Glycosaminoglycans as Potential Regulators of Osteoprotegerin Therapeutic Activity in Osteosarcoma

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Abstract

Osteosarcoma is the most frequent primary bone malignant tumor that develops mainly in children and adolescents. Despite recent improvements in chemotherapy and surgery, survival rate is ~50% after 5 years. Osteoprotegerin (OPG) is a potent inhibitor of osteoclast differentiation and activation, but its use as therapeutic agent in cancer-associated osteolysis remains controversial due to its ability to bind and inhibit the apoptotic effect of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on tumor cells. The therapeutic effects of full-length OPG (1–401) and OPG 1-194 lacking its heparin-binding domain delivered by nonviral gene therapy were compared in a murine model of osteolytic osteosarcoma. Tumor incidence, progression, and associated bone lesions were significantly diminished in the OPG 1-194 group, but not in the OPG 1-401 group, compared with controls. As receptor activator of nuclear factor-κB ligand (RANKL), TRAIL, and glycosaminoglycans (GAG) were shown to be overexpressed in osteosarcoma environment compared with control tissue, OPG 1-401 bioactivity may be modulated by one of these protagonists. Surface plasmon resonance analyses performed with OPG, TRAIL, and GAGs revealed that TRAIL binds both forms of OPG with the same affinity. In addition, as OPG 1-194 and OPG 1-401 similarly inhibit TRAIL-induced apoptosis, it suggests that TRAIL is not involved in the modulation of OPG bioactivity. However, as GAGs inhibit OPG 1-401 but not OPG 1-194 binding to TRAIL or to RANKL, they may represent potent regulators of OPG availability and antitumor activity in bone tumor microenvironment. [Cancer Res 2009;69(2):526–36]

Introduction

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

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

However, the role of OPG in cancer is controversial (11), as, for example, a recent study reported that OPG produced by tumor cells increases tumor growth, whereas OPG-Fc exerts opposite effects (12). Indeed, OPG may play a role in tumor cell survival due to its ability to bind another member of the TNF superfamily, the TNF-related apoptosis-inducing ligand (TRAIL), which induces apoptosis of tumor cells without affecting normal cells (13). To this regard, several in vitro studies have shown that OPG is a survival factor for TRAIL-expressing tumor cells, such as prostate cancer (14), breast cancer (15), or multiple myeloma (16) cells.

As OPG contains a heparin-binding domain, the glycosaminoglycan (GAG) part of proteoglycans may also modulate OPG biological activity. GAGs are unbranched polysaccharides composed of repeated units of alternating uronic acids and hexamine or galactose (17). Most GAGs are covalently bound to core protein (18). Osteosarcoma environment compared with control tissue, OPG 1-401 bioactivity may be modulated by one of these protagonists. Surface plasmon resonance analyses performed with OPG, TRAIL, and GAGs revealed that TRAIL binds both forms of OPG with the same affinity. In addition, as OPG 1-194 and OPG 1-401 similarly inhibit TRAIL-induced apoptosis, it suggests that TRAIL is not involved in the modulation of OPG bioactivity. However, as GAGs inhibit OPG 1-401 but not OPG 1-194 binding to TRAIL or to RANKL, they may represent potent regulators of OPG availability and antitumor activity in bone tumor microenvironment. [Cancer Res 2009;69(2):526–36]
heparin-binding domain in OPG has been reported by Standal and colleagues (19) who showed that myeloma cells internalize and degrade OPG through its binding to syndecan-1. This observation has been strengthened by the fact that syndecan-1 is involved in OPG-induced monocyte chemotaxis (20). More recently, our group showed a key function of heparin and heparan sulfate in the activity of the molecular triad RANK-RANKL-OPG (21). However, no study on GAG modulation of OPG bioactivity has been conducted thus far in vivo.

