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2-Methoxyestradiol-Mediated Anti-Tumor Effect Increases Osteoprotegrin Expression in Osteosarcoma Cells

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ABSTRACT

Osteosarcoma is a bone tumor that frequently develops during adolescence. 2-Methoxyestradiol (2-ME), a naturally occurring metabolite of 17β-estradiol, induces cell cycle arrest and cell death in human osteosarcoma cells. To investigate whether the osteoprotegrin (OPG) protein plays a role in 2-ME actions, we studied the effect of 2-ME treatment on OPG gene expression in human osteosarcoma cells. 2-ME treatment induced OPG gene promoter activity and mRNA levels. Also, Western blot analysis showed that 2-ME treatment increased OPG protein levels in MG63, KHOS, 143B and LM7 osteosarcoma cells by 3-, 1.9-, 2.8-, and 2.5-fold, respectively, but did not affect OPG expression in normal bone cells. In addition, increases in OPG protein levels were observed in osteosarcoma cell culture media after 3 days of 2-ME treatment. The effect of 2-ME on osteosarcoma cells was ligand-specific as parent estrogen, 17β-estradiol and a tumorigenic estrogen metabolite, 16α-hydroxyestradiol, which do not affect osteosarcoma cell cycle and cell death, had no effect on OPG protein expression. Furthermore, co-treating osteosarcoma cells with OPG protein did not further enhance 2-ME-mediated anti-tumor effects. OPG-released in 2-ME-treated cultures led to an increase in osteoblastic activity and a decrease in osteoclast number, respectively. These findings suggest that OPG is not directly involved in 2-ME-mediated anti-proliferative effects in osteosarcoma cells, but rather participates in anti-resorptive functions of 2-ME in bone tumor environment. J. Cell. Biochem. 109: 950–956, 2010.

KEY WORDS: 2-METHOXYESTRADIOL; OSTEOSARCOMA; OPG. OPGL; BONE CANCER

Osteosarcoma is the most common primary malignant bone tumor in children and young adults. It is the 6th leading cancer in children under 15 years. Although the overall incidence is low, about 30% of patients diagnosed with osteosarcoma will develop metastatic diseases [Ward et al., 1994; O'Reilly et al., 1996]. Despite improvement in treatment involving a combination of surgery and chemotherapy in the last few years, a definite therapy is yet to be developed for osteosarcoma

2-Methoxyestradiol (2-ME) is a metabolite of the hormone 17β-estradiol. Unlike the parent compound that stimulates proliferation of estrogen-receptor dependent cancer cells, 2-ME has been shown to inhibit tumor growth in cell lines and tissues, which includes breast cancer, cervical cancer, colon cancer, eye tumor, lung cancer, melanoma, muscle tumor, neural tumor, ovarian cancer, prostate cancer and renal cancer [Fotsis et al., 1994; Mukhopadhyay and Roth, 1997; Seegers et al., 1997; Mukhopadhyay and Roth, 1998; Schumacher et al., 1999; Pribluda et al., 2000; Qadan et al., 2001; Mueck et al., 2002; LaVallee et al., 2003; Lippert et al., 2003; Dobos et al., 2004; Ricker et al., 2004; Sutherland et al., 2005; Garcia et al., 2006; Kang et al., 2006; Cicek et al., 2007]. We have shown that 2-ME does not affect the growth of normal osteoblasts, but induces cell death in osteosarcoma cells as well as in osteoclasts [Maran et al., 2002, 2006, 2008; Shogren et al., 2007]. The mechanism for anti-proliferative actions involves cell-cycle block [Maran et al., 2008], apoptosis [Maran et al., 2002; Shogren et al., 2007].

OPG/RANKL/RANK triad has been implicated in the regulation of bone metabolism [Khosla, 2001; Theoleyre et al., 2004]. OPG has been shown to inhibit tumor-induced osteolysis. OPG blocks cancer cell migration and bone metastasis through the inhibition of RANKL-induced effects in tumors. Alterations in OPG/RANKL ratio and increases in RANKL activity have been demonstrated in osteolytic tumors. The aim of this investigation is to determine whether OPG protein participates in 2-ME-mediated anti-tumor effects in osteosarcoma.

