Osteoprotegerin activates osteosarcoma cells that co-express RANK and RANKL

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ABSTRACT

Background: Osteosarcoma (OS) is an aggressive and often fatal cancer that affects over 1000 humans and 10,000 dogs per year in the United States. Recent evidence suggests deregulation in the signaling triad, receptor activator of nuclear factor kappa B (RANK), its activating ligand (RANKL), and the RANKL inhibitor, osteoprotegerin (OPG) plays a key role in the pathogenesis of OS. This study investigated the expression of RANK and RANKL in osteosarcoma tumors and cell lines and describes an activating effect of OPG on OS cells in vitro.

Results: Canine OS tumors and cell lines co-express mRNA for both RANK and RANKL. Expression of these proteins in OS cell lines was confirmed by Western blot and immunofluorescence microscopy. Expression of the soluble form of RANKL was not detected in media from OS cells. OPG-Fc incubation increased the phosphorylation status of ERK, AKT and the p65 subunit of nuclear factor kappa B (NFκB) and induced NFκB translocation from the cytoplasm to the nucleus in canine OS cells. OPG increased proliferation in both canine and human derived OS cell lines.

Conclusion: RANKL is produced by OS tumors and cell lines that also express RANK. This data provides preliminary evidence for a potential autocrine and paracrine activation pathway in canine OS. An activating effect of exogenous OPG on signal transduction proteins, NFκB and proliferation in OS is described. These data provide new information concerning aberrant signaling in OS and could be important to those considering OPG as a therapeutic agent for osteosarcoma.

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1. Introduction

Osteosarcoma (OS) is an aggressive, highly invasive bone tumor of mesenchymal origin [1]. It is characterized by cells with osteoblastic differentiation, poorly defined borders and cortical destruction that results in bone pain and compromised structural integrity [1,2]. The neoplasm typically arises in a long bone, grows rapidly, and causes early death through metastasis in dogs. Accelerated resorption of bone at the tumor site results in the release of growth factors and cytokines that accelerate tumor progression, vascularization and metastatic potential. The major sites of metastasis are the lungs [3–5].

Because OS occurs more frequently in dogs, its presence in dogs provides a convenient and relevant model for the development of new therapies that may be effective for the treatment of OS in humans [6]. In particular, access to a large number of dogs with OS provides a model system from which an improved understanding of the roles of Receptor Activator of Nuclear Factor κB Ligand (RANKL), and its negative regulator osteoprotegerin (OPG) may be developed [7]. In healthy bone, RANKL is necessary for osteoclast differentiation and regulates osteoclast-mediated bone resorption [8,9]. Blocking RANKL, which exists in both membrane-bound and soluble forms, results in osteoclast death by apoptosis [10]. While RANKL is necessary to osteoclast differentiation, function and survival, the role of RANKL signaling in OS is not well understood.

Aberrant production of RANKL by tumor cells may be responsible for the increased osteoclast number and activity found in both primary and metastatic osteolytic tumors [11]. RANKL has been shown to be expressed in a variety of tumor types including canine osteosarcoma [12] and is implicated in metastasis [13] and tumor progression in several human cancers [11]. These and other studies suggest RANKL may be a critical mechanism by which tumor cells abnormally increase bone resorption [7] and this has led to the idea that OPG could potentially attenuate this effect in osteolytic tumors.
In one study, OPG inhibited tumor progression, osteolysis and osteoclast number in a mouse model of osteosarcoma [5] but other reports describe a protective/permisive effect of OPG in breast, [14], prostate [15] and myeloma cells in vitro [16]. These studies provided evidence for an indirect effect of OPG through binding of TNF-related apoptosis inducing ligand (TRAIL) and OPG was shown to reduce TRAIL-induced apoptosis in cultured cells. Others have shown that OPG is involved in tumor-related angiogenesis [17] and increased OPG expression has been correlated with tumor grade in breast cancer tissue biopsies [18] but few studies have reported a direct effect of OPG on cell function. In this regard, Kobayashi-Sakamoto et al. reported that incubation with OPG rapidly phosphorylated extra-cellular related kinase (ERK) and focal adhesion kinase (FAK) and enhanced invasion in endothelial cells in vitro [19]. Others have described a direct effect of OPG on human osteoblastic and ovarian cancer cells [20,21] suggesting OPG has multiple physiologic actions that have yet to be elucidated.

The current study describes the expression of RANK and RANKL in canine OS tumors and cell lines. Further, we describe an apparent paradoxical activation of proliferation and phosphorylation of cytoplasmic signal transduction proteins including the transcription factor nuclear factor kappa B in OS cells treated with OPG.