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

Materials and Methods

Cell Lines

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

The human osteosarcoma cell line MG63 purchased from the American Type Culture Collection was cultured in DMEM (BioWhittaker) supplemented with 5% FBS and 2 mmol/L L-glutamine. Human embryonic kidney cells (2.93) stably transfected with the cDNA encoding the full-length form of murine RANKL (2.93Rl) were a kind gift of Dr. R. Josien (Institut National de la Sante et de la Recherche Medicale U643, Nantes, France). The pcDNA3, full-length form of murine RANKL, was stably transfected into the 2.93 cells cultured in DMEM that contained 10% FBS by using polyethyleneimine. After 4 d, the cells were transferred to medium containing G418 (Invitrogen) for continuous selection of the surviving cells. Two weeks later, the surviving clones were analyzed by Western blot analysis to detect the expression of RANKL protein.

In vivo Experiments

Plasmids. cDNAs encoding the murine OPG-truncated form (1-194) or full-length sequence (1-401) were kindly provided by Amgen, Inc. and cloned in the pcDNA3 plasmid (Invitrogen), respectively, between the HindIII/XhoI and NotI/XhoI sites using standard cloning procedures. The empty pcDNA3 was used as a control.

Mouse osteosarcoma model. Four-week-old male C3H/HeJ mice (Elevages Janvier) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. The mice were anesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with buprenorphine (0.05 mg/kg; Temgesic, Schering-Plough) before s.c. inoculation of POS-1 cell suspension (2 x 10⁶ in 50 μL PBS) in the hind footpad of the mice. Mice develop a primary tumor at the site of injection in 3 wk that can be transplanted to mice of the same strain as a small fragment (2 x 2 x 2 mm³) in close contact with the tibia, as previously reported (23). Tumors that appeared at the graft site ~8 d later lead to osteolytic lesions that reproduce those observed in human osteosarcoma and are associated with the development of pulmonary metastases over a 3-wk period (23). The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a caliper according to the formula V = 0.5 x L x S², where L and S are, respectively, the largest and smallest perpendicular tumor diameters.

Formulation preparations. Lutrol, a block copolymer consisting of poly(ethyleneglycol)₂₅₀-poly(propyleneglycol)₃₅₀-poly(ethyleneglycol)₂₅₀, was generously provided by BASF. Stock solutions were prepared at 20% (w/v) in water and stored at 4°C. Formulations of DNA with block copolymers were prepared by equivolumetric mixing block copolymer in water and DNA solution at the desired concentration (50 μg/mg), as already reported (24). For i.m. injections, mice were anesthetized with Hypnomidate (etomidate, 40 mg/kg) and 50 μL of block copolymers/DNA formulations were injected into shaved tibial anterior muscles at one site using a microfine syringe.

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

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

Histology Analysis

After sacrifice, mice were perfused with formalin at 4°C, decalcified (PBS-EDTA), and embedded in paraffin for tartrate-resistant acid phosphatase (TRAP) staining. Sections (5 μm) were cut and stained for TRAP to identify osteoclasts by 1-h incubation in a 1 mg/mL naphthol AS-TR phosphate, 60 mmol/L NN-dimethylformamide, 100 mmol/L sodium tartrate, and 1 mg/mL fast red TR salt solution (all from Sigma Chemical Co.). The counterstain was performed with hematoxylin. The number of osteoclasts was evaluated on the cortical bone by manually counting by light microscopy.

RNA Isolation and Real-time PCR

Total RNA was extracted by Trizol reagent (Invitrogen). First-strand cDNA was synthesized at 37°C for 1 h from 5 μg of total RNA in a 50 μL mixture containing reverse transcriptase buffer, 0.5 μg random primers, 0.5 mmol/L deoxynucleotide triphosphate mix, 20 units RNaseOUT, and 400 units of myeloid leukemia virus reverse transcriptase (all from Invitrogen). Sequences of primers used for real-time PCR are listed in Supplementary Table 1. The real-time PCR contained, in a final volume of 10 μL, 10 ng of reverse transcribed total RNA, 300 mmol/L of the forward and reverse primers, and 5 μL of 2X SYBR Green buffer (Bio-Rad). PCRs were carried out in triplicate in 96-well plates using the Chromo4 System (Bio-Rad). Mus musculus hypoxanthine guanine phosphoribosyltransferase 1, β-actin, cyclophilin B, and cytochrome c-1 were used as invariant controls. Analysis was performed using the Vandesompele method (25).

