan Meier. Les résultats sont considérés comme significatifs lorsque $p \leq 0,05$.

Résultats

3.1 Surexpression de RANKL au niveau sérique.

Afin de développer un modèle surexprimant RANKL dosable aisément au niveau sérique, des souris nues athymiques ont reçu une injection de 2 millions de cellules 2.93RL ou 2.93 (servant ici de contrôle négatif) en divers sites : sous cutané, intra-veineux ou intramusculaire. Le sérum prélevé à différents temps montre une surexpression de RANKL 50 jours post-injection (environ 1,6 ng/ml) plus importante dans le groupe de souris injectées avec les cellules 2.93RL en site intramusculaire, par comparaison aux groupes contrôles ou aux autres sites d'injection (Figure 1).

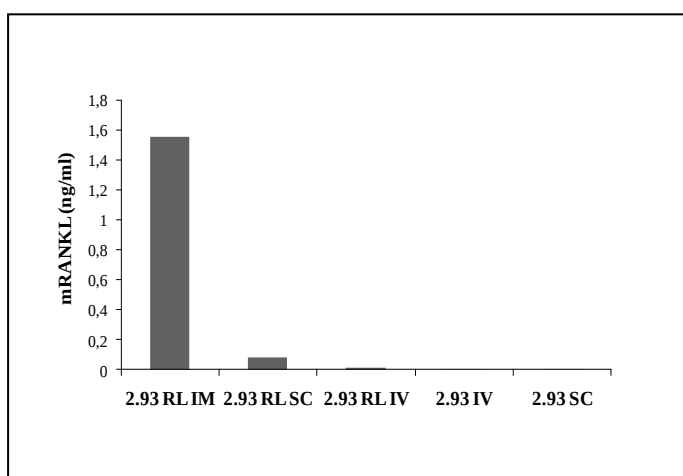


Figure 1 : Surexpression sérique de RANKL à J50 chez des souris ayant reçu une injection de cellules 2.93RL ou 2.93 (servant de contrôle négatif) en site sous cutané (SC), intraveineux (IV) ou intramusculaire (IM).

De plus, il est à noter que les souris ayant reçu une injection de cellules 2.93 ou 2.93RL en site intramusculaire développent, de façon surprenante, une masse tumorale après deux mois par comparaison aux souris injectées en d'autres sites. Le volume de la tumeur est donc plus important lorsque les souris surexpriment RANKL (souris ayant reçu une injection de cellules 2.93RL) en comparaison aux souris ayant reçu une injection de cellules 2.93.

Afin de d'étudier la relation structure-fonction de RANKL dans ce développement tumoral, des cellules 2.93 surexprimant différentes constructions de RANKL ont été injectées en site intramusculaire dans des souris nues athymiques. Les cellules 2.93RL expriment la forme complète mRANKL 1. Les cellules 2.93RL 2.0 expriment la forme mRANKL 2 dépourvu d'un domaine de sa partie membranaire, par conséquent RANKL est moins

exprimés à la membrane que dans les cellules 2.93RL. Les cellules 2.93RL 2.1 expriment l'isoforme mRANKL, sans domaine intracellulaire mimant hRANKL2. De ce fait, après transfection, cette isoforme reste intracytoplasmique car elle n'est pas adressée à la membrane. Puis, les cellules 2.93 qui n'expriment pas RANKL, servent ici de contrôle.

Les résultats montrent que la surexpression sérique de RANKL varie suivant les clones cellulaires injectés. Les résultats obtenus sont en accord avec ceux obtenus *in vitro* lors de la sélection des clones, puisque les différentes isoformes de RANKL ne sont pas exprimées dans les mêmes proportions au niveau membranaire et soluble. En effet, à J50, la surexpression sérique de RANKL est plus élevée dans les souris ayant reçu une injection de cellules 2.93RL (en moyenne 24,24 ng/ml), comparativement aux souris ayant reçu une injection de cellules 2.93RL 2.0 (en moyenne 0,99 ng/ml), alors que l'expression de RANKL est quasi nulle dans les souris ayant reçu une injection de cellules 2.93 ou 2.93RL 2.1 (Figure 2).

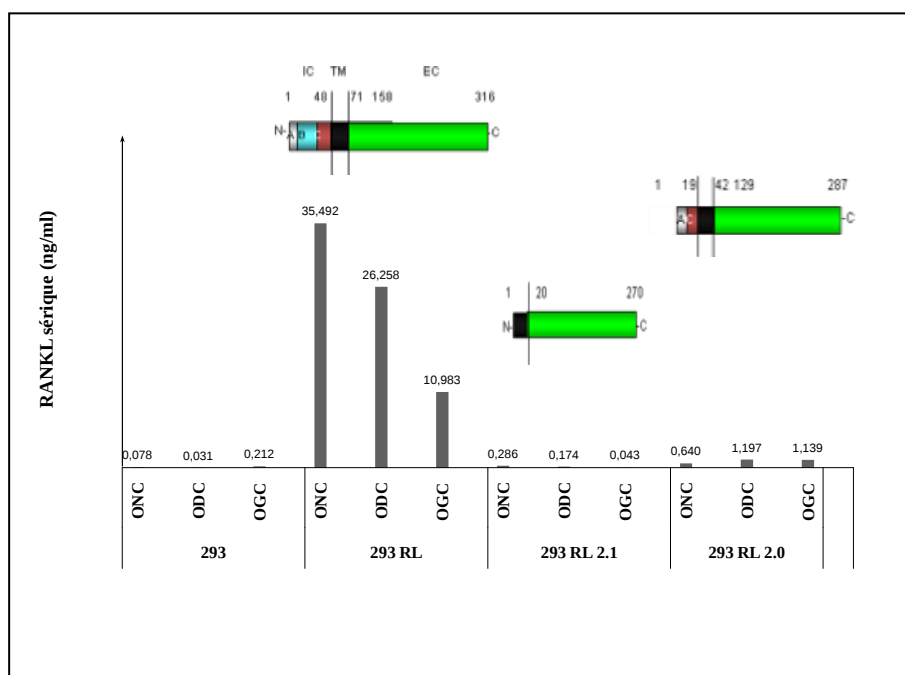


Figure 2 : Comparaison de l'expression sérique de RANKL à J50 après l'injection en site intramusculaire de cellules exprimant les différentes isoformes de RANKL: cellules 2.93RL, 2.93RL 2.0, 2.93RL 2.1 ou 2.93 (n=3).

3.2 RANKL induit une ostéolyse plus sévère.

En parallèle au dosage sérique de RANKL, le remodelage osseux a été étudié par radiographie chez les souris ayant reçu les différentes isoformes de RANKL (Figure 3)

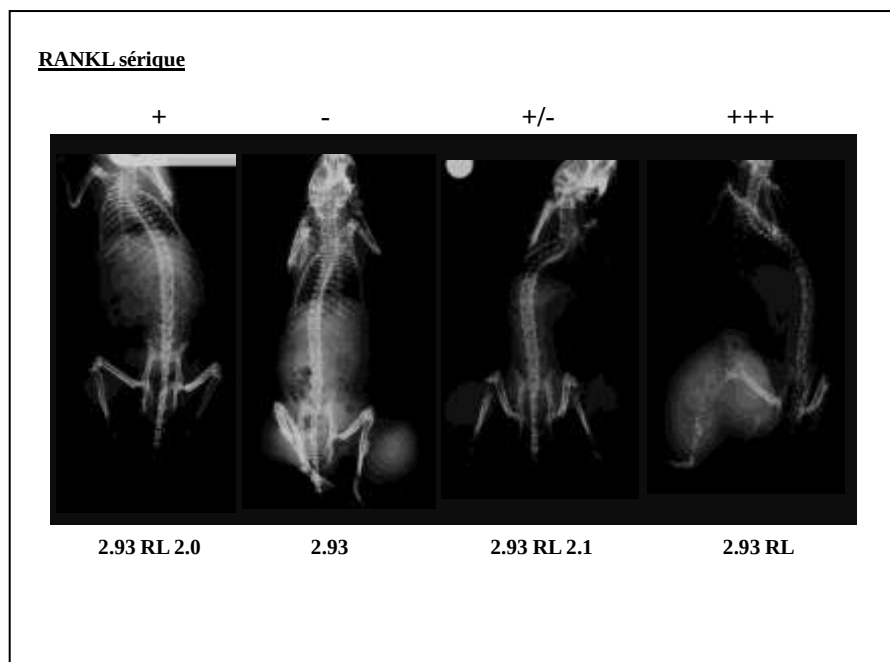


Figure 3 : Radiographies à J50 des souris ayant été injectées avec 2 millions de cellules 2.93 RL, 2.93 RL 2.0, 2.93 RL 2.1 ou 2.93 en site intramusculaire (muscle tibial antérieur, proche du tibia) montrant des lésions ostéolytiques dont la sévérité est corrélée à l'expression de RANKL.

Les analyses radiographiques montrent la présence de lésions ostéolytiques dans chaque modèle, avec une résorption osseuse très prononcée au niveau de la corticale du tibia. Cependant, l'intensité des lésions ostéolytiques semble être étroitement corrélée au niveau d'expression de RANKL. En effet, le groupe 2.93RL surexprimant le plus RANKL présente une ostéolyse très importante avec une disparition quasi-totale du tibia en comparaison aux autres groupes qui montrent une ostéolyse plus ou moins sévère suivant l'expression de RANKL (Figure 3).

3.3 Le niveau de sévérité de l'ostéolyse est associé au développement tumoral

De façon inattendue, la surexpression de RANKL et l'ostéolyse corrélaient avec le développement d'une tumeur. En effet, les souris ayant reçu les cellules 2.93RL présentent un volume moyen plus important (10000 mm^3 à J50) en comparaison aux autres groupes ayant reçu l'injection de cellules 2.93 ou 2.93RL 2.0 (respectivement 2100 et 1600 mm^3 à J50). Par ailleurs, les souris ayant reçu l'injection de cellules 2.93RL 2.1 présentent un volume tumoral intermédiaire (6000 mm^3), alors que l'expression sérique de RANKL n'était pas détectée dans ces souris et que RANKL est humain et non murin.

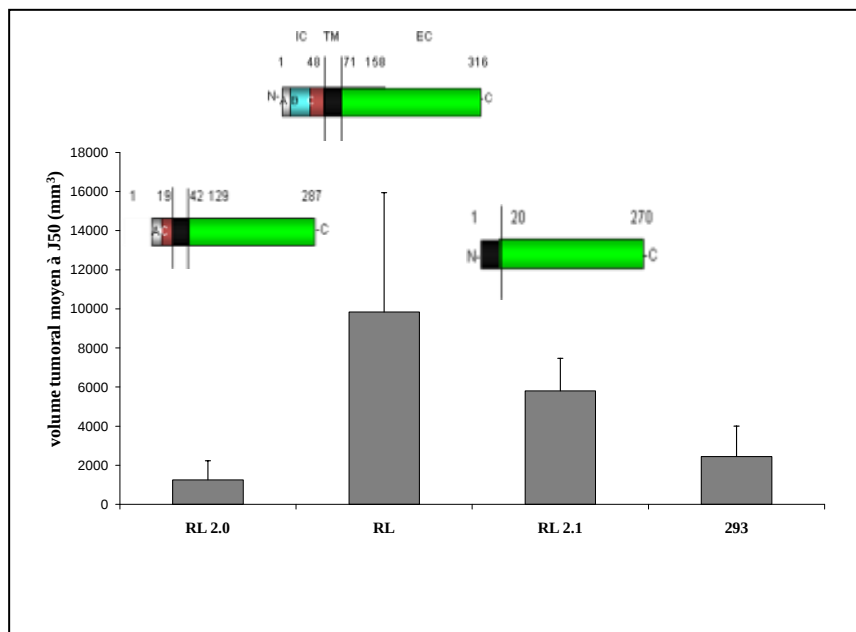


Figure 4 : Comparaisons des volumes tumoraux moyens à J50 des souris nudes athymiques (n=3) ayant été injectées avec des cellules 2.93RL, 2.93RL 2.0, 2.93RL 2.1 ou 2.93 en site intramusculaire.

3.4 Modèle transplantable - Caractérisation histologique de la tumeur

Afin de caractériser cette tumeur qui se développe suite à l'injection de cellules $2.93 \pm RL$, de volume plus important en présence d'une surexpression de RANKL et associée à des lésions ostéolytiques sévères, nous avons, dans un premier temps transplanté un fragment de cette tumeur à différents sites : para-tibial, au contact du crâne, sous-cutané proche de la colonne vertébrale, et à différents sites éloignés d'un site osseux (ex : intra-péritonéal; Figure 5).

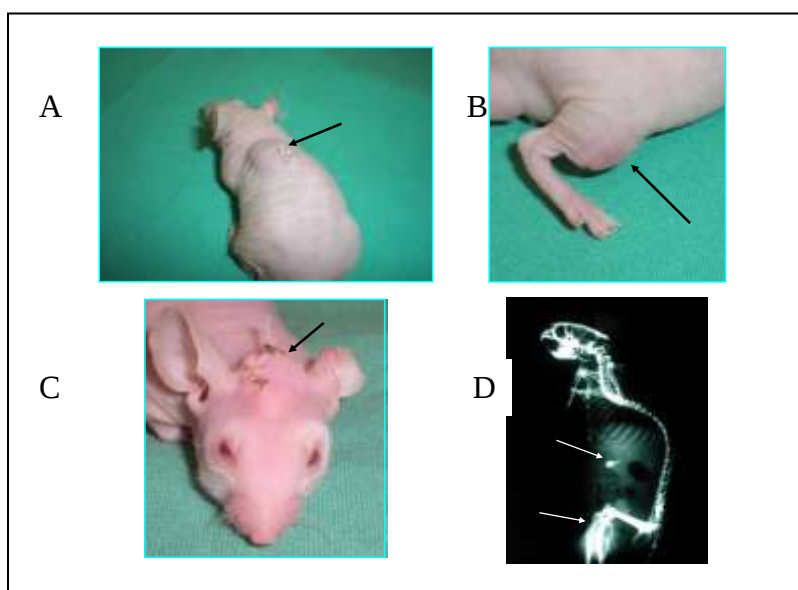


Figure 5 : Développement d'une tumeur à J7 après transplantation d'un fragment d'une tumeur 2.93RL, au niveau de la colonne vertébrale (A), en site para-tibial (B) et au niveau du crâne (C). Radiographie d'une souris transplantée en paratibial et en intra-péritonéal (D).

Nous avons pu observer que cette tumeur est transplantable avec un développement très rapide dès 7 jours après la transplantation (Figure 5A, B et C). De plus, lorsque la transplantation est effectuée en site para-osseux, la tumeur conserve son caractère ostéolytique (Figure 5D). Par contre, et de façon surprenante, nous avons observé, une seule fois, une calcification au niveau du foie à la suite d'une transplantation intra-péritonéale (Figure 5D). A ce jour, les données histologiques n'ont pas confirmé cette observation. L'expression de RANKL sera étudiée prochainement dans ce modèle transplanté afin de vérifier la surexpression sérique de RANKL comme dans le modèle tumoral d'origine.

E. Immunohistochimie

Dans un deuxième temps, la tumeur a été caractérisée au niveau histologique dans le but de définir son origine (Figure 6).

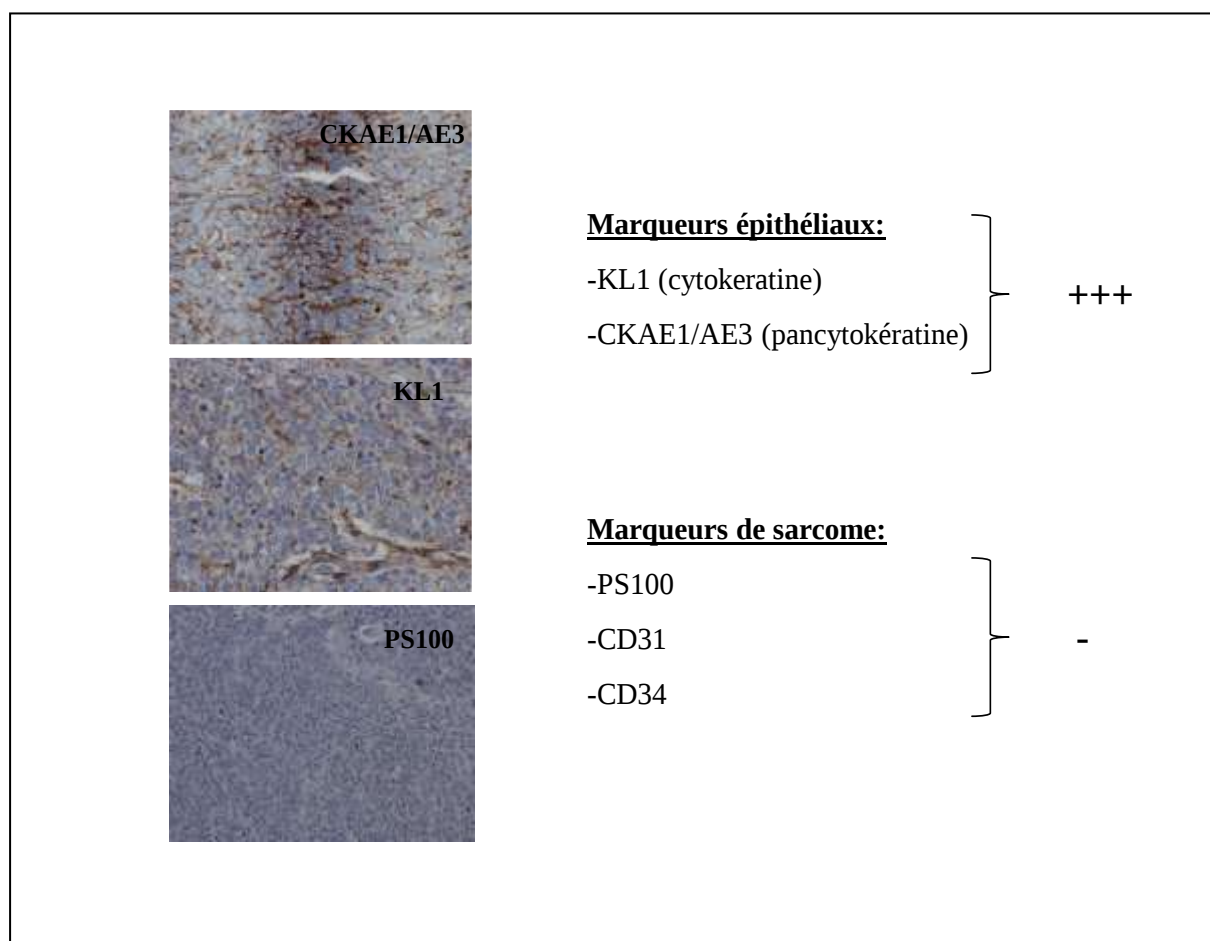


Figure 6 : caractérisation histologique de la tumeur développée suite à l'injection intramusculaire de cellules 2.93RL. Immunohistchimie de marqueurs épithéliaux (KL1, CKAE1/AE3) et sarcomateux (PS100, CD31, CD34) (x40; x20 pour PS100).

Les résultats histologiques montrent un marquage positif pour les marqueurs épithéliaux (cytokératine, pancto-kératine; Figure 6) et négatifs pour des marqueurs de sarcome (CD31, CD34 ; non montré). Les résultats confirment donc que la tumeur est un carcinome et non un sarcome puisque les cellules injectées sont des cellules d'origine épithéliale. Ces résultats doivent être confirmés pour les tumeurs transplantées dans les autres sites.

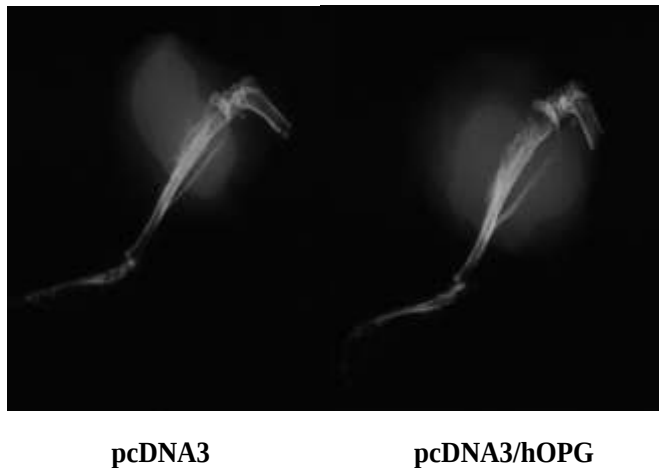
3.5 Approches thérapeutiques

Afin de comprendre le rôle que joue RANKL dans le développement de cette tumeur, nous avons utilisé la même approche thérapeutique que pour l'ostéosarcome, à savoir l'inhibition de RANKL et donc de l'ostéolyse par l'ostéoprotégérine. L'OPG a été utilisée par transfert de gène utilisant un vecteur synthétique (F68) et également par injection de protéine recombinante.

Effet de l'OPG sur la prévention des lésions ostéolytiques associées au développement tumoral

Le modèle tumoral 2.93 RL murin utilisé dans cette étude est associé à de sévères lésions ostéolytiques, d'autant plus fortes que RANKL est surexprimé.