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MATERIALS AND METHODS

METABOLITE TREATMENT AND CELL PROLIFERATION

Human osteosarcoma cells were grown in Dulbecco's modified eagles (DMEM)/F12 medium containing 10% charcoal-stripped fetal bovine serum and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 24-well plates (5×10^4 cells/well) and incubated at 37°C. After allowing the cells to attach overnight, the media was replaced the next day. The estrogen metabolites or the carrier for the estrogen compounds (70% ethanol) were added into the fresh media and maintained for 72 h. At the end of the treatment, the cell survival was determined by MTS-based cell viability assay systems, as per the manufacturer's protocol (Promega, Madison, WI).

2-Methoxyestradiol (2-ME), 17β-estradiol (E2) and 16α-hydroxyestradiol (16-OHE) were purchased from Sigma Chemical Co. (St. Louis, MO), and the stock solutions were made in 95% ethanol.

PREPARATION OF CYTOPLASMIC EXTRACT AND PROTEIN ANALYSIS

Cells harvested after 2-ME treatment were lysed by suspending in cell lysis buffer. After centrifugation at 10,000*g* for 10 min, the supernatant was collected, and the protein concentration was determined by Bradford protein assay. Cytoplasmic extract containing protein (100 μg) was analyzed by Western blot hybridization using anti-OPG, anti-RANKL (R&D Systems, Minneapolis, MN) and anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The expression levels of proteins on the Western blots were quantitated using densitometer and quantity one 4.5.2 software (BioRad, Hercules, CA).

RNA ISOLATION AND cDNA SYNTHESIS

Cells were plated at 10^6 cells per flask in T-75 culture flasks 1 day prior to metabolite treatment. The next day, cells were replaced with fresh medium containing 10 μM concentrations of 2-ME and incubated for different periods of time. The cells were harvested and the cell pellets were used for RNA isolation. Total cellular RNA was extracted and isolated using a modified organic solvent method, and the RNA yields were determined spectrophotometrically at 260 nm. The mRNA was used as a template to synthesize cDNA with the iScript Kit (Bio Rad).

POLYMERASE CHAIN REACTION

The cDNA was diluted and amplified by polymerase chain reaction (PCR) using sense primer sequences (5′GGCAACACAGCTCA CAAGAA3′) and antisense primer sequences (5′CGGTAAGCTTTC CATCAAGC3′) for OPG and using sense primer sequences (5′TGCCTCAGGGCA3′) and antisense primer sequences (5′GCTGT GCTATCCCTGTAC3′) for actin. Amplifications were performed with a hot start at 94°C for 10 min and were carried out for 40 cycles with denaturation at 94°C for 1 min, annealing at 51°C for 1 min and elongation at 72°C for 1 min 30 s. The PCR products were analyzed by agarose gel electrophoresis as described [Turner et al., 1999].

ENZYME-LINKED IMMUNOSORBENT ASSAY

To measure the secreted OPG protein levels, MG63 osteosarcoma cells were plated in T75 flasks and treated with estrogenic compounds for 72 h. Media were collected at different time points and ELISA analysis performed as described (BioVendor, Candler, NC).

OPG PROMOTER CONSTRUCT AND LUCIFERASE ASSAY

Luciferase constructs containing the OPG promoter used in this study were constructed from plasmid pOPG5935βgal, kindly provided by Eli Lilly & Company (Indianapolis, IN) [Thirunavukkarasu et al., 2000]. The 5,935 bp human OPG promoter fragment was excised out using *Kpn*I/*Bgl*II restriction digestions and cloned into pGL3-basic luciferase vector. MG63 osteosarcoma cells plated in 6-well plates (1.5×10^6 cells/well) were transfected at 60% confluence using the transfection reagent, Lipofectamine, as described in the manufacturer's protocol (Invitrogen, Carlsbad, CA). All transfections performed had a control plasmid containing Renilla Luciferase (Promega, Madison, WI).

