# Proteoglycans and osteolysis.

Marc Baud'Huin, Céline Charrier, Gwenola Bougras, Régis Brion, Frédéric Lezot, Marc Padrines, Dominique Heymann

# Proteoglycans and osteolysis

BAUD'HUIN Marc[1,2], CHARRIER Céline[1,2], BOUGRAS Gwenola[1,2],

BRION Régis[1,2], LEZOT Frédéric[1,2], PADRINES Marc[1,2] and HEYMANN Dominique[1,2,3]

1: INSERM, UMR 957, Nantes, F-44035 France

2: Université de Nantes, Nantes Atlantique Universités, Physiopathologie de la Résorption Osseuse et Thérapie des Tumeurs Osseuses Primitives, Nantes, F-44035 France

3: Centre Hospitalier Universitaire, Nantes, F-44035 France

* Corresponding author : INSERM U957 Physiopathologie de la Résorption Osseuse et Thérapie des Tumeurs Osseuses Primitives, Faculté de Médecine, 1 rue Gaston Veil, 44035 Nantes cedex 1. France

Phone: +33 (0) 240 412 845; Fax: +33 (0) 240 412 860

Email: dominique.heymann@univ-nantes.fr

**Abstract**

Osteolysis is a complex mechanism resulting from an exacerbated activity of osteoclasts associated or not with a dysregulation of osteoblast metabolism leading to bone loss. This bone defect is not compensated by bone apposition or by apposition of bone matrix with poor mechanical quality. Osteolytic process is regulated by mechanical constraints, by polypeptides including cytokines and hormones and by extracellular matrix components such as proteoglycans (PGs) and glycosaminoglycans (GAGs). Several studies revealed that GAGs may influence osteoclastogenesis, but data are very controversial: some studies showed a repressive effect of GAGs on osteoclastic differentiation whereas others described a stimulatory effect. The controversy also affects osteoblasts which appear sometimes inhibited by polysaccharides and sometimes stimulated by these compounds. Furthermore, long-term treatment with heparin leads to the development of osteoporosis fueling the controversy. After a brief description of the principal osteoclastogenesis assays, the present chapter summarizes the main data published on the effect of PGs/GAGs on bone cells and their functional incidence on osteolysis.

## 1. Introduction

Bone metabolism is tightly regulated by a balance between two bone cell types combining catabolic and anabolic activities. Bone catabolism is supported by multinucleated cells specialized in bone resorption and named osteoclasts. Osteoclasts originate from the monocyte lineage and differentiate by the action of membranous, soluble and extracellular matrix compounds (1). Among these factors, some are required for proliferation of osteoclast mononuclear progenitors such as macrophage-colony stimulating factor (M-CSF) while other factors such as receptor activator of nuclear factor-kB ligand (RANKL) are more specifically implicated in the commitment of these precursors to their fusion and in the formation of multinucleated resorbing osteoclasts (2-4). Bone catabolism depends on the ability of the osteoclast to generate an acidic extracellular compartment between itself and the bone surface which is essential for solubilization of the alkaline salts of bone mineral (5). This acidic pH is also necessary for the digestion of the organic bone matrix by lysosomal enzymes secreted by osteoclasts (6). According to their ability to solubilize hydroxyapatite crystals and to digest organic matrix, osteoclasts contribute to the orchestration of the phosphocalcic homeostasis together with the second main bone type cells, the osteoblasts. Osteoblasts originate from mesenchymal stem cells and perform anabolic functions consisting in the formation of extracellular matrix composed by 95% type I collagen (7). Osteoblast activities are not limited to the formation of bone extracellular matrix but also extend to the osteolytic process. While osteoblasts produce and secrete gelatinase activities (8) controlling partly the collagenic matrix, they release more particularly cytokines and growth factors regulating osteoclast differentiation and activation (2, 3, 9). Among these polypeptides, RANKL/OPG (osteoprotegerin) is the main molecular couple involved in the communication between osteoblasts and osteoclasts (2, 3). In this system, RANKL expressed by osteoblasts and also by stromal cells binds to its receptor RANK expressed on the surface of osteoclast precursors

and consequently activates TRAF6/NFκB signaling pathway leading to the fusion of osteoclast activity and survival (10, 11). RANK/RANKL interactions are controlled by OPG which is also produced by osteoblasts/stromal cells. OPG acts as a soluble decoy receptor blocking the binding of RANKL to RANK and subsequently the osteoclastogenesis and the osteolytic process (12, 13).

Extracellular matrix components, especially proteoglycans (PGs) and glycosaminoglycans (GAGs) contribute to the bone remodelling and to the maintenance of bone mass (14). Thus, PGs and GAGs are involved in the organization of collagen fibers (14). However, the role of GAGs and PGs in bone metabolism is more complex than initially envisaged and this complexity is mainly related to the structure and the localization of these compounds. PGs exhibit numerous locations and more precisely are associated with intracellular compartments, expressed on the cell surface or anchored in the extracellular matrix and basement membrane in almost all tissues in adults (15). The composition of GAGs is very heterogenous and includes linear polymers which are bound to a core protein to form PGs. There is no unifying feature for core protein structures and then PGs display a great diversity of protein forms. Many core proteins have complex modular structures with protein motifs which have similar sequence to those found in other protein families. GAGs are composed of repeated disaccharidic units of hexosamine and hexuronic acid, except for keratan sulfate in which hexuronic acid is replaced by galactose. According to the epimerization and sulfation of hexosamine and uronic acid, several families of GAGs have been described. All together, this diversity of composition explains in part their very complex biological activities in all tissues and that GAGs/PGs functions are not limited to the control of fibrillogenesis.

The aim of the present review is to better define the function of GAGs and PGs in bone remodeling and more specifically in osteolysis. The first part of the manuscript will

describe the main osteoclastogenesis assays currently used. The review will then focus on the role of PGs in the control of physiological and pathological osteolysis regarding the osteoblastic and osteoclastic components.