A



B

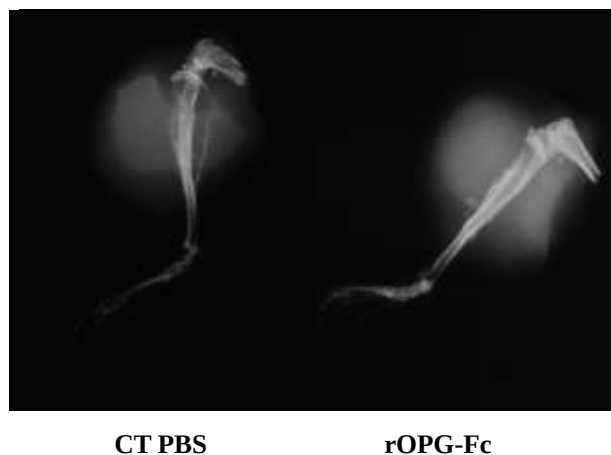


Figure 7 : Radiographie des tibias de souris nude athymiques (n=6) développant une tumeur induite par l'injection de 2 millions de cellules 2.93RL en site intramusculaire. (A) Les souris traitées ont reçu 5 injections (à 7 jours d'intervalle) d'ADN codant hOPG (50µg d'ADN par patte)/F68 en site intramusculaire comparativement aux souris contrôle qui ont reçu le plasmide vide pCDNA3/F68. (B) Les souris ont reçu l'injection d'OPG-Fc (2,5 mg/kg) en site sous cutané 3 fois par semaine durant 6 semaines, comparativement à des souris contrôles injectées avec du PBS.

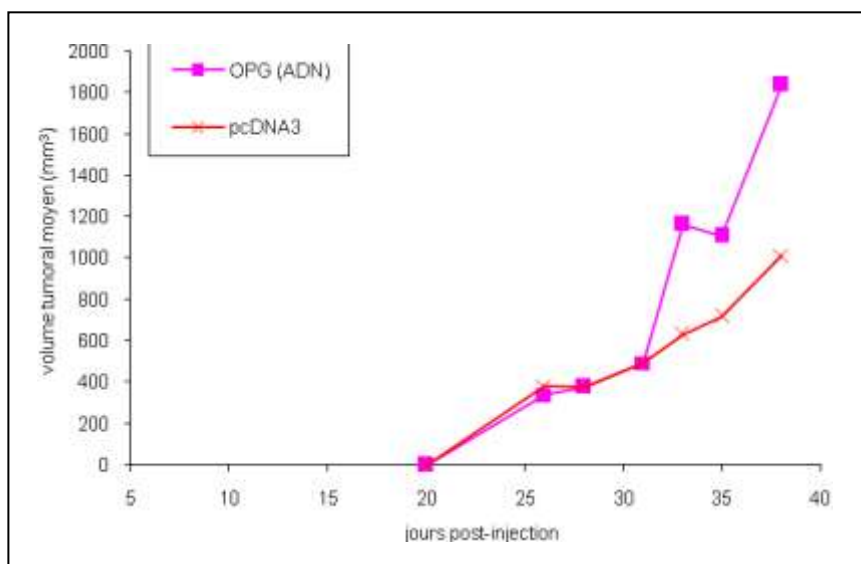
Les radiographies des pattes portant des tumeurs révèlent un remodelage osseux intense avec destruction de la corticale dans les groupes contrôle PBS et contrôle pcDNA3 (Figure 7A et B), résultant d'une intensive interaction entre le tissu osseux et les cellules tumorales. Le groupe traité par l'ADN codant l'OPG présente un profil similaire à celui des groupes contrôles, c'est-à-dire de sévères lésions ostéolytiques marquées par la dégradation importante de la corticale (Figure 7A). La radiographie du groupe traité avec l'OPG sous forme de protéine recombinante révèle un remodelage osseux intense, avec une possible reconstruction de la corticale observable par une intensité osseuse plus importante au niveau

de la corticale que dans le groupe contrôle PBS (Figure 7B). Ces résultats sont à confirmer par analyse de la microarchitecture osseuse par micro-scanner. Cette différence peut s'expliquer par une concentration plus importante en OPG lorsqu'elle est administrée sous forme de molécule recombinante comparativement au transfert de gènes, données qui devraient être confirmées par le dosage sérique d'OPG. En effet, l'expression de RANKL élevée nécessite une quantité plus importante d'OPG pour bloquer RANKL.

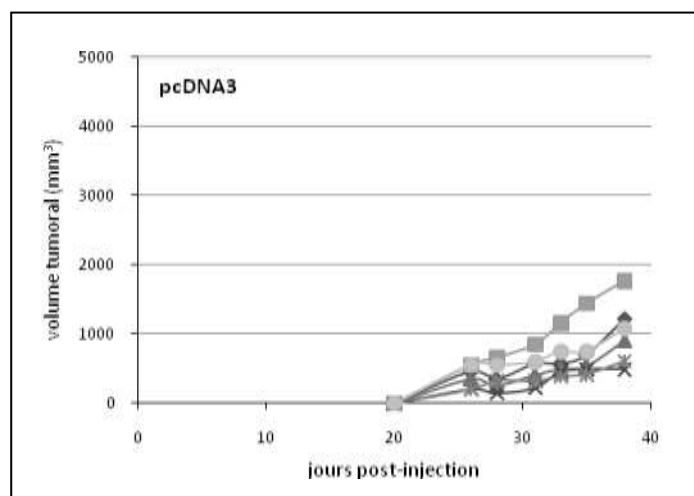
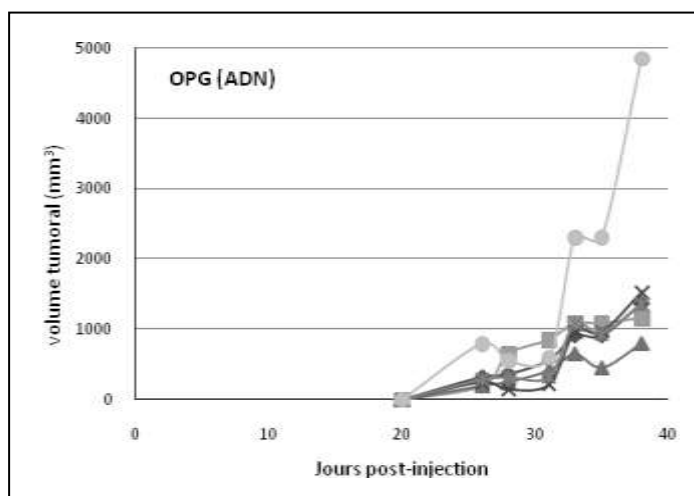
Effet de l'OPG sur la progression tumorale

Parallèlement à l'analyse de l'effet de l'OPG sur les lésions osseuses, son effet (par les deux approches thérapeutiques) sur le développement et la progression tumorale a également été étudié.

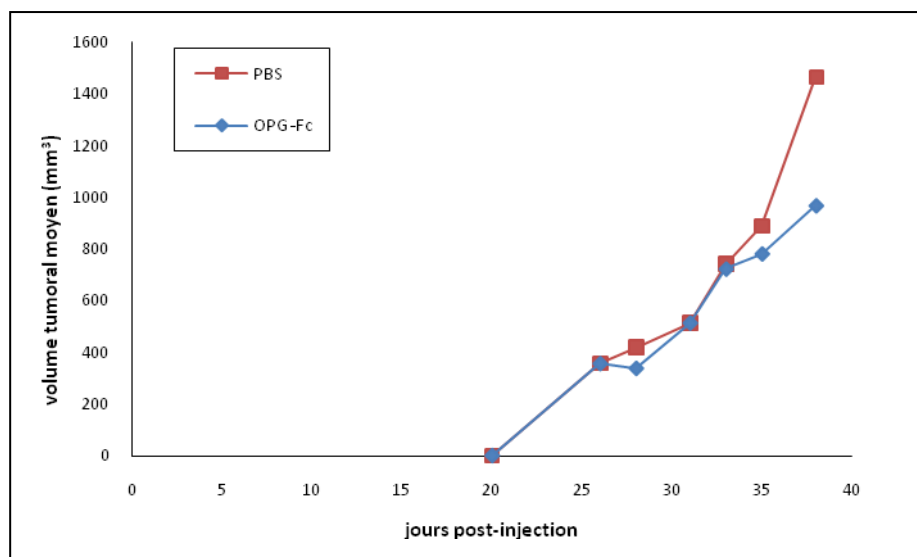
A



B



C



D

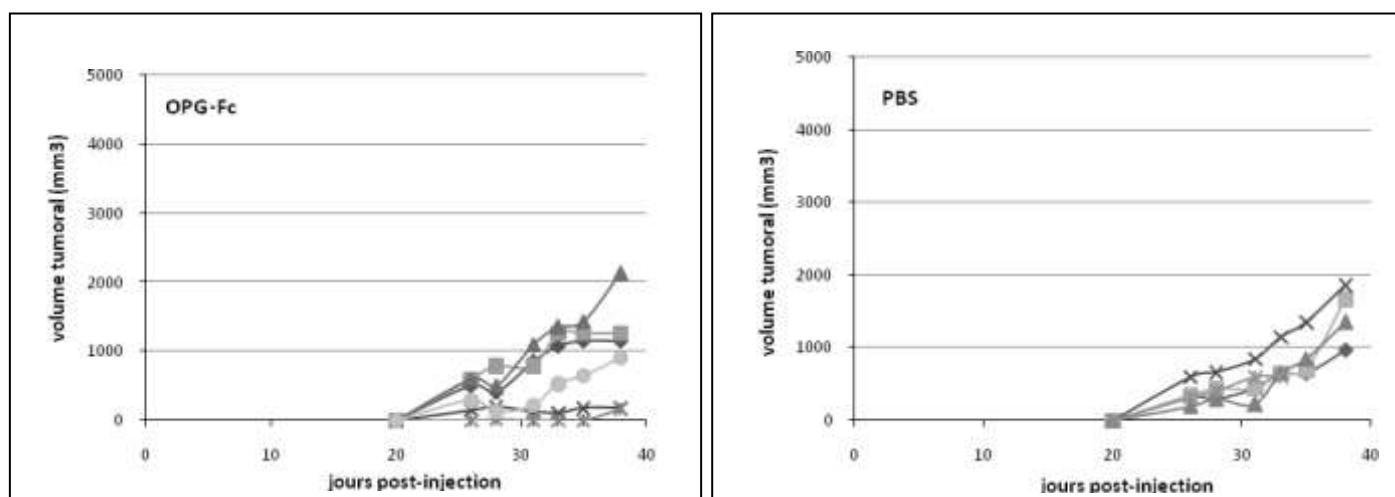


Figure 8 : Evolution des volumes tumoraux en fonction du temps post-injection. Les tumeurs sont induites par l'injection de 2 millions de cellules 2.93RL en site intramusculaire au niveau du tibia et sont traitées par transfert de gène codant l'OPG humaine (50 µg d'ADN/pattes tous les 7 jours; A: volume tumoral moyen; B: volume tumoral par individu) ou par injection de protéine recombinante OPG-Fc de rat (2,5 mg/kg/3 fois par semaine; C: volume tumoral moyen; D: volume tumoral par individu) à partir de J7. Les groupes contrôle respectifs reçoivent le plasmide vide pcDNA3/F68 pour le transfert de gène ou des injections de PBS.

Les résultats ne montrent aucune différence significative du volume tumoral moyen entre les groupes contrôles et les groupes traités que ce soit par l'approche de transfert de gène ou par l'injection de protéines recombinantes (Figure 8 A et C), expliqué par une grande variabilité à l'intérieur des groupes (n=6) (Figure 8 B et D).

Par ailleurs, nous pouvons observer que le traitement par l'OPG délivrée par transfert de gène aurait tendance à favoriser la croissance tumorale (1900 mm^3 pour le groupe traité OPG/F68 contre 1000 mm^3 pour le groupe contrôle pcDNA3) plutôt que de l'inhiber (Figure 8A). Cependant, il faut noter que le traitement était certainement trop tardif. En effet, la tumeur était palpable au moment de la première injection, donc peut être déjà à un stade trop avancé pour un traitement efficace. En effet, dans nos expérimentations dans l'ostéosarcome, nous avons déjà observé qu'il y avait un « effet-seuil », volume tumoral au-delà duquel les quantités d'OPG ou de RANK-Fc étaient insuffisantes pour réverser le développement tumoral. Seuls, les traitements préventifs (dès J1) par transfert de gène non-viral étaient efficaces, ou curatifs seulement avec des constructions adénovirales (production de la molécule d'intérêt en très grande quantité). De plus, le transfert de gène n'est peut être pas suffisant en terme de production d'OPG pour inhiber la quantité importante de RANKL présente.

L'approche thérapeutique par injection sous cutanée de protéines recombinantes OPG-Fc à raison de $2,5 \text{ mg/kg}$ 3 fois par semaine avait pour objectif d'apporter une quantité plus importante d'OPG que le transfert de gène non-viral afin de bloquer RANKL surexprimé. Les résultats obtenus montrent une tendance à diminuer le volume tumoral comparativement au groupe contrôle PBS (en accord avec les observations sur l'ostéolyse vues précédemment) mais sans différence significative (1500 mm^3 dans le groupe contrôle PBS contre 900 mm^3 dans le groupe traité OPG-Fc). Le manque de significativité s'explique par la perte inexplicable de deux souris dans le groupe contrôle PBS, ce qui a mis fin à l'expérimentation. Par ailleurs, comme pour la stratégie par transfert de gène, le traitement par l'OPG-Fc, a peut-être été réalisé peut être trop tardivement pour pouvoir modérer la progression tumorale. Une nouvelle expérimentation est actuellement en cours avec un traitement plus précoce par l'OPG-Fc, et avec un nombre plus important d'animaux par groupe.

L'euthanasie des animaux ayant été effectué au même moment dans chacun des groupes, pour des raisons de résultats significativement non probants et de coût de la protéine recombinante, nous ne disposons d'aucune donnée sur la survie des animaux. Cependant, durant le temps de l'expérimentation, aucune différence n'a été observée. Les souris perdues durant l'expérimentation sont décédées de manière inexplicable et non pas pour des raisons de volume tumoral trop important. De plus, aucune métastase n'a été observée chez ces animaux.

Discussions - perspectives

RANKL joue un rôle pivot dans la destruction osseuse de pathologies d'origine tumorale ou non, et est impliqué dans la mise en place et l'entretien du cercle vicieux s'établissant entre la résorption osseuse et la prolifération tumorale aussi bien dans les tumeurs osseuses primitives que secondaires (Farrugia et al., 2003; Hofbauer et al., 1999; Kitazawa and Kitazawa, 2002; Sezer et al., 2002). RANKL, par la fixation à son récepteur RANK, est le médiateur final qui régule le remodelage osseux entraînant la différenciation, l'activation et la survie des ostéoclastes (Fuller et al., 1998; Lacey et al., 1998; Yasuda et al., 1998b). Différents facteurs tels que PTHrP, TNF, IL-1... induisent une perte osseuse par l'induction de l'expression de RANKL par les ostéoblastes ou les cellules stromales. L'OPG joue un rôle de régulateur de la résorption osseuse en liant RANKL et donc en inhibant l'interaction RANK/RANKL. La balance relative entre les activités de RANKL et OPG régulées par des cytokines est déterminante dans l'activité ostéoclastique. De nombreuses études montrent que l'augmentation de la résorption osseuse observée dans différentes pathologies d'origine tumorale résulte d'une surexpression de RANKL souvent associée à une diminution simultanée de l'expression de l'OPG (Boyle et al., 2003; Hofbauer and Schoppet, 2004; Lacey et al., 1998), soulignant l'importance de la balance RANKL/OPG.

Outre son rôle central dans l'ostéoclastogenèse, il est rapporté que RANKL peut être impliqué dans la migration des cellules tumorales en site osseux. En effet, Jones et collaborateurs ont montré dans une étude récente que RANKL est un facteur déclencheur de la migration de cellules tumorales exprimant RANK, notamment par une modification du cytosquelette de ces cellules (Jones et al., 2006). Cette découverte est confortée par le fait que ces cellules tumorales métastasent préférentiellement en site osseux, renforçant l'hypothèse de l'implication de RANKL présent dans cet environnement (Jones et al., 2006). De plus, l'inhibition sélective de l'interaction RANK/RANKL par l'OPG diminue le développement de métastases osseuses et le volume tumoral (Bakewell et al., 2003; Jones et al., 2006). Ces données montrent que des facteurs de différenciation tels que RANKL peuvent jouer un rôle très important dans la migration cellulaire et le comportement métastatique des cellules tumorales. RANKL peut également intervenir dans le tropisme de ces cellules tumorales pour métastaser à l'os, et constitue donc un facteur favorable au développement et au maintien du cercle vicieux.

Dans la présente étude, nous avons développé un modèle surexprimant RANKL à la suite de l'injection intramusculaire de cellules 2.93 surexprimant RANKL chez la souris. Outre la surexpression sérique de RANKL, une tumeur carcinomateuse détectable au niveau du tibia s'est développée associée à des lésions ostéolytiques sévères. Le facteur étant à l'origine de la tumeur reste à déterminer puisque les souris ayant reçu les cellules 2.93 (contrôle) ont également développé une tumeur, mais dont le volume était nettement inférieur aux souris injectées avec les cellules 2.93RL et avec un remodelage osseux moins important. La présence d'un remodelage osseux sévère au niveau du tibia avec une dégradation totale de la corticale dans le groupe 2.93RL peut s'expliquer par l'injection de ces cellules proches du site osseux. La comparaison des groupes injectés avec des cellules 2.93 exprimant des isoformes de RANKL renforce l'idée que la présence de RANKL dans l'environnement tumoral osseux accentue l'apparition de lésions ostéolytiques et accélère la croissance tumorale, faisant de RANKL un facteur critique dans le développement de la tumeur. Nos données suggèrent que c'est la différence du niveau d'expression de RANKL qui est responsable des différences observées au niveau des lésions ostéolytiques et du développement tumoral. Par ce modèle, nous affirmons le rôle central de RANKL dans le développement tumoral en site osseux par la mise en place du cercle vicieux.

Le développement de ce modèle surexprimant RANKL associée à un développement tumoral accéléré nous a permis d'expérimenter des approches thérapeutiques ciblant RANKL et confirmant son implication dans la mise en place d'un environnement favorable à la croissance tumorale. La première approche a été d'utiliser OPG dans ce modèle, récepteur leurre de RANKL, par deux approches différentes :

- l'OPG libérée par transfert de gène non viral ne prévient pas les lésions ostéolytiques et ne ralentit pas la prolifération tumorale.
- l'OPG injectée sous forme de protéine recombinante montre des résultats encourageants avec une tendance à diminuer la prolifération tumorale et à prévenir le remodelage osseux mais demeurent largement insuffisant.

Ces résultats peuvent s'expliquer par la concentration très importante de RANKL au niveau systémique, laissant imaginer une concentration locale supérieure. Par conséquent, la production d'OPG par transfert de gène utilisant un vecteur synthétique ou la concentration d'OPG-Fc injectée ne sont pas suffisantes pour pallier cette forte surexpression de RANKL (de l'ordre de 20 ng/ml au niveau sérique). De plus, la séquence du traitement n'est peut être pas suffisamment adaptée. En effet, le traitement devra être débuté plus tôt, puisque le temps

associé à l'expression de chaque molécule présente dans l'environnement tumoral osseux devient un facteur essentiel dans le contrôle du développement de la tumeur.

Une autre approche utilisant des siRNA ciblant RANKL est envisagée dans ce modèle, puis sera ensuite utilisée dans des modèles de tumeurs osseuses primitives tels que l'ostéosarcome.

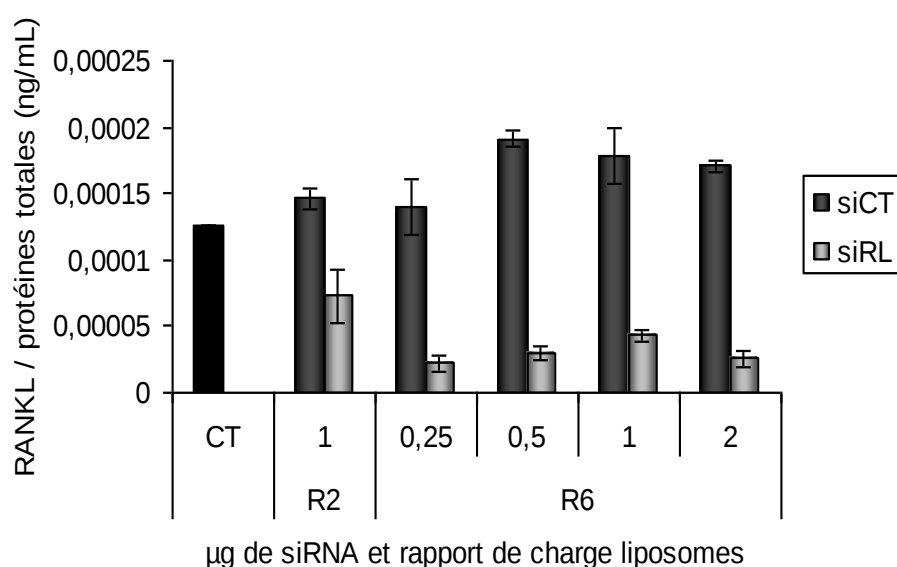


Figure 9 : Concentration de RANKL mesurée par dosage ELISA (en triplicat) dans le lysat cellulaire de cellules 2.93RL 2.0 48h après traitement par 0,25-2 μ g de siRNA CT ou ciblant RANKL associé à un liposome (rapport de charge R2 ou R6).