2. Methods

2.1. Cell culture

One human and four canine OS cell lines were used in this study. The canine cell lines included D17 (ATCC CCL183), Clone 48-4, [22], COS [23], and POS [24]. The human OS cell line (Saos-2) was purchased from American Type Culture Collection Co. (Manassas, VA). Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere in RPMI 1640 (Invitrogen) supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 2 mM HEPES, 1% pen-strep, and 10% fetal bovine serum (from here forward referred to as R10). An osteoprotegrin–immunoglobulin Fc (OPG-Fc) fusion protein produced in yeast containing the below sequence was obtained commercially (GenWay Biotechnology, San Diego) and suspended in sterile water prior to use. OPG portion: ETFPPKYLHEDESHQCLDCCKPCPTVLKQHCJTAHKWT VCACPDPHY TDWSHTDELYSCPYVCWKLYQKCNRTHNRCVECK EGRYLEIEFL HCRSSPCGFCVQAGHTPNVTCKRCDPGFSSHNETSSKA PCKERHTNC VGFLLITQKGNAHNDICSNSESTQKCGIDVT Fc232 portion: EPKSSDKHTCCPPCPAPEFGAPSFLPPKPKDLMIS RTPETVCVVVDV SHEDPEFKNFWYVDGVHEVHNAKTKPREEQYNSTYR VSVLTLHQDWLN GKEYCKCVSNKALPTEKTSKAKGQREPOVVYTLPSSREDLTNKQVSLTC LVKGFYPFDIAVEWSENSQPPENNYKTTPDVLDS DGSFLYSLKTVDKSRRW QQGVNFSVMHEALNHYTQKSLSPGBK

2.2. Tumor samples

Tumor samples were collected from dogs with naturally occurring OS (Table 1). Tumors were excised by amputation during limb salvage surgery, or rib excision and samples were held on ice in RPMI 1640 then rinsed with sterile saline and preserved in an centrifuge (5 min, 300xg). The supernatants were discarded and the cell pellets were re-suspended in “RLT extraction buffer”, sonicated four times (1 s each) using an ultrasonic dismembranator (Fisher, Model 150T) and centrifuged at 10,000xg prior to loading the supernatants on individual extraction columns. RNA was extracted from tumor tissue that had been pulverized under liquid nitrogen using the same procedure except that pulverized frozen tissue was suspended directly in “RLT buffer” (Qiagen #74106). Purified RNA was eluted in sterile water and concentrations were measured by spectrophotometry (Thermo Scientific, ND-1000). One μg of each RNA sample was converted to cDNA by reverse transcription as directed (Applied Biosystems, kit #4368814). Standard PCR was used to amplify signals for RANK and RANKL using the following primers RANK forward; GCCGTGAATTTTGATAGTGTCG, and reverse; GCTCTCTTGCTCCAGTGAAG (product = 346 bp), and RANKL forward; CTGTACTACATGACCAGGT, and reverse, AAACCTCGG- AGTTTGATAGTGTCG (product = 196 bp). PCR products were separated by electrophoresis using 1.5% agarose with added fluorescent dye (Invitrogen #33102) for visualization. PCR products were purified using a magnetic bead method (Invitrogen #C12000) prior to sequencing on an ABI Prism 3130 Genetic Analyzer.

2.4. Western blots

Cells grown in 75 cm² flasks to approximately 80% confluence were rinsed in ice cold PBS, removed with a cell scraper, and pelleted in a tabletop centrifuge (3 min, 1200 x g). Proteins were extracted either directly from whole cell pellets, or from cytoplasmic/nuclear separations using 50 μl of ice cold RIPA buffer with protease and phosphatase inhibitor cocktails (Sigma). Cytoplasmic and nuclear extractions were performed using a commercial kit as directed (Invitrogen, #78835). After solubilization in RIPA buffer, the extracts were sonicated four times (1 s each) using an ultrasonic dismembranator (Fisher, Model 150T) and pelleted at 10,000xg to remove cellular debris. Protein concentration was measured in triplicate using a commercial kit (Pierce, BCA). Proteins (20 μg/lane) were separated on 4–12% SDS polyacrylamide gels and transferred to PVDF membranes using standard methods. The membranes were blocked in 1.5% albumin in TTBS and reacted with indicated primary antibodies diluted 1:400 overnight at 4 °C (antibodies from Santa Cruz Biotechnology (SCBT); RANK, sc-34249; RANKL, sc-9073; AKT, sc-1619; pAKT, sc-33437; ERK, sc-93; pERK, sc-7383; p65, sc-109; pP65, sc-101752). A recombinant form of soluble RANKL was used as a positive control for Western blots (Genway Biotechnology #GWB-B354EC). The membranes were washed, reacted with horseradish peroxidase-linked secondary antibody (SCBT, sc-2005) diluted 40,000:1, exposed to substrate (ECL Select, GE Healthcare) and imaged using a digital imaging system (Image Quant LAS 4000, GE Healthcare). Signals from digital images were assessed for band density using Image Quant TL software (GE Healthcare). Density values (arbitrary units) were