## 2. *In vitro* assays of osteoclastogenesis

Numerous cell culture systems derived from different species have been established to study the molecular and cellular mechanisms of osteoclastogenesis (3). Recently, osteoclasts have been generated from a single-cell suspension of embryonic stem (ES) cells seeded on a feeder monolayer. Bone-resorbing cells expressing osteoclastic markers such as TRAP (Tartrate-Resistant Acid Phosphatase) or RANK were obtained within 11 days (16). However, the main systems used to study the mechanisms of osteoclastogenesis are based on culture of osteoclast progenitors isolated from monocytic cells (peripheral blood monocyte fraction / umbilical cord blood monocytes / spleen cells / monocytic cell lines) in the presence or absence of stromal cells (osteoblastic cells) but after addition of a cytokine cocktail including M-CSF and RANKL. The following paragraphs will describe the most effective methods to study osteoclastogenesis *in vitro*.

### 2.1. Differentiation assay from the murine RAW 264.7 monocytic cell line

#### *Materials and reagents*

- Murine RAW 264.7 monocytic cells (ATCC, Promochem, France) (17)
- Phenol red-free α-Minimal Essential Medium (α-MEM) (Invitrogen, France)
- Fetal calf serum (FCS) (Perbio, Logan, USA), batch specifically selected for osteoclast differentiation
- Non essential amino acids (Invitrogen)

- Solution of trypsin (0.25%) and ethylenediamine tetraacetic (EDTA) (1mM) (Invitrogen)
- Human or murine RANKL (hRANKL or mRANKL, R&D System, UK) is dissolved in phosphate buffer/0.1%BSA at 1 mg/mL and stored in single use aliquots at -80°C until use. Final concentration used is 100 µg/mL (dilution in α-MEM supplemented with 10% FCS)
- Leukocyte (TRAP) staining kit n°387A (Sigma, France)

***Cell culture***

RAW 264.7 cells were routinely cultured in α-MEM supplemented with 10% FCS and 1% non essential amino acids. Fresh medium is replaced twice a week and cell culture amplifications are performed after cell detachment by scraping. RAW 264.7 are frozen in DMSO solution diluted at 20% in FCS and frozen at 5 x $10^6$ cells/mL in liquid nitrogen until use.

***Osteoclast differentiation***

To induce osteoclast formation, RAW 264.7 cells are scraped then incubated at 37°C for 2 minutes to allow adherence of the most differentiated cells. Non adherent cells are then collected and seeded in fresh medium, at a density of 3 x $10^3$ cells/well in a 96-well plate. After 2 hours, medium is changed for a fresh one containing 100 ng/mL hRANKL. After 5 days of culture, multinucleated cells (>3 nuclei) are counted under a light microscope (contrast phase) (Figure 1A) or after TRAP staining according the recommendation of the manufacturer (17, 18). Osteoclasts can be observed from 30 ng/mL of RANKL.

## 2.2 Differentiation from murine CD11b$^+$ monocytes

### *Materials and reagents*

- 4 week-old C57BL6 male mice (Elevage Janvier, France)
- CD11b microbeads and MACS technology (Miltenyi Biotec, Germany)
- Phenol red-free α-Minimal Essential Medium (α-MEM) (Invitrogen, France)
- Fetal calf serum (FCS) (Perbio, Logan, USA), batch specifically selected for osteoclast differentiation
- Murine M-CSF (mM-CSF, R&D System, UK) is dissolved in phosphate buffer /0.1%BSA at 25 µg/mL and stored in single use aliquots at -80°C until use. Final concentration used is 25 ng/mL (dilution in α-MEM supplemented with 10% FCS)
- Human or murine RANKL (R&D System, UK) is dissolved in phosphate buffer /0.1%BSA at 100 µg/mL and stored in single use aliquots at -80°C until use. Final concentration used is 100 ng/mL (dilution in α-MEM supplemented with 10% FCS)
- Leukocyte (TRAP) staining kit n°387A (Sigma, France)

### *Cell preparation and osteoclast differentiation*

CD11b$^+$ monocytes are purified from murine bone marrow cells, obtained by flushing the bone marrow from femora and tibiae of 4 week-old C57BL6 male mice. Mice are anesthetized using isoflurane and euthanized by cervical dislocation. CD11b$^+$ cells are magnetically labelled with CD11b Microbeads and positively selected by MACS technology. CD11b$^+$ cells are seeded in 24-well plates (500 x $10^3$ cells / well) in phenol red-free α-MEM, containing 10% FCS and 25 ng/mL mM-CSF. This step is absolutely necessary to improve adhesion of osteoclast precursor to the plastic and to stimulate their proliferation. After 3 days

of culture, medium is replaced by fresh medium containing 10% FCS, 25 ng/mL mM-CSF, with 100 ng/mL hRANKL. Thereafter, complete medium (with cytokines) are changed every 4 days. The formation of osteoclasts occurred between 15 to 21 days of culture and was detected by TRAP staining (Figure 1B) (21, 22). Fluorescent osteoclasts can be obtained using similar technique with $CD11b^+$ isolated from GFP-mice (Figure 1C).

