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

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

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

As evidenced for bone metastases, a vicious cycle between osteoclasts, bone stromal cells/osteoblasts, and cancer cells has been hypothesized during the progression of primary bone tumors (2). Accordingly, suppression of osteoclasts or inhibition of osteoclast activity would be a promising approach to inhibit local cancer growth. Osteoprotegerin (OPG), a soluble protein of the tumor necrosis factor (TNF) receptor superfamilly, offers considerable promise as a new modality for treating osteolysis associated to bone tumors. OPG acts as a decoy receptor and inhibits osteoclast formation, function, and survival by preventing the binding of receptor activator of nuclear factor-κB ligand (RANKL) to RANK (3). Transgenic mice overexpressing OPG exhibit an osteopetrotic phenotype, whereas OPG knock-out mice have severe osteoporosis (4, 5). The OPG/RANKL/RANK system is also involved in various pathologies associated with tumors in the bone (6, 7). For example, increased expression of RANKL has been observed in osteolytic malignancies, such as breast cancer and multiple myeloma (8–10). A recent study showed that the RANKL/OPG ratio was significantly increased in patients suffering from severe osteolysis from tumor origin or not (7). As a result, the inhibition of osteoclastogenesis has been considered as an intervention strategy in the malignant pathologies that develop in bone: treatment of osteolytic bone metastases with OPG inhibits osteolysis and decreases skeletal tumor burden in myeloma and breast cancer (11, 12). Moreover, a recent publication reports that OPG is able to inhibit cancer cell migration and bone metastasis through the inhibition of the RANKL-induced effects in RANK-expressing cells from tumor origin (13). The relevance of using OPG as a therapeutic approach in bone pathologies including osteolytic tumors has been well documented. Indeed, OPG was shown to inhibit tumor-induced...
osteoclastogenesis and bone tumor growth in osteoporotic mice (14), to reduce bone cancer pain by the blockade of the ongoing osteoclast activity (15), to decrease the number and area of radiographically evident lytic bone lesions in a model of mouse colon adenocarcinoma (12), to exhibit beneficial effects in experimental models of myeloma (11, 16), and to inhibit osteolytic lesions associated with prostate cancer (17). However, no studies were done on OPG effects in primary bone tumors.

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

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

Materials and Methods

Cell Lines

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

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

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

OPGΔ Delivered by Synthetic Vectors in a Mouse Model of Osteosarcoma

Plasmids. The cDNA encoding the truncated form (1-194) of murine OPG (named mOPG) kindly provided by Amgen Inc. was transfected into the mOPG pCEP4 to the pcDNA3 plasmid (Invitrogen) between the HindIII and Xhol sites using standard cloning procedures. The pcDNA3-enhanced green fluorescent protein (EGFP) was used as a control.

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

In vivo experimentations

Mouse osteosarcoma model. Four-week-old male C3H/He mice (Elevages Janvier) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France) in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators. The mice were anesthetized by inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with an i.m. injection of atropine (0.05 mg/kg; Temgégic, Schering-Plough) before s.c. inoculation of POS-1 cell suspension (containing 2 × 10⁶ cells in 50 μL PBS) in the hind footpad of the mice.

Under these conditions, mice develop a primary tumor at the site of injection in 3 weeks that can be transplanted to mice of the same strain as a small fragment (2 × 2 × 2 mm³) in close contact with the tibia. For this purpose, the peristium of the diaphysis was opened and resected along a length of 5 mm, and the underlying bone was intact. The osteosarcoma fragment was placed contiguous to the exposed bone surface without the peristium, and the cutaneous and muscular wounds were sutured. Tumors appeared at the graft site approximately 8 days later associated with the development of pulmonary metastases in a 3-week period. The tumor that develops in contact to the femora leads to osteolytic lesions that reproduce the osteolytic form of human osteosarcoma (26). The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a caliper according to the following formula: V = 0.5 × L × S², where L and S are, respectively, the largest and smallest perpendicular tumor diameters.

Relative Tumor Volumes (RTV) were calculated from the formula RTV = (V21/V15), where V21 is the tumor volume on day 21 and V15 is the tumor volume at day 15. A model of pulmonary metastases was used in a separate experiment, induced in 3 weeks by the i.v. injection of 150,000 POS-1 cells in C3H/He mice.

