Effects of a sulfated exopolysaccharide produced by Alteromonas infernus on bone biology

C Ruiz Velasco2,1, M Baud’huin2,3, C Sinquin4, M Maillasson5, D Heymann2,3,6, S Colliec-Jouault4, and M Padrines1,2,3

1To whom correspondence should be addressed: Tel: +33-240-412-846; Fax: +33-240-412-860; e-mail: marc.padrines@univ-nantes.fr

The growth and differentiation of bone cells is controlled by various factors, which can be modulated by heparan sulfates. Here, we investigated the effects of an oversulfated exopolysaccharide (OS-EPS) on the bone. We compared the effect of this compound with that of a native EPS. Long-term administration of OS-EPS causes cancellous bone loss in mice, in part, to an increase in the number of osteoclasts lining the trabecular bone surface. No significant difference in cancellous bone volume was found between EPS-treated mice and age-matched control mice, underlying the importance of sulfation in trabecular bone loss. However, the mechanism sustaining this osteoporosis was unclear. To clarify OS-EPS activities, we investigated the effect of OS-EPS on osteogenesis. Our results demonstrated that OS-EPS inhibited osteoclastogenesis in two cell models. Using the surface plasmon resonance technique, we revealed that OS-EPS can form a heteromolecular complex OS-EPS/receptor activator of NF-κB ligand (RANKL)/RANK and that RANK had a higher affinity for RANKL pre-incubated with OS-EPS than for RANKL alone, which would be in favor of an increase in bone resorption. However, in vitro, OS-EPS inhibited the early steps of osteoclast precursor adhesion and therefore inhibited the cell fusion step. In addition, we showed that OS-EPS reduced proliferation and accelerated osteoblastic differentiation, leading to strong inhibition of mineralized nodule formation, which would be in favor of an increase in bone resorption. Taken together, these data show different levels of bone resorption regulation by EPSs, most of them leading to proresorptive effects.

Keywords: bone metabolism / bone remodeling / exopolysaccharide / glycosaminoglycan / heparin

Introduction

Bone is a specialized connective tissue that is continually remodeled according to physiological events. This remodeling is the result of the activities of many cell lineages including mainly osteoblasts, osteocytes and osteoclasts. Their cell interactions control their cell activities and bone remodeling intensity. These interactions can be established either through cell–cell contact or by the release of many polypeptide factors and/or their soluble receptor chains (cytokines and growth factors). These factors can act directly on osteogenic cells and their precursors to control differentiation, formation and functions (e.g. matrix formation, mineralization and resorption; Kwan Tat et al. 2004). Of these factors, proteoglycans (PGs) appear critical for maintaining an appropriate number of osteoblasts and osteoclasts by modulating their proliferation and/or differentiation (Lamoureux et al. 2007).

PGs are composed of a core protein with covalently attached glycosaminoglycan (GAG) chains. These GAGs, linear polymers of repeated disaccharidic units, are sulfated, and the number and the position of the sulfates are extremely variable in sulfated GAGs, depending on the tissue, cell and metabolic context, ensuring structural variability in these polysaccharides (Bernfield et al. 1999). These PGs are ubiquitous, being present as cell surface molecules anchored in the plasma membrane, as components of the insoluble extracellular matrix or as soluble molecules present in the extracellular matrix and serum. PGs function in both cell–cell and cell–extracellular matrix adhesion and can also act to promote the assembly of extracellular matrix molecules. Additionally, PGs bind to a wide range of bioactive molecules, such as growth factors and chemokines, which regulate cell behavior in normal and pathological processes. Thus, the PGs or GAGs associated with the cell membrane or resident in the extracellular bone matrix regulate bone growth and remodeling (Ruiz Velasco et al. 2010).

The osteoprotegerin (OPG)/receptor activator of the NF-κB ligand (RANK)/RANK ligand (RANKL) molecular triad has been identified as a member of a ligand-receptor system that directly regulates osteoclast differentiation and osteolysis (Simonet...
et al. 1997). Although RANKL is a powerful inducer of bone resorption through its interaction with RANK (Kong et al. 1999), OPG acts as a decoy receptor for RANKL, thereby strongly inhibiting osteoclast differentiation (Yasuda et al. 1998). Any dysregulation in their respective expression leads to pathological conditions (Wittrant et al. 2004). OPG contains a heparin-binding domain and strongly binds to GAGs with a high affinity ($K_D$: 0.28 nM for heparin; Theoleyre et al. 2006). PGs therefore decrease the bioavailability of OPG, inducing its internalization (Standal et al. 2002; Kwan Tat et al. 2006), and enhance the RANKL half-life at the cell membrane (Kwan Tat et al. 2006). Like OPG, PGs interact with RANKL and abolish osteoclastogenesis (Shinmyouzu et al. 2007). These data demonstrate that PGs must be considered as essential co-factors modulating bone remodeling. PGs are thus not simply passive structural components of cells and extracellular matrices, but instead multifunctional molecules that regulate cell behavior by fine-tuning the function of many regulatory proteins (Lamoureux et al. 2009).

Heparin has several kinds of biological activity, binding to various extracellular molecules. However, the role of heparin in the biological activity of bone remains unclear. Recently, Ariyoshi et al. (2008) showed that heparin suppressed osteoclastogenesis. In contrast, Irie et al. (2007) showed that heparin enhanced osteoclastic bone resorption by inhibiting OPG activity. Long-term heparin treatment causes cancellous bone loss in rats due in part to an increase in the number of osteoclasts lining the trabecular bone surface. However, the results from various other groups suggest that GAGs, and in particular heparan sulfate (HS) and heparin, are potent co-stimulators of osteogenic signaling pathways (Zhao et al. 2006; Jackson et al. 2007; Ling et al. 2010). Thus, the opportunity arises to leverage the stimulatory properties of HS-derived compounds as adjuvants for osteo-inductive therapies. However, the strong anticoagulant property limits applicability in other therapeutic indications. A further disadvantage of heparins is their animal origin and the increasing demand for heparins is in conflict with the limited resources.