### 2.3 Differentiation assay from human $CD14^+$ cells

***Materials and reagents***

- Human peripheral blood from healthy volunteer donors and collected on EDTA or citrate buffer
- CD14 microbeads and MACS technology (Miltenyi Biotec, Germany)
- α-Minimal Essential Medium (α-MEM) (Invitrogen, France)
- Fetal calf serum (FCS) (Perbio, Logan, USA), batch specifically selected for osteoclast differentiation
- Human M-CSF (R&D System, UK) is dissolved in phosphate buffer /0.1%BSA at 25 µg/mL and stored in single use aliquots at -80°C until use. Final concentration used is 25 ng/mL (dilution in α-MEM supplemented with 10% FCS)
- Human or mouse RANKL (R&D System, UK) is dissolved in phosphate buffer /0.1%BSA at 100 µg/mL and stored in single use aliquots at -80°C until use. Final concentration used is 100 ng/mL (dilution in α-MEM supplemented with 10% FCS)
- Ficoll® solution, d = 1,077 (Sigma, France)
- Leukocyte (TRAP) staining kit n°387A (Sigma, France)

***Selection of CD14$^+$ cells***

Blood samples are first diluted with phosphate buffer at 50% and diluted samples are layered onto Ficoll solution in a centrifuge tube. Human peripheral blood mononuclear cells (PBMCs) were then isolated by centrifugation over Ficoll gradient for 25 minutes at 500 g. Whether osteoclasts can be differentiated directly from PBMC or from purified monocytes obtained after 45 min adhesion followed by a differentiation step in the presence of M-CSF and RANKL, enrichment and purification of osteoclast precursors ($CD14^+$) allow the differentiation of high number of osteoclasts. $CD14^+$ cells are magnetically labeled with CD14 Microbeads and positively selected by MACS technology.

***Osteoclast differentiation***

To induce osteoclast formation, $CD14^+$ cells are seeded at 250 x $10^3$ cells/well in 24-well plates or 45 x $10^3$ cells/well in 96-well plates in α-MEM supplemented with 10% FCS and 25 ng/ml M-CSF. From day 3 of the culture, medium is changed twice a week with fresh medium containing 10% FCS, 25 ng/mL human M-CSF and 100 ng/mL human RANKL. The formation of osteoclasts occurs after around 11 days and is confirmed by TRAP staining (Figure 1D) (21, 22).

The two main factors involoved in osteoclast differentiation process and survival are: i) M-CSF which modulates cell adhesion, differentiation, fusion, resorbing activity and ii) RANKL which is dedicated to the osteoclast fusion, activation and survival. Osteoclastogenesis can be observed from 30 ng/mL and mRANKL can replace human RANKL with 2 fold higher concentration. RANKL and M-CSF then represent the canonical pathway of osteoclastogenesis and they can be substituted by other protagonists (23). It has been shown that several cytokines can be substituted for RANKL to promote osteoclastogenesis *in vitro* (TNF-α, IL-11, IL-8) (23). However, osteoclast differentiation is

absolutely dependent on RANKL *in vivo* as confirmed by RANKL knock-out mice which completely lack TRAP-positive immature and mature multinucleated osteoclasts [24]. In contrast, M-CSF can be replaced *in vitro* and *in vivo* by VEGF, HGF, FLt-3 ligand or IL-34 for instance (23, 25).

***Resorption assay***

The best validation of the osteoclastic phenotype is to assess the ability of differentiated cells to resorb a mineralized matrix *in vitro*. For this, $CD14^+$ cells are cultured on dentine or cortical bone slices (for bovine bone for instance, animal dentine: horse, bovine, etc) in the conditions previously described. At the end of the culture period, osteoclasts are removed by bleach; dentin/bone slices are fixed with 4% glutaraldehyde in 0.2 M sodium cacodylate solution for 30 minutes, followed by staining with 1% toluidine blue in 0.5% sodium tetraborate solution for 3 minutes (26). Resorption lacunae are identified by light stereomicroscopy (Zeiss, STEMI 2000-C, Göttingen, Germany) and area of resorbed surfaces are measured using QWin software (Leica, France).

To study the resorption ability of fully mature osteoclasts, technique established by Fuller *et al* (27) can be used. Briefly, after formation of osteoclasts as described above, the medium are removed and the cell layer is washed three times with PBS without calcium and magnesium. Six hundred microliters of 0.02% EDTA are added per well (6-well plate) and cells are incubated for 20 min at room temperature. EDTA is then removed from the well and replaced by 600 µl of calcium/magnesium-free PBS. A cell scraper is used to harvest the cells in PBS, and the resulting cell suspension is mixed using a pipette to ensure uniform cell dispersal. Two hundred and fifty microliters of this cell suspension is then added to each well (24-well plate) on a dentin slice in 250 µl αMEM, 10% FCS. Cells are allowed to sediment for 20 min at 37°C before dentin/bone slices are washed. Cells are incubated in 500 µl

αMEM, 10% FCS in the presence or the absence of tested compounds/drugs. After incubation, resorption surfaces are assessed as described above (Figure 1E) (28). Resorption lacunae and resorbed surface area can be also revealed and measured by scanning electron microscopy (29, 30).

In all models described, the main markers used to determine the presence of osteoclasts are TRAP, calcitonin receptor, vitronectin receptor, cathepsin K, and the capacity for resorbing mineralized matrix. In all models, RANKL-induced osteoclastogenesis is specifically inhibited by addition of recombinant OPG or RANK-Fc (4).

## 3. Functional activities of PGs and GAGs on osteoclasts

Numerous growth factors/cytokines/receptors carry a heparin binding domain and consequently can bind to isolated GAGs or GAGs from PGs. Thus, GAGs have many biological activities by holding various extracellular molecules which play key roles in bone metabolism and in bone remodelling. Indirect evidence of the role of GAGs in bone remodelling has been published by Kram *et al* (31). These authors have shown that heparanase, a heparin sulfate-degrading endoglycosidase, is weakly expressed throughout the bone marrow with a substantial increase in osteoblasts and osteocytes and in contrast heparanase is absent from osteoclasts. Interestingly, heparanase transgenic mice exhibit a marked increase of trabecular bone mass, cortical thickness, and bone formation rate, but no difference in osteoclast number. Their data suggest that proteoglycans in bone reduce osteoblast function and heparanase limits this reduction by degrading heparan sulfate (31).