GAG Content in Tumor Tissue

Small animal imaging. GAG imaging was performed using a γ camera especially devised for small animal imaging (γ IMAGER, Biospace Mesures). The γ camera consists of a R3292 Hamamatsu position-sensitive photomultiplier having a continuous 4-mm-thick × 120-mm-diameter CsI(Na) crystal leading to a 10-cm field of view. The energy resolution and intrinsic planar resolution of the basic system are given as 11% at 140 keV and <2 mm full width at half maximum, respectively. For mouse planar imaging,
the camera was equipped with a 1.3/0.2/20 collimator (hole diameter/septum thickness/height in mm) leading to a sensitivity given as 330 cpm/μL. For imaging procedures, mice were anesthetized by i.p. injection of 40 μL of a solution of ketamine and xylazine solution (1:1). Anesthetized animals i.v. injected with the tracer 99mTc-N-[triethylammonium]-3-propyl-[15]ane-N5 (99mTc-NTP15-5) (25 MBq), which was previously shown to bind to proteoglycans, were placed supine over the collimator of the γ camera, and a posterior whole-body image started 15 min after tracer administration. Acquisition was performed in List Mode for a 10 min duration, with a 15% window centered on the 140 keV photopeak of 99mTc.

**Radiolabeled tracers.** The NTP-15-5 was prepared and radiolabeled with 99mTc by the stannous chloride method as previously described, with a specific radioactivity of 25 MBq/μmol (26). The quality control was performed with Whatman PartiSize KCF18F strip TLC using methanol/acetonitrile/tetrahydrofuran/ammonium acetate (1N; 3:3:2:2) as eluent.

**Alcian blue staining.** The tumor was fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 μm) were cut using microtome, dehydrated, and stained with Alcian blue (0.1%) and then counterstained with Groat's hematoxylin and phosphomolybdic acid/orange G. The sections were examined by light microscopy.

**Cell proliferation analysis.** Replicate subconfluent cultures of MG63 cells seeded in 96-well plates were treated for 24 to 72 h with 50 ng/mL of recombinant human TRAIL (Promokine) in the presence or absence of OPG. In this assay, two forms of recombinant murine OPG were used: a truncated 22-194 form (generous gift from Amgen) and a complete form (22-401) purchased from R&D Systems. Cell viability was determined by the sodium-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation reagent assay kit (Roche Molecular Biomedicals). The effects of heparin (50 nmol/L; Sigma) were studied in the same experimental conditions.

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

**Surface Plasmon Resonance Analysis**
All experiments were performed on a BIACore 3000 instrument (BIACore). All experiments were performed at 25°C. For kinetic analysis, 260 resonance units (RU) of TRAIL were covalently coupled to a research-grade CM5 chip (BIACore) as recommended by the manufacturer. Increasing concentrations of OPG 1-401 or OPG 1-194 ranging from 1 to 520 nmol/L in HEPES-buffered saline were injected at a flow rate of 30 μL/min for 5 min and dissociation was monitored for 15 min. Regeneration was achieved with NaOH (4.5 mmol/L) after each cycle. For binding analysis, RANKL was immobilized at 1,600 RU on a CM5 chip. Binding of OPG 1-401 alone or preincubated for 60 min with oligosaccharide 16 (16 sugar units, kindly provided by Pr Gallagher, University of Manchester, Manchester, United Kingdom) was assayed for 3 min at a flow rate of 20 μL/min followed by dissociation for 3.5 min. The resulting sensograms were fitted using BiaEval 4.1 software (BIACore).

**Data Analyses**
For in vivo experiments, the ANOVA test was used. The differences of actuarial survival were determined by the log-rank test on a Kaplan-Meier survival curve. Statistical evaluation of the in vitro proliferation data was performed using the Student’s t test. Results are given as mean ± SD and results with P < 0.05 were considered significant.