A broad range of polysaccharides has emerged as an important class of bioactive products (Franz and Alban 1995; Mayer and Lehmann 2001; Bernas 2003). Polysaccharide-producing marine microorganisms occur widely in nature in different types of habitat (Sutherland 1996), and certain thermophilic and mesophilic polysaccharide-producing strains have been isolated from deep-sea hydrothermal vents (Guezennec 2002; Nichols et al. 2005). Alteromonas infernus, classified as a non-pathogenic microorganism by the Institut Pasteur, secretes a water-soluble acidic heteropolysaccharide (Raguénès et al. 1997). The composition of this high-molecular-weight polysaccharide ($10^6$ g mol$^{-1}$) differs in monosaccharide content and/or ratio and sulfate content (10%) from other polysaccharides isolated from deep-sea hydrothermal bacteria and from polysaccharides of other origins. Structural characterization has shown that this exopolysaccharide (EPS) is a highly branched acidic heteropolysaccharide composed of neutral sugars (glucose and galactose) and uronic acids (glucuronic acid and galacturonic acid; Roger et al. 2004). The high-molecular-weight EPS was chemically depolymerized and sulfated, with a view to obtaining a bioactive compound compatible with therapeutic use (Roger et al. 2004). Highly sulfated low-molecular-weight EPS (40% sulfate groups, MW: 24,000; Figure 1), with uronic acid and sulfate content comparable with that of heparin, was obtained without altering the sugar composition of this polymer (Guezennec et al. 1998). In contrast to the EPS secreted by A. infernus, this oversulfated EPS (OS-EPS) has anticoagulant properties, a heparin-like activity. It is less potent than low-molecular-weight heparin and unfractionated heparin (2.5 and 6.5 times, respectively) and should therefore carry a lower risk of bleeding (Colliec-Jouault et al. 2001). Like heparin, OS-EPS increases the angiogenic properties of FGF-2 (fibroblast growth factor-2) or vascular endothelial growth factor; however, it also inhibits the effect of FGF-2-induced cell migration (Matou et al. 2005). These angiogenic properties of OS-EPS are related to its sulfate content because no effect was observed with the native low-molecular-weight EPS. FGFs also play a major role in controlling cell proliferation,

![Fig. 1. Branched nonasaccharidic repetitive unit of native EPS.](image_url)
differentiation and survival in several tissues, including the bone (Marie 2003). However, no data concerning the structure and biological activity of these EPSs on bone cell proliferation and differentiation were available until now. To explore its ability to promote bone resorption or formation, the influence of OS-EPS on bone cell proliferation and differentiation was determined. OS-EPS was compared with EPS in order to study the effect of sulfate content on the biological properties of the EPS.

Results

**OS-EPS inhibits the proliferation of bone marrow stem cells during osteoblastic differentiation**

To study the effect of OS-EPS on osteoblast precursors, we used the bone marrow stem cell (BMSC) model, in which dexamethasone is known to induce osteoblast differentiation. Proliferation of BMSCs during osteoblastic differentiation was analyzed in the presence of OS-EPS; 25 µg mL$^{-1}$ of OS-EPS significantly inhibited cell proliferation (Figure 2A and B) in contrast to EPS. To determine whether these effects were due to the inhibition of cell proliferation and/or the induction of cell death, we used time-lapse microscopy to monitor the mitosis events. The results demonstrated a 70% inhibition of cell mitosis in the presence of 25 µg mL$^{-1}$ of OS-EPS after 48 h of culture when compared with the control (Figure 2C). To determine whether the OS-EPS induced death in BMSCs by apoptosis, Hoechst staining and caspase-3 activation were investigated. Hoechst staining showed no modification in nuclear morphology in the presence of OS-EPS when compared with control cells (data not shown). Concerning the caspase-3 activity in BMSCs, the results showed that OS-EPS did not induce any activation of caspases (data not shown). Flow cytometry of DNA content was performed to identify cell cycle perturbations following treatment with OS-EPS over a 48 h period in BMSCs. Although 24 h of OS-EPS treatment did not modulate the cell cycle in BMSCs (data not shown), the results showed a significant inhibition of cell proliferation in the presence of OS-EPS.

![Figure 2](link)
shown), 48 h of OS-EPS treatment induced cell cycle arrest in G1 phases (Figure 2D). The number of cells in G1 phases increased from 73 ± 2 to 83 ± 3% when treated with OS-EPS (Figure 2D). This observation was concomitant with a reduction in cells in S and G2/M phases: 25 ± 3 vs. 17 ± 2% (Figure 2D). EPS had no effect in any of the experimental conditions, highlighting the importance of the sulfated polysaccharide on proliferation.

**OS-EPS inhibits mineralized nodule formation in pre-osteoblasts**

To examine the role of OS-EPS in extracellular matrix mineralization in BMSC cultures, BMSCs were treated for 17 days with OS-EPS, after which the mineral was visualized with von Kossa staining. As shown in Figure 3A, OS-EPS dose-dependently inhibited mineralization with maximal inhibition occurring at 50 µg mL⁻¹. In BMSC cultures, OS-EPS reduced the formation of bone nodules. To further understand the mechanism by which OS-EPS inhibits mineralization, the cultures were treated with 25 µg mL⁻¹ of OS-EPS on days 3–17. OS-EPS reduced the formation of bone nodules when added in the first 2 weeks (D3→D14) but not later (D17; Figure 3B). This result shows that OS-EPS is effective during the osteoblast differentiation period (D3→D14), suggesting that OS-EPS blocks culture mineralization mainly by adversely affecting osteoblast differentiation and matrix depositing during the early matrix assembly stage and not by direct binding to hydroxyapatite crystals during the mineralization stage.

Based on reduced mineralization, it was expected that OS-EPS would reduce the expression of osteoblastic markers in cells. This study was performed on BMSCs under conditions where runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and α-1 type I collagen expression (collα1) were observed by preosteoblast stage, followed by bone sialoprotein (BSP) and osteocalcin (OC) by mature osteoblast stage. Unexpectedly, after 17 days of treatment, OS-EPS induced BSP and OC (the mature osteoblast stage) in a dose-dependent manner (Figure 3C). In contrast, OS-EPS reduced ALP, Runx2 and collα1 mRNA levels (preosteoblast stage) in a dose-dependent manner (Figure 3C). This result clearly demonstrated that OS-EPS had an effect on osteoblastic differentiation. OS-EPS acted in the early stages of the process by affecting the preosteoblasts and accelerating osteoblastic differentiation.