<table>
<thead>
<tr>
<th>Case ID</th>
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<th>Sex</th>
<th>Age</th>
<th>Weight</th>
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<tbody>
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<tr>
<td>E</td>
<td>German Shepherd mix</td>
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<td>6 yrs</td>
<td>24 kg</td>
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</tr>
<tr>
<td>F</td>
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<td>9 yrs</td>
<td>35 kg</td>
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<tr>
<td>G</td>
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<td>11 yrs</td>
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normalized in order to present data in histogram form. Normalization was accomplished by dividing the density values obtained from the phosphorylated blot (untreated sample) by the values from the total protein blot from the same sample. This baseline value was used as a divisor of subsequent ratios to show changes due to OPG-Fc treatment.

Example: density of p-AKT band = 2700
density of total AKT band = 5600
baseline ratio (2700/5600) = 0.4821
normalization untreated (0.4821/0.4821) = 1

Data presented are representative of at least two independent experiments.

2.5. Immunofluorescence

Cells grown on coverslips were rinsed with PBS then fixed in a 50/50 solution of cold acetone and methanol for 60 seconds, allowed to air dry, blocked in 1.5% albumin in TTBS (blocking buffer) and reacted with indicated primary antibodies, or isotype controls, diluted 1:50 in blocking buffer for 1 hour at room temperature (antibodies from Santa Cruz Biotechnology; RANK, sc-34249; RANKL, sc-9073). Normal mouse or rabbit IgG were used as isotype controls for RANK and RANKL stained cells respectively (SCBT, sc-2025, sc-3888). After blocking, the slides were rinsed in PBS and incubated for 45 min at room temperature with fluorophore-linked secondary antibodies (anti-rabbit/mouse IgG) for detection. Slides were rinsed again with PBS, stained with dimethyl-formamide diluted to 100 ng/ml in PBS, to illuminate nuclei, cover-slipped and viewed using the fluorescence microscope with attached camera (Nikon Eclipse ti). Slides stained with primary antibodies and isotype controls had identical camera settings, exposure times and digital processing. Images are representative of two independent experiments.

2.6. Proliferation assay

The effects of OPG-Fc on cellular proliferation were determined using the COS and SAOS-2 cell lines. Cells in R10 medium were seeded in 96-well plates, at a density 5000 cells per well, and allowed to adhere overnight. The medium was then replaced with an identical medium but without serum (Hereafter referred to as R0). The cells were incubated for an additional 24 h in R0 medium which was then replaced with fresh R0 containing either 0, 1.5, 3, 6 or 10 μg/ml OPG-Fc. After 24 h incubation with OPG-Fc, cell viability in triplicate wells was assessed using an MTS assay (Cell Titer 96 One Solution Assay, Promega). RANKL/OPG-Fc combination incubations used 100 ng/ml RANKL and 10 μg/ml OPG-Fc in R0 medium. Cells were incubated overnight in R0 prior to use and the incubation periods were 48 h. RANKL was purchased commercially (#6449-TEC-010, R&D Systems, Minneapolis, MN) and suspended in sterile PBS+0.5% albumin (Invitrogen) prior to use. Absorbance was measured at λ=490 following 60 min incubation at 37 °C with the MTS product. Data presented are representative of three independent experiments.

2.7. Invasion chamber assay

Invasion studies with the COS cell line were performed using reduced growth-factor invasion chambers containing a membrane with 8 μm pores (BD Biosciences, Bedford, MA) according to the manufacturer’s instructions. Briefly, cells were seeded at a density of 25,000/well into the top chamber in R0 medium. The bottom chambers contained R10 medium. OPG-Fc was added to both the top and bottom chambers, when appropriate, so the only difference between the top and bottom chambers was the 10% serum. After 24-h, the membranes were removed, stained with Diff Quik, mounted on glass slides and imaged on a microscope. All cells that had migrated through each membrane were counted and each experiment was performed three times.

2.8. Statistics

Statistical comparisons using a one-way ANOVA with Tukey’s post hoc tests were performed, when appropriate, using Graphpad Prism software (La Jolla, CA). Statistical significance was set at p < 0.05 for all comparisons.