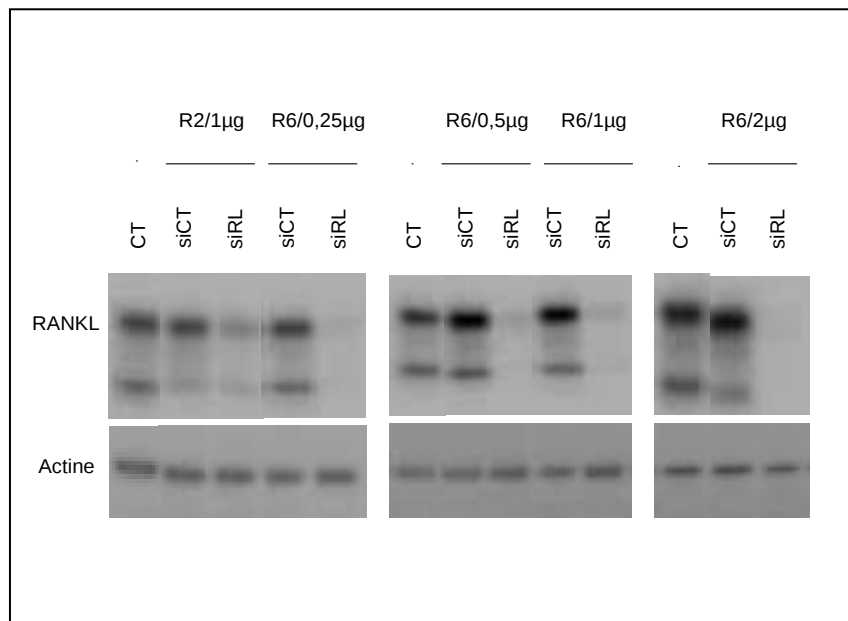


Figure 10 : Evaluation de l'expression de la protéine RANKL par western blot dans le lysat cellulaire de cellules 2.93RL 2.0 48h après traitement avec 0,25-2µg de siRNA CT ou ciblant RANKL associé à un liposome (rapport de charge R2 ou R6).

Les résultats préliminaires obtenus *in vitro* sont encourageants et montrent une inhibition de l'expression de RANKL dans les cellules 2.93RL 2.0 par dosage ELISA (Figure 9) confirmés par western blot (Figure 10). La mise au point de la transfection de siRNA *in vivo* est actuellement en cours avec un siRNA anti-luciférase dans des modèles induits par l'injection de cellules tumorales modifiées pour exprimer la luciférase, dont le développement peut être quantifié par bio-luminescence *in vivo* avant d'utiliser les siRNA anti-RANKL dans ces mêmes modèles.

Conclusions générales et perspectives

La mise en place de nouveaux protocoles pour le traitement des pathologies osseuses tumorales nécessite une meilleure connaissance de la physiologie du tissu osseux. La découverte de la triade moléculaire OPG/RANK/RANKL et de ses activités biologiques dans la régulation du remodelage osseux a permis de mieux comprendre le rôle déterminant joué par cette triade dans le développement tumoral en site osseux. En effet, le développement de lignées cellulaires et de modèles animaux transgéniques ont permis de mettre en avant le rôle pivot de RANKL comme un des principaux responsables de la résorption osseuse et de la mise en place d'un contexte favorable au développement tumoral en site osseux. L'implication de la triade moléculaire OPG/RANK/RANKL dans de nombreuses pathologies osseuses d'origine tumorale ou non conforte l'hypothèse que cette triade représente la cible thérapeutique privilégiée dans le développement de nouvelles stratégies thérapeutiques.

Comme cela a été décrit dans le cadre des tumeurs osseuses secondaires, la problématique biologique des tumeurs osseuses primitives repose sur l'existence d'un cercle vicieux entre la résorption osseuse et la prolifération tumorale. La première partie de cette thèse a permis de confirmer l'existence de ce cercle vicieux dans les ostéosarcomes par l'utilisation d'agents anti-résorptifs (OPG et RANK-Fc) qui, en bloquant l'interaction RANKL/RANK ont permis d'inhiber indirectement la prolifération tumorale. Les deux études présentées dans ce manuscrit ont mis en évidence le potentiel thérapeutique réel de ces deux molécules OPG et RANK-Fc dans le traitement des ostéosarcomes, et qui doit maintenant être étendu à d'autres tumeurs osseuses primitives telles que le sarcome d'Ewing.

Néanmoins, l'utilisation de l'OPG dans le traitement des tumeurs osseuses est controversée car son rôle dans le développement tumoral est plus complexe qu'il ne paraît. En effet, l'OPG est capable de fixer TRAIL et ainsi d'inhiber l'apoptose des cellules tumorales induites par TRAIL, pouvant devenir un agent pro-tumoral. La deuxième partie de cette thèse a été consacrée à la relation structure/fonction de l'OPG dans le développement tumoral et, montre pour la première fois *in vivo*, l'importance de sa structure dans la modulation de son activité biologique. En effet, nous avons démontré la capacité thérapeutique de la forme OPG 1-194 comparativement à la forme native OPG 1-401 à prévenir les lésions osseuses et inhiber la progression tumorale dans le traitement de l'ostéosarcome. L'hypothèse retenue est que la forme complète de l'OPG reste stockée dans la matrice osseuse par son domaine de liaison à l'héparine. Par ailleurs, la régulation de l'activité biologique de l'OPG 1-194 semble également être finement régulée par les protagonistes cellulaires et moléculaires contenus

dans le microenvironnement tumoral osseux, et plus particulièrement de la balance RANKL/TRAIL. En effet, la détermination de l'activité anti-tumorale ou pro-tumorale de l'OPG est liée à la présence, la concentration, la localisation et le « timing » de l'expression de RANKL, TRAIL dans le microenvironnement tumoral osseux. Notre étude montre *in vivo* et *in vitro* que chacun de ces facteurs est capable de réguler l'activité biologique de l'OPG, et suggère que la concentration relative de chacun est primordiale pour orienter l'activité de l'OPG dans la voie anti- ou pro-tumorale.

Au niveau physiologique, il est bien admis que RANKL joue un rôle important dans la différenciation, l'activation et la survie des ostéoclastes. Les données bibliographiques sont claires sur son implication dans de nombreuses pathologies osseuses malignes ou non. En effet, l'expression de RANKL se trouve augmentée dans ces pathologies alors que l'expression de l'OPG est au contraire diminuée. De ce fait, il est évident que RANKL est un facteur critique dans le développement tumoral par sa capacité à créer un environnement favorable à la croissance tumorale. Nous avons développé un modèle animal permettant de corréler le comportement tumoral à l'expression de RANKL. Nos résultats ont montré *in vivo* qu'une surexpression de RANKL dans l'environnement tumoral osseux accélérât la dégradation osseuse, et par voie de conséquence la progression tumorale en site osseux. Ces résultats démontrent clairement pour la première fois le lien « direct » entre RANKL et le développement tumoral en site osseux. RANKL représente donc une cible privilégiée dans l'inhibition de la résorption osseuse et indirectement dans l'inhibition de la progression tumorale nécessitant de nouvelles approches thérapeutiques. Notre modèle tumoral surexprimant RANKL est un atout dans le développement de ces nouvelles approches ciblant RANKL tels que l'utilisation de siRNA, dont les résultats préliminaires sont prometteurs *in vitro*.

L'ensemble des résultats obtenus avec l'OPG dans le traitement de l'ostéosarcome sont très encourageants et mettent en évidence le réel potentiel thérapeutique de l'OPG dans la prévention des lésions osseuses associé à un effet anti-tumoral indirect. Cependant, différents points demandent à être éclaircis et constituent les perspectives de ce travail :

- La différence d'activité biologique entre l'OPG 1-194 et l'OPG native 1-401 reste intrigante et nécessite de poursuivre les études sur la relation structure/fonction de

l'OPG dans les tumeurs osseuses. Pour cela, d'autres constructions doivent être désignées et testées *in vivo* dans nos modèles animaux d'ostéosarcome. En effet, la partie 195-401 différenciant les deux formes d'OPG devra être découpée et de nouvelles constructions testées *in vivo* dans l'objectif de déterminer la contribution de chaque domaine composant cette partie dans l'activité biologique de l'OPG, et dans sa fixation avec les différents protagonistes susceptibles d'intervenir dans la modulation de l'activité biologique de l'OPG présents dans l'environnement tumoral osseux (RANKL, TRAIL et PGs). Il serait donc intéressant de comparer la forme 1-194 ($\Delta D567$) à la forme 1-361 ($\Delta D7$), c'est-à-dire contenant les domaines de mort, puis d'ajouter ensuite le domaine de liaison à l'héparine (forme 1-400) sans la cystéine terminale responsable de la dimérisation de l'OPG afin d'étudier la fonction de chaque domaine dans l'activité biologique de l'OPG. Différentes constructions peuvent être envisagées afin de tester toutes les associations entre les différents domaines de l'OPG *in vivo* comme dans l'étude de Yamaguchi et collaborateurs qui avaient caractérisé chaque domaine de l'OPG *in vitro* (Figure A).

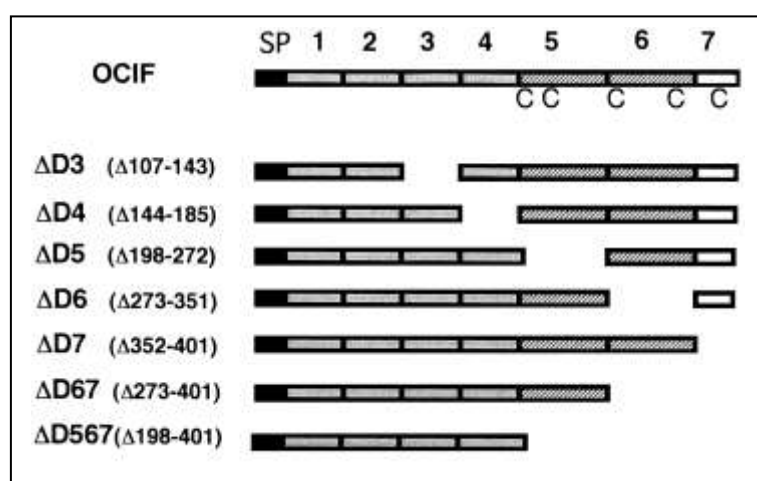


Figure A : Représentation schématique des mutants OCIF (= OPG). SP, peptide signal ; C, résidue cystéine dans les domaines 5-7; 1-4: domaines riches en cystéines; 5 et 6: domaines de mort; 7: domaine de liaison à l'héparine (Yamaguchi et al., 1998).

- Il est clairement admis que RANKL joue un rôle majeur dans le développement tumoral et nos résultats préliminaires utilisant des modèles surexprimant RANKL le confirment, mais il serait intéressant de mieux comparer son rôle dans le développement de tumeurs en site osseux et non osseux, notamment par la comparaison du comportement de ces tumeurs au niveau de leur cinétique de progression, du développement de métastases, au niveau histologique en corrélation avec l'expression de RANKL. En effet, notre étude préliminaire a montré qu'un fragment de tumeur 2.93RL pouvait être transplanté et croître aussi bien en site osseux que non osseux, alors que l'injection de cellules tumorales 2.93RL induit une tumeur seulement lorsque les cellules sont injectées en site intramusculaire c'est-à-

dire proche d'un environnement osseux soulignant l'importance de l'environnement pour initier le développement tumoral. Par ailleurs, une fois la tumeur développée, il semble que la prise tumorale après transplantation d'un fragment ne nécessite pas d'environnement spécifique, mais le comportement de la tumeur s'avère différent (très agressive et lytique en site osseux tandis qu'une tumeur ostéocondensante a été observée lorsque la transplantation s'effectue en site intrapéritonéal). Cependant, l'expérimentation de transplantation n'a été réalisée qu'une seule fois, et nécessite d'être reproduite afin de déterminer si RANKL est toujours surexprimé dans les deux cas, en quelle proportion, et s'il joue un rôle aussi important dans le développement tumoral en site non osseux qu'en site osseux. Dans cette expérimentation réalisée sur peu d'animaux, aucune métastase n'avait été observée, alors que RANKL a été décrit pour jouer un rôle dans la dissémination métastatique. C'est pourquoi de nouvelles études incluant un plus grand nombre d'animaux sur des temps peut-être plus longs permettraient de préciser le rôle de RANKL dans le développement de métastases. Le développement d'approches thérapeutiques ciblées, actuellement en cours utilisant les siRNA anti-RANKL permettra probablement, par l'inhibition de l'expression de RANKL, de mieux définir son rôle pivot dans le développement tumoral.

De plus, en utilisant différentes isoformes de RANKL, nos résultats suggèrent qu'une différence d'expression de RANKL est responsable des variations d'intensités des lésions ostéolytiques et de la progression tumorale. Cependant, nous ne pouvons pas exclure que la structure de RANKL pourrait jouer un rôle dans le développement tumoral, les différentes isoformes pouvant être impliquées dans la différence de niveau d'expression de RANKL. En effet, l'isoforme RANKL 2 dépourvue de la partie centrale du domaine intracytoplasmique est moins présente à la membrane comparativement à l'isoforme RANKL 1, donc moins clivée et moins exprimée dans l'environnement tumoral osseux. La mise en place de clones cellulaires 2.93 transfectés avec les différentes isoformes de RANKL exprimées dans les mêmes proportions permettrait de déterminer l'implication de chaque isoforme dans le développement tumoral. De plus, après avoir déterminé les cellules qui expriment RANKL dans l'environnement tumoral, il serait intéressant de caractériser les isoformes exprimées dans ces pathologies, et s'ils diffèrent par rapport à la situation physiologique.

- Puis il serait intéressant d'élargir la stratégie thérapeutique utilisant l'OPG à d'autres tumeurs osseuses primitives telles que le sarcome d'Ewing. Comme nous l'avons vu dans l'introduction, le sarcome d'Ewing est la deuxième tumeur osseuse primitive maligne de

l'enfant après l'ostéosarcome avec environ 80 nouveaux cas recensés chaque année en France. Il s'agit d'une tumeur très agressive caractérisée par la présence de lésions ostéolytiques plus sévères que dans l'ostéosarcome. Rare après 30 ans, le sarcome d'Ewing touche principalement l'enfant et l'adulte jeune avec un âge médian de 12 ans. Comme pour l'ostéosarcome, le traitement associe chimiothérapie et chirurgie conservatrice d'exérèse. Le pronostic est identique à celui de l'ostéosarcome, la survie à 5 ans étant limitée à 30% en présence de métastases osseuses ou d'une non réponse à la chimiothérapie préopératoire. C'est pourquoi, le développement de nouvelles stratégies thérapeutiques devient nécessaire.

Au laboratoire nous disposons d'un modèle murin de sarcome d'Ewing induit suite à l'injection de cellules tumorales humaines A673 en site intramusculaire dans le muscle tibial antérieur ou en site intra-osseux dans la cavité médullaire du tibia. Ce modèle est caractérisé par un remodelage osseux important avec une destruction totale du tibia (Figure B).



Figure B : radiographie d'un tibia de souris 40 jours après l'injection de 3 millions de cellules tumorales A673 en site intra-osseux

Les mêmes approches thérapeutiques que dans l'ostéosarcome ont été initiées dans ces modèles. Les résultats préliminaires du traitement par des injections d'OPG-Fc recombinante (22-194) de rat a montré à 3 reprises un effet pro-tumoral de cette molécule (Figure C), que la tumeur soit induite par l'injection de cellules tumorales en site intramusculaire ou intra-osseux. Nous avons confirmé que cette OPG-Fc (22-194) de rat était capable de prévenir les lésions osseuses et d'inhiber la progression tumorale dans nos modèles d'ostéosarcomes syngéniques et chez des souris nudes.

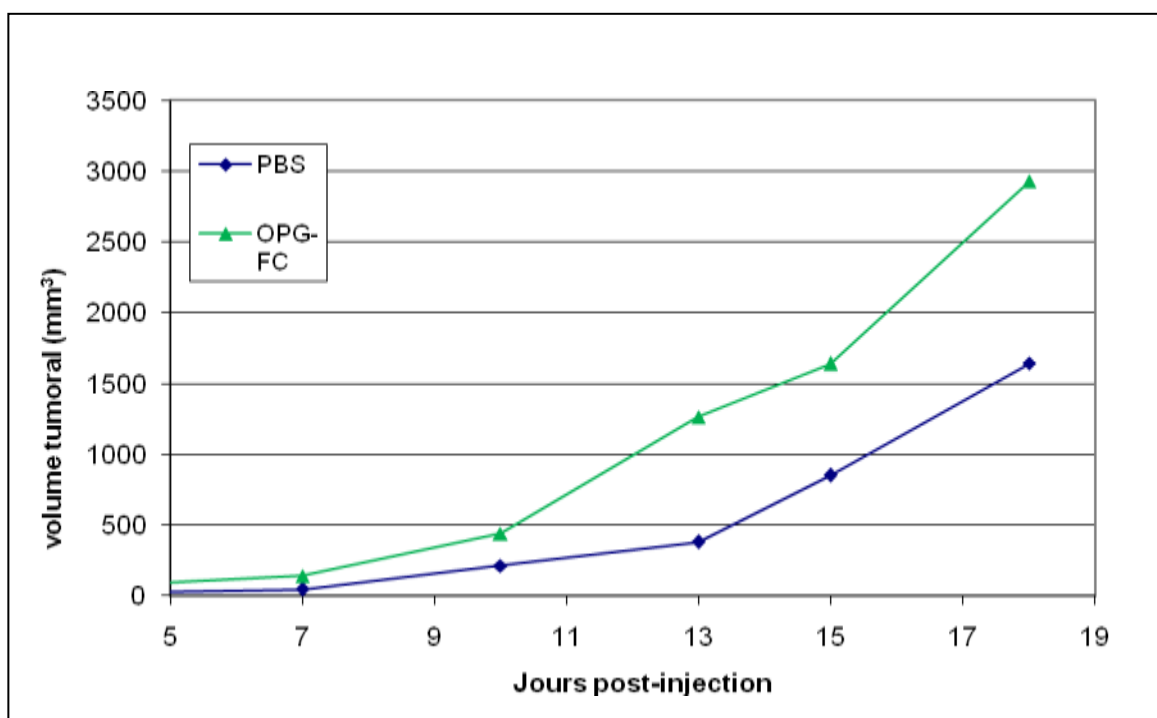


Figure C : Evolution des volumes tumoraux moyens en fonction du temps post-injection. Les tumeurs sont induites par l'injection de 3 millions de cellules tumorales A673 en site intra-osseux au niveau de la cavité médullaire du tibia et sont traitées par injection de protéines recombinantes OPG-Fc (2,5 mg/kg / 3 fois par semaine) ou PBS (contrôle) à partir de J5.

Si nous suivons notre hypothèse, à savoir que l'activité biologique de l'OPG dépend du rapport RANKL/TRAIL et des PGs, ce résultat surprenant doit être corrélé aux concentrations des différents protagonistes présents dans l'environnement tumoral osseux. Cependant, dans notre cas, l'utilisation de l'OPG-Fc (22-194) écarte l'hypothèse de l'implication des protéoglycanes. Les analyses préliminaires révèlent une expression de TRAIL mais pas celle de RANKL par les cellules tumorales de sarcome d'Ewing *in vitro*. Cependant, ces résultats ne sont pas représentatifs de l'expression de ces molécules dans l'environnement tumoral, puisque nous avons déjà observé que RANKL n'était pas exprimé par les cellules d'ostéosarcome *in vitro* alors que son expression était augmentée dans l'environnement tumoral *in situ*. Cependant Lau et collaborateurs ont montré par PCR semi-quantitative que des tumeurs d'Ewing issues de deux patients expriment l'OPG, TRAIL et RANKL (Lau et al., 2007). Cependant, ces expressions restent à être confirmées dans notre modèle animal aux niveaux qualitatif et quantitatif. La balance RANKL/TRAIL pourrait expliquer la différence d'activité biologique de l'OPG 22-194 observée entre l'ostéosarcome

et le sarcome d'Ewing puisque ces deux molécules se fixent à l'OPG avec la même affinité. En effet, si RANKL est plus exprimé que TRAIL comme cela semble être le cas dans l'ostéosarcome, l'OPG jouerait alors un rôle anti-tumoral. Dans le cas où la concentration de TRAIL serait supérieure à celle de RANKL (sarcome d'Ewing ?), l'OPG aurait plutôt un rôle pro-tumoral (Figure D). C'est pourquoi, il est très important de bien connaître, quel que soit le type de tumeur, la concentration relative de TRAIL et RANKL dans le but de déterminer si l'OPG jouera ou non un rôle thérapeutique.

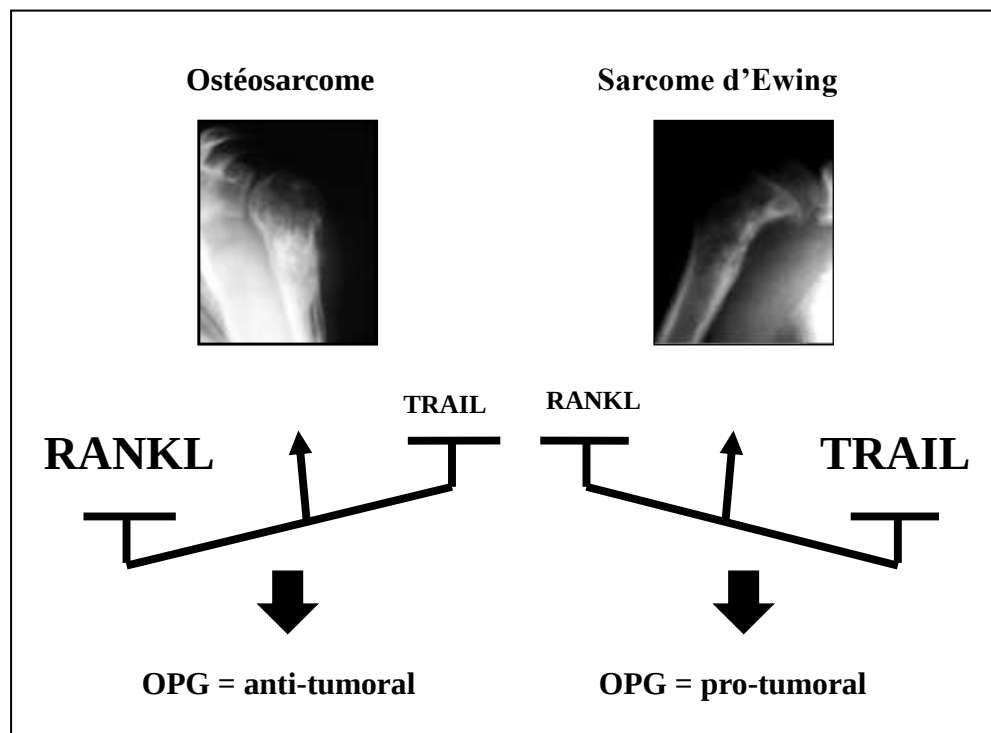


Figure D : Hypothèse biologique du rôle de l'OPG régulé par la balance RANKL/TRAIL dans l'ostéosarcome et le sarcome d'Ewing.