Formulation preparations. For i.m. injections, mice were anesthetized with hypnomidate (40 mg/kg, i.p. injection). About 50 μL of block copolymers/DNA formulations were injected into shaved tibial anterior muscles at one site using a microfine syringe (UL100, Becton Dickinson). Lutrol, a block copolymer consisting of poly(ethyleneoxide) j-poly(propyleneoxide) k-poly(ethyleneoxide) m was generously provided by BASF (Mount Olive, NJ, USA). Stock solutions were prepared at 20% (w/v) in water and stored at 4°C. Formulations of DNA with block copolymers were prepared by equimetric mixing block copolymers in water and DNA solution at the desired concentration (50 μg per muscle) as already reported (22).

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

OPGΔ Delivered by Adenoviral Vectors in a Rat Osteosarcoma Model

In vitro experimentation

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

In vivo experimentations

Rat osteosarcoma model. The osteosarcoma was initially induced by a local injection of colloidal radioactive ⁴⁴ Ca-radium in rats (28). The evolution of the tumor is comparable at the temporal (ratio 1:100 between rats and humans) and physiologic levels to the development of human osteosarcoma. The tumor can be regrafted as described above and maintained for many months, or fragments can be frozen until reuse. Lung metastases develop in contact to the femora leads to osteolytic lesions that reproduce the osteolytic form of human osteosarcoma. The tumor volume (V) was calculated from the measurement of two perpendicular diameters using a caliper according to the following formula: V = 0.5 × L × S², where L and S are, respectively, the largest and smallest perpendicular tumor diameters.
inhalation of a combination isoflurane/air (1.5%, 1 L/min) associated with a
i.m. injection of buprenorphine (0.05 mg/kg). A 10-mm² fragment of
osteosarcoma was placed contiguous to the exposed bone surface as
previously described, and the cutaneous and muscular wounds were
sutured. Tumors appeared at the graft site approximately 7 to 10 days later.

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

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

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

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

**Microscanner and radiological analyses.** Analysis of architectural
parameters was done using the high-resolution X-ray micro-computed
tomography (CT) system for small animal imaging SkyScan-1072 (SkyScan).
Relative volume (BV/TV) of the femora [total bone (cortical + trabecular)
or trabecular bone] was quantified at necropsy in the osteosarcoma groups
that received mOPGΔ (associated with adenosine or synthetic vectors),
compared with that of control mice. Radiographs on anesthetized animals
(Nesdoral, 50 mg/kg) were taken every week and at the time of necropsy
to compare the transfection efficiency between murine myoblasts
POS-1 and OSRGa cells.

**Gene and protein expression analysis**

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

**Osteoprotegerin ELISA**

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

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

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

**In vitro analyses**

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

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

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

**OPG binding on osteosarcoma cells.** A coupling of the FITC on
the mouse OPG-Fc was achieved by using a FluoroTag kit (Sigma). About 1 mg
of OPG-Fc was incubated in the presence of 0.3 μg of FITC protected from
light during 2 h at room temperature. The purification of the OPG-Fc-FITC
was done using a Sephadex G-25M column. The OPG-Fc-FITC was then
quantified by the ELISA technique (R&D Systems). POS-1, OSRGa, and
293LR 2.0 (2.93 cells transfected with the cDNA encoding the full-length
form of hRANKL were used as positive controls) cells were seeded in a 96-
well plate (1 × 10⁴ cells per well) in the presence of 2 μg of OPG-Fc-FITC in
PBS/bovine serum albumin 1% in 1 h at 4°C. At the end of the incubation
period, the cells were washed with PBS and analyzed by flow
cytometry (FACScalibur, data base Biosciences) using the CellQuest
software to analyze the fixation of OPG.

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

**Results**

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

**Transfection efficiency of osteosarcoma cells.** A pilot experiment was designed in vitro using a pcDNA3-EGFP construct to compare the transfection efficiency between murine myoblasts
C2C12 and osteosarcoma cells POS-1 to determine which cell type
shows the best transfection efficiency to direct the injections in the further in vivo experimentations. POS-1 cells showed poor transfection efficiency whatever the method used (synthetic vectors: cationic or amphiphilic biopolymers, lipoplexes, and calcium phosphate). Therefore, the i.m. site was chosen for the further in vivo protocol.