3. Results

3.1. Expression of RANK and RANKL

The canine OS tumors and cell lines tested showed widespread expression of both RANK and RANKL mRNA (Fig. 1A). Only one tumor sample was negative for mRNA signal for either protein and this was a rare telangiectatic osteosarcoma [25] (Table 1 and Fig. 1: Case ID ‘A’). The results from the sequencing reactions using PCR products from the COS cell line and tumor samples B–D matched exactly with the published sequences for the canine RANK and RANKL genes (NCBI gene ID; 483957 and 609418 respectively, Fig. 1B). No mutations or single nucleotide polymorphisms were noted in the sequenced regions (data not shown). RANK and RANKL protein expression in 4 cell lines, shown by Western blot, corroborates the mRNA data (Fig. 1C). Immunofluorescence staining in 4 cell lines suggests RANK and RANKL proteins are co-expressed in the same cell (Fig. 2).

3.2. Activating effects of OPG-Fc

Our findings of widespread RANK and RANKL expression in canine OS led us to hypothesize the expression of the soluble form of RANKL might act as a paracrine or autocrine signal that could drive tumor growth. Further, we hypothesized this mechanism might represent an option for therapeutic intervention because OPG is a commercially available drug. In this regard, we were unable to detect the soluble form of RANKL in conditioned media using commercially available ELISA kits (Amsbio, Cambridge, #E08R0005: RnDSystems, Minneapolis, #MTR00, data not shown) or by Western blot (Fig. 1D). Having detected no evidence of soluble RANKL, we speculated the membrane form of RANKL could potentially retain the ability to bind its receptor through cell-cell contact. Consequently we attempted to inhibit this signal using OPG-Fc. The activating effect of OPG-Fc on proliferation in these cells is shown in Fig. 3. After only 24 h incubation, the cells exposed to OPG-Fc were visually more dense (data not shown) and the MTS assay shows a dose response relationship (p < .05) in the COS cell line for every data point up to and including the highest concentration tested (10 μg/ml, Fig. 3A). Fig. 3B shows a similar but less pronounced effect of OPG-Fc on Saos-2 cells.

3.3. Mechanisms of OPG-Fc stimulation

Western blots, using whole cell lysates and lysate proteins from the COS cell line that had been separated into cytoplasmic and nuclear fractions, were used to begin to describe the mechanisms of action of OPG-Fc in canine OS. The cytoplasmic signal transduction proteins AKT and ERK both showed an increase in phosphorylation after 30 min incubation with OPG-Fc (Fig. 4). Histogram representation of densitometry data from the Western blots shown in Fig. 4A shows OPG-Fc incubation caused a 4–5 fold increase in the ratio of phosphorylated versus total AKT and ERK in
the cytoplasm (Fig. 4B). ERK and AKT were found in both the cytoplasmic and nuclear fractions and the pattern of increased phosphorylation was similar in the nuclear fractions but of smaller magnitude (Fig. 4B). Nuclear/cytoplasmic separations were carried out because NFkB moves, when activated, from the cytoplasm to the nucleus. This study used the P65 sub-unit of NFkB as an indicator of NFkB activity. The Western blot data shown in Fig. 4 suggests total P65 protein declined in the cytoplasm after 30 min exposure to OPG-Fc but the expected concomitant increase in nuclear P65 was not observed at this time point. Consequently we increased the OPG-Fc incubation time to 24 h. After 24 h, the cells exposed to OPG-Fc showed a slight decrease in total P65 Western blot signal in the cytoplasmic fraction and a dramatic increase in the nuclear fraction suggesting the protein was activated and

Fig. 1. RANK and RANKL expression in canine osteosarcoma. A) PCR amplifications of RANK and RANKL transcripts indicate strong expression in both OS cell lines and primary tumors. Signalment for patients A through J are shown in Table 1. B) PCR products were sequenced and, in all cases, confirmed homology with published sequences (NCBI gene IDs: 483957 and 609418). C) Western blots from OS cell lines confirm protein expression of RANK and RANKL. D) The soluble form of RANKL was not detected in cell lysates by western blot or in conditioned media by ELISA (data not shown).

Fig. 2. Immuno-fluorescence. Cells stained with green- (FITC) and red- (TRITC) secondary linked fluorophores show co-expression of RANK and RANKL respectively. The merged images include blue-stained nuclei. The orange color indicates where RANK and RANKL appear co-expressed. Bars equal 10 μm. There was mild positive signal (green) from the isotype control sera matching the RANK primary antibody (data not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
transported from the cytoplasm to the nucleus (Fig. 5A and B). A dramatic increase in the phosphorylated form of P65 was also observed in the nuclear fraction (Fig. 5B) providing strong evidence that OPG-Fc activates P65 and induces its transport to the nucleus.