OSTEOSARCOMA/OSTEOBLAST CO-CULTURE

Estrogen-receptor positive human fetal osteoblast cells expressing estrogen receptor alpha (hFOB/ER9) [Robinson et al., 1997] were plated at a density of 9×10^4 cells/well in a 12-well plate. MG63 cells were plated above the hFOB/ER9 cells in a transwell at a density of 5×10^4 cells/well and treated with ethanol vehicle control or 10 μM 2-ME. After 72 h of treatment, alkaline phosphatase assay was performed in hFOB/ER9 cells.

OSTEOCLAST CELL CULTURE

Primary rat osteoclast precursor cells were plated in 96-well plates (5×10^4 cells/well) and maintained in culture for 2 days, as described (B-Bridge International, Mountain View, CA). Following this, the cells were treated with a 1:1 mixture of media plus conditioned media (collected from MG63 cultures treated with Veh, 2-ME or 16α-hydroxyestradiol) supplemented with 10 ng/ml RANKL (R&D Systems) and 10 ng/ml m-CSF (R&D Systems) and incubated for an additional 2 days. The cells were stained for tartrate-resistant acid phosphatase (TRAP), as described by the manufacturer (B-Bridge International), and the TRAP activity was normalized to cell number.

STATISTICAL ANALYSIS

All values are expressed as means ± standard error. The data are representative of three independent experiments. Significant differences between groups were determined by Fisher's protected least significant difference post hoc test for multiple-group comparisons following detection of significance by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

2-ME TREATMENT INDUCES OPG TRANSCRIPTION

To determine whether OPG protein plays a role in 2-ME-mediated anti-tumor actions in osteosarcoma cells, we have studied the effect

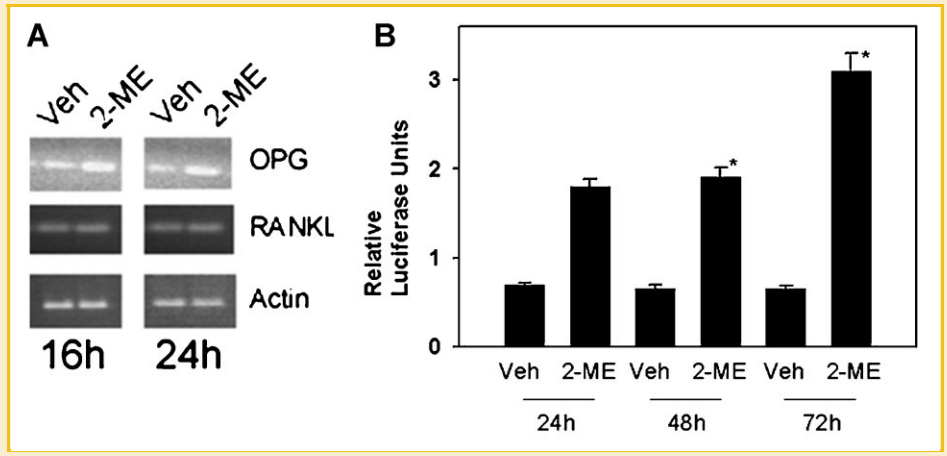

Fig. 1. A: 2-ME treatment increases OPG mRNA levels. MG63 cells were treated for 16 and 24 h with vehicle control (Veh), 2-ME (10 μM) and used for RNA isolation. Total RNA isolated from the cells was analyzed by RT-PCR, as described in Methods. B: 2-ME induces OPG-Luciferase activity in osteosarcoma cells. Cells transiently transfected with OPG 5935 pGL3-luciferase were treated for 24, 48, and 72 h with vehicle control (Veh), 2-ME (10 μM) and the luciferase reporter activity was analyzed. Values are the mean ± SE (n = 6 replicate cultures). $^{*}P < 0.05$ versus veh.

of 2-ME on OPG mRNA levels. Reverse transcription and polymerase chain reaction (RT-PCR) analysis in Veh and 2-ME-treated MG63 cells show that 2-ME treatment increased OPG mRNA levels by 2.4- and 2-fold at 16 and 24 h, respectively (Fig. 1A), compared to vehicle control. However, Veh and 2-ME did not have any effect on RANKL and actin mRNA levels.