Validation of transgene expression in vitro and in vivo. The aim of this study was to show the induction of mOPGΔ expression using the polyethyleneimine in vitro and the amphiphile block copolymer Lutrol in vivo, which showed the best efficiency, respectively, in vitro and in vivo (21, 29). Preliminary experiments validated the induction of mOPGΔ expression in vitro in C2C12 cells transfected with p-mOPGΔ associated to polyethyleneimine at the mRNA and protein levels, whereas the osteosarcoma POS-1 cells showed a poor transfection efficiency. According to these in vitro results, mice were treated with Lutrol associated with p-mOPGΔ or pcDNA3 alone by i.m. injections (in the tibial anterior muscle). Induction of mOPGΔ transgene expression was evidenced at the trabecular bone level by microscanner analysis (B), BV, bone volume; TV, total volume (n = 8). **, P < 0.001, POS-1/pcDNA3 versus CT; *, P < 0.01, POS-1/mOPGΔ versus POS-1/pcDNA3. Relative bone loss was calculated as [(BV/TV)CT − (BV/TV)POS or mOPGΔ]/(BV/TV)CT.

Protective effect of OPG transgene expression on bone resorption associated with osteosarcoma development. The mouse osteosarcoma POS-1 model used in this study has been previously associated with osteolytic lesions by microscanner analysis (26). Because control tumors and pcDNA3-treated mice exhibit similar bone alterations, only radiographs and microscanner analysis of the tibiae of mice treated with pcDNA3 alone are shown. Radiographs of the POS-1 implanted mouse tibiae revealed a high bone remodeling, resulting in cortical destruction and intensive interactions between altered bone tissue and tumor cells, as compared with control mice (Fig. 1A). Osteolytic lesions were no more observed in osteosarcoma-bearing mice treated with p-mOPGΔ associated with Lutrol, and the long bones exhibited high bone density as observed on radiographies, reflecting decreased bone resorption. These results were confirmed and quantified by bone microarchitecture analysis, showing bone osteolysis both at the total and trabecular bone levels in the POS-1 osteosarcoma group that was abrogated in mOPGΔ-treated animals (Fig. 1B). The specific trabecular bone volume was significantly decreased in osteosarcoma-bearing mice as compared with control animals (respectively, 16.41% versus 27.38%, P < 0.001). OPG treatment induced a partial recovery of the bone microarchitecture (23.51± 4.01 versus 16.41% in osteosarcoma-bearing mice; P < 0.01). The relative bone loss has been deduced from these values and estimated as 14.1% and 40%, respectively, in POS-1–bearing mice treated or not with mOPGΔ.
Overall, these results showed the antibone resorption effect of mOPGΔ delivered by gene transfer using synthetic vectors in a preventive protocol developed in an osteolytic mouse model of osteosarcoma.

**OPG transgene expression limits osteosarcoma progression and augments animal survival.** A preventive protocol was designed to determine not only a potential protective effect of mOPGΔ on bone resorption associated with osteosarcoma development, but also on the tumor progression itself. The results of tumor development of a representative series out of three (six mice per group in the series presented in Results) show both a decrease of the tumor incidence and a diminution of the tumor volume itself (Fig. 2A and B). Indeed, when each animal is considered individually, only 30% of mice developed a primary tumor in the p-mOPGΔ group versus more than 80% in the pcDNA3 group (Fig. 2A and B). Moreover, in the mice that develop primary tumors, the mean tumor volume is smaller in the mOPGΔ-treated mice than in control mice (for example, mean volume of 984 mm³ at day 21, versus 3,166 in the controls; *P* < 0.005). The tumor progression calculated between days 15 and 21 is also strongly inhibited by the mOPGΔ-transgene expression: 1,394 mm³ versus 2,603 mm³ in pcDNA3 mice (*P* < 0.05, Fig. 2C). These results show that mOPGΔ expression diminished the tumor incidence and progression in an osteolytic model of osteosarcoma. As a consequence, overall survival was significantly increased in the mOPGΔ-treated mice: 100% survival 28 days posttumor implantation versus 33% in pcDNA3 (Fig. 2D, *P* < 0.001). The control and pcDNA3 mice died or were sacrificed because of the huge tumor development or respiratory distress caused by pulmonary metastasis dissemination. In this experiment, OPG-treated mice were sacrificed at the same time as control/pcDNA3 mice (28 days posttumor implantation). No pulmonary metastases could be evidenced in the mice that received the mOPGΔ transgene (0/6 at the time of necropsy versus 5/6 in controls).