3.4. Effects of OPG-Fc on invasion

Others have shown that OPG acts through αv integrins to induce invasion in endothelial cells [26]. This effect was not observed in COS cells exposed to 2 μg/ml OPG-Fc (Fig. 6A). Higher concentrations were not tested.

3.5. Effects of RANKL alone and in combination with OPG-Fc on proliferation

RANKL alone (100 ng/ml) had no apparent effect on proliferation in the COS or Saos-2 cell lines (Fig. 6B). Proliferation was not different between cells incubated with OPG-Fc alone and cells incubated with OPG-Fc/RANKL combined.

4. Discussion

The osteolytic invasion that characterizes OS may be driven by RANKL-producing tumor cells that activate NFκB in osteoclasts and drive their development and function. The resulting degradation of bone likely causes the release of sequestered growth factors to create a feed-forward loop [27]. The potential downstream consequences of this cascade of events are numerous and the literature contains multiple references to the effects of aberrant activation of the NFκB pathway in tumor cells. These include proliferation, invasion, angiogenesis, inflammation and metastasis, many of which are hallmarks of malignant cancer [28,29]. This study describes widespread expression of RANK and RANKL in canine OS tumors and cell lines however, the function of these in OS has not been determined. Alternative tests such as, siRNA knockdown of RANK, are needed to further elucidate the importance of the co-expression of RANK and RANKL in OS.

Novel information suggesting incubation with OPG-Fc phosphorylates signal transduction proteins, activates NFκB and stimulates the growth of OS cells in vitro is presented. A recent finding that OPG is elevated in the serum of human patients with malignant or benign bone tumors [30] suggests OPG could be one component of a complex system that drives bone tumor growth. Kobayashi-Sakamoto and colleagues showed OPG activates Src and FAK in endothelial cells and suggest this mechanism may be relevant to tumor angiogenesis, vascular disease and periodontitis [19]. Similar to other published studies [19], the OPG concentrations required to elicit a significant response in the current work were higher than could be achieved pharmacologically. Nonetheless, the current study provides additional information.
concerning the OPG activation pathway and suggests OPG signaling could culminate in the phosphorylation and transport of NFκB from the cytoplasm to the nucleus. Whether this is directly linked to the expression or activation of RANK and RANKL remains to be determined.

Both the membrane and soluble forms of RANKL are likely capable of activating OS cells, however the soluble form was not detected in the cells used in the present study. This could be because the soluble form is not produced in these cell lines, or because the conditions for its production are not replicated under the current cell culture conditions. Additional tests using alternative model systems are likely needed to establish the significance of the current result.

The mechanism and conditions where RANK is activated through membrane-bound RANKL is not well described. In this regard, and based on a large body of literature suggesting the primary function of OPG is to reduce RANK signaling, we sought to block RANK activation using exogenous OPG-Fc. Thus we were surprised to find OPG-Fc activated rather than suppressed growth in these cells. Lamoureaux and colleagues described a suppressive effect of OPG on tumor growth using gene transfer methods in two models of OS in rodents [5] however their report did not show a direct effect of OPG on murine OS cells. In the current study, the effects of OPG-Fc on two cell lines are described therefore no generalization to OS is warranted and further studies are needed. The present data describe a dramatic increase in OPG-induced phosphorylation of AKT and ERK which suggests OPG either directly or indirectly activates a membrane bound receptor tyrosine kinase upstream of these signaling proteins. Future efforts to identify this receptor are warranted.

The lack of an observed growth stimulatory effect of 100 ng/ml RANKL on the COS and Saos-2 cell lines suggests the receptor RANK could be saturated, or not relevant to the proliferative pathway in these cell lines. In this regard, Mori et. al. published two papers in 2007 showing the same concentration of RANKL (100 ng/ml) phosphorylates ERK and alters gene expression in Saos-2 cells but no changes to proliferation, invasion or other functions were observed [31,32]. The current data corroborates these prior reports and provides new information showing that OPG has pro-proliferative effects on OS cells that are independent of RANKL.

5. Conclusion

The co-expression of RANK and RANKL in OS may have important implications concerning the aggressive, fast growing and metastatic nature of this neoplasm. The pro-proliferative effect of OPG-Fc on these cells warrants further investigation in additional OS cell lines and tumors.
Competing interests

The authors declare they have no competing interests.

Authors’ contributions

KM was responsible for conception of the study, design of the experiments, data collection and interpretation and drafted the manuscript. SB was responsible for design of experiments, interpretation of data and revision of manuscript. BS was responsible for design of experiments, interpretation of data and revision of manuscript. All authors read and approved the final manuscript.

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