To determine whether 2-ME has any effect on OPG gene, we have studied the effect of 2-ME on OPG-driven luciferase activities by transient transfection assays in MG63 osteosarcoma cells. Relative to vehicle control, 2-ME treatment stimulated the luciferase activity by 1.9-, 3.8-, and 4-fold at the end of 24, 48, and 72 h, respectively (Fig. 1B).

2-ME TREATMENT INDUCES OPG PROTEIN LEVELS

The effect of 2-ME treatment on OPG protein levels was investigated by Western blot hybridization in osteosarcoma cells. OPG protein levels were increased by 3- and 2.8-fold in MG63 cells in the presence of 2-ME treatment at 16 and 24 h, respectively (Fig. 2A), compared to the vehicle control. Our results also show that 2-ME treatment increased OPG protein levels in KHOS, 143B and LM7 human osteosarcoma cells by 1.9-, 2.8-, and 2.5-fold, respectively, compared to vehicle (Fig. 2B). Both Veh and 2-ME did not have any effect on actin control.

To determine whether 2-ME treatment leads to OPG protein secretion, we have carried out ELISA using cell culture media collected at 16, 24, 48, and 72 h following treatment. The results show that 2-ME treatment leads to an increased OPG protein levels in cultured media following 24, 48, and 72 h of treatment (Fig. 3).

OPG REGULATION AND CELL KILLING

In order to determine the specificity of 2-ME-mediated OPG regulation, we investigated the effect of various estrogenic compounds on OPG protein levels in MG63 osteosarcoma cells. Our results show that only 2-ME treatment stimulated OPG expression by 2.4-fold compared to vehicle control; whereas,

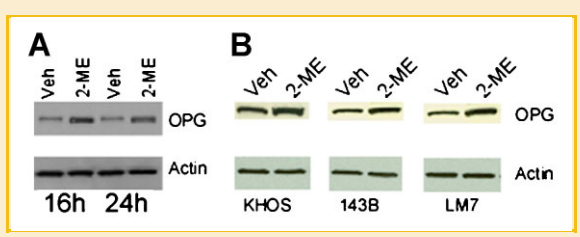

Fig. 2. 2-ME induces OPG protein levels in osteosarcoma cells. Osteosarcoma cells were treated with vehicle control (Veh), 2-ME (10 μM). The cytoplasmic extracts prepared were analyzed by Western blot hybridization using anti-OPG, anti-RANKL (R&D systems) and anti-actin (Santa Cruz Biotechnology) antibodies. A: Effect of 2-ME on OPG protein in MG63 osteosarcoma cells at 16 and 24 h. B: Effect of 2-ME on OPG protein in KHOS, 143B and LM7 osteosarcoma cells at 24 h.

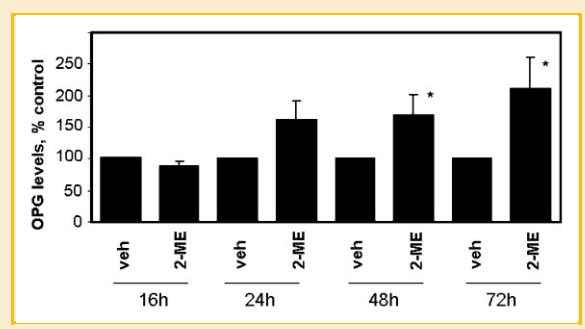

Fig. 3. 2-ME treatment results in OPG release in cultured osteosarcoma cells. MG63 osteosarcoma cells were treated for 24, 48, and 72 h with vehicle control (Veh) and 2-ME (10 μM), and the media were collected and analyzed by ELISA for OPG levels, as described in Methods.