**Results**

**OPG 1-194 but not OPG 1-401 transgene expression decreases osteosarcoma development.** Mice received an i.m. injection of DNA formulation encoding murine OPG 1-194 or OPG 1-401 as a preventing treatment 7 days before osteosarcoma implantation followed by one injection per week during 3 weeks. This time schedule has been chosen as the OPG transgene production is optimal 7 days after the injection of the DNA formulation, leading to a high OPG production at the time of osteosarcoma induction by tumor transplantation. Overexpression of mOPG (1-194 and 1-401) was confirmed by ELISA in muscle of the corresponding mice versus mice injected with pcDNA3 alone or control osteosarcoma-bearing mice (data not shown). All animals injected i.m. with the plasmid construct encoding mOPG 1-194 exhibit a significant decrease of the tumor volume at day 20 (633.1 mm3) compared with the animals that received pcDNA3 alone or OPG 1-401 in the same conditions [3,048.8 mm3 (P < 0.01) and 2,674 mm3 (P < 0.05), respectively] or with control osteosarcoma-bearing mice that developed progressive tumor (3,848.4 mm3, P < 0.001; Fig. 1A). In addition, when each animal is considered individually, the tumor incidence of mice developing a progressive primary tumor (tumor volume, ≥1,000 mm3) is significantly diminished at day 18 in OPG 1-194–treated animals (0/5) compared with controls, pcDNA3-treated, or OPG 1-401–treated mice (6/6, 5/5, and 5/5, respectively; Fig. 1B). These results reveal that the OPG antitumor activity is structure dependent: the antitumor effect of OPG 1-194 was confirmed as previously reported in osteosarcoma (10), whereas OPG 1-401 overexpression did not significantly affect tumor growth (tumor volume was similar to pcDNA3-injected mice). A significant increase of survival rate was observed in the OPG 1-194 group (P < 0.01, Fig. 1C) as a consequence of decreased pulmonary metastases dissemination compared with the other groups. Indeed, at day 20, all mice died or were euthanized due to a high tumor burden in the group treated with OPG 1-401, 80% in the pcDNA3 group and 100% in the control group, compared with the OPG 1-194–treated group where all mice were still alive (Fig. 1C).

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

Quantification of the osteolytic lesions by microcomputed tomography analysis confirmed radiographic data (Fig. 2B). POS-1 tumors are associated with bone lesions with cortical degradation in pcDNA3-treated mice compared with naive animals, whereas OPG 1-194–treated mice showed a protective effect on bone resorption associated with osteosarcoma development (Fig. 2B). Indeed, the specific trabecular bone volume is strongly decreased in pcDNA3-treated mice compared with control naive mice (27.3% versus 50.28%; P < 0.001), whereas bone volume/total volume of OPG 1-194–treated mice is closer to those of control mice (42.2% versus 50.28%, P = not significant). However, animals treated with OPG 1-401 show intermediary results compared with OPG
1-194–treated and pcDNA3–treated mice (32.1% versus 42.2% and 27.3%, respectively). As a consequence, the relative bone loss calculated as percent of controls reached 45.7% in the pcDNA3.1 group, and 36.5% and 16.1%, respectively, in OPG 1-401–treated and OPG 1-194–treated mice.

In addition, TRAP staining performed on osteosarcoma sections revealed an increase of osteoclast recruitment to the tumor-bone interface associated with osteosarcoma development (pcDNA3 mice; Fig. 2C), whereas the presence of TRAP-positive osteoclasts was totally absent in OPG 1-194–treated mice and only partially in OPG 1-401–treated mice (Fig. 2C). Osteoclast counting confirms these analyses, with 2 and 15 osteoclasts/tumor-bone interface surface (TBIS) observed, respectively, in the OPG 1-194 and OPG 1-401 groups compared with 38 osteoclasts/TBIS in controls (Fig. 2C), revealing a higher protective effect of OPG 1-194 on tumor-induced bone lesions compared with OPG 1-401–treated mice.