The effect of GAGs on osteoclastogenesis *in vitro* is controversial. For example, Ariyoshi *et al* (32) and Shinmyouzu *et al* (33) showed an inhibition of osteoclastogenesis after a direct interaction of GAGs with RANKL. In contrast, Irie *et al* (34) showed a stimulation of

osteoclastic bone resorption by inhibiting OPG activity. However, more recently, using unfractionated osteoblast-derived GAGs that reflect the complex tissue microenvironment within which osteoclasts reside, Ling *et al* (35) demonstrate that GAGs block the osteoclastogenic activity of RANKL. Similarly, Baud'huin *et al* (28) demonstrated using three various models of osteoclastogenesis (RAW264.7, murine $CD11b^+$ cells and human $CD14^+$ cells) that GAGs downregulate RANKL-induced osteoclastogenesis. The mechanism by which GAGs control osteoclastogenesis remains unclear. Baud'huin *et al* (28) gave some arguments indicating that GAGs inhibit consecutively osteoclast precursor-adhesion and the fusion of these precursors. Size and sulfation of GAGs are key parameters for the inhibition of RANKL-induced osteoclastogenesis (28) but GAGs (heparin, chondrotin sulfate, dermatan sulfate, heparin sulfate or oligosaccharides) do not bind to RANKL as studied by surface plasmon resonance experiments (28, 36, 37). Shinmyouzu *et al* (33) published that dermatan sulfate inhibits osteoclast formation by binding to RANKL. However, these authors used non-relevant physiological concentrations of dermatan sulfate (300 µg/ml) and non-purified osteoclast precursors to study osteoclastogenesis. To continue in the controversial data, Ariyoshi *et al* (32) observed that hyaluronic acid increases osteoclastogenesis through activation of CD44 signaling pathway whereas Chang *et al* (38) demonstrated opposite activities and showed an activation of TLR4 signaling pathway without any involvement of CD44. Finally, using fully differentiated osteoclasts derived from human peripheral blood monocytes, Pivetta *et al* (39) revealed that hyaluronan inhibits their migration on collagen as well as their ability to resorb bone matrix. These effects are mainly due to a decrease of TRAP, MMP-9 and cathepsin K activities and to the increased levels of TIMP-1. The role of CD44 was confirmed by using blocking anti-CD44 antibodies which fully abrogated hyaluronan effects. Hyaluronan then hampers osteoclast migration through its activity on CD44 (40). Overall, the data published show that GAGs inhibit osteoclastogenesis and their

resorption activity by inhibiting the adhesion and fusion of osteoclast precursors. These activities appear independent of RANKL signaling pathway but may involve CD44 and TLR4 depending on the GAGs used.

In contrast to RANKL, OPG has a heparin binding domain. OPG belongs to the family of the TNF receptor family and contains three structural domains specifically influencing its biological activities. The first one is a cysteine-rich domain in the N-terminal position which is essential for the inhibition of osteoclastogenesis as well as for the dimerization of OPG *via* the Cys400. The second domain corresponds to two death domain homologous regions. The third domain is a heparin-binding domain which is able to interact with numerous proteoglycans (41). Full length OPG binds to GAGs with a high affinity (Kd: 0.28 nM for heparin) in contrast to OPG-Fc in which the heparin-binding domain is lacking (36, 37). Therefore, the first role of the OPG heparin-binding domain has been revealed by Standal *et al* (42) who demonstrated that myeloma cells internalize and degrade OPG through its binding to syndecan-1 and consequently induce osteolysis in patients. Thus, PGs control the bioavailability of OPG one of the main inhibitor of osteoclastogenesis and bone resorption. PGs are involved in OPG-induced chemotaxis of monocytes (43). Indeed, OPG can interact with syndecan-1 expressed by monocytes (potential osteoclast precursors) and can stimulate the cell migration. In this context, OPG is a chemotactic factor for monocytes which can be recruited in inflammatory context or during osteolysis process. In light of these studies, PGs and GAGs exert a very complex pattern of activities which are not arguable if the biological context is taken into account (inhibition of osteoclastogenesis *in vitro*, bioavailability of OPG and monocyte chemotaxis in favour of pro-osteolytic activity).

## 4. Functional activities of PGs and GAGs on osteoblasts

Bone remodelling is a balance between osteoblast and osteoclast activation and the functional activity of the first is influenced by the other one. In this context, similarly to their activities on osteoclasts, PGs and GAGs strongly modulate osteoblast metabolism (14, 44). In bone microenvironment, membrane or soluble forms of RANKL are mainly expressed by stromal cells and osteoblasts which control osteoclastogenesis by this pathway (2, 4). OPG regulates the half-life of membrane RANKL and GAGs inhibit the OPG-induced shortened half-life of RANKL (36). In this specific context, GAGs may increase the half-life of RANKL by inhibiting OPG activity and thus act as a pro-osteoclastic factor. Furthermore, RANKL significantly reduces ERK activity, a putative suppressor of osteoclastogenesis but unfractionated osteoblast-derived GAGs abolish the inhibitory effects of RANKL on ERK activity (35) underlining the fact that osteoblast microenvironment is a potent source of GAGs that promote bone anabolic activities. Although the exact mechanism by which GAGs regulate RANKL activity remains unclear, Cao *et al* (45) showed that hyaluronan increases RANKL expression in bone marrow stromal cells through CD44 which in turn could stimulate osteoclast activity.