These results suggest that OS-EPS led to significant inhibition of mineralized nodule formation that could be in favor of an increase in bone resorption. As RANKL is considered to be a powerful stimulator of bone resorption produced by mature osteoblasts, we also examined the effect of OS-EPS on RANKL expression by these cells. Figure 3D shows that OS-EPS did not modulate the RANKL expression.

**OS-EPS inhibits RANKL-induced osteoclastogenesis in human models**

CD14⁺ cells are human monocyte cells, which can differentiate into tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells in 21 days with RANKL stimulation. As shown in Figure 4A, in contrast to EPS, adding 0.5 µg mL⁻¹ of OS-EPS, at the same time as the RANKL was added, almost completely inhibited RANKL-induced osteoclastogenesis (P < 0.01). We confirmed this effect in a second model for osteoclastogenesis. RAW 264.7 cells are murine monocyte/macrophage cells, which can differentiate into TRAP-positive multinucleated cells in 5 days with RANKL stimulation. OS-EPS completely inhibited RANKL-induced osteoclastogenesis in RAW 264.7 (Figure 4B). To better characterize the mechanisms by which OS-EPS inhibits osteoclast formation, the effect of OS-EPS was assessed at different times in the culture period. When OS-EPS was added to the culture 3 days before RANKL (D3), no osteoclasts were generated after 17 days (Figure 4A) and the number of adherent CD14⁺ osteoclast precursors was lower than in the control condition. To determine whether these effects were due to induced loss of adhesion, the effects of OS-EPS were evaluated by counting viable cells, as assessed by Trypan blue exclusion. After the adherence of CD14⁺ cells in the wells, these cells were incubated with EPS or OS-EPS for 5 days. Trypan blue counting revealed that OS-EPS caused a loss of adherence in this type of cell (Figure 4C). In this culture condition, fewer osteoclast precursors thus adhered to the plastic surface and less osteoclasts were generated in the presence of RANKL. The addition of OS-EPS after 1 week of the culture stage (D7) did not completely inhibit osteoclast formation (Figure 4A). OS-EPS acted at two distinct levels of osteoclastogenesis: (i) in the early stages of the process by affecting and decreasing cell adherence and (ii) at the end of the osteoclastogenesis process by inhibiting the spreading of the preformed osteoclasts. This result clearly demonstrated that OS-EPS had an effect on RANKL-induced osteoclastogenesis. OS-EPS acted in the early stages of the process by affecting and decreasing cell adherence.

**OS-EPS binds to RANKL**

OPG/RANK and RANKL have been identified as members of a ligand-receptor system that directly regulates osteoclast differentiation and osteolysis. Although RANKL is a powerful inducer of bone resorption through its interaction with RANK, OPG is a soluble decoy receptor and acts as a strong inhibitor of osteoclastic differentiation.

Surface plasmon resonance (SPR)-binding assays showed that recombinant full-length OPG binds immobilized heparin (Theoleyre et al. 2006). To determine the involvement of OS-EPS in the binding of OPG, we determined the kinetic parameters by injecting different concentrations of OS-EPS over immobilized OPG and thus demonstrated the high-affinity OPG binding to OS-EPS (dissociation constant K_D: 0.22 nM; Figure 5). To explore the molecular mechanism underlying the effect of OS-EPS on RANKL-induced osteoclastogenesis, we investigated the molecular interactions between RANKL and OS-EPS by SPR. Surprisingly, OS-EPS was also able to bind to immobilized-RANKL, whereas EPS was not (Figure 5A). We next compared OS-EPS activity with that of heparin, HS, chondroitin sulfate and dermatan sulfate. None of the GAGs analyzed were able to bind to RANKL, with the exception of OS-EPS (Figure 5A). Furthermore, using a single cycle kinetic assay, the K_D of OS-EPS for RANKL was 91 nM (Figure 5).
Fig. 3. OS-EPS inhibits the extracellular matrix mineralization from BMSCs in a dose-dependent manner. (A) BMSCs were incubated in OM for 3 weeks in the presence or the absence of increased concentrations of EPS and OS-EPS. (B) EPS or OS-EPS at 25 µg mL\(^{-1}\) were added at different times during the culture period as indicated in the presence of 50 µg mL\(^{-1}\) of ascorbic acid and 10\(^{-8}\) M dexamethasone. Controls were done in the absence of EPS and in the presence (CT+) or the absence (CT-) of 50 µg mL\(^{-1}\) of ascorbic acid (Sigma) and 10\(^{-8}\) M dexamethasone. At the end of the culture period, Alizarin red staining was used (original magnification ×10). (C) Gene expression profiles of the bone-specific markers for osteoblastic differentiation were determined by qPCR in the absence (CT) or the presence of different OS-EPS concentrations. The data show fold differences in expression following normalization against Cyc1. (D) The RANKL expression profile was determined by qPCR in the absence (CT) or the presence of different OS-EPS concentrations after 17 days. The data show fold differences in expression following normalization against Cyc1.
As RANKL is the natural ligand of RANK, we wondered whether OS-EPS could inhibit the RANKL/RANK complex present at the osteoclast membrane. We investigated the molecular interactions between OS-EPS, RANKL and RANK using the SPR technique. We confirmed that RANK or RANKL bound to immobilized RANKL or immobilized RANK, respectively (Figure 5B and C). Although, in our conditions, OS-EPS did not bind to immobilized RANK (Figure 5C), it bound to the preformed RANKL/RANK complex (Figure 5B) thereby forming a ternary complex OS-EPS/RANKL/RANK. In addition, the pre-incubation of OS-EPS (30 min at room temperature) with RANK or RANKL did not inhibit the capacity of OS-EPS to further bind to the preformed ternary complex OS-EPS/RANKL/