Nous avons montré par des études préliminaires réalisées dans notre laboratoire sur les cellules de sarcome d'Ewing A673 à l'origine du modèle utilisé que ces cellules sont très sensibles à TRAIL et qu'elles expriment les récepteurs activateurs de TRAIL (DR4 et DR5; Figures E et F).

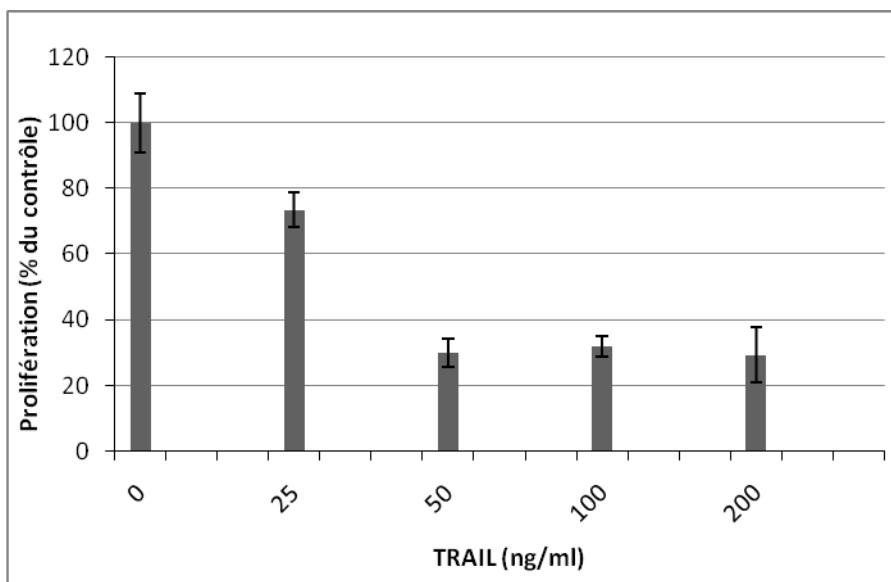


Figure E : Effet de la concentration croissante de TRAIL (0, 25, 50, 100 et 200 ng/ml) durant 72 heures sur la prolifération des cellules tumorales A673.

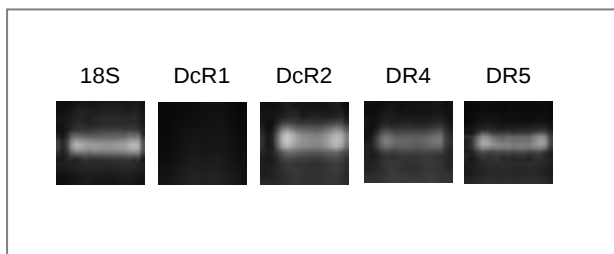


Figure F : Expression des récepteurs activateurs (DR4 et DR5) et inhibiteurs (DcR1 et DcR2) de TRAIL analysée par RT-PCR dans les cellules tumorales A673.

L'effet pro-tumoral de l'OPG observé dans le sarcome d'Ewing est peut être la conséquence de la fixation de l'OPG à TRAIL, probablement plus présent dans l'environnement tumoral que RANKL, empêchant ainsi l'apoptose des cellules tumorales induite par TRAIL. L'expression de TRAIL et de RANKL sera comparée dans l'environnement tumoral de différents modèles d'ostéosarcome et de sarcome d'Ewing afin de déterminer avec précision l'implication de la balance RANK/TRAIL sur la modulation de l'activité biologique de l'OPG dans les tumeurs osseuses primitives.

Annexes

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ANNEXES

Proteoglycans: key partners in bone cell biology

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Summary

The diversity of bone proteoglycan (PG) structure and localisation (pericellular, extracellular in the organic bone matrix) reflects a broad spectrum of biological functions within a unique tissue. PGs play important roles in organizing the bone extracellular matrix, taking part in the structuring of the tissue itself as active regulators of collagen fibrillogenesis. PGs also display selective patterns of reactivity with several constituents including cytokines and growth factors, such as transforming growth factor- β or osteoprotegerin thereby modulating their bio-availability and biological activity in the bone tissue. In this review, the complex PG composition in bone will be addressed together with the specific role played by PGs (or their GAGs chains) in bone biology, as regulatory molecules for bone resorption and their involvement in bone tumor development. These roles have been determined after modulation of PG expression or mutations in their corresponding genes, which revealed specific roles for these compounds in bone pathologies (e.g. perlecan or glypican-3 mutations observed respectively in chondrodysplasia or dysmorphic syndrome). Finally, the potential therapeutic interest of PGs is discussed based on recent data, more particularly on bone tumor-associated osteolysis as these molecules are involved both in bone resorption and tumor development. *BioEssays* 29:758–771, 2007.

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Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; FGF, fibroblast growth factor; GAG, glycosaminoglycan; HP, heparin; HS, heparan sulfate; KS, keratan sulfate; LRR, leucine-rich repeat; OPG, osteoprotegerin; PG, proteoglycan; RANK, receptor activator of nuclear factor- κ B; RANKL, receptor activator of nuclear factor- κ B ligand; SLRP, small leucine-rich repeat proteoglycan; TGF- β , transforming growth factor- β .

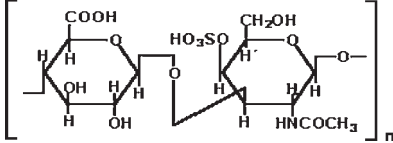
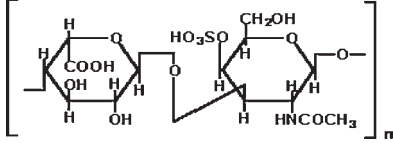
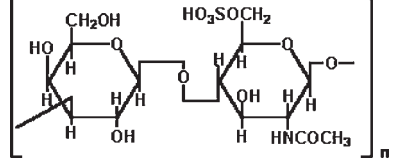
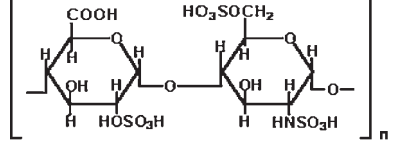
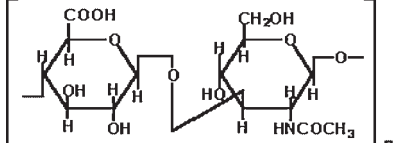
Introduction

Proteoglycans (PGs) are a ubiquitous family of biomolecules that are composed of a core protein and one or more covalently attached sulfated glycosaminoglycan (GAG) chains. Synthesis and sulfation of GAG occur on genetically distinct acceptor core proteins within the Golgi, followed by rapid translocation to the cell surface.⁽¹⁾ The GAGs are linear polymers of repeated disaccharidic units of hexosamine and hexuronic acid, except for keratan sulfate in which hexuronic acid is replaced by galactose. The presence of either two hexosamine isomers, D-glucosamine or D-galactosamine, divides the GAGs into two groups: glucosaminoglycans [heparin (HP)/heparan sulfate (HS) and keratan sulfate (KS)] and the galactosaminoglycans [chondroitin (CS)/dermatan sulfate (DS)] (Table 1). Hexuronic acid is also present as two 5' epimers: D-glucuronic acid and L-iduronic acid. Hyaluronic acid is a non-sulfated, non-attached to protein GAG composed of D-glucuronic acid and D-glucosamine. The degree and position of sulfate as well as the degree and position of 5' epimerisation are extremely variable in sulfated GAG depending on the tissular/cellular/metabolic context, ensuring structural variability of these polysaccharides.⁽¹⁾

There is no unifying feature for core protein structures as they display a great diversity of protein forms. Many core proteins have complex modular structures with protein motifs that are of similar sequence to those found in other protein families.^(2,3) The core protein size ranges from 10 kDa to >500 kDa, and each corresponding protein is encoded by a lonely gene belonging to different gene families.⁽⁴⁾ These genes encode multidomain core proteins whose structures share similarities with known proteins or specific binding domains. This huge variability of protein structure provides specific functions for the different PG families.

PGs have been found associated with intracellular compartments, the cell surface, extracellular matrices and basement membranes in almost all tissues in adults (Table 2). The importance of the extracellular matrix in skeletal development has been overlooked until the development of improved extraction procedures and recombinant DNA technology. It is now well known that bone extracellular matrix is a dynamic network of molecules secreted by cells. PGs together with collagens are the major constituents of the organic matrix of

Table 1. Disaccharide composition of the different glycosaminoglycan families

Repeating disaccharide of GAG	Sulfation position
	chondroitin sulfate (glucuronic acid + N-acetylgalactosamine) GalNAc-4SO ₄ GalNAc-6SO ₄ GalNAc-4,6SO ₄
	dermatan sulfate (glucuronic acid or iduronic acid + N-acetylgalactosamine) GalNAc-4SO ₄ GalNAc-6SO ₄ (IdoA-2SO ₄)
	keratan sulfate (galactose + N-acetylglucosamine) GlcNAc-6SO ₄ Gal-6SO ₄
	heparin and heparan sulfate (glucuronic acid or iduronic acid + N-acetylglucosamine) (IdoA-2SO ₄) GlcNAc-3,6SO ₄ (GlcA-2SO ₄)
	hyaluronic acid (glucuronic acid + N-acetylglucosamine) no sulfation

The glycosaminoglycan chains are composed of repeating disaccharide units of uronic (glucuronic or iduronic) acid and N-acetylated hexosamine (glucosamine or galactosamine), except for keratan sulphate, which is composed of galactose that can be modified (sulfation position) at irregular intervals along the chain.

bone, constituting about 5–7 per cent of the non-collagenous matrix components.⁽⁵⁾

Many different aspects within PG metabolism in skeletal tissue are emerging. The first part of this review describes the complex composition of PGs present in the bone tissue, together with their associated functions. Then the review focuses on the role of PGs in mediating osteoclastic activity in physiological and pathological conditions and, more particularly, in bone tumor-associated osteolysis. Finally, the potential therapeutic interest of PGs in bone tumors is discussed, based on recent data.

Specific functions associated with the complex proteoglycan composition in bone

Bone matrix contains different PG families of various structures, including small leucine-rich PG (the most abundant),

heparan sulphate PG, aggrecan and hyaluronic acid (Fig. 1) that exert specific functions.

Small leucine-rich repeat proteoglycans (SLRPs)

This class of PG represents the most-abundant kind of PG in the bone matrix. Indeed, decorin, biglycan, fibromodulin, lumican, osteoadherin and PG-Lb have been well described in this tissue. In line with other members of the leucine-rich superfamily, their core proteins are proposed to adopt a horseshoe structure that may facilitate protein–protein interaction with a range of other matrix components (mainly collagen fibres) and with the mineral phase during the formation of calcified tissues. Classically decorin and biglycan within mineralized matrices have been described to be predominantly substituted with the GAG chondroitin sulfate while those identified in soft connective tissues predominantly

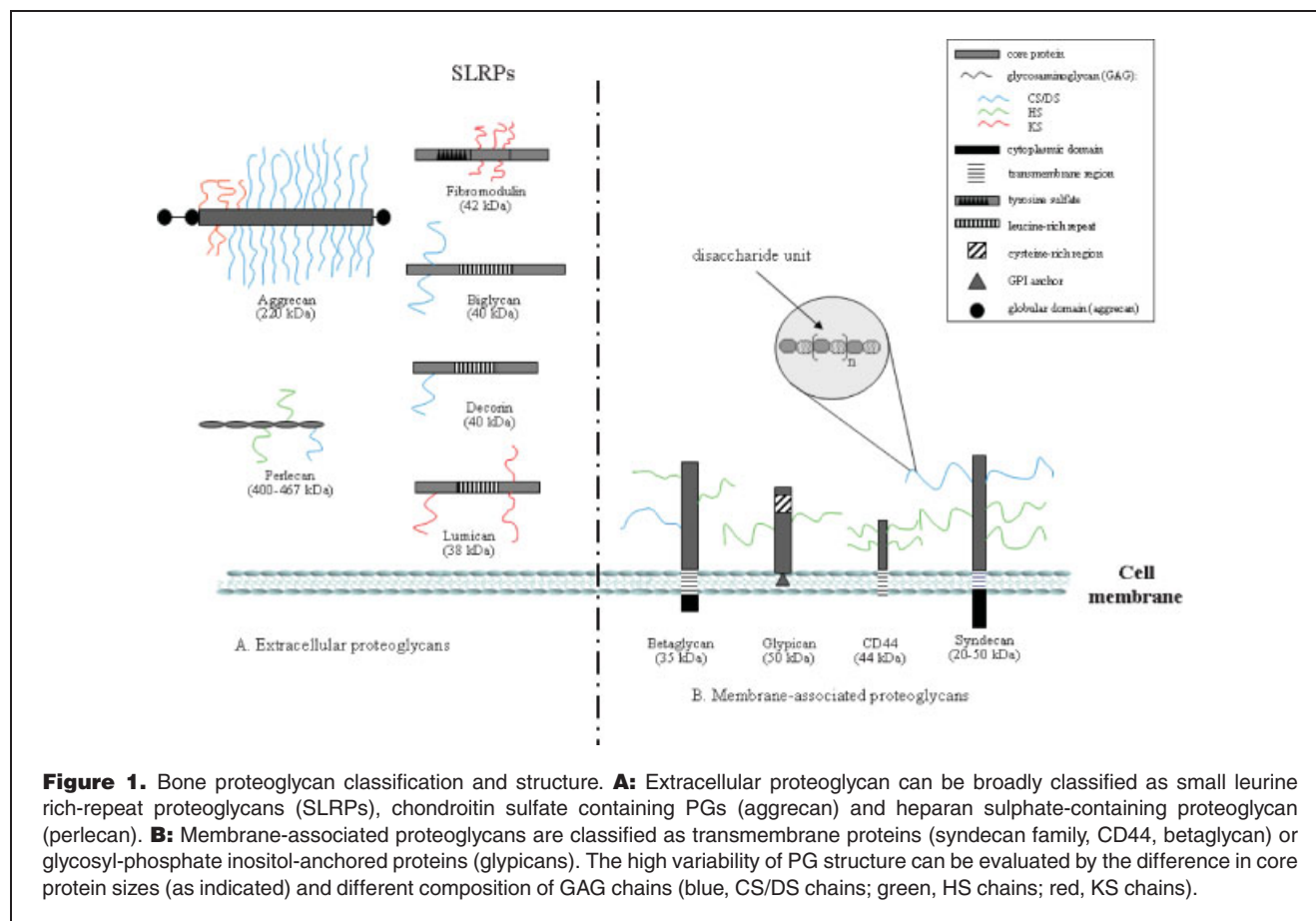
Table 2. Expression data for the main proteoglycans in adults

Proteoglycan	Main expression in adults	References
Syndecan-1	Epithelia, plasma cells	Bernfield et al. <i>Annu Rev Cell Biol</i> 1992, 8:365–93
Syndecan-2	Endothelia, fibroblasts	Spring J et al. <i>Proc Natl Acad Sci USA</i> 1994, 91:3334–8
Syndecan-3	Neural crest derivatives, CNS	Bernfield M et al. <i>Development</i> 1993, suppl. 205–12.
Syndecan-4	Ubiquitous on adherent cells	
Glypican-1	Ubiquitous prevalent in CNS	Litwack ED et al. <i>Dev Dyn</i> 1998, 211:72–87.
Glypican-2	None in adults (CNS in embryo)	Arribas J and Massagué J. <i>J Cell Biol</i> 1995, 128:433–41
Glypican-3	Markedly reduced in CNS (ubiquitous in embryo)	Veugeliers M et al. <i>Genomics</i> 1998, 53:1–11.
Glypican-4	Reduced in CNS (kidney, CNS, adrenal in embryo)	Selleck S. <i>Matrix Biol</i> 1998, 14:473–6.
Glypican-5,-6		Veugeliers M et al. <i>J Biol Chem</i> 1999, 274: 26968–77
Hyalactans:		Wlad H et al, <i>J Biol Chem</i> 1994, 269:24538–41.
aggrecan	Cartilage	
versican	Fibroblasts	Mallein-Gérin F et al. <i>Dev Biol</i> 1988, 126:337–45.
brevican, neurocan	CNS	Lebaron RG, <i>Perspect Dev Neurobiol</i> 1996, 3:261–71
		Grover J and Roughley PJ. <i>Biochem J</i> 1993, 291:361–7
		Zimmermann DR and Ruoslahti E. <i>EMBO J</i> 1989, 8:2975–81
		Rauch U et al. <i>J Biol Chem</i> 1992, 267:19536–47
		Yamaguchi Y. <i>Perspect Dev Neurobiol</i> , 1996, 3:307–17
Basement membrane PGs:		Noonan DM et al. <i>J Biol Chem</i> 1991, 266:22939–47
perlecan, agrin, bamacan	Vascular and epithelial basement membrane	Tsen G et al. <i>J Biol Chem</i> 1995, 270:3392–99.
		Wu RR and Couchman JR. <i>J Cell Biol</i> 1997, 136:433–44.
SLRPs:		
decorin	Skin, bone, tendon	Corsi A et al. <i>J Bone Min Res</i> 2002, 17:1180–9.
biglycan	Bone, skin, dentin, tendon	Danielson KG et al. <i>Mamm Genome</i> 1999, 10: 201–3
fibromodulin	Tendon, articular cartilage, growth plate	Svensson L et al. <i>J Biol Chem</i> 1999, 274:9636–47
lumican	Cornea, dermis, tendon	Chakravarti S et al. <i>J Cell Biol</i> 1998, 141:1277–86
keratocan	Cornea	Ezura Y et al. <i>J Cell Biol</i> 2000, 151:779–88.
PRELP ^c	Cartilage, osteoblasts	Liu CY et al. <i>J Biol Chem</i> 1998, 273:22584–8
osteoaderhin	Bone	Grover J et al, <i>Genomics</i> 1996, 38:109–17.
Epiphycan/PG-Lb	Epiphyseal cartilage	Sommarin Y et al. <i>J Biol Chem</i> 1998, 273:16723–9
osteolectin/mimecan	Bone, vascular matrix, cornea	Deere M et al. <i>Genomics</i> 1996, 38:399–404

carry the isomeric form dermatan sulfate. However, a recent study highlights the identification of DS bearing SLRPs in osteoid.⁽⁶⁾ The localisation of these small leucine-rich repeat PGs varies within the bone tissue. Studies on mature bone have revealed the presence of decorin in specific bone matrix areas while biglycan appears to be evenly distributed throughout the bone matrix.⁽⁷⁾ The small keratan sulfate proteoglycan fibromodulin was first isolated in tendons and articular cartilage, and throughout the growth Plate⁽⁸⁾ suggesting that fibromodulin could play a role during endochondral ossification. Pericellular deposition of fibromodulin is strong around the late-hypertrophic chondrocytes of the secondary ossification centre and in the growth plate.⁽⁹⁾ Lumican, a fibril-associated molecule is implicated in regulating the progression of fibril assembly through a series of steps leading to structurally and mechanically mature fibrils in tendons.⁽¹⁰⁾ Moreover, it has been shown to play an essential role in the regulation of collagen fibril formation in the bone matrix, as it represents a significant proteoglycan component of the bone matrix, secreted by differentiating and mature osteoblasts only.⁽¹¹⁾ Osteoadherin, a bone PG containing keratan sulfate was detected in mature osteoblasts located superficially on

trabecular bone. Immunohistochemical studies of the bovine fetal rib growth plate revealed that osteoadherin is exclusively identified in the primary bone spongiosa.⁽¹²⁾ PG-Lb is a chondroitin/dermatan sulfate proteoglycan first isolated from chick embryo limb cartilage that showed a higher homology to human osteoinductive factor rather than to other small PGs, suggesting the possible participation of this PG in osteogenic processes.⁽¹³⁾

Numerous studies have demonstrated that SLRPs are involved in the structural organisation of the bone matrix. To better understand their functions in vivo, mice deficient in one or two of the four most prominent and widely expressed SLRPs (biglycan, decorin, fibromodulin and lumican) have been generated. Consistent with the high level of expression of decorin in the dermis, decorin-deficient mice have fragile skin,⁽¹⁴⁾ but decorin single deficiency also introduces changes in collagen fibril size and shape in bone.⁽¹⁵⁾ The modulation of collagen fibrillogenesis by DCN is linked to the specific three-dimensional DCN structure as horse-shaped SLRPs interact with collagen molecules by their concave surface and the space inside the horseshoe accommodates a single triple helix of collagen.⁽¹⁶⁾ However, in contrast to biglycan deficiency,



decorin deficiency does not affect bone mass and does not appear to lead to any major phenotypic changes in bone at macroscopic or histological levels. The targeted disruption of biglycan confirmed that it was involved in the regulation of postnatal skeletal growth.⁽¹⁷⁾ Biglycan knockout mice fail to achieve peak bone mass due to a decrease in bone formation explained by a lower osteoblast number and a lower osteoblast activity.⁽¹⁷⁾ The cellular and molecular mechanisms leading to osteoporosis in the absence of biglycan have been partially identified.⁽¹⁸⁾ Biglycan-deficient mice have diminished capacity to produce bone marrow stromal cells, and this deficiency increases with age. The cells also have reduced response to TGF- β , reduced collagen synthesis and relatively more apoptosis than cells from normal littermates. In addition, the absence of biglycan introduces quantitative variations in the range, mean and distribution profiles of the collagen fibril diameters compared to the wild type, increasing the average fibril diameter in bone.⁽¹⁵⁾ The phenotypic analysis of the double knockout decorin/biglycan reveals that the bone phenotype is more severe and develops earlier than in the biglycan single-deficient mice, with cortical and trabecular bone mass severely reduced compared to the wild type.⁽¹⁵⁾ Thus the effects of decorin and biglycan double deficiency are

synergistic in bone even if bone is not affected by the decorin deficiency. Despite their distinct functions (demonstrated by their distinct phenotypes developed by the decorin and biglycan single-deficient mice), biglycan and decorin are therefore still sufficiently similar to partly rescue or compensate their absence in the single-deficient mice.