GAGs can be considered as polysaccharides containing protein-binding domains that coordinate mesenchymal stem cell commitment and growth, and ultimately, osteoblast phenotype (44). Among the heparan sulfate-binding factors known to be essential in to this process, FGFs, their receptors and members of the TGF superfamily are the most important molecule families. Fibroblast growth factor-2 (FGF2) is a crucial growth factor family driving the proliferation of osteoblasts as many other cell types. Robinson *et al* demonstrated that GAGs, heparin and heparan sulfate are essential for the activity of the fibroblast growth factor (FGF) family (46). GAGs promote FGF oligomerization that, in turn, triggers FGFRs dimerization and signal transduction (47). Like OPG, heparan sulfate PGs (HSPGs) mediate cell internalization of FGF and possibly its nuclear delivery (48). When FGF binds to free

heparin/HSPGs, FGF is sequestered in the extracellular environment. Similar observations have been made for BPM2 and the depletion of cell surface HSPGs by enzymatic treatment enhances BMP2 bioavailability and osteogenic activity (49). FGFs also bind to transmembrane HSPGs and then enhance osteoblast proliferation and mineralization, effect partly abolished by an anti-syndecan 4 antiboby (49). Another example is given by the paper of Haupt *et al* (50). These authors demonstrated that MC3T3-E1 cells under osteogenic conditions decrease their chondroitin and dermatan sulfate PGs (biglycan, decorin, and versican) but increase glypican-3. This shift in expressed HSPGs is concomitant to the switch of FGR1 to FGR3 expression related to the commitment to osteoblast differentiation (51). Similarly to FGF, TGFs stored into the bone matrix could be released during bone resorption and modulate in turn osteoblast and ostoclast metabolism (52). Bi *et al* (53) revealed that the absence of the critical TGFβ-binding proteoglycans, biglycan and decorin, prevents TGFβ from proper sequestration within the extracellular matrix. Thus proteoglycans appear essential for maintaining an appropriate number of osteoblasts and osteoclasts by modulating their proliferation and/or differentiation. More recently, Bi *et al* revealed that biglycan deficiency upmodulates osteoclast differentiation and activation due to defective osteoblasts but independently of RANKL and OPG production (53). The effects of GAGs on osteoblast lineage are dependent on their sulfation. Indeed, sulfation strongly enhances the biological activity of BMPs (TGF member family) by continuously binding the ligands to their receptors and by enhancing osteoblast differentiation (54). In agreement with these data, desulfation of GAGs expressed by MG63 cells delayed *in vitro* mineralization process (55). Overall, these data point out the key role of GAGs in bone formation and their ability to modulate osteoblast differentiation by indirect mechanism and more specifically by controlling bone cytokines/growth factors. It is also important to keep in mind, that osteolysis is the result of

both osteoblast and osteoclast activity and even if osteoblasts are bone cells specialized in bone formation they contributes in part to the degradation of osseous organic matrix.

## 5. PGs/GAGs and bone remodelling: a complex dysregulation of the anabolic/catabolic balance

It is well known that long-term administration of heparin was shown to lead to the development of osteoporosis (56-58). Thus, 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. Barbour *et al* (59) also showed that 36% of pregnant women undergoing long-term heparin treatment had a 10% reduction in femoral bone mineral density. However, the mechanism sustaining this osteoporosis was unclear and it was difficult to determine if the effects on bone resorption were due to the direct effect of heparin on osteoclasts or indirectly via its osteoblast activity. Furthermore, these controversial findings on GAG effects on osteoclastogenesis are intensified by the study of Folwarczna *et al* (60) who showed that in a rat model, low concentrations of heparin increased the formation of osteoclasts, whereas it decreased with the highest concentrations. In mouse bone marrow cell cultures, heparin suppressed the formation of osteoclasts, with the exception of low concentrations of standard heparin which intensified this process (60). In fact, heparin activity on bone remodelling probably results from a more complex mechanisms altering simultaneously osteoclast and osteoblast metabolisms. Heparin may increase the resorption process through the release of pro-resorptive factor by osteoblast/stromal cells (62) explaining in part the discrepancy between the *in vivo* and *in vitro* results and heparin may also exert an inhibitory activity on bone formation by decreasing osteoblast number and by inhibiting the mineralization process (56-58, 61).

## 6. Conclusions

GAGs and PGs exert a broad panel of action targeting simultaneously osteoblasts and osteoclasts. Unfortunately long-term administration of heparin leads to the development of osteoporosis. In this context, although the mechanisms of action of low-molecular-weight heparins are not yet totally elucidated their use is preferred to unfractionated heparin (61). More specifically, the effect of low-molecular-weight heparins on osteoblasts and on osteoblast–osteoclast communications needs to be investigated and complementary studies to determine whether the effects of heparin on bone are reversible are needed.

**References**

1. Bruzzaniti A and Baron R (2006) Molecular regulation of osteoclast activity. Rev Endocr Metab Disord 7: 123-139.

2. Theoleyre S, Wittrant Y, Kwan Tat S, Fortun Y, Redini F and Heymann D (2004) The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 15: 457-475

3. Heymann D, Guicheux J, Gouin F, Passuti N and Daculsi G (1998) Cytokines, growth factors and osteoclasts. Cytokine 10: 155-168.

4. Baud'huin M, Lamoureux F, Duplomb L, Rédini F and Heymann D (2007) RANKL, RANK, osteoprotegerin: key partners of osteoimmunology and vascular diseases. Cell Mol Life Sci 64: 2334-2350

5. Rousselle AV and Heymann D (2002) Osteoclastic acidification pathways during bone resorption. Bone 30: 533-540.

6. Georges S, Ruiz Velasco C, Trichet V, Fortun Y, Heymann D and Padrines M (2009) Proteases and bone remodelling. Cytokine Growth Factor Rev 20: 29-41.

7. Marie PJ and Fromigué O. Osteogenic differentiation of human marrow-derived mesenchymal stem cells. Regen Med 1: 539-548.

8. Damiens C, Fortun Y, Charrier C, Heymann D and Padrines M (2000) Modulation by soluble factors of gelatinase activities released by osteoblastic cells. Cytokine 12: 1727-1731.