Fig. 4. OS-EPS inhibits RANKL-induced osteoclastogenesis and cell adhesion of human CD14⁺ monocytes. (A) CD14⁺ purified monocytes were first cultured for 3 days in 25 ng mL⁻¹ of hM-CSF to attain complete cell adhesion. For osteoclastogenesis experiments, the cells were then incubated for 15 days in the presence of 25 ng mL⁻¹ of hM-CSF with or without hRANKL (100 ng mL⁻¹). EPS or OS-EPS (0.5 µg mL⁻¹) were added at different time points during the culture period, as indicated: -3 days indicates the culture condition with only 25 ng mL⁻¹ of hM-CSF (cellular adhesion period); 0 → 14 days indicate the period with 25 ng mL⁻¹ of hM-CSF and 100 ng mL⁻¹ of RANKL (osteoclastic differentiation period). At the end of the culture period, TRAP staining was performed (original magnification ×40) and TRAP-positive multinucleated cells (more than three nuclei) were counted under a light microscope. (B) RAW 264.7 cells were cultured in the presence of hRANKL (100 ng mL⁻¹) and EPS or OS-EPS (0.5 µg mL⁻¹). After 5 days, the cells were stained for TRAP expression. (C) For adherence experiments, the cells were incubated for 5 days in the presence of EPS or OS-EPS at 1 µg mL⁻¹. The non-adherent cells in the supernatant were counted in triplicate by using Trypan blue exclusion dye. The adherent cells were washed three times in 1× DPBS, trypsinized and counted as above.
However, sensorgrams with RANKL, pre-incubated or not with OS-EPS, immobilized on the surface of a CM5 sensor chip revealed different binding response units for RANK (Figure 5B). To determine the biophysical binding parameters for the ternary complex interactions, real-time SPR spectroscopy was performed (Figure 6). Surprisingly, the kinetic study showed a significant increase in the association rate of RANK to RANKL pre-incubated with OS-EPS facilitating the complex formation. RANK had a higher affinity for RANKL pre-incubated with OS-EPS surface sensor chips than for RANKL surface sensor chips (>2-fold). This result would be in favor of an increase in bone resorption, which was not observed during the study of osteoclastogenesis (Figure 4).

RANK is not only a ligand for RANK but also acts as a ligand for the decoy receptor OPG. To determine whether or not OS-EPS could affect the OPG/RANKL complex, RANKL was immobilized on a sensor chip, and the capacity of OS-EPS to bind to complex OPG/RANKL was analyzed. As with the RANKL/RANK complex, OS-EPS was able to bind to a preformed OPG/RANKL complex (Figure 5D and E), forming another ternary complex, OS-EPS/RANKL/OPG. Also, the pre-incubation of OS-EPS (30 min at room temperature) with OPG or RANKL did not inhibit the capacity of OS-EPS to further bind to the preformed ternary complex OS-EPS/RANKL/OPG (data not shown). However, no modification was observed in the kinetic parameters for the interaction of OPG with RANKL pre-incubated with OS-EPS.

**OS-EPS increases the collagenolytic activity of cathepsin K at physiological plasma pH**

Cathepsin K is abundantly and predominantly expressed in osteoclasts and is considered to be the principal protease responsible for the degradation of most of the bone matrix. Cathepsin K can cleave most extracellular substrates, including the collagen triple helix and it has been reported that the collagenolytic activity of cathepsin K at acidic pH depends on
a complex formation with chondroitin sulfate, which increases the activity and stability of the enzyme (Li et al. 2002). Recently, Novinec et al. (2010) showed that heparin also increased the collagenolytic activity of the enzyme at physiological plasma pH. Therefore, we also examined the activity of cathepsin K on collagen in the presence of OS-EPS. Collagenolytic assays (Figure 7) show that cathepsin K is capable of digesting type I collagen on its own. Heparin and OS-EPS increased the collagenolytic activity of cathepsin K, whereas chondroitin sulfate and dermatan sulfate decreased the extent of collagen digestion.

OS-EPS induces trabecular bone loss
During the 28 days of this study, all the mice from the different treatment groups (OS-EPS, EPS and heparin-treated) gained weight. No significant differences with respect to weight gain were found between those mice treated with either OS-EPS or EPS or heparin or age-matched controls. However, at the femur level, micro-computed tomography (µ-CT) scanner analysis revealed that the mouse femur was characterized by major remodeling activities when compared with the control femurs (Figure 8A). OS-EPS thus resulted in a reduction in cancellous bone volume when compared with controls. The effect of OS-EPS on the cancellous bone was similar to that of our positive control (heparin). OS-EPS did not modify cortical bone volume (data not shown) but significantly reduced trabecular bone volume ($P < 0.01$; Figure 8A). The trabecular number (Tb.N) was significantly reduced by OS-EPS ($P < 0.05$), whereas the trabecular space (Tb.Sp) increased ($P < 0.05$). Trabecular thickness (Tb.Th) was not significantly altered (Figure 8). However, no significant difference in cancellous bone volume (BV/TV) was found between EPS-treated mice and age-matched control mice, highlighting the importance of sulfation in trabecular bone loss.

Histologic sections were stained with TRAP or ALP to quantify osteoclast or osteoblast surface-based data, respectively. Figure 8B shows that the osteoblast surface parameter was not significantly different when comparing OS-EPS or EPS-treated mice and age-matched controls. In contrast, OS-EPS produced an increase in the percentage of cancellous bone covered by osteoclasts. Whereas mice given 6 mg kg$^{-1}$ of OS-EPS showed a $125 \pm 23\%$ ($P < 0.05$) increase in osteoclast surface, the osteoclast surface was unaffected by treatment with EPS (Figure 8B).

**Discussion**
In the present study, we have shown that the long-term administration of OS-EPS, a “heparin-like” component (with uronic acid and sulfate content comparable to that of heparin) with “heparin-like” activity (anticoagulant properties), causes cancellous bone loss in mice due, in part, to an increase in the number of osteoclasts lining the trabecular bone surface. Our results are therefore similar to the results published many years ago, in which the long-term administration of heparin was shown to lead to the development of osteoporosis (Muir et al. 1996). Similarly, Barbour et al. (1994) showed that 36% of pregnant women undergoing long-term heparin treatment had a 10% reduction in femoral bone mineral density. Both heparin (Muir et al. 1996, 1997; Rajgopal et al. 2008) and OS-EPS increase the process of
bone resorption. Although heparin and OS-EPS have similar effects on osteoclast numbers, heparin was found to decrease osteoblast numbers (Muir et al. 1996, 1997; Rajgopal et al. 2008), whereas OS-EPS has no effect. These effects resulting in bone loss begin early in the course of OS-EPS treatment (data not shown). However, the mechanism sustaining osteoporosis was unclear and it was difficult to determine whether these effects on bone resorption were due to the direct effect of OS-EPS on osteoclasts or indirectly via its osteoblast activity.

