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Characterization of osteoprotegerin binding to glycosaminoglycans by surface plasmon resonance: Role in the interactions with receptor activator of nuclear factor κB ligand (RANKL) and RANK

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Abstract

Osteoprotegerin (OPG) is a decoy receptor for receptor activator of nuclear factor κB ligand (RANKL), a key inducer of osteoclastogenesis via its receptor RANK. We previously showed that RANK, RANKL, and OPG are able to form a tertiary complex and that OPG must be also considered as a direct effector of osteoclast functions. As OPG contains a heparin-binding domain, the present study investigated the interactions between OPG and glycosaminoglycans (GAGs) by surface plasmon resonance and their involvement in the OPG functions. Kinetic data demonstrated that OPG binds to heparin with a high-affinity (K_D: 0.28 nM) and that the pre-incubation of OPG with heparin inhibits in a dose-dependent manner the OPG binding to the complex RANK–RANKL. GAGs from different structure/origin (heparan sulfate, dermatan sulfate, and chondroitin sulfate) exert similar activity on OPG binding. The contribution of the sulfation pattern and the size of the oligosaccharide were determined in this inhibitory mechanism. The results demonstrated that sulfation is essential in the OPG-blocking function of GAGs since a totally desulfated heparin loses its capacity to bind and to block OPG binding to RANKL. Moreover, a decasaccharide is the minimal structure that totally inhibits the OPG binding to the complex RANK–RANKL. Western blot analysis performed in 293 cells surexpressing RANKL revealed that the pre-incubation of OPG with these GAGs strongly inhibits the OPG-induced decrease of membrane RANKL half-life. These data support an essential function of the related glycosaminoglycans heparin and heparan sulfate in the activity of the triad RANK–RANKL–OPG.

Keywords: Osteoprotegerin; RANKL; RANK; Heparin-binding domain; Glycosaminoglycan; Proteoglycan; Bone; Bone matrix; Surface plasmon resonance

Bone is a specialized connective tissue formed by a mineralized matrix which is continually remodelled according to pathophysiological events. This remodelling maintains homeostasis of phosphorus and calcium through coordinated phases of bone formation by osteoblasts and resorption by osteoclasts. The bone extracellular matrix is composed mostly of collagen associated with proteoglycans and hydroxyapatite crystals as mineral component. Chemically, proteoglycans refer to dichotomous structure: a protein core on which polyanionic linear polysaccharides called glycosaminoglycans (GAGs) are attached [1]. During the past decade, it has been shown that GAGs are

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involved in numerous biological processes including the organization of the extracellular matrix, the modulation of cell adhesion and migration, the control of proliferation and differentiation, and the cytokine presentation/binding [2–7]. Heparin (HP) and heparan sulfates (HS) [5], chondroitin sulfates [6], and dermatan sulfates [7] belong to the GAG family, each member differing from the others by the polysaccharide chain structure and by the *N*- and *O*-sulfation pattern. Thus, HS and heparin are structurally related as they are both composed of the same linear array of alternative disaccharide unit (D-glucuronic acid/L-iduronic acid and D-glucosamine) but differ by the epimerization rate and by the degree and pattern of sulfation. Numerous proteins containing heparin-binding domains specifically interact with GAGs. Such observations have been done for several growth factors including the basic fibroblastic growth factor (bFGF) [8], the macrophage inflammatory protein 1α (MIP1α) [9], the interleukin-8 [10], the stromal cell-derived factor-1α (SDF1) [11], and the β chemokine regulated on activation normal T cell expressed and secreted (RANTES) [12]. In all cases, the binding of these growth factors to GAGs modified their biochemical characteristics (i.e., oligomerization) as well as their biological activities (i.e., bio-availability, presentation to cell membrane high-affinity receptors). Furthermore, proteoglycans are involved in collagen organization and then in the maintenance of bone mass. Thus, Corti et al. [13] demonstrated that the targeted disruption of decorin and biglycan genes, two members of the small leucine-rich proteoglycan family, results in reduced skeletal growth and bone mass leading to generalized osteopenia.