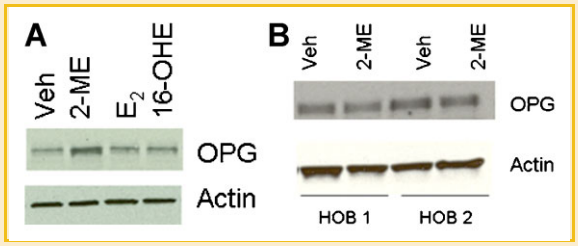

Fig. 4. OPG induction is specific to anti-tumor effects of 2-ME. MG63 osteosarcoma cells, and normal primary human osteoblasts (HOB 1 and 2) cells were treated with control vehicle (Veh) or 10 μM of 2-ME and 16-OHE (16α-hydroxyestradiol) for 24 h. The cytoplasmic extracts prepared were analyzed by Western blot hybridization using anti-OPG (R&D systems) and anti-actin (Santa Cruz Biotechnology) antibodies. A: Effect of estrogenic compounds on OPG protein expression. B: Effect of 2-ME on OPG protein levels in normal primary human osteoblasts.

parent estrogen, 17β-estradiol and 16α-hydroxyestradiol do not affect OPG expression (Fig. 4A). None of these additions had an effect on actin protein levels (Fig. 4A).

We have investigated whether 2-ME induces OPG expression in normal human osteoblast cells that are resistant to anti-growth effects of 2-ME treatment [Maran et al., 2002]. Our results, in two separate primary osteoblast cultures, show that 2-ME does not influence the OPG and control actin expression in these normal osteoblast cells (Fig. 4B).

ADDITION OF OPG PEPTIDE DOES NOT INFLUENCE OSTEOSARCOMA CELL DEATH

We have studied the direct effect of OPG treatment on osteosarcoma cell growth (Fig. 5). Our results show that the addition of OPG protein to osteosarcoma cultures neither affects cell proliferation, nor does have any additive effect in the presence of 2-ME-treatment (Fig. 5).

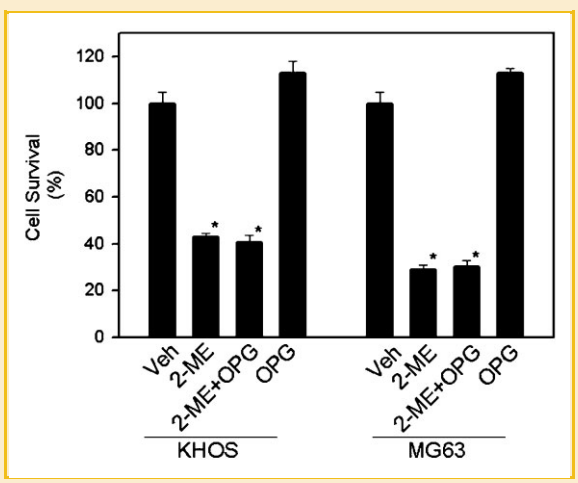

Fig. 5. The addition of OPG protein does not enhance 2-ME effects. MG63 cells were treated with vehicle control (Veh) and 2-ME (5 μM) in the presence and absence of OPG (100 ng/ml) for 72 h. The cells were harvested and the proliferation was determined by MTS assay. Values are the mean ± SE (n = 6 replicate cultures). *$P < 0.05$ versus veh.

2-ME-MEDICATED OPG RELEASE ENHANCES OSTEOBLAST ACTIVITY

To understand the biological significance of OPG release in 2-ME-treated condition, we investigated the direct contact of OPG on osteoblasts by co-culturing MG63 osteosarcoma and human fetal osteoblast cells. 2-ME treatment in osteosarcoma cells leads to an increase in osteoblastic effect, as shown by increases in alkaline phosphatase activities (Fig. 6A).

2-ME-MEDIATED OPG RELEASE BLOCKS OSTEOCLAST GROWTH

To further determine whether the 2-ME mediated OPG induction might have any biological significance in vivo, we studied the effect of conditioned media from Veh-, 2-ME-, and 16α-hydroxyestradiol-treated osteosarcoma cultures on osteoclast cells (Fig. 6B). Our results show that conditioned media from 2-ME-treated cultures decreased the number of tartrate-resistant acid phosphatase (TRAP)-positive cells, whereas conditioned media from Veh and 16α-hydroxyestradiol-treated cultures did not have similar effects on osteoclast cells (Fig. 6B).