GAGs exhibit several kinds of biological activities by binding to various extracellular molecules and play a pivotal role in bone metabolism. Indirect proof of HSPG involvement in osteoclast function was described in a publication that demonstrated that heparanase, a HS-degrading endoglycosidase expressed in osteoblastic cells, stimulates bone formation and bone mass (Kram et al. 2006). However, the mechanism sustaining osteoporosis was unclear and it was difficult to determine whether these effects on bone resorption were due to the direct effect of OS-EPS on osteoclasts or indirectly via its osteoblast activity.

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Ariyoshi et al. (2008) and Shinmyouzu et al. (2007) showed inhibition of osteoclastogenesis after direct interaction between GAGs and RANKL. On the contrary, Irie et al. (2007) showed that osteoclastic bone resorption was stimulated by inhibiting OPG activity. Our results clearly demonstrated that OS-EPS inhibited osteoclastogenesis in the two systems tested. Furthermore, we demonstrated the importance of the sulfation of the EPS in their inhibitory effect. Sulfation also plays a key role in the biological activities of GAGs, as revealed by the present work. Sulfated polysaccharides enhance the biological activity of both the homodimers and heterodimers in bone morphogenetic protein (BMP) by continuously serving the ligands to their signaling receptors expressed on cell membranes, similar to oversulfated chondroitin sulfate which binds to BMP-4 and enhances osteoblast differentiation (Miyazaki et al. 2008). In addition, Kumarasuriyar et al. (2009) showed that the chlorate-induced desulfation of GAGs expressed by MG63 cells delayed in vitro osteogenesis.

As our in vitro data are in favor of OS-EPS having direct inhibitory activity on osteoclastogenesis, we analyzed the effects of OS-EPS on the adhesion of osteoclast precursors, demonstrating a sequential effect of OS-EPS on RANKL-induced osteoclastogenesis. OS-EPS inhibits the early stage of osteoclast precursor adhesion and consequently...
inhibits the cell fusion stage. The alteration in cell adhesion and morphology prevents the cell fusion of osteoclast precursors and blocks osteoclast resorption that is particularly sensitive to cell morphology to develop their brush border (Rousselle and Heymann 2002). However, the question remains of how we can explain the major discrepancies between the in vivo and the in vitro results.

In this context, we first analyzed the interaction of OS-EPS with the OPG/RANK/RANKL molecular triad and the effect of OS-EPS on the adhesion of osteoclast precursors. OPG contains a heparin-binding domain and, like heparin (K$_D$: 0.28 nM; Theoleyre et al. 2006), strongly binds to OS-EPS (K$_D$: 0.22 nM). The sulfation is essential as a totally desulfated heparin loses its capacity to bind OPG. Here, we clearly showed that OS-EPS can form a hetero-molecular complex (OS-EPS/RANKL or OS-EPS/RANKL/OPG), as demonstrated using the SPR technique, and that RANK had a higher affinity for RANKL pre-incubated with OS-EPS than for RANKL alone. On the contrary, OS-EPS did not interfere in the binding of RANKL by OPG. These results also revealed that RANKL had higher affinity for OS-EPS (K$_D$: 91 pM) than for OPG (K$_D$: 0.27 nM; Kwan Tat et al. 2006). In conclusion, the preferential binding of RANKL to OS-EPS made it easier for the binary OS-EPS/RANKL complex to form, which, in turn, may facilitate ternary OS-EPS/RANKL/ RANK complex formation, which would be in favor of an increase in bone resorption. Secondly, we analyzed the collagenolytic activity of cathepsin K at pH 7.40. Like heparin, OS-EPS increased the collagenolytic activity of cathepsin K. This shows that the molecular mechanism behind the unique collagenolytic activity of cathepsin K depends on the environment and suggests that it may also be an important factor in cathepsin K regulation in vivo. Given that cathepsin K is one of the major factors playing a part in osteoporosis (Stoch and Wagner 2008), it seems a plausible target for orally administered EPSs, which would be also in favor of an increase in bone resorption.

The effect observed in vivo may be also explained by its effects on the bone osteoblast compartment and then by the dysregulation of the balance between osteoblasts and osteoclasts or by a slow down in bone remodeling. Osteoblastic cells produce a complex extracellular matrix composed of a mixture of PGs, collagens and non-collagenous proteins. The interaction of PGs with matrix effector macromolecules via either their GAG chains or their protein core is critical in regulating a variety of cellular events. Alterations in the structural composition of the GAG/PG components of the extracellular matrix may have important consequences on cell proliferation and/or differentiation. Recently, Hauert et al. (2009) demonstrated the dependence of osteogenesis on specific HS chains, in particular those associated with glypican-3. The differentiating osteoblast-committed cells produced a homogenous HS species (21 kDa), which correlated with an increase in HSPG glypican-3. Abrogation of glypican-3 reduced the expression of the osteogenic transcription factor Runx2. The data demonstrated close links between HS modifications and progression of osteogenic precursors through their developmental program.

Our results showed that (i) OS-EPS reduced expression of ALP, coll1a1 and Runx2 (preosteoblast stage) in BMSC cells and enhanced OC and BSP (mature osteoblast stage); (ii) OS-EPS did not modulate expression of RANKL by mature osteoblasts; (iii) exogenous application of OS-EPS to cultures of primary BMSCs during in vitro differentiation completely blocked BMSC mineralization; (iv) OS-EPS strongly reduced the proliferation of differentiating osteoblast BMSCs. This inhibition of proliferation was not due to the induction of apoptosis. These results suggest that OS-EPS reduces proliferation and accelerates osteoblastic differentiation, leading to strong inhibition of mineralized nodule formation that would be in favor of an increase in bone resorption. However, no significant difference in osteoblast surface was found between OS-EPS-treated mice and age-matched control mice. Although the explanation for these conflicting results is unclear, the findings in a whole bone organ culture system are more likely to reflect events in vivo than those that occur in isolated osteoblast cultures. It is possible that this reflects the propensity of OS-EPS to bind to other critical growth factors, such as FGF, BMP and tumor growth factor-$\beta_1$ (TGF-$\beta_1$).