Comparing the different effects exerted by OPG1-194 and OPG 1-401 toward prevention of bone degradation and tumor development, it may be suggested that OPG 1-401 exerts diminished effects due to loss of bioactivity. Two molecules are potential candidates to interfere with OPG 1-401 bioactivity, TRAIL and GAGs, which

Figure 1. mOPG 1-194 transgene expression significantly diminishes tumor volume and progression and augments survival rate in an osteolytic model of mouse osteosarcoma. Mice were transplanted with POS-1 osteosarcoma fragments as described in Materials and Methods. Lutrol/DNA formulations were injected as a curative protocol into both tibial anterior muscles once a week. The tumor volume (V) was calculated from the measurement of two perpendicular diameters according to the following formula: 

\[ V = 0.5 \times L \times S^2 \]

The mean (A) or the individual (B) tumor volume of mice treated with p-mOPG 1-194/Lutrol formulation is compared with that of mice that received p-mOPG 1-401/Lutrol, pcDNA3/Lutrol, or control mice without tumors. ***, P < 0.005; **, P < 0.01; *, P < 0.05. i, tumor incidence. C, overall survival rate was compared between mice that received p-mOPG 1-194/Lutrol, p-mOPG 1-401/Lutrol, pcDNA3/Lutrol, and control mice over a 20-d period. One representative experiment of three is shown. *, P < 0.01.
binding domain to OPG has been described (GAG) or hypothesized (TRAIL) in the 195-401 domain.

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

TRAIL binds both forms of OPG with the same affinity. To determine whether TRAIL is the protagonist involved in the modulation of OPG bioactivity in vivo, two sets of experiments...
were performed: (a) TRAIL binding to both forms of mOPG was compared by BIAcore analysis and (b) OPG ability to inhibit TRAIL proapoptotic activity was compared between both forms of mOPG.

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

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

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

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

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

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

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

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

GAGs were then tested for their ability to modulate OPG 1-401 bioactivity as assessed in a model of 2.93 cells stably transfected to overexpress RANKL. OPG 1-401 binds RANKL present in the supernatant of 2.93 RANKL cells, decreasing its detection by 75% (Fig. 6C). When heparin or oligo 16 was coincubated with OPG 1-401, RANKL detection decrease was only of 24% and 60%, respectively, suggesting that GAGs inhibit OPG-RANKL interaction.

**Discussion.**

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

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

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

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

Furthermore, because OPG is also composed of a heparin-binding domain at its COOH-terminal portion, the cytokine is able to bind the GAG part of PGs, which may regulate its bioavailability and biological activity in the bone environment. Indeed, PGs or GAGs are associated to the cell membrane or resident in the extracellular matrix and one of their main property is their ability to store growth factors or cytokines at both levels (17). In the present study, higher GAG expression and production were evidenced in osteosarcoma environment compared with control tissue (muscle), analyzed by Alcian blue staining, in vivo imaging using a radiotracer binding to PG, and real-time PCR, suggesting that the PGs present in the tumor environment may modulate OPG 1-401 bioavailability and consequently its biological activity. Using

Figure 5. PGs are highly expressed in osteosarcoma tissue. A, the presence of GAGs was evaluated by Alcian blue staining on the osteosarcoma tumor tissue compared with chondrosarcoma as a positive control; unstained chondrosarcoma is given as a negative control. B, whole-body planar scintigraphy of a representative POS osteosarcoma bearing mouse at the stage day 23. Planar static image (10-min duration) was acquired 15 min after i.v. injection of $^{99m}$Tc-NTP 15-5 radiotracer (25 MBq/animal). A clear uptake of the radiotracer was observed within the tumor-bearing paw (arrow) to the contralateral paw. C, quantitative analysis of $^{99m}$Tc-NTP 15-5 tumoral uptake at early (day 9) and late stage of study (day 23). Columns, mean TBR values at each time point; bars, SD. *, statistical significance ($P < 0.05$). D, GAG expression (biglycan, syndecan-1, syndecan-2, and syndecan-4) was analyzed at the transcript level by real-time PCR in the tumor tissue compared with control tissue (muscle without tumor). Results are expressed as fold increase compared with the POS-1 cells. Experiments were performed at least thrice in triplicate.
quantitative reverse transcription-PCR, we showed that syndecan-1 is the main proteoglycan overexpressed in osteosarcoma tumor with a 340-fold increase compared with contralateral muscle, whereas decorin was not expressed at all and the three other PGs studied were less increased. It is quite surprising that biglycan is not the most overexpressed PG because osteosarcoma is characterized by the direct synthesis of osteoid substance by tumor cells and that biglycan is the most PG present in the bone matrix (17). However, overexpression of syndecan-1 has been already reported in tumors, especially in myeloma (19). Among several examples of OPG bioactivity regulation by PGs reported in the literature, Irie and colleagues (40) have reported that heparin was able to bind OPG, thereby preventing OPG-mediated inhibition of osteoclastic bone resorption \textit{in vitro}. However, another study revealed that dermatan sulfate is able to inhibit osteoclastogenesis, such as OPG, by binding to RANKL (41). In pathologic situations, it has been shown that OPG is able to bind to syndecan-1 expressed at the surface of myeloma cells (42) and that these tumor cells then