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

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

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

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

* **P** < 0.01. D, overall survival rate was compared between mice that received pcDNA3/Lutrol formulation (pcDNA3) and p-mOPGΔ/Lutrol (p-mOPGΔ) over a 28-d period.
OPG\(\Delta\) expression protects from bone remodeling associated with tumor development. Ad-mOPG\(\Delta\) (5 \(\times\) 10^9 pfu) was injected i.m. and i.v. to rats bearing osteosarcoma following a curative protocol, when the tumor volume is superior to 1,200 mm^3 (14–16 days posttumor implantation), corresponding to progressive tumors. Microscanner analyses of hemisagittal sections of tibias taken at the time of necropsy revealed that osteosarcoma-bearing rats or receiving Ad-EGFP showed intense bone remodeling with a huge increase of specific total bone volume (respectively, 52.88% and 47.75% compared with 11.21% in normal rats, \(P < 0.001\), Fig. 3B). Because the bone formation process is always preceded by bone resorption (osteolytic lesions have been previously shown in this model by our group ref. 30), a therapeutic strategy using an ant bonere sorption agent is relevant in this model. Rats that received an i.v. injection of Ad-mOPG\(\Delta\) displayed less bone remodeling, as revealed by a significant decrease of the specific bone volume when compared with that of osteosarcoma-bearing control rats (23.97% as compared with 47.75% and 52.88%, respectively, for Ad-EGFP and control rats bearing osteosarcoma, \(P < 0.01\)). OPG treatment contrasts to the increased bone formation associated with tumor development, but cannot totally inhibit it. Both osteolytic lesions and bone formation, characteristic of osteosarcoma development, were clearly evidenced on radiographs of osteosarcoma-bearing rats (Fig. 3C). These results validated the biological activity of the OPG\(\Delta\) transgene at the bone level.

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

<table>
<thead>
<tr>
<th>BV/TV (%) (mean (\pm) SD)</th>
<th>Control</th>
<th>Osteosarcoma</th>
<th>Osteosarcoma Ad-EGFP</th>
<th>Osteosarcoma Ad-mOPG(\Delta)</th>
</tr>
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<tbody>
<tr>
<td>Total bone</td>
<td>11.21 (\pm) 2.1</td>
<td>52.88 (\pm) 7.92</td>
<td>47.75 (\pm) 8.59</td>
<td>23.97 (\pm) 3.84 $^\dagger$</td>
</tr>
<tr>
<td>Trabecular bone</td>
<td>7.77 (\pm) 1.32</td>
<td>34.65 (\pm) 5.89</td>
<td>27.83 (\pm) 4.56</td>
<td>18.61 (\pm) 3.25 $^\dagger$</td>
</tr>
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\(P < 0.001\), osteosarcoma or osteosarcoma/Ad-EGFP versus control. $^\dagger\, P < 0.01$, Osteosarcoma/Ad-mOPG\(\Delta\) versus osteosarcoma/Ad-EGFP.
developed progressive tumor (Fig. 4A, mean volume of 3,410 mm³ 43 days postinoculation, range 1,372–6,534 mm³, versus 0 for Ad-mOPGΔ; \( P < 0.001 \); Fig. 4B). When the adenovirus construct was injected i.m., intermediary results were observed: 50% of tumors were progressive (mean tumor volume of 3,137 mm³, 43 days postinoculation), 25% stopped their progression (936 and 850 mm³ at day 43), and 25% totally regressed (Fig. 4C). As a result, animals that received an i.v. injection of Ad-mOPGΔ showed a high increase in survival (100% versus 50% in the control group, 65 days posttumor implantation, \( P < 0.001 \), Fig. 4D). However, overall survival was not significantly modified between the Ad-OPGΔ i.m. and control groups (62.5% and 50%, respectively, 65 days posttumor implantation).