Fibromodulin-deficient mice have abnormal and less numerous collagen fibril bundles in the tail than wild-type animals. When compared with decorin, differences in the expression and distribution profiles suggest different roles for fibromodulin and decorin in the regulation of collagen fibrillogenesis, maintenance of the fibril organization and matrix mineralization.⁽⁹⁾ It is hypothesized that, in the absence of fibromodulin, lumican binds to the sites normally used by fibromodulin, suggesting that they compete for the same binding sites on collagen fibrils, which has been confirmed by binding competition experiments.⁽¹⁹⁾ Lumican deficiency has profound effects on the structure of collagen fibril as shown by the phenotype of mice homozygous for a null mutation in the lumican gene.⁽²⁰⁾ However, no alteration in bone structure was reported in lumican-deficient mice. Only the double-null lumican^{-/-} fibromodulin^{-/-} mice are smaller than their wild-type littermates and display age-dependent osteoarthritis.⁽²⁰⁾

The high expression of lumican in mature osteoblasts in vitro does not correlate with abnormalities in the bone structure of lumican-deficient mice. Overall, the tissue distribution of SLRPs indicating that biglycan is the major PG of bone matrix is confirmed by main alterations in bone structure only in biglycan-deficient mice, while mice deficient in other SLRPs (decorin, lumican, fibromodulin) show more extensive alterations in skin, tendons and cornea, where they are mainly produced.

Apart from their involvement in the structural organisation of the bone matrix, SLRPs play essential roles in the regulation of growth factor activity, among them TGF- β . High concentrations of TGF- β are found in the bone matrix, reflecting a pivotal role of this factor in the coupling of bone resorption and formation. Decorin, together with other small PGs of the SLRP family, biglycan and fibromodulin, possess the capacity for TGF- β binding.⁽²¹⁾ Using extracts from bovine mineralized bone matrix, TGF- β -binding experiments have showed that the growth factor was almost exclusively bound to decorin,⁽²²⁾ via the core protein. In this system, the core protein of decorin specifically binds TGF- β 1 with high affinities and the binding of TGF- β 1 to decorin increases TGF- β 1 binding to its receptors and enhances its bioactivity. It has been suggested that the collagen-bound decorin sequesters TGF- β in the extracellular matrix without inactivating it completely, the interaction between decorin and TGF- β neutralizing only particular functions of the growth factor depending on the cellular context.^(23,24) The predominant effect of the increased TGF- β signalling in decorin/biglycan-deficient bone marrow stromal cells is a switch from growth to apoptosis, leading to decreased number of osteoprogenitor cells and subsequently reduced bone formation.⁽²⁵⁾ Thus, biglycan and decorin appear to be essential for maintaining an appropriate number of mature osteoblasts by modulating the proliferation and survival of bone marrow stromal cells. Apart from TGF- β , evidence is now emerging that biglycan can modulate the response of other growth factors and cytokines such as TGF- α ,⁽²⁶⁾ TNF- α ,⁽²⁷⁾ EGF,⁽²⁸⁾ BMP-4⁽²⁹⁾ and BMP-2.⁽³⁰⁾ Moreover, SLRP such as decorin can interact with cell surface EGF receptors, activating them through its dimerization and increased phosphorylation.⁽³¹⁾

Part-time SLRPs, where the GAG chains are absent must also be mentioned; for example, decorin core proteins have been identified in the conversion predentine-dentine mineralising bone matrix.⁽³²⁾ Such differences in structure may imply that these PGs perform differing functions in matrix formation.

Hyaluronan/CD44, aggrecan and chondroitin sulfate containing proteoglycan

A few reports indicate that the expression of aggrecan is not restricted to cartilage and may be expressed in certain conditions by osteoblasts. Wong et al. have demonstrated its expression in embryo osteoblasts in vivo by in situ

hybridization of membranous bone tissue sections, and in calvaria using Northern blot technology.⁽³³⁾ Unexpectedly, this study provides evidence that aggrecan is indeed expressed in membranous bone as well as cartilage. Furthermore, pathological conditions that can be reproduced by the overexpression of the scleraxis transcription factor in osteosarcoma ROS17/2.8 cells leads to the enhanced expression of the aggrecan gene, which is not normally expressed at high levels in these osteoblastic cells.⁽³⁴⁾ Hyaluronan was observed in regional areas of the periosteum and endosteum, around osteoblasts, osteoprogenitor cells, osteoclasts and osteocyte lacunae. CD44, a cell surface hyaluronan receptor was identified using in situ hybridization and immunochemistry techniques on osteoclasts, chondroclasts, osteocytes and hematopoietic marrow cells.⁽³⁵⁾ Versican, another chondroitin sulphate containing PG from the hyalactan family has been suggested to be present in early osteoid formation.⁽³⁶⁾

Heparan sulfate proteoglycans

Immunohistochemical studies of HSPG localization in rat tibiae suggest that osteoblasts and osteoclast lineage cells synthesize HSPG, that both membrane and matrix HSPG are localized in bone tissue and that HSPG may play an important role in cell–cell interactions between fibroblast-like cells and osteoclast-lineage cells by reserving heparin-binding growth factors and/or heparin-binding adhesion molecules, such as fibronectin.⁽³⁷⁾

In bone cells, HSPGs purified from the osteosarcoma cell line UMR 106-01 are covalently bound to the plasma membrane by a GPI structure, suggesting the presence of a member of the glypican family.⁽³⁸⁾ For the other HSPGs, syndecan-3 is expressed in the growth plates of developing long bones in chick and murine embryos.⁽³⁹⁾ Its transcripts are very abundant in the presumptive periosteum surrounding the diaphysis of early chondrocytic skeletal elements in chick limb.⁽⁴⁰⁾ In addition, syndecan-3 gene expression is low in osteoblasts and osteocytes located deep into trabecular bone. A significant development in proteoglycan biology has been the realization that many HS polysaccharide chains of the proteoglycans by their negatively charged groups are able to bind growth factors and thereby modulate their activity.⁽⁴¹⁾ Work from Gallagher and coll. has reported that the sulfation and epimerisation levels of HS dictate biological activity and binding of growth factors.⁽⁴²⁾ Indeed, the sulphated regions are likely to display considerable conformational versatility as a result of the presence of iduronate residues,⁽⁴³⁾ and this property may be important for the growth factor-binding properties of the GAG. Many signalling molecules, such as FGFs, VEGF, TGF- β 1, TGF- β 2 and several cytokines, bind to HS present on cell surface PGs, the PGs acting as co-receptors for these growth factors/cytokines.^(44,45) The binding of proteins to HS chains serves a variety of functional purposes, from simple immobilization or protection against

proteolytic degradation to distinct modulation of biological activity. The binding to PGs in the extracellular matrix or at the cell surface then provide a local tissue-bound reservoir of growth factors (Fig. 2). HSPGs are known to regulate the availability and the biological activity of TGF- β s and FGFs, two major growth factor families involved in the regulation of bone biology.

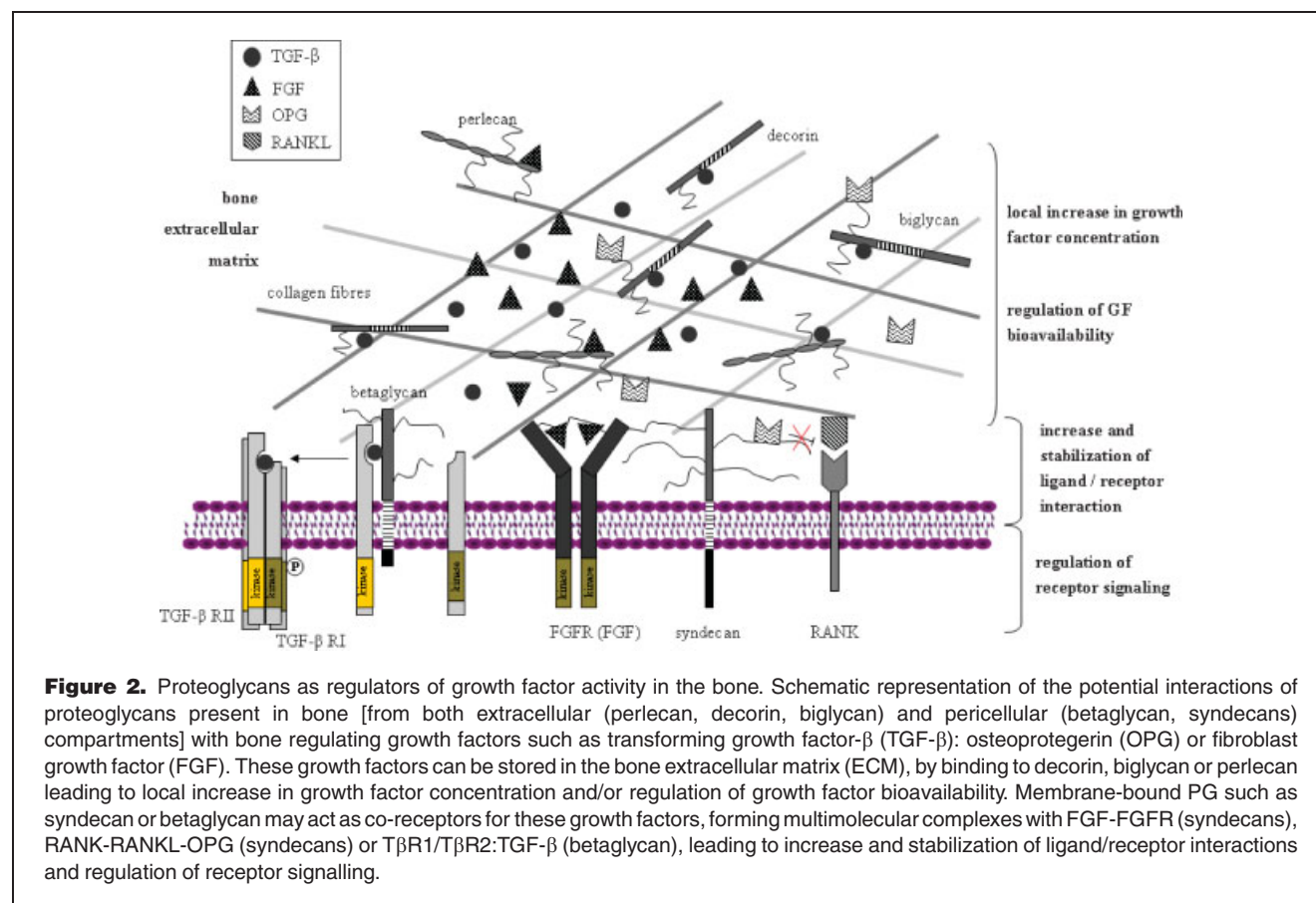
Perlecan, a pericellular hybrid HS/CSPG that was first discovered in a murine tumor cell line, is also present in the extracellular matrix of the growth plate and in articular cartilage,⁽⁴⁶⁾ playing an important role in bone structure. Only a few perlecan null fetuses survive to birth (about 12%) and most die prenatally about mid-gestation with severe abnormalities affecting many tissues. Indeed, the perlecan-null mutation in mice has been independently accomplished by two research groups and they reported similar perinatal lethal chondrodysplasia: they develop skeletal dysplasia characterized by shortened long bones and craniofacial abnormalities and then die shortly after birth.^(47,48) Histological evaluation of the perlecan^{-/-} mice revealed a disorganised growth plate with reduced chondrocyte proliferation and differentiation and defective endochondral ossification.^(47,48) Initially, no significant abnormalities were found prior to embryonic day 13.5, at which time endochondral ossification starts and thereafter

disruption of the matrix became obvious and progressively severe, accounted for the shortened long bones. Moreover, it has been shown that heparan and chondroitin sulphate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors⁽⁴⁹⁾ suggesting that perlecan may act as a regulator of FGF activity in bone.

Minor bone PGs

Several extracellular PGs do not belong to any family. They are often presented as “part time” or “facultative” PGs. In bone, a keratan sulfate proteoglycan has been isolated under denaturing conditions from the mineral compartment of rabbit cortical bone.⁽⁵⁰⁾ The core protein has several common properties with the bone sialoprotein II molecule of bovine and human bone that do not possess GAG chains in these species (human, bovine and rat). However, its role is not well defined currently.

Overall, these data show that the bone tissue is composed of several PG families. However, each member of these PGs is not specific to the bone tissue as, for example, BGN, the major PG species found in bone, has been also detected in almost every organ.⁽⁵¹⁾ Therefore, its currently proposed function in bone appears to be dependent on the particular bone microenvironment.



Dynamic expression of proteoglycans during bone morphogenesis, development and growth

Morphogenesis of skeletal tissues is accompanied by dramatic changes in the types and amounts of extracellular macromolecules synthesized. The extracellular matrix of bone and cartilage shows extensive variations in the expression of their constituents during development, reflecting a dynamic expression of collagens and proteoglycans. In developing bones, spatially restricted patterns of gene expression and/or matrix localization of decorin and biglycan were identified in articular regions, epiphyseal cartilage, vascular canals, subperichondral regions and periosteum; this indicated the association of each molecule with specific developmental events at specific sites.⁽⁵²⁾ For example, within developing bone, biglycan is localized to the walls of the osteocyte lacunae and bone cell surface, whilst decorin is present throughout the osteoid matrix and associated with bone cells associated with osteogenic and non-osteogenic layers of the periosteum.⁽⁵²⁾ The observed differences in the distribution of these two SLRPs in mature and immature bone provide the first indication that decorin and biglycan play different roles in the development of the initial osteoid matrix and also during bone mineralization. Another SLRP, fibromodulin is developmentally expressed in cartilage and bone cells during endochondral and intramembranous ossification.⁽⁵³⁾ Fibromodulin pericellular deposition is strong around the late-hypertrophic chondrocytes of the secondary ossification centre and in the growth plate. Taken together, in contrast to decorin, fibromodulin and biglycan can be detected throughout the growth plate though their abundance is decreased in the proliferative and hypertrophic zones.⁽⁸⁾

The fundamental roles played by HSPG, such as perlecan, in the ECM during cartilage development and skeletal growth have been highlighted.⁽⁵⁴⁾ Syndecans are developmentally regulated with syndecan-1 weakly expressed in mesenchymal cells and transiently expressed in the limb bud. Syndecan-2 is abundant in putative precursor cells of hard and connective tissue. Its expression is high in prechondrogenic cells, decreases in differentiating chondrocytes, and persists in the perichondrium and periosteum at the onset of osteogenesis.⁽⁵⁵⁾ Syndecan-4 is widely expressed in various tissues and cell types, including chondrocytes and osteoblasts.⁽⁵⁶⁾ Syndecan-3 participates in limb outgrowth and proliferation, mediates cell–matrix and/or cell–cell interactions involved in regulating the onset of chondrogenesis, may be involved in regulating the onset of osteogenesis and joint formation, and plays a role in regulating the proliferation of epiphyseal chondrocytes during endochondral ossification.⁽⁵⁷⁾ Syndecan-3 plays important roles in restricting mitotic activity to the proliferative zone of the growth plate,⁽³⁹⁾ and their transcripts are very abundant in the presumptive periosteum surrounding the diaphysis of early chondrocytic skeletal elements in chick limb.⁽⁴⁰⁾ As the elements developed further, syndecan-3 gene expression decreases in the diaphyseal periosteum, whereas

it becomes stronger around the early epiphysis and within the forming articular cells. However, as the diaphyseal periosteum initiates osteogenesis and gives rise to the intramembranous bone collar, syndecan-3 gene expression increases again. Concerning perlecan, its greatest deposition during embryonic development is found in cartilage undergoing endochondral ossification in the growth plate during long-bone development.⁽⁵⁸⁾

The molecular bases of hereditary proteoglycan defects in animal models as well as some human bone disorders collectively are providing a better understanding of the role of PGNs in the development and maintenance of the skeletal elements (Table 3).

Regulatory molecules for bone resorption

An emerging role of PGs in the bone biology is in the control of osteoclast activity and bone resorption (Fig. 3). The molecular triad osteoprotegerin (OPG)–receptor activator of nuclear factor- κ B ligand (RANKL)–RANK is involved in the orchestration of pathophysiological bone remodeling.⁽⁵⁹⁾ OPG, a 55 kDa protein that belongs to the tumor necrosis factor (TNF) receptor family contains three structural domains specifically influencing its biological activities, including a heparin-binding domain potentially capable of interacting with proteoglycans.⁽⁶⁰⁾ OPG is considered as a decoy receptor that inhibits the binding of RANKL, a member of the tumor necrosis factor family required for osteoclast differentiation, to its membrane receptor RANK, thereby inhibiting the terminal stage of osteoclastic differentiation, suppressing the activation and inducing apoptosis of mature osteoclasts.⁽⁶¹⁾ Therefore, the molecular triad OPG–RANKL–RANK represents the final effectors of bone resorption regulation. The role of the OPG heparin-binding domain has been revealed by Standal et al. who demonstrated that myeloma cells internalize and degrade OPG through its binding to syndecan-1.⁽⁶²⁾ This observation has been strengthened more recently by Moshimer et al. who reported that syndecan-1 is involved in OPG-induced monocyte chemotaxis.⁽⁶³⁾ Our group also demonstrated a key function of the related glycosaminoglycans heparin and heparan sulfate in the activity of the molecular triad RANK–RANKL–OPG.⁽⁶⁴⁾ Indeed, kinetic data demonstrated that OPG binds to heparin with a high affinity (K_d : 0.28 nM) and that the preincubation of OPG with heparin inhibited, in a dose-dependent manner, the OPG binding to the complex RANK–RANKL. GAGs from different structure/origin (HS, DS and CS) exert similar activity on OPG binding. Sulfation is essential in the OPG-blocking function of GAGs and a decasaccharide is the minimal structure that totally inhibits the OPG binding to the complex RANK–RANKL. Western blot analysis performed in 293 cells overexpressing RANKL revealed that the pre-incubation of OPG with these GAGs strongly inhibits the OPG-induced decrease of membrane RANKL half-life.⁽⁶⁴⁾ Therefore, GAGs are able to

Table 3. Mouse knock-out phenotypes for PGs in bone and cartilage

Gene affected	Disorder	Phenotype	Reference
<i>Aggrecan</i>	Chondrodystrophy, nanomelia Cartilage matrix deficiency (cmd)	Severely reduced ECM, disruption of the columnar organization of the growth plate, shortened and broader bones	Li H et al. J Biol Chem 1993, 268:23504 Watanabe H et al. Nat Genet 1994, 7:154–7 Finkelstein JE et al. Am J Hum Genet 1991, 48:97–102 Doege KJ et al. J Biol Chem 1997, 272:13974–9
	Murine brachymorphism (bm) Defective PAPS synthesis	Dome-shaped skull, shortened limbs and short tail	Kurima K et al. PNA SUSA 1998, 95: 8681–5 Schwartz NB et al. Biochem Biophys Res Commun 1978, 82:173–8
	Spondylo-epimetaphyseal dysplasia	Short and bowed lower limbs, enlarged knee joints, early onset degenerative joint disease in the hands in the knees	UI Haque MF et al. Nat Genet 1998, 20:157–62 Ahmad M et al. Am J Med Genet 1998, 78:468–73
<i>DTDST (sulphate transporter)</i>	Recessive chondrodysplasia: Diastrophic dysplasia Atelosteogenesis type II Achondrogenesis type 1B	Skeletal abnormalities, dwarfism, spinal deformation, specific joint abnormalities, neonatally lethality, bowed lower limbs	Hastbacka J et al. Cell 1994, 78:1073–87 Hastbacka J et al. Am J Hum Genet 1996, 58:255–62 Superti-Furga A et al. Nat Genet 1996, 12:100–2
<i>Sox-9</i>	Campomelic dysplasia	Skeletal abnormalities, bowed lower limbs	Mansour S et al. J Med Genet 1995, 32: 415–20 Wagner T et al. Cell 1994, 79:1111–20
<i>Glypican-3</i>	Simpson-Golabi-Behmel syndrome	Pre- and postnatal overgrowth, coarse face, visceral and skeletal abnormalities	Pilia G et al. Nat Genet 1996, 12:241–7 Neri G et al. Am J Med Genet 1998, 79:279–83
<i>EXT1/EXT2</i>	Hereditary multiple exostoses	Formation of cartilage-capped exostoses, short stature	Lind T et al. J Biol Chem 1998, 273: 26265–8 Wuyts W et al. Am J Hum Genet 1998, 62:346–54 Kitagawa H et al. J Biol Chem 1999, 274: 13933–7
<i>Perlecan</i>	Dyssegmental dysplasia Silverman-Handmaker type (DDSH)	Disproportionate dwarfism, disorganized growth plate, cleft palate, perinatal lethality	Arikawa-Hirasawa E et al. Nat Genet 2001, 27:431–4
	Perlecan-null embryo Chondrodysplasia	Shortened collagen fibrils, reduced fibrillar network, elevated expression of cartilage ECM genes	Arikawa-Hirasawa E et al. Am J Hum Genet 2002, 70:1368–75
<i>SLRPs (Decorin, Byglican, Fibromodulin, Lumican)</i>	Osteoporosis Osteoarthritis Muscular dystrophy Ehler-Danlos syndrome	Decrease in bone mass, in bone trabecular formation	Xu T et al. Nat Genet 1998, 20:78–82 Ameye L et al. FASEB J 2002, 16+: 673–80 Ameye L et al. Glycobiology 2002, 12:107R–16R Nielsen KL et al. J Bone Miner Res 2003, 18:2152–8 Seidler DG et al. J Mol Med 2006, 84:583–94 Ekman S et al. Matrix 1990, 10:402–11
<i>FGFR</i>	Crouzon's syndrome	Pathological skull bone development	Bodo M et al. Eur J Cell Biol 1999, 78:323–30

diminish OPG availability and bioactivity that may indirectly increase bone resorption through the inhibition of interaction with RANKL. These results are particularly important and demonstrate another regulation level of bone resorption through the modulation of OPG bio-availability and indirectly of RANKL activity.