9. Lamoureux F, Baud'huin M, Duplomb L, Heymann D and Rédini F (2007) Proteoglycans: key partners in bone cell biology. Bioessays 29: 758-771.

10. Boyle WJ, Simonet WS and Lacey DL (2003) Osteoclast differentiation and activation. Nature 423: 337-342.

11. Wittrant Y, Theoleyre S, Couillaud S, Dunstan C, Heymann D and Redini F (2004) Relevance of an in vitro osteoclastogenesis system to study receptor activator of NF-kB ligand and osteoprotegerin biological activities. Exp Cell Res 293*:* 292-301.

12. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R et al (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89, 309-319.

13. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N et al. (2004) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 139, 1329-1337.

14. Lamoureux F, Baud'huin M, Duplomb L, Heymann D and Rédini F (2007) Proteoglycans: key partners in bone cell biology. Bioessays 29: 758-771.

15. Corsi A, Xu T, Chen XD, Boyde A, Liang J, Mankani M et al (2002) Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. J Bone Miner Res 17: 1180-1189.

16. Duplomb L, Dagouassat M, Jourdon P and Heymann D. (2007) Concise review: embryonic stem cells: a new tool to study osteoblast and osteoclast differentiation. Stem Cells 25: 544-552.

17. Raschke WC, Baird S, Ralph P and Nakoinz I (1978) Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15: 261-267.

18. Theoleyre S, Wittrant Y, Couillaud S, Vusio P, Berreur M, Dunstan C et al (2004) Cellular activity and signaling induced by osteoprotegerin in osteoclasts: involvement of receptor activator of nuclear factor kappaB ligand and MAPK. Biochim Biophys Acta 1644: 1-7.

19. Blin-Wakkach C, Breuil V, Quincey D, Bagnis C and Carle GF (2006) Establishment and characterization of new osteoclast progenitor cell lines derived from osteopetrotic and wild type mice. Bone 39: 53-60.

20. Lézot F, Thomas BL, Blin-Wakkach C, Castaneda B, Bolanos A, Hotton D, Sharpe PT, Heymann D, Carles GF, Grigoriadis AE, Berdal A (2010) Dlx homeobox gene family expression in osteoclasts. J Cell Physiol 223: 779-787.

21. Baud'huin M, Duplomb L, Téletchéa S, Charrier C, Maillasson M, Fouassier M and Heymann D (2009) Factor VIII-von Willebrand factor complex inhibits osteoclastogenesis and controls cell survival. J Biol Chem 284: 31704-31713

22. Duplomb L, Baud'huin M, Charrier C, Berreur M, Trichet V, Blanchard F and Heymann D (2008) Interleukin-6 inhibits receptor activator of nuclear factor kappaB ligand-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of Serine727 phosphorylation of signal transducer and activator of transcription 3. Endocrinology 149: 3688-3697

23. Knowles HJ and Athanasou NA (2009) Canonical and non-canonical pathways of osteoclast formation. Histol Histopathol **24**:337-346.

24. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C et al. (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397:315-323.

25. Baud'Huin M, Renault R, Charrier C, Riet A, Moreau A, Brion R, Gouin F, Duplomb L, Heymann D (2010) Interleukin-34 is expressed by giant cell tumours of bone and plays a key role in RANKL-induced osteoclastogenesis. J Pathol 221: 77–86.

26. Chu K, Snyder R, Econs MJ and Facp MD (2006) Disease status in autosomal dominant osteopetrosis type 2 is determined by osteoclastic properties. J Bone Miner Res 21: 1089–1097.

27. Fuller K, Kirstein B and Chambers T.J (2006) Murine osteoclast formation and function: differential regulation by humoral agents. Endocrinology 147, 1979–1985.

28. Baud'huin M, Ruiz-Velasco C, Jego G, Charrier C, Gasiunas N, Gallagher J et al (2011) Glycosaminoglycans inhibit the adherence and the spreading of osteoclasts and their precursors: Role in osteoclastogenesis and bone resorption. Eur J Cell Biol 60: 49-57

29. Gouin F, Couillaud S, Cottrel M, Godard A, Passuti N and Heymann D (1999) Presence of leukaemia inhibitory factor (LIF) and LIF-receptor chain (gp190) in osteoclast-like cells cultured from human giant cell tumour of bone. Ultrastructural distribution. Cytokine 11: 282-289.

30. Guicheux J, Heymann D, Rousselle AV, Gouin F, Pilet P, Yamada S et al (1998) Growth hormone stimulatory effects on osteoclastic resorption are partly mediated by insulin-like growth factor I: an in vitro study. Bone 22: 25-31.

31. Kram V, Zcharia E, Yacoby-Zeevi O, Metzger S, Chajek-Shaul T, Gabet Y et al (2006) Heparanase is expressed in osteoblastic cells and stimulates bone formation and bone mass J Cell Physiol 207: 784-92.

32. Ariyoshi W, Takahashi T, Kanno T, Ichimiya H, Shinmyouzu K, Takano H et al (2008) Heparin inhibits osteoclastic differentiation and function. J Cell Biochem 103:1707-1717.

33. Shinmyouzu K, Takahashi T, Ariyoshi W, Ichimiya H, Kanzaki S and Nishihara T (2007) Dermatan sulfate inhibits osteoclast formation by binding to receptor activator of NF-kappa B ligand. Biochem Biophys Res Commun 354: 447-452.

34. Irie A, Takami M, Kubo H, Sekino-Suzuki N, Kasahara K and Sanai Y (2007) Heparin enhances osteoclastic bone resorption by inhibiting osteoprotegerin activity. Bone 41: 165-174.