FGFs control the proliferation and differentiation of osteoblast cells (Xiao et al. 2010). FGF-2 is thus a powerful promoter of bone growth, enhancing mineralized nodule formation (Downey et al. 2009). This effect is mediated via HSPGs that coordinate the interaction of FGFs with their high-affinity tyrosine kinase receptors, the FGFRs (Eswarakumar et al. 2005). The interaction of PGs with FGFs provides a physiological mechanism for regulation of FGF signaling, FGFR1 and the extracellular signal-regulated kinase pathway. Furthermore, osteoclast differentiation and activity are regulated by GAGs at different levels, as revealed in previous studies. FGF-2 induces, after binding to HS, the expression of RANKL and osteoclast maturation by rheumatoid synovial fibroblasts. FGF-2 not only increases the proliferation of rheumatoid synovial fibroblasts, but is also involved in osteoclast maturation, which leads to bone destruction in rheumatoid arthritis (Nakano et al. 2004). Matou et al. (2005) showed the binding of OS-EPS to FGF-2; therefore, we hypothesize that FGF-2 can induce bone resorption after binding to OS-EPS. HS and chondroitin sulfate directly regulate the BMP-mediated differentiation of mesenchymal stem cells (hMSCs) into osteoblasts. BMPs, which have been shown to be heparin-binding proteins, induce osteoblast differentiation in hMSCs (Manton et al. 2007). The role of heparin in the biological activity of BMP remains unclear. Heparin inhibits the binding of BMP-2 to BMP receptor and subsequent mRNA expression of Runx2, as well as phosphorylation of Smad and mitogen-activated protein kinases signal transduction. Furthermore, heparin has been found to suppress the differentiation of osteoblastic MC3T3-E1 cells treated with BMP-2 (Kanzaki et al. 2008). In contrast, Zhao et al. (2006) showed that heparin enhanced BMP-induced osteoblast differentiation by protecting BMPs from degradation and inhibition by BMP antagonists. TGF-$\beta_1$ is a known inhibitor of osteoprogenitor growth, which has a higher affinity than several other bone-related, heparin-binding growth factors (Manton et al. 2006). This binding suggests that GAGs play a critical role in regulating TGF-$\beta_1$ availability.
Overcoming such sugar-mediated inhibition may prove important for wound repair.

Given the importance of OS-EPSs for bone metabolism, it can be anticipated that OS-EPSs, thanks to its structural similarity to HS chains, somehow interferes with the biological activities of these cell surface- and extracellular matrix-associated molecules. Taken together, these data show different levels of bone resorption regulation by GAGs or EPSs, most of them leading to representative effects.

Materials and methods

Materials

Human macrophage colony stimulating factor (hM-CSF), human RANK and human OPG were obtained from R&D Systems (Abington, UK). Human RANKL (hRANKL) was kindly provided by Amgen Inc. (Thousand Oaks). Heparin sodium salt, HS from bovine kidney, HS from porcine intestinal mucosa, chondroitin sulfate from shark cartilage, dermata suggest from porcine intestinal mucosa and hyaluronic acid were purchased from Sigma (St Quentin Fallavier, France). The two low-molecular-weight EPSs (EPS and OS-EPS) were obtained from high-molecular-weight EPS (GY 785). The isolation procedure and characteristics of the GY 785 A. infernus strain have been reported previously (Raguénès et al. 1997). The GY 785 was produced, purified and characterized as described previously (Guezennec et al. 1998). The preparation of EPS and OS-EPS derivatives was performed as described previously. Briefly, GY 785 was first depolymerized by a process using a modification of the procedure of Nardella et al. (1996). Four hundred milligrams of GY 785 was dissolved in water (95 mL) in a reaction vessel, and 5 mL of 3 \times 10^{-3} M cupric acetate monohydrate was added. The temperature was kept at 60°C. A 0.07% (w/w) hydrogen peroxide solution was then added at a flow rate of 60 mL h^{-1}. The reaction was stopped after 2.5 h, and the contaminating copper ions were removed from the product by chromatography on Chelex 20 resin (in Na. form), with water as the eluent. The solution was concentrated, desalinated by ultrafiltration with a 1000 Da cutoff membrane and then freeze-dried. EPS was chemically oversulfated according to a previously described direct sulfation procedure (Nishino and Nagumo 1992). After sulfation, OS–EPS with a 40% sulfate group (w/w) was obtained.

The purities of the final products (EPS and OS-EPS) were estimated at >80%, as judged by the use of well-described chemical methods (Colliec-Jouault et al. 2001): monosaccharide content was determined by GC analysis of trimethylsilyl derivatives after acidic methanolysis; protein content was determined by Pierce BCA Protein Assay Reagent (Fischer Scientific, Illkirch, France); nitrogen, hydrogen, carbon and sulfur contents were determined by elemental analysis by the Central Microanalysis Department of the CNRS (Gif/Yvette, France). Sulfate content (sodium salt) was deduced from sulfur analysis according to the following relation: sulfate group% = 3.22 \times S%. Sulfate content was also determined by the Fourier transform infrared analysis. Pellets were obtained by careful grinding of a mixture of 2 mg of EPS with 200 mg of dry KBr. Infrared spectra were recorded on a BOMEM M100 Fourier Transform Infrared Spectrometer with resolution of 4 cm^{-1}. EPS and OS-EPS are hygroscopic so in their dry state they may contain between 10 and 20 wt% of water.

Culture medium and preparation of BMSCs

BMSCs were isolated from the bone marrow of 4-week-old Sprague-Dawley rats (Janvier, Le Genest Saint Isle, France). After anesthetization and cervical dislocation, femurs and tibias were dissected aseptically and cleaned of soft tissues. The BMSCs were then flushed out with a syringe fitted with a 22-gage needle containing maintenance media (MM) consisting of \( \alpha \)-minimal essential medium (\( \alpha \)-MEM, Invitrogen, Ergbay, France) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U mL^{-1} penicillin and 100 mg L^{-1} streptomycin). After centrifugation (5 min at 1000g), the cells were resuspended in 20 mL of MM. For osteogenic media (OM) culture conditions, this medium was supplemented with 50 µg mL^{-1} of ascorbic acid (Sigma) and 10^{-8} M dexamethasone (Sigma). Cells between passages 0 and 3 were used for all experiments. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Proliferation of BMSCs

Cells were plated (5 \times 10^{4} well^{-1}) into a 6-well plate in MM overnight. Then the MM was replaced by fresh OM in the presence of the absence of 25 µg mL^{-1} of EPS or OS-EPS, and the cells were left to recover for 7 days (the medium was changed every 3 days). Then, the cells in each well were washed three times in 1× Dulbecco phosphate-buffered saline (DPBS), trypsinized and counted in triplicate at days 3 and 7 using Trypan blue exclusion dye.