Osteoclast differentiation and activation may also be regulated by heparan sulfate type GAGs at different levels, as revealed in previous studies. In an in vitro model of osteoclastogenesis, FGF-2 upregulated the expression of RANKL on rheumatoid arthritis synovial fibroblasts, which was diminished by the removal of HS with heparitinase.⁽⁶⁵⁾ HSPG

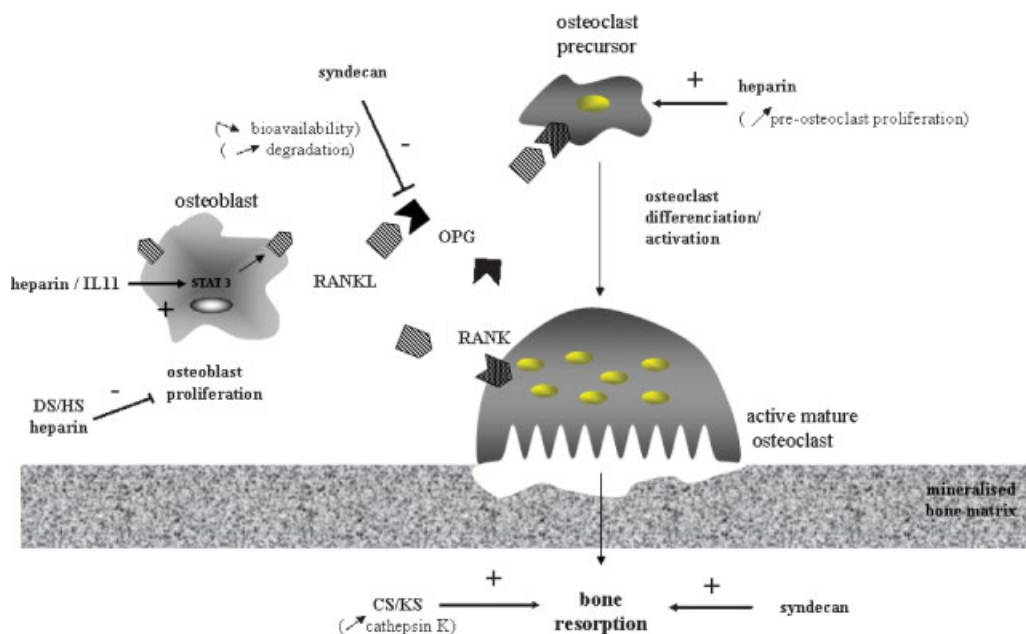


Figure 3. PGs as regulators of bone remodelling by interfering with osteoblast and osteoclast biological activities. Glycosaminoglycans (GAGs) or PGs mainly act as pro-resorptive agents: dermatan sulfate (DS)/heparan sulfate (HS) and heparin diminish osteoblast proliferation, heparin associated with Interleukin-11 (IL-11) enhances RANKL expression via STAT3, syndecan-1 inhibits osteoprotegerin (OPG) bioavailability, heparin enhances pre-osteoclast proliferation, syndecan activates bone resorption, and chondroitin sulfate (CS)/keratan sulfate (KS) enhance cathepsin K activity. All these effects lead to osteoclast differentiation and activation, then to bone resorption.

can also participate in bone resorption regulation through the modulation of cathepsin K activity, as demonstrated by the study of Li Z et al.⁽⁶⁶⁾ Indeed, cathepsin K, a lysosomal papain-like cysteine protease mainly involved in bone matrix destruction forms complexes with chondroitin sulfate therefore demonstrating a highly collagenolytic activity. GAGs predominantly expressed in bone and cartilage, such as CS and KS enhance the collagenolytic activity of cathepsin K, whereas DS, HS and HP selectively inhibit its activity. In another set of experiments, CS and DS modulate osteoblast functions by the inhibition of its proliferation.⁽⁶⁷⁾ DS shows a stronger inhibitory effect than CS, probably due to the presence of flexible IdoA residues that provide a greater variety in conformation of these macromolecules. Glycosaminoglycans (heparin/heparan sulfate) have also been shown to influence bone pathophysiology. For example, it is well known that long-term administration of heparin can lead to development of osteoporosis. Rats treated once daily by subcutaneous injections of heparin exhibited decreased trabecular bone volume both by decreasing the rate of bone formation and increasing the rate of bone resorption.⁽⁶⁸⁾ In vitro experiments showed that low concentrations of heparin increased bone resorption in isolated chick and rat osteoclasts.⁽⁶⁹⁾ The stimulation of resorption is associated with an increase of osteoclasts number on bone slices. One suggested mechanism resides in the fact that heparin acts synergistically with interleukin-11 to induce STAT3 activation

and in vitro osteoclast formation, through enhancement of IL-11's ability to induce the expression of RANKL and gp130.⁽⁷⁰⁾ Bone marrow cell cultures are also sensitive to heparin, and differences in the sensitivity to standard and low-molecular-weight heparins have been reported between mouse and rat species.⁽⁷¹⁾ An indirect proof of HSPGs involvement in osteoclast functions is given in a recent publication that demonstrated that heparanase, a HS-degrading endoglycosidase expressed in osteoblastic cells stimulates bone formation and bone mass.⁽⁷²⁾ The implication of glypican-3 (GPC-3) has also been demonstrated in bone formation. The loss-of-function mutations in the gene encoding glypican-3 causes a X-linked disorder in humans known as Simpson-Golabi-Behmel Syndrome, including both prenatal and postnatal overgrowth and skeletal abnormalities.⁽⁷³⁾ GPC-3 knockout animals show a marked reduction in calcified trabecular bone and an abnormal persistence of hypertrophic chondrocytes at embryonic day 16.5. These cells normally downregulate type X collagen and undergo apoptosis; however, their replacement by mineralized bone is delayed in association with a marked delay in the appearance of osteoclasts in the bone in vivo.

Glypican mutant animals exhibit an increase in osteoclast differentiation and activity due to the defective osteoblasts.⁽⁷⁴⁾ Indeed, glypican-deficient osteoblasts from neonatal murine calvaria are less sensitive to BMP-4 stimulation,⁽²⁹⁾

demonstrating that biglycan modulates BMP-4-induced signalling to control osteoblast differentiation.

Endostatin represents the C-terminal fragment of type XVIII collagen, a component of basement membranes with the structural properties of both collagens and proteoglycans. Endostatin plays a major role in angiogenesis which is intimately associated with bone development and remodelling. In a recent study, endostatin inhibits the VEGF-A stimulated osteoclastic bone resorption, whereas endostatin alone had no effect on the basal resorption level in the absence of VEGF-A.⁽⁷⁵⁾ In addition, endostatin could inhibit osteoclast differentiation *in vitro* independently of VEGF-A, and osteoclastogenesis is also inhibited by endostatin alone. The regulatory effect of endostatin, however, is not critical since endostatin alone does not modify the basal bone resorption.

Although CD44 is involved in the fusion process of osteoclast precursor cells, *in vivo* studies do not support this yet, since an osteopetrotic phenotype has not been described for CD44 k.o. mice. This discrepancy may suggest that the role of CD44 in fusion may depend on the microenvironment of osteoclast formation.⁽⁷⁶⁾

Taken together, these data show different levels of bone resorption regulation by GAGs, most of them leading to pro-resorptive effects. Therefore, modulation of PG synthesis or structure during pathological states may also interfere with bone content through dysregulation of bone resorption.

Proteoglycans and bone tumor development

It has become increasingly clear that heparan sulfates and HSPGs play important roles in regulating disease processes including cancer. Although early studies provided evidence that HS acts to suppress the malignant phenotype, evidence is mounting that HS can also promote tumor growth and metastasis. Within the extracellular matrix, HS facilitates metastasis by sequestering chemokines or growth factors within close proximity to migrating tumor cells.⁽⁷⁷⁾ Other potential mechanisms for HS in promoting metastasis include aiding in protection and localization of proteases and blocking interactions between tumor cells and other adhesion molecules.⁽⁷⁸⁾ Importantly, regulation of tumor cell behaviour by PGs is not restricted to the activity of HS chains as there is increasing evidence that core proteins of HSPGs have biological activities independent of HS. Such activities likely include co-receptor interactions with integrins⁽⁷⁹⁾ and core protein–growth factor interactions.⁽⁸⁰⁾ Indeed, it has been shown that syndecans can act as co-receptors to modulate integrin-mediated cell–matrix adhesion as, for example, syndecan-4 co-localizes in focal adhesions with integrin β 1 subunits.⁽⁸¹⁾ Proteoglycans can accumulate within the extracellular matrix or diffuse into the circulation and reach high levels in some cancer patients.

Recent data highlighted the importance of HSPGs as promoters of osteolytic tumor metastasis. Syndecan-1

(CD138) is the dominant HSPG expressed on the surface of myeloma cells and is used as a standard marker for identification and purification of these tumor cells.⁽⁸²⁾ Cell surface syndecan-1 is shed from myeloma cells and is present at high levels in the serum of some myeloma patients, being an indicator of poor prognosis.⁽⁸³⁾ In addition to the soluble form of syndecan-1 in the serum, the shed PG also becomes trapped within the bone marrow extracellular matrix and within regions of bone marrow fibrosis.⁽⁸⁴⁾ An *in vivo* experiment in SCID mice demonstrated that myeloma cells transfected with the soluble syndecan-1 grew faster than tumors modified by control-transfected cells or by cells expressing the cell surface form of syndecan-1,⁽⁸⁵⁾ providing evidence that soluble syndecan-1 enhances tumor growth *in vivo*. It is likely that the promotion of growth mediated by syndecan-1 is due to its interaction with HS-binding effector molecules, therefore representing a key mediator of cross-talk between the myeloma tumor cells and the bone marrow stroma. Similar findings have been reported for metastatic breast carcinomas, where a high expression of syndecan-1 is associated with a poor prognosis and an aggressive phenotype, syndecan-1 being localized both at the cell surface and within stroma.^(86,87) The proposed mechanisms that govern syndecan-1-mediated bone tumor growth suggest that bone tumor (myeloma or carcinoma cells) cells shed syndecan-1, which accumulates adjacent to cells and within bone marrow stroma. The heparan sulfate chains of syndecan-1 act to trap and concentrate growth and chemotactic factors within the tumor microenvironment (Wnt, FGF-2, VEGF). These factors enhance angiogenesis and promote tumor growth and osteoclastogenesis resulting in bone destruction. Enhanced osteoclastogenesis, in turn, leads to the release of factors stocked in the bone matrix that promote bone cancer cell growth.⁽⁸⁸⁾ The evidence indicates that syndecan-1 is a key mediator of cross-talk between tumor cells and bone, thereby sparking events that ignite the “vicious cycle” of lytic bone disease where tumor cells undergo rampant growth with associated increased osteoclastogenesis.⁽⁸⁹⁾ Syndecan-1 also regulates bone resorption in multiple myeloma through OPG modulation. The balance between the pro-resorptive agent RANKL and the anti-resorptive agent OPG is of major importance. Serum OPG levels are lower in myeloma patients than in healthy individuals. Indeed, OPG through its heparin-binding domain is bound, internalized and degraded by multiple myeloma cells that express high levels of syndecan-1.¹¹⁴ The syndecans have also been implicated in osteosarcoma cell viability, as the regulation of syndecans expression differed from osteosarcoma cells to osteoblasts.⁽⁹⁰⁾ In addition, syndecan-2 by its cytoplasmic domain controls the growth of normal human osteoblastic cells⁽⁹¹⁾ by inducing osteoblastic cell apoptosis through the JNK/Bax apoptotic pathway.⁽⁹²⁾

The finding that soluble syndecan-1 promotes growth and metastasis of tumors and perhaps angiogenesis, provides a

striking parallel to the known effects of heparanase on tumor behaviour. The expression of heparanase-1 that can act on extracellular heparan sulfate is rare in normal tissues but becomes evident in many human tumors where it significantly increases both the angiogenic and metastatic potential of tumor cells.⁽⁹³⁾ The mechanisms involve several distinct activities: (i) cleavage of HS within the extracellular matrix may be required for migration of metastasizing tumor cells and for remodelling the vasculature during angiogenesis, (ii) heparanase directly promotes angiogenesis by releasing heparin-binding angiogenic growth factors such as FGF-2 and VEGF that are trapped within the extracellular matrix,⁽⁹⁴⁾ (iii) heparanase produces HS fragments that are 10–20 residues long and more biologically active than the native HS chain from which they are derived.^(95,96) For example, in the mouse model of MDA-MB-231 breast carcinoma, cells transfected with the cDNA encoding human heparanase induced tumors that initially grow faster compared to control cells.⁽⁹⁷⁾ Moreover, in the same model, although metastases to bones are not detected, high bone resorption is stimulated in animal bearing tumors formed by the heparanase-transfected cells, suggesting that soluble factors or factors that stimulate osteoclastogenesis are specifically produced when heparanase expression is elevated.

Future direction as therapeutic approaches in bone tumors

As decorin is the product of a tumor suppressor gene, its role in tumor progression was assessed in term of angiogenesis regulation in several tumor xenograft models including osteosarcoma.⁽⁹⁸⁾ Decorin-expressing tumor xenografts grew at markedly lower rates and showed a significant suppression of neovascularization as compared to their wild-type counterparts, certainly caused by the reduced rate of VEGF production in decorin-expressing cells. These results provide a novel mechanism of action for decorin and indicate that decorin could adversely affect in vivo tumor growth by suppressing the endogenous tumor cell production of a powerful angiogenic stimulus. Based on in vitro analysis, a complex picture is emerging, indicating that SLRP control cell proliferation and cell differentiation in a cell-specific fashion.⁽⁹⁹⁾ Concerning decorin, it controls cell division as the ectopic expression of decorin in vitro in a wide variety of malignant cell lines and in normal fibroblast stops cell growth.^(31,100) Another interesting role for decorin as anti-tumor agent is given by the study of Teicher et al.⁽¹⁰¹⁾ Decorin was used as a naturally occurring TGF- β inhibitor to reverse the in vivo drug resistance that takes place in EMT-6/CTX and EMT-6/CDDP murine mammary tumors. Administration of decorin increased the sensitivity of the EMT-6/CTX tumor to cyclophosphamide and the EMT-6/CDDP to CDDP so that the drug resistance of these tumors was nearly ablated.

The recent findings that HSPG and heparanase act to fuel growth of osteolytic tumors present an opportunity for the development of new therapies aimed at controlling these tumors. Strategies such as modifying or neutralizing HS functions or inhibiting HS or PG synthesis all have potential. For example, modification of HS with bacterial heparitinase III generates biologically active fragments of HS that inhibit tumor cell growth and metastasis.⁽¹⁰²⁾ Other enzymes that modify HS structure, such as sulfatase-1 may also have growth-promoting or growth-inhibiting effects depending on the growth factor examined.^(103,104) Neutralizing HS functions in vivo may be possible using rationally designed peptides that bind to HS, thereby blocking its interactions with its natural ligand.⁽¹⁰⁵⁾ Inhibiting HS or HSPG synthesis represents another possible therapeutic approach by the knockdown of expression of the various heparan sulfate synthetic enzymes using antisense or siRNA technologies. Another approach is to inhibit assembly of heparan sulfate chains onto their core protein by exposing cells to β -D-xylosides. Indeed, it has been found that tumor growth was inhibited when mice were fed with a combination of naphthalene methanol β -D-xyloside and α -difluoromethylornithine.⁽¹⁰⁶⁾ Interestingly, there is evidence that some heparanase inhibitors may also inhibit heparan sulfate function. The use of low molecular weight heparins that have numerous inhibitory effects on tumor growth and metastasis inhibit heparanase activity as well.⁽¹⁰⁷⁾

Conclusion

Bone proteoglycans, through their compositional diversity, play a broad variety of roles in this specific tissue: (1) acting as crucial structural elements by the control of collagen fibrillogenesis, or (2) acting as co-receptors for some cytokines, playing important roles for the localization of cytokines, acting both as a tissue store and as a mediator of morphogen gradient formation in development. Among these functions, PGs play important roles as modulators of bone resorption. The overall data strengthen a pro-bone-resorptive role for GAGs, through the control of OPG availability and of RANKL bioactivity. In parallel, PGs participate in the control of tumor development at different levels, including bone tumor development and bone metastases dissemination. This dual role makes them good candidates as regulatory molecules in the vicious cycle between tumor proliferation and bone resorption that takes place during tumor development in bone site (Fig. 4). Taken together, the roles of HSPG families as bone pro-resorptive agents and promoters of osteolytic tumors make them good targets for new therapeutic approaches to these osteolytic bone tumors. However, these conclusions cannot be extended to all bone PGs as, for example, decorin is the product of a tumor suppressor gene that may on the contrary be considered as a therapeutic agent itself. The complex composition of bone PGs

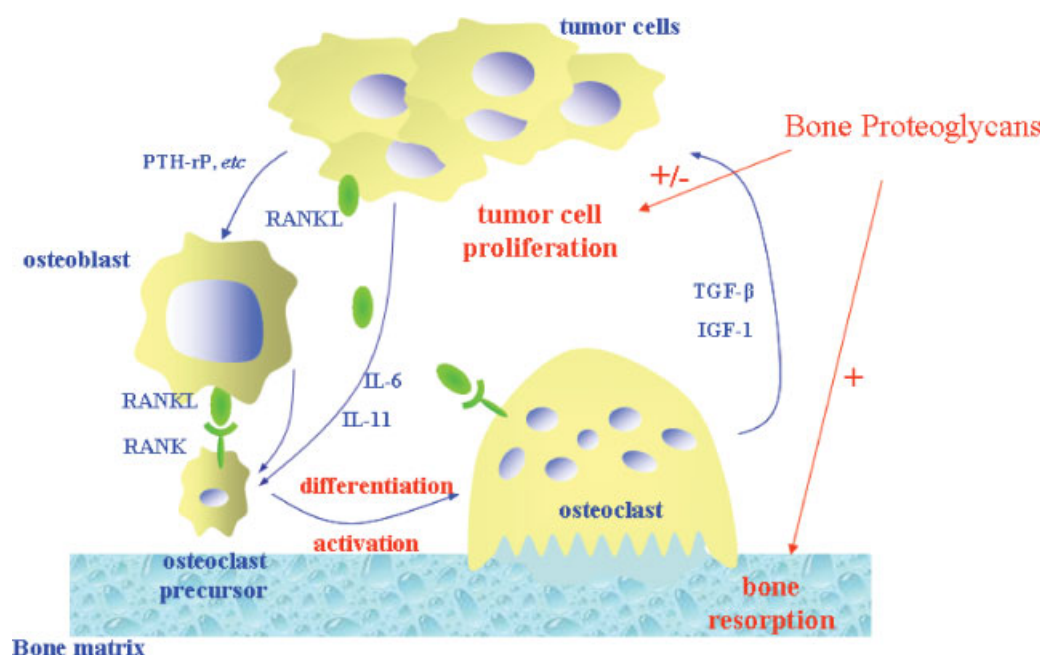


Figure 4. PGs present in the bone matrix can interfere with the vicious cycle that takes place during tumor development in bone site. Tumor cells secrete bone-modulating compounds such as parathyroid hormone-related protein (PTH-rP) or cytokines such as Interleukin-6 (IL-6) or IL-11 that directly or indirectly (via osteoblasts) activate bone resorption via the expression of the pro-resorptive cytokine receptor activator of NF- κ B ligand (RANKL). Growth factors such as Transforming Growth Factor- β (TGF- β) and Insulin-like Growth Factor-1 (IGF-1) are released from the bone matrix further stimulating tumor growth. PGs may be involved in this vicious cycle as both pro-resorptive agents and modulators of tumor development.

must be carefully considered before determining any therapeutic interventions in bone pathologies.

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RANKL directly induces bone morphogenetic protein-2 expression in RANK-expressing POS-1 osteosarcoma cells

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Abstract. The POS-1 murine model of osteolytic osteosarcoma was used to elucidate the molecular and cellular mechanisms involved in the development of primary bone tumors and associated lung metastasis. The POS-1 cell line is derived from an osteosarcoma tumor which develops spontaneously in C3H mice. The POS-1 cell line was characterized *in vitro* by mineralization capacity and expression of bone markers by semi-quantitative RT-PCR, compared to primary osteoblasts and bone marrow cells. POS-1 cells showed no mineralization capacity and exhibited an undifferentiated phenotype, expressing both osteoblastic and unexpected osteoclastic markers (TRAP, cathepsin K and RANK). Thereby, experiments were performed to determine whether RANK was functional, by studying the biological activity of murine RANKL through the receptor RANK expressed on POS-1 cells. Results revealed a RANKL-induced increase in ERK phosphorylation, as well as BMP-2 induction at the mRNA and protein levels, and a decrease of POS-1 cell proliferation in the presence of 10 ng/ml RANKL. BMP-2 induction is dependent on the ERK 1/2 signal transduction pathway, as its expression is abolished in the presence of UO126, a specific synthetic inhibitor of the ERK 1/2 pathway. Moreover, a 2-fold molar excess of soluble RANK blocks the RANKL-induced BMP-2 expression, demonstrating that the biological effects of RANKL observed in POS-1 cells are mediated by RANK. This is the first report describing a functional RANK expressed on osteosarcoma cells, as shown by its ability to induce signal transduction pathways and biological activity when stimulated by RANKL.