![Graphs showing binding interactions](cite Graphs)

\textbf{Figure 6.} GAGs inhibit OPG binding to TRAIL (A) or RANKL (B) and modulate OPG bioactivity \textit{in vitro}. Surface plasmon resonance was used to study interactions between OPG (1-401 and 1-194), RANKL, and GAGs (oligosaccharides containing 16 sugar units) or OPG (1-401 and 1-194), TRAIL, and GAGs. Recombinant OPG 1-401 or OPG 1-194 previously preincubated or not at room temperature for 45 min with 400 nmol/L GAGs was injected over immobilized RANKL (A) or TRAIL (B). Flow rate, 20 μL/min; injected volume, 200 μL. C. RANKL production in the supernatant of 2.93 cells stably transfected with full-length murine RANKL was detected by ELISA in the presence or absence of OPG 1-401 (50 ng/mL) alone or preincubated with heparin (50 nmol/L) or oligo 16 (200 nmol/L) during 45 min at room temperature. **, \( P < 0.001 \); *, \( P < 0.05 \), when compared with control; $, \ P < 0.05 \), when OPG + oligo 16 is compared with OPG alone; *, \( P < 0.001 \), when OPG + heparin is compared with OPG alone.
internalize and degrade OPG through its binding to syndecan-1 (19). A similar process could be explained to the decreased biological activity of full-length OPG in osteosarcoma expressing syndecan-1. Our group recently showed by kinetic studies of molecular interactions that OPG binds to heparin with a high affinity ($K_d = 0.28$ mmol/L) and that the preincubation of OPG with heparin inhibited in a dose-dependent manner the further OPG binding to the RANK-RANKL complex (21). In addition, plasmon resonance analyses reveal in the present report that GAGs strongly interfere with full-length OPG binding to RANKL or TRAIL, suggesting that GAGs may modulate OPG bioavailability and consequently block the inhibitory effects of OPG 1-401 on RANKL and TRAIL biological activities. The present results associated to bibliographic data suggest that OPG 1-401, contrary to OPG 1-194, can be stored in the extracellular matrix of bone tumor by binding to PGs, thereby diminishing its biological activity.

However, other hypotheses cannot be excluded. We do not rule out the possibility that the two death domains present in the 195-401 OPG domain are involved in this phenomenon. Another hypothesis is that OPG 1-401 is able to dimerize, and Schneeweis and colleagues (43) have reported that the exact role of these domains is not elucidated in OPG, one study showed that they are active with a high potential for mediating a cytotoxic signal when they are fused with the transmembrane region of Fas (30). Another hypothesis is that OPG 1-401 is able to insert or take up by endocytosis into cell membrane and induce apoptosis (31) in cancer cells. However, other hypotheses cannot be excluded. We do not rule out the possibility that the two death domains present in the 195-401 OPG domain are involved in this phenomenon.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 7/11/2008; revised 9/19/2008; accepted 10/8/2008.

Grant support: Association pour la Recherche sur le Cancer (fellowship for F. Lamoureux), Ligue Contre le Cancer (comité régional des Pays de la Loire), Fondation d’Europe, and Institut National du Cancer (n°030722N; fellowship for G. Picardia).

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