Induction of apoptosis

Programmed cell death was monitored microscopically following Hoechst staining. BMSCs were seeded at 10^{4} cells well^{-1} in a 24-well plate and cultured for 24 h in MM. Then, the MM was replaced with fresh OM in the presence of 25 µg mL^{-1} of OS-EPS for 24, 48 and 72 h or 100 nM staurosporine (Sigma) for 16 h as a positive control. At the end of the culture period, the cells were stained with 10 µg mL^{-1} Hoechst reagent for 30 min at 37°C, and then observed under UV microscopy (DMRXA; Leica, Wetzlar, Germany).

Induction of apoptosis was also investigated by cleavage of caspase-3 substrates in supernatants of cultures with or without OS-EPS treatment. BMSCs were seeded at 15 \times 10^{3} cells well^{-1} (in a 24-well plate), then incubated with OS-EPS (25 µg mL^{-1}) for 24, 48 and 72 h. Cells incubated with 1 µM staurosporine for 6 h were used as positive controls. At the end of the incubation period, the cells were lysed with 50 µL of Radio-Immuno Precipitation Assay (RIPA) buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min. The cells were then scraped off and protein content was quantified in parallel samples using the BCA (bicinchoninic acid + Copper II sulfate) assay. Caspase-3 activity was assessed in 10 µL of the cell lysate with the CaspACEi Assay System kit (Fluorometric, Promega, Madison) following the manufacturer’s instructions.
**Time-lapse microscopy**

The BMSCs were plated (2 × 10^3 cells well⁻¹) in triplicate into a 24-well plate in MM overnight. Then, MM was replaced with fresh OM in the presence or the absence of 25 µg mL⁻¹ of EPS or OS-EPS. Immediately post-treatment, the cultured dishes were placed onto a time-lapse instrument (Leica) designed to capture transmission-phase images every 10 min from multiwell plates. Images were taken and edited using the MetamorphTM software. Cell divisions were then scored manually.

**Cell cycle analysis**

Cells were plated in triplicate (2 × 10^3 cells well⁻¹) in a 6-well plate in MM overnight. Then, MM was replaced with fresh OM in the presence or the absence of 25 µg mL⁻¹ of EPS or OS-EPS. After 48 h, the cells were trypsinized and centrifuged at 1600 rpm for 3 min. Cell pellets were fixed in 70% ice-cold ethanol for 30 min and then washed twice in 100 µL of DPBS. The cells were then centrifuged, washed twice and incubated in 50 µg mL⁻¹ of propidium iodide for 20 min at 4°C. Cell cycle distribution was analyzed by flow cytometry F500 (Beckman Coulter France) based on 2N and 4N DNA content using the MultiCycle software.

**Mineralization assay**

The BMSCs were plated (3.5 × 10^6 cells well⁻¹) in duplicate in a 24-multiwell plate in MM. After 24 h of culture, the supernatant (MM) containing the non-adherent hematopoietic cells was removed. At confluence (72 h later), the MM was replaced by OM in the presence of 25 µg mL⁻¹ of EPS or OS-EPS (the medium was changed twice a week). After 1 week of culture, the OM was supplemented with 10 mM β-glycerophosphate for 2 weeks (the medium was changed twice a week).

**Osteogenic differentiation assay**

The BMSCs were plated (15 × 10^6 cells well⁻¹) in a 6-multiwell plate in MM. After 24 h of culture, the supernatant (MM) containing the non-adherent hematopoietic cells was replaced by OM in the presence of 25 µg mL⁻¹ of EPS or OS-EPS. After 1 week of culture, the OM was supplemented with 10 mM β-glycerophosphate for 2 weeks (the medium was changed twice a week).

**Reverse transcribed—qualitative polymerase chain reaction analysis**

Total RNA was isolated from osteogenic cultures at different times using the NucleoSpin II kit (Macherey-Nagel). RNA (500 ng) was reverse transcribed using the ThermoSript System (Invitrogen). cDNA synthesis was performed using total RNA with oligo(dT) at 50°C for 1 h. Qualitative polymerase chain reaction (qPCR) was performed in triplicate for each sample using 5 µL of 2× SYBR Green Supermix buffer (Bio-Rad, Marnes la Coquette, France), 1 µL of cDNA, 300 nM of each primer and DEPC (diethylenepyrocarbonate) H₂O to a final volume of 10 µL. After denaturing the cDNA at 98°C for 30 s, amplification and fluorescence determination were carried out in two steps: denaturation at 95°C for 15 s and annealing and extension at 60°C for 30 s. The detection of SYBR Green was during the annealing process. The sequence of the primers used in the PCRs is shown in Table I. Cyc1 cDNA as internal controls was used to normalize the data to determine the relative expression of the target genes. PCRs were carried out in 96-well plates using the Chromo4 System (Bio-Rad).

**Table I. Primer sequence for osteogenic markers and RANKL**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene (full name)</th>
<th>Accession number</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
<td>NM_013059.1</td>
<td>Forward: agggcagactccttttgg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: tggtaacagcgtggtgg</td>
</tr>
<tr>
<td>Runx2</td>
<td>Run-related transcription factor 2</td>
<td>NM_053470.1</td>
<td>Forward: cagcagctttaaagtgcaatggg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: aaacaaattagttgagctcaage</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
<td>NM_012587.2</td>
<td>Forward: ccacttttattcctctcaagc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse: tegcagctctattttcetctc</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcin</td>
<td>NM_013414.1</td>
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<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>α-1 type I collagen</td>
<td>NM_053304.1</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>NM_001130491.1</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RANKL</td>
<td>transf11 (Mus musculus)</td>
<td>NM_011613.3</td>
<td>Reverse: ggcgcccaaatggtgtgta</td>
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</table>
Collagen digestion
Soluble calf-skin collagen was diluted in 50 mM HEPES (acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique), pH 7.40, containing 1 mM EDTA to a final concentration of 0.5 mg mL⁻¹. The solutions were supplemented with 2.5 mM DTT (dithiothreitol) and 0.2 mM GAGs and digestion started by addition of cathepsin K (final concentration 0.25 μM). All reactions were incubated for 12 h at 25°C and then stopped by addition of SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample buffer. Polypeptides were separated by SDS–PAGE (8% gels) and stained with Coomassie brilliant blue R-250.