35. Ling L, Murali S, Stein GS, van Wijnen AJ and Cool SM (2010) Glycosaminoglycans modulate RANKL-induced osteoclastogenesis. J Cell Biochem 109: 1222-1231

36. Théoleyre S, Kwan Tat S, Vusio P, Blanchard F, Gallagher J, Ricard-Blum S et al (2006) Characterization of osteoprotegerin binding to glycosaminoglycans by surface plasmon resonance: role in the interactions with receptor activator of nuclear factor kappaB ligand (RANKL) and RANK. Biochem Biophys Res Commun 347: 460-467.

37. Lamoureux F, Picarda G, Garrigue-Antar L, Baud'huin M, Trichet V, Vidal A et al (2009) Glycosaminoglycans as potential regulators of osteoprotegerin therapeutic activity in osteosarcoma. Cancer Res 69: 526-536.

38. Chang EJ, Kim HJ, Ha J, Kim HJ, Ryu J, Park KH et al (2007) Hyaluronan inhibits osteoclast differentiation via Toll-like receptor 4. J Cell Sci 120: 166-176

39. Pivetta E, Scapolan M, Wassermann B, Steffan A, Colombatti A and Spessotto P (2011) Blood-derived human osteoclast resorption activity is impaired by hyaluronan-CD44 engagement via a p38-dependent mechanism. J Cell Physiol, in press

40. Spessotto P, Rossi FM, Degan M, Di Francia R, Perris R, Colombatti A and Gattei V (2002) Hyaluronan-CD44 interaction hampers migration of osteoclast-like cells by down-regulating MMP-9. J Cell Biol 158: 1133-1144.

41. Yamaguchi K, Kinosaki M, Goto M, Kobayashi F, Tsuda E, Morinaga T et al (1998) Characterization of structural domains of human osteoclastogenesis inhibitory factor. J Biol Chem 273: 5117–5123.

42. Standal T, Seidel C, Hjertner O, Plesner T, Sanderson RD, Waage A et al (2002) Osteoprotegerin is bound, internalized, and degraded by multiple myeloma cells. Blood 100: 3002– 3007.

43. Mosheimer BA, Kaneider NC, Feistritzer C, Djanani AM, Sturn DH, Patsch JR et al (2005) Syndecan-1 is involved in osteoprotegerin-induced chemotaxis in human peripheral blood monocytes, J Clin Endocrinol Metab 90 : 2964–2971.

44. Cool SM and Nurcombe V. The osteoblast-heparan sulfate axis: control of the bone cell lineage (2005) Int J Biochem Cell Biol 37: 1739-1745.

45. Cao JJ, Singleton PA, Majumdar S, Boudignon B, Burghardt A, Kurimoto P et al (2005) Hyaluronan increases RANKL expression in bone marrow stromal cells through CD44. J Bone Miner Res 20: 30-40

46. Robinson CJ, Harmer NJ, Goodger SJ, Blundell TL and Gallagher JT (2005) Cooperative dimerization of fibroblast growth factor 1 (FGF1) upon a single heparin saccharide may drive the formation of 2:2:1 FGF1.FGFR2c.heparin ternary complexes. J Biol Chem 280: 42274-42282.

47. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R and Rusnati M (2005) Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 16: 159-178.

48. Jiao X, Billings PC, O'Connell MP, Kaplan FS, Shore EM and Glaser DL (2007) Heparan sulfate proteoglycans (HSPGs) modulate BMP2 osteogenic bioactivity in C2C12 cells. J Biol Chem 282: 1080-1086.

49. Song SJ, Cool SM and Nurcombe V (2007) Regulated expression of syndecan-4 in rat calvaria osteoblasts induced by fibroblast growth factor-2. J Cell Biochem 100: 402-411.

50. Haupt LM, Murali S, Mun FK, Teplyuk N, Mei LF, Stein GS et al (2009) The heparan sulfate proteoglycan (HSPG) glypican-3 mediates commitment of MC3T3-E1 cells toward osteogenesis. J Cell Physiol 220: 780-791.

51. Fox SW and Lovibond AC (2005) Current insights into the role of transforming growth factor-beta in bone resorption. Mol Cell Endocrinol 243: 19–26.

52. Bi Y, Stuelten CH, Kilts T, Wadhwa S, Iozzo RV, Robey PG, Chen XD et al (2005) Extracellular matrix proteoglycans control the fate of bone marrow stromal cells. J Biol Chem. 280, 30481–30489.

53. Bi Y, Nielsen KL, Kilts TM, Yoon A, Karsdal MA, Wimer HF et al (2006) Biglycan deficiency increases osteoclast differentiation and activity due to defective osteoblasts, Bone 38 (2006) 30481–30489.

54. Miyazaki T, Miyauchi S, Tawada A, Anada T, Matsuzaka S, Suzuki O (2008) Oversulfated chondroitin sulfate-E binds to BMP-4 and enhances osteoblast differentiation. J Cell Physiol 217: 769-777.

55. Kumarasuriyar A, Lee I, Nurcombe V and Cool SM (2009) De-sulfation of MG-63 cell glycosaminoglycans delays in vitro osteogenesis, up-regulates cholesterol synthesis and disrupts cell cycle and the actin cytoskeleton. J Cell Physiol 219: 572-583.

56. Muir JM, Andrew M, Hirsh J, Weitz JI, Young E, Deschamps P, Shaughnessy SG (1996) Histomorphometric analysis of the effects of standard heparin on trabecular bone in vivo. Blood 88: 1314-1320.

57. Muir JM, Hirsh J, Weitz JI, Andrew M, Young E, Shaughnessy SG (1997) A histomorphometric comparison of the effects of heparin and low-molecular-weight heparin on cancellous bone in rats. Blood 89: 3236-3242.

58. Ruiz-Velasco C, Baud'huin M, Sinquin C, Maillasson M, Heymann D, Colliec-Jouault S, Padrines M (2011) Effects of a sulphated 'heparin-like' exopolysaccharide produced by *Altermonas infernus* on bone biology. Glycobiology, in press.