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

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

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

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

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

![Figure 4](https://example.com/image4.png)

**Figure 4.** Ad-mOPG administered as a curative protocol by i.v. injection induces a total regression of tumor development in a rat model of osteosarcoma. Rats implanted with osteosarcoma fragment (as described in Fig. 3) were treated with Ad-EGFP (5 × 10⁹ pfu, \( n = 8 \); A) or with 5 × 10⁹ pfu Ad-mOPGΔ i.v. (\( n = 4 \); B) or i.m. (\( n = 8 \); C) 16 d posttumor implantation, i.e., when tumors were progressive. The tumor volume was calculated as described in Fig. 3. D, overall survival rate was compared between control rats that received Ad-EGFP (CT) and rats that received Ad-mOPGΔ i.v. or i.m. over a 65-d period. ***, \( P < 0.001 \).
showed no modulation of gene transcription in POS-1 and OSRGa cells in the presence of 100 ng/mL OPG-Fc (data not shown).

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

**Indirect Inhibitory Antitumor Effect of OPG via RANKL Inhibition**

**Apoptosis induced by OPG in vivo.** TUNEL staining on biopsies of POS-1 tumors revealed the presence of few apoptotic cells in the tumor itself, corresponding to the necrosis areas. An increase of the number of apoptotic cells was evidenced in OPG-treated mice, which may explain the inhibition of tumor development previously observed (Fig. 6A).

**Osteoclast number as measured by TRACP 5b activity.** Two forms of TRACP circulate in blood, known as TRACP 5a and TRACP 5b; TRACP 5b being derived from osteoclasts and TRACP 5a from macrophages. Osteoclasts secrete TRACP 5b into the blood circulation. Recent studies have shown that TRAP 5b indicates the number of osteoclasts rather than their activity (31, 32). Figure 6B shows that OPG-treated mice evidenced a 51.6% reduction in
osteoclast number as compared with osteosarcoma-bearing mice ($P < 0.001$).

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

**Discussion**

Osteosarcomas represent the most frequent primary malignant bone tumor, with a 5-year survival rate of about 60% to 70%, decreasing to only 20% when pulmonary metastases are detected at the time of diagnosis. The vicious cycle that has been described in osteolytic metastases consists of release of osteolytic mediators by tumor cells, bone degradation, release of growth factors from degraded bone, enhanced tumor cell growth, and further release of osteolytic mediators (2). Inhibitors of bone resorption thus appear one of the more promising tools to manage skeletal metastases. One can speculate that this vicious cycle may also apply in the case of the primary bone tumors, and that inhibitors of bone resorption such as OPG may interfere with primary tumor development at a skeletal site. Delivery of Fc-OPG as a recombinant protein has shown promise as a potential therapy through experiments in animal models in that OPG limits hypercalcemia and osteolysis induced by myeloma, breast, lung, or prostate cancer and reduces tumor establishment in bone (11, 12, 33, 34).

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

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

![Figure 6](image.png)

*Figure 6.* Comparison of tumor cell apoptosis, osteoclast number, and RANKL expression between POS-1 osteosarcoma bearing mice treated or not with p-mOPGΔ. A, tumor cell apoptosis was investigated *in vivo* by TUNEL staining and compared between POS-1 mice treated or not with OPG (POS-1/p-mOPGΔ). Original magnification, ×400. B, the effect of p-mOPGΔ on osteoclast number was studied by TRACP 5b activity in the serum of POS-1 osteosarcoma bearing mice. C, RANKL production was measured by ELISA test in the serum of POS-1 osteosarcoma-bearing mice treated or not with OPG (POS-1/p-mOPGΔ) as compared with control mice (CT). Delta, localization of RANKL expression was investigated by immunohistochemistry in the tumor microenvironment of POS-1 osteosarcoma-bearing mice treated or not with OPG (POS-1 and POS-1/p-mOPGΔ). Original magnification, ×640.
together with direct bone formation by tumor cells (30). In this model, even if the modifications of bone remodeling result in an overall bone formation as quantified by microarchitectural parameters (BV/TV), the initial process is always osteolysis. In these conditions, the use of OPG as an antibone resorption molecule is justified. Because OPG is a decoy receptor for TRAIL, blocking TRAIL-induced apoptosis of several tumor cell lines, it could confer a survival advantage on cancer cells (35, 36). Indeed, recent data from the literature highlighted the differences of biological activity between full-length OPG produced by tumor cells and therapeutic administered Fc-OPG (37). Therefore, to avoid a potential survival role of OPG on osteosarcoma cells, the DNA construct encoding the truncated form of OPG (1-194) was used in this study.