Differentiation of human CD14⁺ cells into osteoclasts
Human peripheral blood mononuclear cells were isolated by centrifugation over Ficoll gradient (Sigma Chemicals Co., St Louis, MO). CD14⁺ cells were magnetically labeled with α-MEM containing 10% FBS and 25 μM hRANKL. Then, the medium was changed every 4 days. The formation of osteoclasts occurred at around 14 days and was observed by TRAP staining. In this experiment, EPS or OS-EPS were added at different times during the culture period. Then, the medium was changed once. Then, non-adherent cells in the supernatant were counted in triplicate using Trypan blue exclusion dye. The adherent cells were washed three times in DPBS, trypsinized and counted as above.

SPR-binding assays
Experiments were carried out on a BIAcore 3000 instrument (BIAcore). RANKL, OPG [5 μg mL⁻¹ in 10 mM acetate buffer, pH 4.0 and pH 5.0 (1:1; v/v)] and RANK (10 μg mL⁻¹ in 10 mM acetate buffer, pH 5.0) were covalently immobilized to the dextran matrix of a CM5 sensor chip (BIAcore) via its primary amino groups at a flow rate of 30 μL min⁻¹. Immobilization levels ranging between 4000 resonance units (RU) (RANKL and OPG) and 5000 RU (RANK) were obtained. EPS and OS-EPS KD values for OPG, RANK and RANKL were determined using single cycle kinetics, starting with 1 nM OS-EPS or 100 nM EPS (OPG), 250 nM OS-EPS or 2 μM EPS (RANKL) and 100 nM OS-EPS or 2 μM EPS (RANKL), then 1/2 dilutions. For binding analysis over the immobilized RANKL, OPG or RANK chip, the concentrations of 1 μg mL⁻¹ OPG, 2 μg mL⁻¹ RANKL, 0.5 μg mL⁻¹ RANKL, 20 μg mL⁻¹ GAGs (heparin, or HS, or chondroitin sulfate, or dermatan sulfate or hyaluronic acid), 20 μM EPS and 200 nM OS-EPS were used. Binding assays were performed at 25°C in 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl and 0.005% P20 surfactant (HSB-P buffer, BIAcore) at a flow rate of 30 μL min⁻¹. Control sensorgrams (flow cell without RANKL, OPG and RANK) were automatically subtracted from the sensorgrams obtained with immobilized RANKL or OPG or RANK to yield true binding responses. The resulting sensorgrams were fitted using BiaEval 4.1 software (BIAcore).

Animal studies
Twenty-four 4-week-old male C3H/HeN mice (Janvier) were housed in pathogen-free conditions at the Experimental Therapy Unit (Nantes Faculty of Medicine, France) in accordance with the institutional guidelines by the French Ethical Committee and under the supervision of authorized investigators. After 7 days of acclimatization, the animals were assigned randomly to four groups: control (CT), heparin (Sigma; H4784, 140 U mg⁻¹), 50 mg mL⁻¹), EPS and OS-EPS. All mice received a (6 mg kg⁻¹) daily s.c. injection for a period of 28 days, consistent with the period of rapid growth in young mice. On the 29th day, the animals were anesthetized with isoflurane (0.2% air, delivered via nosecone) and sacrificed by cervical dislocation. Bilateral femurs from each animal were dissected for histological studies and micro-architectural parameter quantification. Two independent experiments were performed.

Histological analysis
After sacrifice, the left femurs were cleaned from adjacent tissues and fixed in 10% buffered formaldehyde. Then, samples were decalcified in 4% EDTA 0.2% paraformaldehyde (pH 7.4) buffer for 4 weeks and embedded in paraffin wax for TRAP and ALP staining. Five-micrometer thick sections were cut through the femur (microtome: Leica SM 2500; Leica Instruments GmbH) and mounted on glass slides. Analysis and quantification of osteoblastic and osteoclastic areas were done using a Leica Q500 image analysis system.
Micro-computed tomography
After sacrifice, the right femur from each animal was dissected from the soft tissues, fixed in 10% buffered formaldehyde and the distal metaphysis was used for μ-CT on a SkyScan-1072 (SkyScan, Aartselaar, Belgium). CT-Analyser software (from SkyScan) was used to analyze the structure of the sample, using the global segmentation method. Two-dimensional images were used to generate three-dimensional reconstructions and to calculate morphometric parameters with the SkyScan CtAn 3D creator software supplied with the instrument. Analysis was performed for the trabecular bone, whereby the trabecular region was precisely contoured in each single cross section manually. Bone volume ratio (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular space (Tb.Sp) were assessed.

Statistical analysis
All analyses were performed using GraphPad InStat v3.02 software (La Jolla, CA). The mean ± SD was calculated for all conditions and compared by ANOVA. In vivo experimentation results were analyzed with the unpaired nonparametric Mann–Whitney U-test using two-tailed P-values. Differences relative to a probability of two-tailed P < 0.05 were considered significant.

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Conflict of interest
None declared.

Abbreviations
ALP, alkaline phosphatase; α-MEM, α-minimal essential medium; BMP, bone morphogenic protein; BMSC, bone marrow stem cell; BSP, bone sialoprotein; coll1, α-1 type I collagen expression; DPBS, Dulbecco phosphate-buffered saline; EPS, exopolysaccharide; FBS, fetal bovine serum; FGF, fibroblast growth factor; GAG, glycosaminoglycan; hMSC, human M-CSF; hMC, mesenchymal stem cell; hRANKL, human RANKL; HS, heparan sulfate; MM, maintenance media; RU, resonance unit; TRAP, tartrate-resistant acid phosphatase; μ-CT, micro-computed tomography; OC, osteocalcin; OM, osteogenic media; OPG, osteoprotegerin; OS-EPS, oversulfated EPS; PG, proteoglycan; qPCR, qualitative polymerase chain reaction; RANK, receptor activator of NF-κB; RANKL, RANK ligand; Runx2, runt-related transcription factor 2; SPR, surface plasmon resonance; TGF-β, tumor growth factor-β; VEGF, vascular endothelial growth factor.

References
Effects of EPS on bone biology