Introduction

Osteosarcomas (OS), the most frequent primary bone tumor that develops mainly in the young, the median age of diagnosis being 18 years, are classified into several subgroups according to their localisation and histology (1). The unifying histological feature present in all types and subtypes of OS is the presence of osteoid produced by neoplastic cells (2). The origin of osteosarcoma is not fully understood, as the tumor cells may have different origins depending on the dominant histological element. A preference for pulmonary metastases compared with other metastatic sites is a distinct feature of osteosarcoma, and 5-year survival rates after the detection of lung metastasis are less than 30% (3). Despite recent improvements in chemotherapy and surgery, the problem of non-response to chemotherapy remains and current strategies for the treatment of high-grade osteosarcoma fail to advance in its prognosis. Therefore, the developments of new therapies are needed.

Osteoprotegerin (OPG), the receptor activator of NF- κ B (RANK) and RANK ligand (RANKL) are the main molecules involved in the regulation of bone metabolism (reviewed in ref. 4). In the case of bone tumors, Grimaud *et al* demonstrated an increase in the RANKL:OPG ratio in the serum of patients with high-grade osteosarcoma (5), suggesting that the molecular triad, OPG/RANK/RANKL, might play a crucial role in tumorigenic osteolysis. RANKL augmentation directly produced by tumor cells (6) or indirectly via osteoblasts (7) contributes not only to an increased bone resorption but also to the release of growth factors stored in the bone matrix, creating a suitable context for tumor expansion as they increase tumor cell proliferation (8). In addition, it has been reported that tumor cell proliferation is often followed by a loss of differentiation markers or adhesion molecules, such as integrins, allowing the spreading of tumor cells to other organs leading to metastasis (9).

To elucidate the molecular and cellular mechanisms involved in the development of primary bone tumors and associated lung metastasis, the POS-1 murine model of osteosarcoma was used. The POS-1 cell line is derived from osteosarcoma which developed spontaneously in C3H mice. The tumor can be successfully transplanted in C3H mice or

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POS-1 cells inoculated into the hind footpad of mice and shows spontaneous metastasis to the lungs (10). Tumors were first recognized macroscopically at 2 weeks after inoculation and developed in more than 90% of inoculated mice at 5 weeks, lung metastasis being observed in all mice that developed tumors.

In this study, the *in vitro* phenotype of the POS-1 cell line was compared to those of the primary murine osteoblasts and bone marrow cells, in terms of mineralization capacity and bone marker expression. POS-1 cells exhibit an undifferentiated phenotype as they express osteoblastic and surprisingly osteoclastic markers, such as tartrate resistant acid phosphatase (TRAP), cathepsin K (Cat K) and RANK. Data from the literature reported the involvement of RANKL, either directly expressed by tumor cells themselves (6) or indirectly by osteoblasts or bone stromal cells (7), in bone tumor progression. In the osteolytic tumor-bone 'vicious cycle' described for bone metastasis from breast carcinoma (8), RANKL may mediate the release of growth factors from bone matrix and the production of growth factors, cytokines or chemokines by the activated osteoclast, or it may directly induce the production of angiogenic, invasion, chemoattractant and osteotropic factors by RANK positive cells. The influence of murine RANKL was therefore determined to see whether RANK may act as a functional receptor at the surface of osteosarcoma cells. The potential biological activity of RANKL was assayed on POS-1 cells in terms of phenotype modulation, signalling pathway and cell proliferation.

Materials and methods

Cell culture. The murine osteosarcoma cell line, POS-1, derived from osteosarcoma which spontaneously developed in mice was kindly provided by the Kanagawa Cancer Center (Kanagawa, Japan). The cells were cultured in RPMI-1640 medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Dominique Dutscher, Brumath, France), at 37°C in a humidified atmosphere (5% CO₂/95% air). The cells were harvested at confluence with trypsin (0.5 g/l)/EDTA (0.2 g/l) (Cambrex BioSciences, Verviers, Belgium). Mouse macrophage precursors RAW 264.7 cells (obtained from Professor Matsumoto, Japan) were used as osteoclast-like cells as they are able to differentiate into multinucleated cells in the presence of RANKL (11). Bone marrow cells (BMC) and primary osteoblasts were isolated from C3H/HeN mice (IFFA-CREDO, L'Arbresles, France). Briefly, femora and tibiae were collected and minced in RPMI-1640 after the removal of soft tissue. Bone fragments were cultured as explants in one side, whereas bone marrow was cultured separately, to lead respectively to osteoblasts and bone marrow cell cultures, both cell types being cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics, at 37°C in a humidified atmosphere.

RNA extraction and semi-quantitative RT-PCR analysis. POS-1 cells and osteoblasts were seeded in a 6-well plate at a density of 10⁴ cells/cm² in RPMI-1640 supplemented with 10% FBS. RAW 264.7 cells were seeded at the same density and maintained in phenol red-free α -MEM supplemented 1% nonessential amino acids and 10% FBS. At confluence, total

RNA was isolated from POS-1 cells, primary osteoblasts and the differentiated RAW 264.7 cells using TRIzol reagent (Invitrogen, Eragny, France). Total RNA were quantified by measuring the OD₂₆₀ and integrity was checked by 1% agarose/formaldehyde gel electrophoresis. First, RNA was reversed-transcribed (RT), using 400 U MMLV-RT from Invitrogen. To determine the expression of OPG, RANKL, collagen I (Coll I), osteocalcin (OC), Bone Sialo-Protein (BSP), bone morphogenetic protein-2 (BMP-2), core binding factor 1 (Cbfa1), RANK, calcitonin receptor (CTR), tartrate resistant acid phosphatase (TRAP) and cathepsin K (Cat K), two microliters of the RT reaction mixture were subjected to PCR using upstream and downstream primers (30 pmoles each, Table I) and 0.25 μ l of 5 U/ μ l Taq polymerase (Eurobio, Les Ulis, France). After the number of PCR cycles was increased, a plot was made for each sample, and the cycle values corresponding to the linear part of the amplification curve were determined (30 cycles for all primers, except 40 for CTR) and used to quantify the messages versus the 18S signal determined in the same way (27 cycles). The PCR products were electrophoresed in 1% agarose gel containing ethidium bromide. The band densities were measured using the ImageQuant computer software program (Molecular Dynamics). The relative expression of each gene was calculated as the ratio to 18S signal. Three independent experiments were performed for each gene and a representative experiment is shown in the Results section. To study the influence of RANKL on the POS-1 phenotype, mRNA expression was quantified in RANKL-treated POS-1 cells. When cells reached 70-80% confluence, they were washed and cultured in serum-free RPMI for 6 to 24 h in the presence of 1-200 ng/ml human soluble RANKL, kindly provided by Amgen Inc. (Thousand Oaks, USA). In a separate experiment, POS-1 cells were treated for 6 h with 100 ng/ml human RANKL preincubated for 1 h at room temperature with increasing concentrations of soluble human RANK (100-400 ng/ml, R&D Systems).

Cell proliferation assays. POS-1 cells were seeded in a 96-well-plate at a density of 2,800 cells per well, and cultured for 72 h in RPMI-1640 medium supplemented with 0.5% heat inactivated serum in the absence or presence of 1-200 ng/ml human soluble RANKL, or 1-100 ng/ml hBMP-2 (Abcys, Paris, France). The cell proliferation was determined by an XTT-based method, using the Cell Proliferation Kit II (Sigma, St. Quentin Fallavier, France) according to the supplier's recommendations. The OD was finally determined at 450 nm using a Wallac 1420 VICTOR 2™ multilabel counter (Perkin-Elmer, USA).

Mineralization assay. Mineralization assays were performed as previously described (12). Briefly, bone marrow cells and POS-1 cells were seeded in a 6-well plate at a density of 10⁴ cells/well, in RPMI-1640 medium supplemented with 10% FBS, in the absence or presence of 50 μ g/ml ascorbic acid (Sigma) and 10⁻⁸ M dexamethasone (Sigma). The medium was changed twice a week. After 7 days in culture, 10 mM of Na- β -glycerophosphate (Sigma) was added to each well in association with ascorbic acid and dexamethasone, and maintained in culture for 14 days. At the end of the incubation period, supernatant was removed and ice-cold ethanol was

Table I. Oligonucleotide primers used for RT-PCR.

Molecule	Primers	Strand	Size (bp)	Conditions (T _m , cycle number)
RANKL	ACAGCGCTTCTCAGGAGTTC	+	465	60°C
	TGGACGCTAATTTCTCACC	-		30 cycles
OPG	TGACCAAGACACCTTGAAGG	+	200	60°C
	CTAGTTATAAGCAGCTTATT	-		30 cycles
OC	TGACAAAGCCTTCATGTCCA	+	174	60°C
	TTTGTAGGCGGTCTTCAAGC	-		30 cycles
BSP	GGGAGGCAGTGACTCTTCAG	+	482	60°C
	GTTCTTCTGCACCTGCTTC	-		30 cycles
Coll I	ATGACCAGGTTACCTTTTCG	+	469	60°C
	AAGAGGCGAGAGAGGTTTCC	-		30 cycles
Cbfa1	GGACCGTGTTACCGTCAT	+	242	60°C
	ATGCGCCCTAAATCACTGAG	-		30 cycles
BMP-2	AGATCTGTACCGCAGGCACT	+	458	60°C
	TCTAAATGGGCCACTTCCAC	-		30 cycles
RANK	AAGATGGTTCCAGAAGACGGT	+	350	60°C
	CATAGAGTCAGTTCTGCTCGGA	-		30 cycles
TRAP	AAATCACTCTTTAAGACCAG	+	316	60°C
	TTATTGAATAGCAGTGACAG	-		30 cycles
CTR	CTGCTCCTAGTGAGCCCAAC	+	444	61°C
	AGTGAGGAACAGCTAACGAC	-		40 cycles
Cat K	GGGCCAGGATGAAAGTTGTA	+	324	60°C
	CCGAGCCAAGAGAGCATATC	-		24 cycles
18S	TCAAGAACGAAAGTCGGAGGTTTCG	+	462	62°C
	TTATTGCTCAATCTCGGGTGGCTG	-		27 cycles

^aPrimers are presented in a 5' to 3' orientation for the coding strand (+) and 3' to 5' for the non-coding strand (-). The product size generated by RT-PCR is indicated together with the experimental conditions used (T_m and cycle number corresponding to the linear part of the amplification curve used to quantify the messages versus the 18S signal, determined in the same way). All the primers are against mouse molecules, except for RANKL (rat).

added to the wells for 1 h. The cells were then washed twice with distilled water, and the presence of mineralized nodes was revealed by microscopic observation (Leica, DM IRB) using a 40 mM alizarin red staining solution, pH 7.4. In a separate experiment, POS-1 cells were co-cultured with bone marrow under the same experimental conditions to determine whether POS-1 cells could interfere with the mineralization capacity of bone marrow cells.

Protein quantification and alkaline phosphatase assays. POS-1 cells were seeded in a 6-well plate at a density of 10⁴ cells/well and cultured as described above (§ Cell culture). After 96 h of culture, POS-1 cells were washed with PBS and lysed in ice-cold buffer [NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, leupeptine 10 mg/ml, aprotinin 10 mg/ml, phenylmethylsulfonylfluoride (PMSF) 0.5 mM, and glycerol 10%]. The total amount of proteins for each sample was determined using a bicinchoninic acid (BCA, Sigma) based method. Ten microliters of cellular

lysis or standard BSA solution were added to 200 µl of reagent (Copper II solution 1/50 diluted in bicinchoninic acid) and incubated for 30 min at 37°C. OD was determined at 570 nm as well as the protein concentration compared to the standard curve. The same amounts of proteins were used to perform alkaline phosphatase assays using Enzyline PAL kit according to the supplier's recommendations (BioMérieux, Marcy l'Etoile, France). At the end of the incubation period (10 min at room temperature), the reaction was stopped and OD was determined at 405 nm using a Wallac 1420 VICTOR 2TM multilabel counter.

Signal transduction analysis by Western blotting. POS-1 cells were seeded at a density of 10⁵ cells/well in a 6-well plate in RPMI-1640 supplemented with 10% FBS. When POS-1 cells reached 70-80% confluence, they were washed 3 times with RMPI-1640 and cultured in serum-free RMPI-1640 for 24 h, then incubated for 1, 2, 5, 10, 15 and 30 min in the absence or presence of 100 ng/ml human RANKL. POS-1

cells were lysed in ice-cold buffer (NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, NaVO_4 1 mM, leupeptine 10 mg/ml, aprotinin 10 mg/ml, PMSF 0.5mM, glycerol 10%). The same amounts of proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a polyvinylidene fluorure (PVDF) membrane (Millipore, Saint-Quentin en Yvelines, France). After washing twice with 0.05% Tween-20/PBS, the membrane was incubated in a saturating solution (0.05% Tween-20/3% BSA/PBS) for 30 min at room temperature with appropriate antibodies. The levels of phosphorylated and total forms of ERK1/2, I κ B and p38 were detected by respective specific antibodies (Ozyme, Saint-Quentin en Yvelines, France, and R&D) and revealed using the BM Chemiluminescence Western Blotting substrate (Roche Applied Science, USA). In another set of experiments, POS-1 cells were cultured in the presence of 25 μM UO 126 (Calbiochem Merck, Fontenay-aux-Roses, France), a synthetic inhibitor of the ERK pathway to confirm the involvement of the ERK signalling pathway in RANKL-induced biological activity.

BMP-2 quantification. ELISA assay was used to quantify BMP-2 levels in culture supernatants of POS-1 cells treated or not with increasing concentrations of RANKL. Experiments were realized using the Human/Mouse/Rat BMP-2 Quantikine ELISA kit (R&D Systems) according to the supplier's recommendations.

Caspase activity. POS-1 cells (2×10^4) grown in 24-well plates were treated with 1-100 ng/ml hBMP-2 for 24-72 h, washed once with PBS and lysed with 50 μl of RIPA-buffer for 30 min. The cells were then scraped off and the protein amount was quantified using the BCA (bicinchomonic acid + Copper II sulfate) test (Pierce Chemical Co., Rockford, IL, USA). Caspase-3 activity was assessed on 10 μl of cell lysate with the CaspACE™ assay kit (Promega, Madison, WI, USA) following the manufacturer's recommendations. Cells treated with UV light for 30 sec, 24 h before harvesting, were used as a positive control for caspase activity.

In vivo characterization of osteosarcoma induced by POS-1 cell inoculation. Four-week-old male C3H/He mice (IFFA-CREDO, L'Arbresle, France) were housed under pathogen-free conditions at the Experimental Therapy Unit (Medicine Faculty of Nantes, France), in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. POS-1 cells (2×10^6) were inoculated s.c. in the hind footpad of the mice. A tumor develops at the site of injection within three weeks that can be transplanted as a small fragment ($2 \times 2 \times 2 \text{ mm}^3$) in contact with the femora, leading to the development of a primary tumor and pulmonary metastases in three weeks. The tumor was then characterized by micro-architectural studies.

Micro-architectural quantification. Analysis of architectural parameters was performed using the high resolution X-ray micro-CT system for small animal imaging SkyScan-1072 (SkyScan, Aartselaar, Belgium). Relative volume (BV/TV) of the femora [total bone (cortical + trabecular) or trabecular

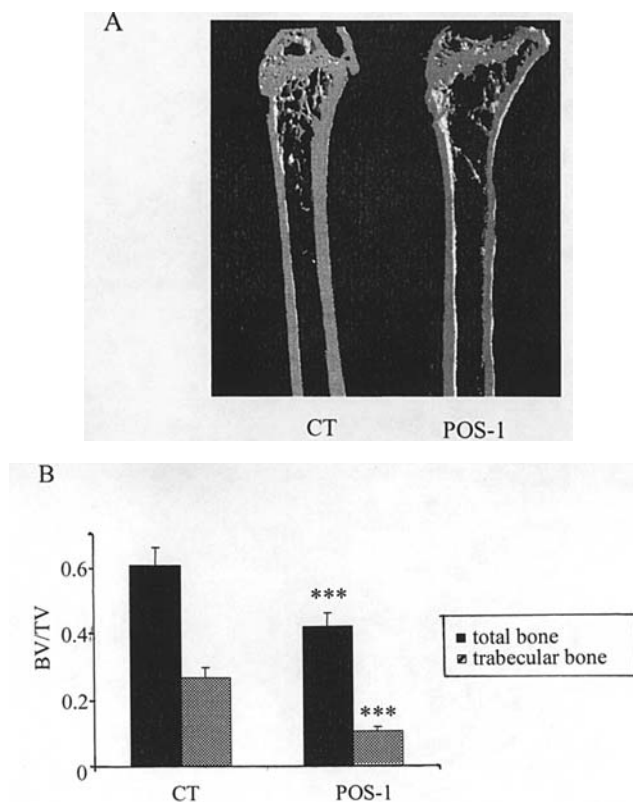


Figure 1. Micro-CT scans of mouse femora 4 weeks after implantation with POS-1 osteosarcoma as compared to controls. A, Hemi-sagittal sections of control (CT) and osteosarcoma (POS-1) femora are shown. B, Quantification of relative bone volume (BV/TV) of total and trabecular bone compared between controls (CT) and osteosarcoma-bearing mice (POS-1). *** $p < 0.005$.

bone] was quantified in the osteosarcoma group and compared to that of control mice.

Statistical analyses. Statistical evaluation of the *in vitro* proliferation data was performed by the ANOVA test. Results with $p < 0.05$ were considered significant.

Results

Characterization of osteosarcoma induced by POS-1 cell injection. After injection of 2×10^6 POS-1 cells in the hind footpad of C3H/He mice, a primary tumor develops locally within approximately 21 days. This tumor can be re-grafted in contact with the femora, to see whether the primary tumor exhibits rather osteocondensant or osteolytic features. The tumor develops locally within two weeks. The mice were sacrificed 28 days post-implantation, and the femora were harvested and analysed for their microarchitectural parameters. The results presented in Fig. 1 demonstrate that the POS-1-induced osteosarcoma is osteolytic as it reduces the cortical thickness and dramatically diminishes the relative trabecular bone volume, as quantified by $\text{BV/TV} = 0.106$ versus 0.27 for the control (-61% , $p < 0.005$) and total bone volume ($\text{BV/TV} = 0.42$ versus 0.61 for the control, -32% , $p < 0.005$).

POS-1 cells' characterization in vitro. POS-1 cells exhibit an undifferentiated phenotype, with expression of both osteo-

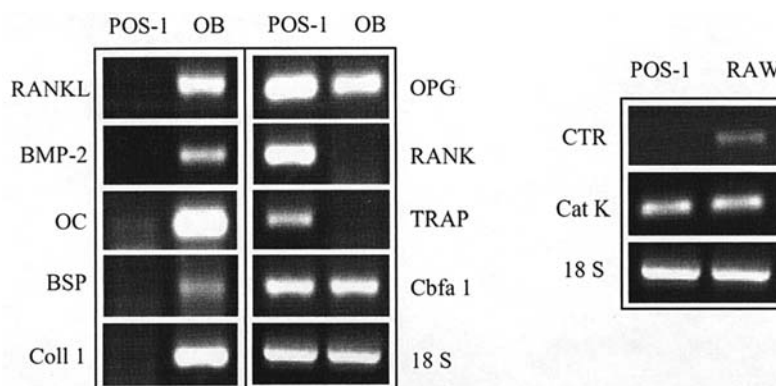


Figure 2. Bone marker expressed by POS-1 cells *in vitro*, as compared to mouse primary osteoblasts (OB) and mouse RAW 264.7 cells differentiated in osteoclast-like cells (RAW). The expression of several bone parameters, among them receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG), osteocalcin (OC), Bone SialoProtein (BSP), type I collagen (Coll I), bone morphogenetic protein-2 (BMP-2), RANK, tartrate resistant acid phosphatase (TRAP), cathepsin K (Cat K), Core binding factor α 1 (Cbfa1) and calcitonin receptor (CTR) was analysed in POS-1 cells by semi-quantitative RT-PCR according to the conditions described in Table I, and compared to osteoblasts or RAW 264.7 cells.

blastic and osteoclastic markers. Mature osteoblasts are characterised by the expression of phenotypic markers, including Coll I, OC, BSP, BMP-2, OPG, RANKL, and the transcription factor Cbfa1. The constant positive expressions of these markers were confirmed in the mouse osteoblasts tested in this study (Fig. 2). In comparison, semi-quantitative RT-PCR analysis showed that POS-1 cells do not express several osteoblastic markers, such as BMP-2, OC, BSP, Coll I and RANKL. However, the expression of Cbfa1 and OPG transcripts was still observed (Fig. 2). As osteosarcoma may be heterogeneous in its cell origin and composition, the expression of other markers was analysed, including osteoclastic cathepsin K, TRAP, CTR and RANK. Interestingly, POS-1 cells expressed Cat K, TRAP and RANK, but not CTR, which represents one of the most specific osteoclastic markers (Fig. 2). It can be concluded that POS-1 cells exhibit an undifferentiated phenotype, expressing markers from both osteoblastic and osteoclastic lineages.

POS-1 cells' biological activity in vitro. In addition to their phenotype, POS-1 cells were assessed for their capacity to demonstrate alkaline phosphatase activity and to induce mineralization nodules *in vitro*. Results presented in Fig. 3A show that POS-1 cells did not demonstrate any alkaline phosphatase activity as compared to primary osteoblasts. Bone marrow cells (BMC) isolated from C3H mice contain osteoblastic cell precursors that can differentiate into mature osteoblasts when cultured with dexamethasone (12). Both cell types were incubated with ascorbic acid, dexamethasone and Na- β -glycerophosphate or not. After 21 days of culture, the results of alizarin red staining revealed that POS-1 cells were not able to form mineralization nodules in the presence of β -glycerophosphate, whereas BMC showed marked mineralization nodules (Fig. 3B). Moreover, the nodule formation induced in BMC alone was totally inhibited when POS-1 cells were co-cultured with bone marrow cells (not shown).