Osteoprotegerin (OPG), a 55 kDa protein which belongs to the family of the tumor necrosis factor (TNF) receptor family [14], contains three structural domains specifically influencing its biological activities. The first one is a cysteine-rich domain in the N-terminal position that is essential for the inhibition of osteoclastogenesis as well as for the dimerization of OPG via the Cys^{400}. The second domain is a heparin-binding domain potentially capable of interacting with numerous proteoglycans and the third one corresponds to a death domain homologous (DDH) region [15]. OPG is considered as a decoy receptor which inhibits the binding of Receptor Activator of Nuclear factor-κB Ligand (RANKL), a member of the tumor necrosis factor family to its membrane receptor RANK. This molecular triad OPG–RANKL–RANK is involved in the orchestration of pathophysiological bone remodelling. Indeed, RANKL is preferentially expressed on committed preosteoblastic cells, whereas its specific receptor RANK is expressed on hematopoietic osteoclast progenitors [16,17]. RANKL is required for osteoclast differentiation and acts as a survival factor for osteoclast precursors. In this context, OPG produced by osteoblast lineage cells blocks the interaction between RANKL and RANK, and inhibits the terminal stage of osteoclastic differentiation, suppresses the activation, and induces apoptosis of mature osteoclasts [18–20]. The first role of the OPG heparin-binding domain has been revealed by Standal et al. [21] who demonstrated that myeloma cells internalize and degrade OPG through its binding to syndecan-1. This observation has been strengthened more recently by Mosheimer et al. [22] who reported that syndecan-1 is involved in OPG-induced monocyte chemotaxis.

The aim of this work was to study the interactions of osteoprotegerin with several glycosaminoglycans, (i) to further characterize the role of the OPG heparin-binding domain, (ii) to determine the involvement of GAGs in the interactions between OPG, RANKL, RANK and (iii) to analyze the influence of GAGs on OPG biological activity (regulation of membrane RANKL half-life).

Materials and methods

Reagents. Soluble human RANK (RANK) was purchased from Promocell (Heidelberg, Germany). Human OPG was obtained from R&D Systems (Abington, UK), human RANKL and OPG-Fc were kindly provided by Amgen Inc. (Thousand Oaks, USA). Heparan sulfate from bovine kidney (bHS), heparan sulfate from porcine intestinal mucosa (pHS), chondroitin sulfate from shark cartilage (CS), dermatan sulfate from porcine intestinal mucosa (DS), and cycloheximide were purchased from Sigma (St. Quentin Fallavier, France). Enhanced chemiluminescence reagents were obtained from Roche (Mannhein, Germany). Heparin from porcine intestinal mucosa was biotinylated at its reducing end according to the technique described by Amara et al. [10]. Heparin-derived oligosaccharides of defined size were prepared by digestion of porcine mucosal heparin with heparinase I followed by gel filtration chromatography on a Bio-Gel P-10 column [11]. Heparin initially contained 97.7% of *N*-sulfate groups, 89.3% of 2-*O*-sulfate groups, and 92.4% of 6-*O*-sulfate groups. De-*N*-sulfated/re-*N*-acetylated heparin contained 90.5% of 2-*O*-sulfate groups, 85.3% of 6-*O*-sulfates, and a very low amount of remaining *N*-sulfate groups (2.4%). De-2-*O*-sulfated heparin contained 80.2% of 6-*O*-sulfate groups, 91.4% of *N*-sulfate groups, and a residual 2.2% of the 2-*O*-sulfates. De-6-*O*-sulfated heparin contained 98.2% of *N*-sulfate groups, 54.7% of 2-*O*-sulfate groups, and a residual 4.2% of 6-*O*-sulfates [23].

Surface plasmon resonance-binding assays. These experiments were performed with the BIAcore 2000 optical biosensor (BIAcore, Uppsala, Sweden). Biotinylated heparin was injected over a SA (streptavidin)-sensor chip (BIAcore) in 10 mM Hepes, pH 7.4, 0.3 M NaCl, and 0.005% P20 surfactant at a flow rate of 10 μl/min. An immobilization level ranging of approximately 120 resonance units (RU) was obtained. RANK (10 μg/ml in 10 mM acetate buffer, pH 5.0) was covalently immobilized to the dextran matrix of a CM5 sensor chip (BIAcore) via its primary amine groups at a flow rate of 5 μl/min. An immobilization level ranging of approximately 4900 resonance units (RU) was obtained. 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 (HBS-P buffer, BIAcore) at a flow rate of 10 μl/min for immobilized-heparin, and 5 μl/min for immobilized-RANK. Control sensorgrams (flow cell without heparin or RANK) were automatically substracted from the sensorgrams obtained with immobilized heparin or RANK to yield true binding responses. The resulting sensorgrams were analyzed using the BIAevaluation 3.2 software (BIAcore).

Western blot studies. The 293 human embryonic kidney cells transfected with the cDNA encoding the full length form of murine RANKL (293-RANKL cells) [24,25] were cultured in serum-free DMEM for 24 h and then incubated for 2, 3, 4 or 6 h in the presence or absence of 4 μg/ml cycloheximide, with or without 0.90 nM OPG. In some cases, OPG was pre-incubated with 90 nM heparin or HP-derived oligosaccharides before being added on 293-RANKL cells. 293-RANKL cells were then lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, sodium 0.25% deoxycholate, 1 mM NaF, 1 mM $NaVO_4$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM phenymethylsulfonyl fluoride, and 10% glycerol). Equal amounts of proteins were subjected to electrophoresis on 10% SDS–PAGE, transferred electrophoretically onto a PVDF