59. Barbour LA, Kick SD, Steiner JF, LoVerde ME, Heddleston LN, Lear JL, Baron AE, Barton PL (1994) A prospective study of heparin-induced osteoporosis in pregnancy using bone densitometry. Am J Obstet Gynecol 170: 862-869.

60. Folwarczna J, Sliwiński L, Janiec W and Pikul M (2005) Effects of standard heparin and low-molecular-weight heparins on the formation of murine osteoclasts *in vitro*. Pharmacol Rep 57: 635-645

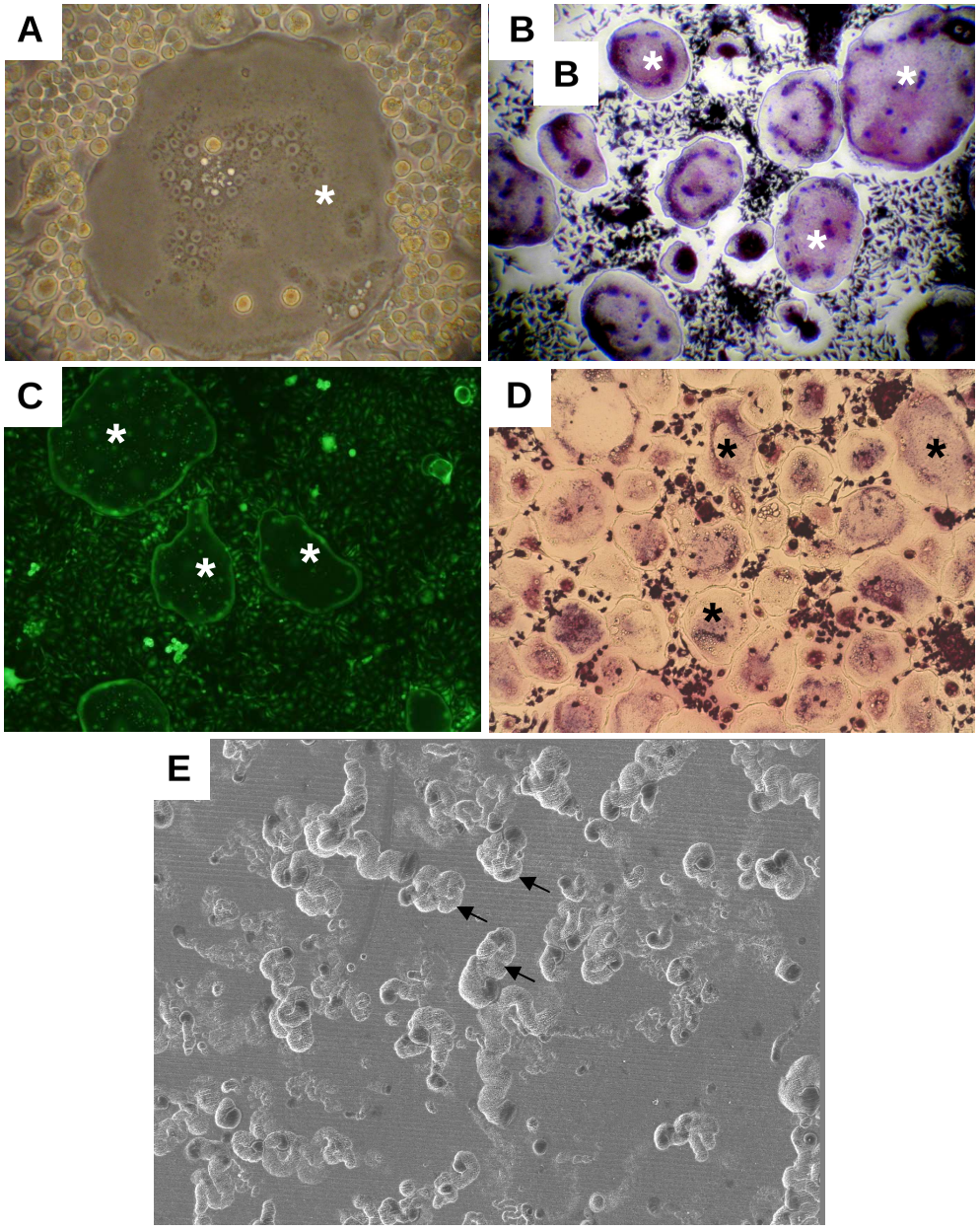
61. Rajgopal R, Bear M, Butcher MK, Shaughnessy SG (2008) The effects of heparin and low molecular weight heparins on bone. Thromb Res 122, 293–298.

62. Nakano K, Okada Y, Saito K and Tanaka Y (2004) Induction of RANKL expression and osteoclast maturation by the binding of fibroblast growth factor 2 to heparan sulfate proteoglycan on rheumatoid synovial fibroblasts. Arthritis Rheum 50: 2450-2458.

**Figure legend**

**Figure 1: Study of osteoclastogenesis and osteoclast function from human and murine models.**

**A)** Osteoclasts formed from murine RAW264.7 cells after 5 days of culture in the presence of human RANKL (light microscopy, phase contrast); **B)** Osteoclasts formed from murine $CD11b^+$ cells cultured in the presence of murine M-CSF and human RANKL for 15 days (after TRAP staining); **C)** Osteoclasts obtained from bone marrow of GFP-mice in the presence of murine M-CSF and human RANKL for 15 days; **D)** Human $CD14^+$ cells differentiated in osteoclasts in the presence of human M-CSF and human RANKL for 11 days (after TRAP staining); **E)** Typical resorption lacunae formed by osteoclasts derived from human $CD14^+$ cells cultured on dentin slice and observed by scanning electron microscopy. *: osteoclast; arrow: resorption lacuna.


A
B
B
C
D
E