RANKL inhibits cell proliferation and induces BMP-2 expression in osteosarcoma POS-1 cells. As POS-1 cells express RANK, RANKL-induced biological activities were assayed to determine whether the receptor RANK is functional

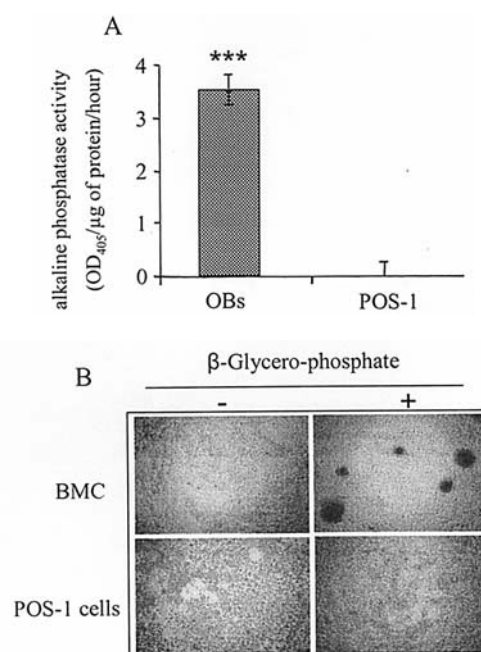


Figure 3. *In vitro* characterization of osteogenic properties of POS-1 cells. A, alkaline phosphatase activity in cell lysates of POS-1 cells and primary mouse osteoblasts (OBs) was performed using p-nitrophenyl phosphate as a substrate and corrected for protein content. The results are presented as the OD value measured at 405 nm per μ g of protein per hour. *** p <0.005. B, mineralized nodule formation in POS-1 cells. POS-1 cells and bone marrow cells (BMC) were cultured in a medium containing 50 μ g/ml ascorbic acid, 10 mM Na β -glycerophosphate, 10^{-8} M dexamethasone for 21 days. Typical alizarin red-S⁺ nodules were observed in phase contrast microscopy. Magnification x200.

on osteosarcoma cells and could mediate biological activity. First, the effects of RANKL were studied on POS-1 cell proliferation after 72 h of culture. Using an XTT-based method, 1-200 ng/ml hRANKL decreased POS-1 cell proliferation in the presence of 0.5% inactivated FCS, the maximal effect being observed at 100 ng/ml (-31%, p =0.003, Fig. 4). In parallel, the effects of RANKL were studied on POS-1 cell phenotype modulation by the analysis of osteoblastic and osteoclastic marker expression by semi-quantitative RT-PCR.

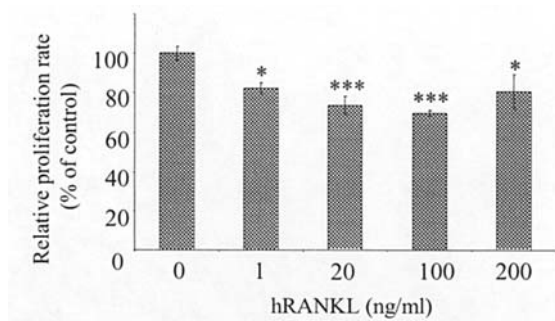


Figure 4. Effects of soluble human RANKL on POS-1 cell proliferation *in vitro*. POS-1 cells were seeded in a 96-well plate and cultured for 72 h in RPMI-1640 medium supplemented with 0.5% heat-inactivated serum in the absence or presence of human soluble RANKL at the indicated concentrations. Proliferation was measured using the XTT quick proliferation kit as described in Materials and methods. Changes in absorbance at 490 nm were measured and results are presented as percentage of untreated cell proliferation. * $p < 0.05$, *** $p < 0.005$.

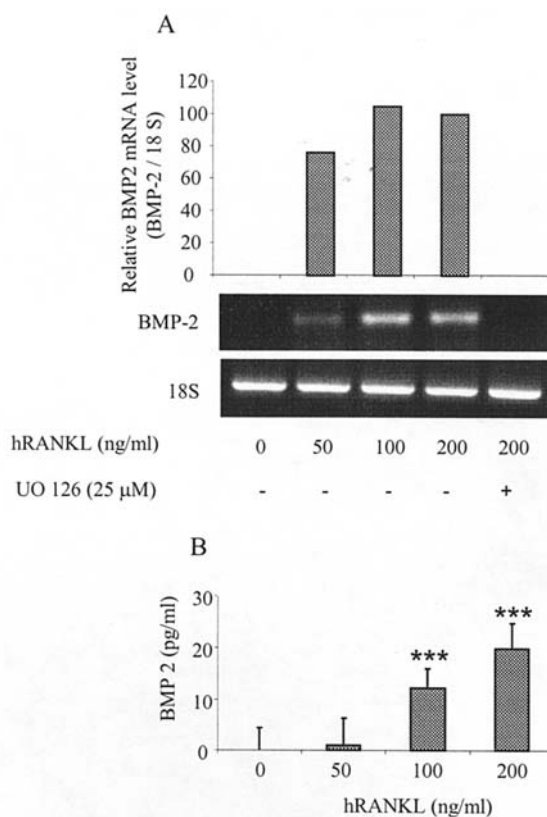


Figure 5. RANKL induces BMP-2 expression in POS-1 cells. A, relative BMP-2 mRNA levels were measured by semi-quantitative RT-PCR in POS-1 cells treated for 6 h in the presence of increasing concentrations of human RANKL. In a separate experiment, POS-1 cells were incubated with 200 ng/ml RANKL in the presence of 25 μ M UO 126, a synthetic inhibitor of the ERK pathway. B, ELISA assay was used to quantify BMP-2 levels in culture supernatants of POS-1 cells treated or not with increasing concentrations of RANKL for 24 h. *** $p < 0.005$.

Whereas POS-1 cells did not express BMP-2 at the mRNA level (Fig. 2), the induction of the BMP-2 transcripts was observed when these cells were cultured in the presence of 50 ng/ml hRANKL for 6 h with a maximal effect at 100 and 200 ng/ml (Fig. 5A). The RANKL-induced BMP-2 mRNA

expression was then confirmed at the protein level by ELISA, with a dose-dependent augmentation of BMP-2 in the supernatant of POS-1 treated with 50, 100 and 200 ng/ml RANKL (respectively 1.83, 12.66 and 19.05 pg/ml; Fig. 5B). The expression of other bone markers was not significantly modified by RANKL treatment (not shown). The ability of RANKL to induce mineralization nodules was further tested *in vitro* to ascertain whether this factor may induce other activities in POS-1 cells related to enhanced osteogenic potential. The results revealed no induction of mineralization nodules in POS-1 cells (not shown).

RANKL-induced BMP-2 expression depends on ERK signalling pathways. As RANKL was shown to induce biological activities in POS-1 cells, Western blot analysis was performed to determine the signalling pathways involved in these effects. p38, ERK 1/2 and I κ B, the three most reported signalling pathways for RANKL were analysed (13). The results revealed a specific induction of the phosphorylated ERK 1/2 form after 5-, 10- and 15-min incubation with 100 ng/ml hRANKL (Fig. 6), as compared to the total protein (not shown). The phosphorylation signal then returned to basal level after an incubation period of 30 min in the presence of RANKL. A weaker induction of I κ B and p38 phosphorylations was also observed after 5 and 10-15 min of incubation with hRANKL respectively (Fig. 6). As ERK seemed to be the major transduction pathway activated by RANKL in POS-1 cells, the effect of a synthetic ERK inhibitor, UO 126, has been studied in the RANKL-induced BMP-2 expression. The results showed a specific blockade of RANKL-induced BMP-2 expression in the presence of 25 μ M UO 126 (Fig. 5A).

Collectively, these results demonstrate that soluble RANKL acts on POS-1 cells by inducing BMP-2 mRNA and protein expression through the ERK-dependent signalling pathway.

RANKL-induced BMP-2 expression has no effect on POS-1 cell behaviour *in vitro*. To determine whether RANKL-induced BMP-2 expression may alter POS-1 cell behaviour, the effects of hBMP-2 (1-100 ng/ml) were assessed *in vitro* by proliferation test, caspase-3 activation or alkaline phosphatase activity. Using the XTT-based method, 1-100 ng/ml hBMP-2 did not significantly modify the POS-1 cell proliferation (not shown). As this factor has been described as promoting osteoblast cell death (14), we analysed the effects of hBMP-2 on caspase-3 activity in POS-1 cells. The results showed a weak up-regulation of caspase-3 activity in the presence of 10 and 50 ng/ml BMP-2 for 72 h, which was not significant (+5.8% and 5.9% respectively, not shown). To see whether BMP-2 may modify POS-1 cell biological activity, alkaline phosphatase activity was studied in the presence of 1-100 ng/ml hBMP-2 for 24-72 h. No induction of alkaline phosphatase activity could be detected in POS-1 cells in the presence of BMP-2 (not shown), as observed in the presence of RANKL (Fig. 2).

RANKL-induced BMP-2 expression in osteosarcoma POS-1 cells is RANK-dependent. To demonstrate that cell surface RANK expressed by POS-1 cells is involved in the RANKL-induced BMP-2 expression, we blocked the RANKL activity

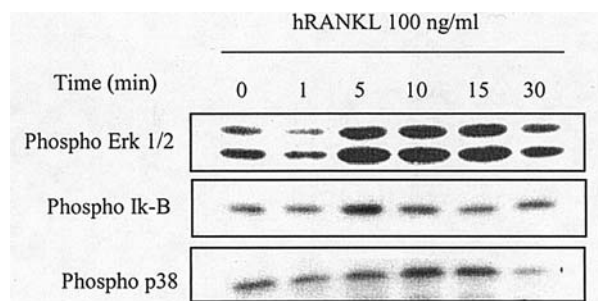


Figure 6. RANKL induces signal transduction pathways in POS-1 cells. At 70-80% confluence, pOS-1 cells were serum-starved for 24 h and then incubated for 1, 2, 5, 10, 15 and 30 min. in the absence or presence of 100 ng/ml human RANKL. Aliquots of whole cell lysates were analysed by immunoblotting for phospho-ERK 1/2, phospho IκB and phospho p38.

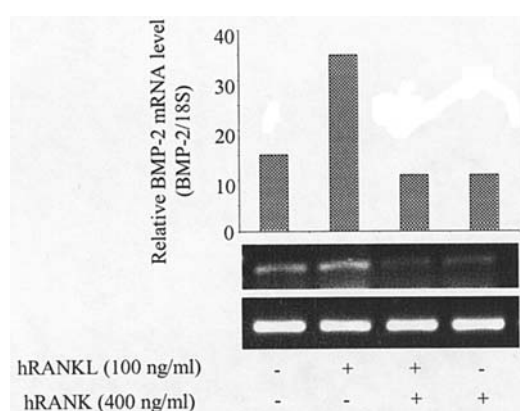


Figure 7. RANKL-induced BMP-2 expression in POS-1 cells is inhibited by soluble RANK. At 70-80% confluence, pOS-1 cells were treated for 6 h with 100 ng/ml hRANKL that was first pre-incubated for 1 h with 400 ng/ml human soluble RANK (2-fold molar excess). Relative BMP-2 mRNA levels were measured by semi-quantitative RT-PCR and corrected with the 18S mRNA levels. A representative experiment is shown.

with an excess of soluble RANK by preincubating 100 ng/ml hRANKL with increasing concentrations of human RANK before adding to POS-1 cells. The results presented in Fig. 7 showed a complete inhibition of RANKL-induced BMP-2 expression in the presence of a two-fold molar excess of soluble hRANK.

Discussion

A comprehensive multidisciplinary approach has transformed osteosarcoma from a disease with a modest long-term survival to one in which 50-70% of patients will be cured. Unfortunately, some groups of patients who present with overt metastatic disease, patients with tumors that recur after treatment or that show low degrees of necrosis after administration of chemotherapy, remain at high risk of eventual relapse and continue to have an unsatisfactory outcome. These patients may benefit from future investigations into novel agents, such as biological response modifiers, anti-angiogenesis factors or growth receptor modulation. There is an obvious need for an adequate model for the study of osteosarcoma pathophysiology and to investigate important problems related to the therapeutics of human bone tumors.

Permanent osteosarcoma cell lines represent an important tool for the study of primary bone tumors. The POS-1 cell line is derived from osteosarcoma which developed spontaneously in C3H mice and parallels with humans clinical data, leading to spontaneous metastasis to the lungs (10). This experimental model was used in the present study to evaluate cellular characteristics *in vitro* and then correlate them with the *in vivo* compartment of the tumor. Conventional osteosarcomas are classified as fibroblastic, chondroblastic or, more often, osteoblastic, depending on the predominant cellular component. However, Narita *et al* demonstrated that several tumor cell lines can lose some of their properties when cultured for a long time *in vitro* (9). Another study from Yoshikawa *et al* demonstrated that HMOS-1, a human mandibular osteosarcoma cell line with an osteoblastic phenotype, could induce osteoid formation and ALP activity but did not express collagen I (15). Taking advantage of our osteosarcoma model, both at the *in vitro* and *in vivo* level, we wanted to characterize these cells *in vitro* in relation with the *in vivo* behavior of the tumor. In our model, POS-1 osteosarcoma cells express very few osteoblastic markers: OPG and the transcription factor, Cbfa1, predominantly described in cells of the osteoblast lineage (16), but not osteocalcin, Bone Sialoprotein, BMP-2 or RANKL, and they show no ALP activity. In fact, POS-1 osteosarcoma cells exhibit an undifferentiated phenotype. This is in agreement with the well-described characteristics of tumor cells showing an undifferentiated state associated with a high proliferative capacity, as POS-1 cells were shown to highly proliferate *in vitro*. Moreover, these tumor cells express specific tumor antigens, such as SART3 (not shown), and still exert tumorigenicity as they induce pulmonary metastatic lesions when re-injected into mice, and they also induce bone destruction *in vivo* contiguously to the tumor itself. It appears, therefore, that osteosarcoma POS-1 cells in culture express an undifferentiated phenotype but keep their capacity to induce tumor growth and bone destruction *in vivo*. In humans, osteosarcomas frequently penetrate and destroy the cortex of the bone and extend into the surrounding soft tissue. The unifying histological feature present in all types and subtypes of OS is the presence of malignant osteoid produced by the neoplastic cells (2). As POS-1 cells express some 'osteoclast' markers *in vitro* (TRAP, Cat K and RANK) and induce bone destruction *in vivo*, one can suggest that mouse POS-1 osteosarcoma is more osteolytic than osteosclerotic. It can be hypothesized that POS-1 cells can induce osteolytic lesions, as they express cathepsin K and MMP-9 mRNA, two major enzymes involved in bone destruction (17).

However, the expression of TRAP and RANK is not restricted to osteoclasts as TRAP has been already described in osteoblast-like cells (18) including osteosarcoma (19). RANK expression has been described in osteosarcoma-derived cell lines (20) together with bone marrow stromal cells and osteoblasts (21). Cathepsin K, a cysteine protease expressed by osteoclasts, appears to be essential for osteoclast-mediated collagen type-I degradation (22). Cathepsin K is also expressed in breast cancer cells (23), and Brubaker *et al* have recently detected cathepsin K and its proteolytic activity in prostate cancer tissue and cell lines (24). Therefore, it can be hypothesized that cathepsin K expressed directly by osteosarcoma

cells participates in the development of osteolytic lesions observed in our POS-1 model.

The OPG/RANK/RANKL triad is an important therapeutic axis in pathologies involving a dysregulation in bone remodelling, including tumor-associated osteolysis (4). A number of studies provide evidence for the direct production of RANKL by tumor cells themselves, as reported in multiple myeloma (25), prostate cancer (26), carcinoma cell lines (6) or human neuroblastoma (27). RANKL can then bind to its cognate receptor, RANK, at the surface of osteoclast precursors acting directly on osteoclast differentiation and activation. In the present POS-1 osteosarcoma model, POS-1 cells express RANK, not RANKL. RANK is known to be predominantly present at the surface of osteoclasts and some immune cells (28), but its expression has also been revealed in marrow stromal cells and osteoblasts and was strongly up-regulated when activated by T-cell conditioned medium (21). Another publication from Miyamoto *et al* reported that human osteosarcoma-derived cell lines expressed both RANK and RANKL mRNAs but the functionality of the receptor, RANK, was not investigated (20). However, the presence of a functional receptor, RANK, at the surface of cancer cells is in agreement with the results of Tometsko *et al* who recently reported the direct effects of RANKL on RANK-expressing human breast cancer cells, MDA-MB-231, and prostate PC3 (29). They demonstrated that RANKL treatment of both MDA-231 and PC3 cells led to the activation of signal transduction pathways (p38 MAPK, p42/44 MAPK, NF- κ B) and upregulated the expression of 194 mRNA as assessed by micro-array. From these data, we can hypothesize that, while neither cell line (MDA-231, PC3 and POS-1) expresses RANKL *in vitro*, it is probable that the locally increased RANKL within the bone microenvironment could activate tumor cell-expressed RANK in a paracrine manner.

In our experimental model, RANKL decreased POS-1 cell proliferation and induced the expression of BMP-2 through its receptor, RANK, expressed on osteosarcoma cells. The *in vitro* induction of BMP-2 by RANKL does not reflect an enhancement of the osteogenic properties of the cells, as mineralization was not induced by RANKL, nor expression of other bone anabolic molecules. BMPs are a group of related proteins which are capable of inducing the formation of cartilage and bone, but are now regarded as multifunctional cytokines. A study by Hay *et al* (14) demonstrated that BMP-2 promoted apoptosis in primary human calvaria osteoblasts by increasing the Bax/Bcl-2 ratio, the release of cytochrome c to the cytosol and caspase-9, -3, -6 and -7 activity. As BMP-2 induces apoptosis in myeloma cell lines and primary samples from patients with multiple myeloma *in vitro*, it has been suggested that this cytokine may have the potential to be a novel therapeutic agent for the treatment of patients with multiple myeloma (30). As BMP-2's receptors have been described on several cancer cell lines, its effects as an anticancer drug is now emerging (31). However, in the present study, BMP-2 did not induce POS-1 cell apoptosis nor modulate POS-1 cell proliferation. Therefore, further study is needed to explore the potential biological activity of BMP-2 in osteosarcoma.

The RANK signalling mechanisms involved in RANKL responses have been well described and include the recruitment

of TNF receptor-associated factor proteins, the activation of transcription factors (NF- κ B, AP-1, and NFAT2), the cascades of mitogen-activated protein kinases (ERK, JNK, and p38), and the induction of Src- and phosphatidylinositol 3-kinase-dependent Akt activation (13). In the present study, RANKL predominantly induced ERK phosphorylation with a weak activation of NF- κ B and p38 signalling pathways. As synthetic inhibition of the ERK pathway totally blocked the RANKL-induced BMP-2 expression in POS-1 cells, this signal transduction pathway seemed to be the major pathway activated by RANKL through its receptor, RANK, at the surface of osteosarcoma cells, whereas NF- κ B and p38 pathways were less involved.

This study describes for the first time the presence of a functional receptor RANK at the surface of osteosarcoma cells, that may mediate the establishment of osteolytic lesions induced by the tumor *in vivo*. Targeting RANKL/RANK interaction by the use of OPG or soluble RANK may offer new therapeutic approaches for osteosarcoma, as it has for other malignant osteolytic pathologies (32).

Acknowledgements

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Ciblage Thérapeutique des tumeurs osseuses primitives par transfert de gène

Mots-clés :

ostéosarcome, cercle vicieux, OPG, RANKL, microenvironnement tumoral osseux

L'ostéosarcome est la plus fréquente des tumeurs osseuses primitives affectant principalement une population jeune. Bien que les traitements actuels aient contribué à la progression de la survie des patients, ils montrent désormais leurs limites d'où la nécessité de développer de nouvelles thérapies. L'hypothèse biologique de l'ostéosarcome repose sur l'existence d'un cercle vicieux s'instaurant entre la prolifération tumorale et la résorption osseuse. Récemment découverte, la triade moléculaire Receptor Activator of NF κ B (RANK) / RANK ligand / Ostéoprotégérine (RANK/RANKL/OPG) est fortement impliquée dans la régulation de la résorption osseuse et du développement tumoral, ainsi l'inhibition de l'interaction RANK/RANKL représente une cible thérapeutique majeure. L'objectif de cette thèse a été de développer de nouvelles approches thérapeutiques dans des modèles expérimentaux d'ostéosarcomes afin de rompre ce cercle vicieux, notamment par l'utilisation de l'OPG ou de RANK-Fc, molécules anti-résorption osseuse, libérés par transfert de gène non viral, puis de tenter de comprendre la rôle de l'OPG et de RANKL dans le microenvironnement tumoral osseux.

Primary bone tumors therapeutic targeting by gene transfer

Key words: osteosarcoma, vicious cycle, OPG, RANKL, tumor bone microenvironment

Osteosarcoma is the most frequent primary bone tumor that affects mainly young patients. Despite current treatments have contributed to increase the survival rate, they show now their limitations with the need to develop new therapeutic approaches. The biological hypothesis of osteosarcoma is based on the existence of a vicious cycle between tumor proliferation and bone resorption. Recently discovered, the molecular triad Receptor Activator of NF κ B (RANK) / RANK ligand / Osteoprotegerin (RANK / RANKL / OPG) is strongly implicated in the regulation of bone resorption and tumor development, and the inhibition of the interaction RANK / RANKL represents a major therapeutic approach. The objectives of this thesis were to develop new therapeutic approaches in experimental models of osteosarcoma in order to break this vicious cycle, including the use of OPG and RANK-Fc, anti-bone resorption molecules, released by non-viral gene transfer, and then to try to understand the place of OPG and RANKL in the tumor bone microenvironment.

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