The preventing effect of OPG on osteosarcoma-associated osteolysis is the first report of its efficacy as an antibone resorption molecule in vivo in a model of primary bone tumor. OPG has been reported to exert antibone resorption activities through RANKL inhibition in several pathologic osteolyses. For example, OPG was shown to augment bone volume with reduced osteoclast number (18), reverse established osteopenia (38) in ovariectomized mice, or prevent osteoclastic resorption in wear debris-induced osteolysis (39). OPG inhibitory activity (inhibition of osteoclast formation and bone-resorbing activity) has also been reported in osteolysis associated with tumor pathologies as in giant cell tumor of bone, in which both the osteostat formation and activation processes are promoted by RANKL (40). In multiple myeloma, tumor cells express RANKL, and treatment of mice with recombinant OPG protein prevented the development of osteolytic lesions (11). OPG also inhibits osteoclastogenesis in prostate cancer (17, 34) and mouse models of experimental bone metastasis (12). In most of the osteolysis models associated with tumor development, OPG also shows antitumor activity. Because the RANKL system may be abnormally regulated in bone tumor, targeting this system may represent a novel therapeutic approach. For example, besides murine models of myeloma (11, 16) and prostate cancer (17, 34), targeting this system with OPG has been shown to inhibit the development of bone disease in vivo in established osteolytic prostate cancer bone metastases (41). A recent study of Corey et al. (42) revealed that OPG overexpression in prostate cancer cells does not directly affect proliferation of tumor cells, but indirectly decreases growth of prostate tumors in the bone environment. The experiments described in the present study revealed similar results in two models of primary bone tumor (osteosarcoma), with no direct effect of OPG on tumor cells as revealed by the absence of OPG binding on osteosarcoma cells and confirmed by the absence of OPG effect on cell proliferation, apoptosis, cell cycle distribution, or gene expression, but an indirect preventing effect on tumor development via the inhibition of associated osteolysis. The hypothesis relies on the fact that OPG, by inhibiting bone resorption, causes not only a reduction in the extent of osteolytic lesions, but also deprives tumor cells of bone-derived growth factors that are required for tumor-cell proliferation (2). This OPG inhibitory effect on tumor progression may be the consequence of the RANK-RANKL axis blockade because RANKL concentration is increased in the serum of osteosarcoma-bearing mice, reflecting an increase of RANKL production in the tumor microenvironment caused by a direct RANKL expression by osteosarcoma cells. This hypothesis was indeed confirmed in our osteosarcoma models, as an inhibition of RANKL expression was evidenced in OPG-treated animals. In other bone tumor models, RANKL can be directly produced by tumor cells (11, 34) or is present in the tumor environment, produced by neighboring cells (8, 43). We previously reported that POS-1 osteosarcoma cells express RANK but not RANKL in vitro (26), and the present results suggest that in vivo, some factors present in the bone tumor environment induce RANKL expression by tumor cells themselves, such as growth factors released by bone degradation. The therapeutic hypothesis that is emerging for bone tumors focuses on targeting bone microenvironment rather than tumor cells themselves. A recent study from Jones et al. (13) shows that in vivo neutralization of RANKL by OPG results in complete protection from paralysis and a marked reduction in tumor burden in bones in a mouse model of melanoma metastasis, but not in other organs. In the 5T2MM murine model of myeloma, the OPG-like peptidomimetic OP3-4 that selectively inhibits RANKL but not TRAIL prevented the tumor-induced decrease in the cancerous bone area and the development of osteolytic lesions, but also reduced tumor burden when compared with the controls (44). The importance of the microenvironment of the local host tissue that actively participates in the propensity of certain cancers to metastasize to the bone has been studied, confirming the "seed-and-soil" hypothesis proposed by Paget (45): metastasis depends on cross-talks between selected cancer cells (the seeds) and specific organ microenvironment (the soil). Among the homeostatic factors that promote tumor cell growth in the bone, RANKL is a good candidate influencing the local bone resorption process that is necessary for tumor development in bone site (46).

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

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