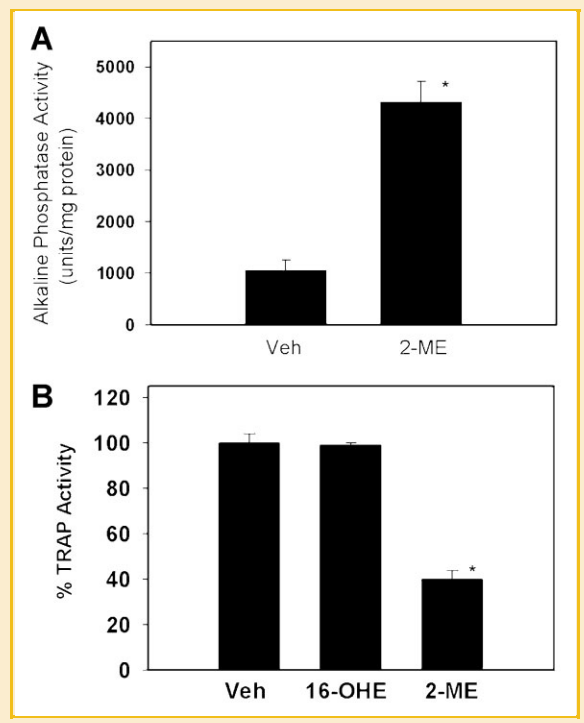

Fig. 6. A: 2-ME-mediated OPG release stimulates alkaline phosphatase activity in osteoblasts. MG63 osteosarcoma and FOBER cells were co-cultured for 72 h in the presence of (Veh) and 2-ME (5 μM) treatment. At the end of treatment, FOBER cells were harvested and the alkaline phosphatase activity was measured. Values are the mean ± SE (n = 4 replicate cultures). *$P < 0.05$ versus veh. B: 2-ME-mediated OPG release blocks osteoclast growth. Osteoclast cells were maintained in conditioned media from osteosarcoma cultures treated with vehicle (Veh) or 10 μM of 2-ME and 16-OHE (16α-hydroxyestradiol) for 2 days. Cells were fixed and stained for TRAP. *$P < 0.05$ versus veh.

DISCUSSION

The metabolite of mammalian estrogen, 2-ME, acts as an anti-cancer agent in vitro and in vivo [Ward et al., 1994; Seegers et al., 1997; Zhu and Conney, 1998; Schumacher et al., 1999; Maran et al., 2002, 2008; LaVallee et al., 2003; Shogren et al., 2007]. We have shown that 2-ME does not affect normal osteoblasts, but induces apoptosis in osteosarcoma cells. In this report, we demonstrate that 2-ME treatment stimulates OPG gene expression in human osteosarcoma cells. 2-ME treatment leads to increases in OPG gene promoter activity, mRNA, protein levels, and secretion in osteosarcoma cells. This effect is ligand specific as other estrogenic compounds do not have similar effects in osteosarcoma cells. 2-ME-mediated induction of OPG is associated with anti-proliferative actions of 2-ME, since OPG expression is not altered by 2-ME treatment in normal cells, which are resistant to growth-inhibitory actions of 2-ME.

Anti-tumor effects of 2-ME involve multiple pathways involving disruption of microtubles, oncogenes, cytokines, cell-cycle arrest, apoptosis and angiogenesis [Ward et al., 1994; Seegers et al., 1997; Zhu and Conney, 1998; Schumacher et al., 1999; Maran et al., 2002, 2008; LaVallee et al., 2003; Shogren et al., 2007]. 2-ME has been shown to act through P53-dependent and -independent pathways [Mukhopadhyay and Roth, 1997, 1998; Schumacher et al., 1999; Pribluda et al., 2000]. In addition, 2-ME-mediated anti-cancer actions include the induction of vascular endothelial growth factor (VEGF) in breast cancer cells [Sweeney et al., 2001], the inhibition of hypoxia-inducible factor, (HIF)-1α the VEGF in head and neck squamous cell carcinoma and breast cancer [Mabjeesh et al., 2003; Ricker et al., 2004], and finally the induction of tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-dependent death receptor (DR)-5 proteins in several cancers [LaVallee et al., 2003]. 2-ME-mediated apoptosis in osteosarcoma cells has been shown to be dependent on RNA-dependent protein kinase, PKR [Shogren et al., 2007]. In this article, we show that OPG is induced by 2-ME-treatment in osteosarcoma cells.

OPG/RANK/RANKL triad has been implicated in bone cell regulations and skeletal resorption [Khosla, 2001; Theoleyre et al., 2004; Pivonka et al., 2008]. Under normal conditions, OPG interferes with the binding of RANKL to RANK by acting as a decoy receptor for RANKL, and thereby prevents excessive bone destruction. It has been demonstrated that osteoclastic bone resorption contributes to the establishment of tumors in the skeleton and is the major skeletal regulatory event in patients with bone metastases.

OPG expression is frequently altered in cancers. Investigations by several groups have shown that OPG levels hold promise as markers of cancer progression or as prognostic indicators. OPG expression increases in prostate [Brown et al., 2001; Eaton et al., 2004; Jung et al., 2004]. Similarly, serum OPG levels have been positively correlated with progression and bone metastasis in prostate cancer [Brown et al., 2001; Jung et al., 2004]. However, serum OPG levels are decreased in patients with advanced prostate cancer who responded to androgen ablation therapy, compared with patients with advanced prostate cancer, but whose disease was progressing as determined by serum PSA levels [Jung et al., 2004]. The expression and potential involvement of OPG in breast cancers have been widely demonstrated [Brown et al., 2001; Guise, 2002; Lipton et al., 2002], although there is only limited data on serum OPG levels. Serum OPG levels are increased in pancreatic and colorectal cancers, and Hodgkins's lymphoma [Lipton et al., 2002]. Increased serum OPG levels are associated with the increased tumor stage, grade and early recurrence in bladder cancers [Mizutani et al., 2004].

In preclinical animal models, OPG controls tumor progression by blocking bone resorption through inhibition of RANKL activation. In primary and metastatic bone tumors, the OPG and RANKL levels play a key role in the vicious cycle involving bone destruction and tumor growth [Guise, 2002]. In multiple myeloma animal models, treatment with OPG protein reduces tumor burden and increases survival. In osteosarcoma animals, OPG exerts an indirect inhibitory effect on tumor progression through the inhibition of RANKL, whose production is often enhanced in the bone tumor environment. OPG has been shown to regulate the RANKL-induced epithelial cancer cell migration and bone metastasis [Jones et al., 2006]. Thus, OPG has been shown to be a potential therapeutic molecule, due to its anti-resorptive properties.

Although these above studies demonstrate the potential benefits associated with OPG, the direct role of OPG in cancer is not fully understood. One of the main reasons is that OPG also binds to TRAIL, another member of TNF superfamily, resulting in the inhibition of TRAIL-induced apoptosis [Emery et al., 1998]. This indicates that OPG could also function as a survival factor for tumor cells. Hence, the role of OPG in tumor, which depends on relative in vivo concentrations and expression of OPG, TRAIL and RANKL, remains to be elucidated.

Current studies support that the stimulation of OPG is specific to 2-ME treatment as other compounds, namely, the parent estrogen, 17β estradiol, and the tumorigenic metabolite, 16α-hydroxyestradiol, do not induce OPG gene activity and expression. This indicates that OPG responds to only anti-tumor effects in osteosarcoma cells.

The 2-ME-mediated OPG induction is specific to tumor cells, as OPG is not induced in normal cells which do not respond to the cell killing effects of 2-ME. Our results indicate that OPG does not affect osteosarcoma cell death, as the addition of OPG protein to cultured osteosarcoma cells neither blocked osteosarcoma cell growth nor enhanced 2-ME actions. These data imply that OPG may not have a direct role in 2-ME-mediated anti-tumor activities in osteosarcoma. This is in agreement with the previous observations where it has been shown that OPG does not have direct effects on osteosarcoma cell proliferation and cell cycle [Lamoureux et al., 2007]. Our results show that 2-ME treatment does not affect RANKL mRNA levels. However, it is possible that 2-ME-treatment could control RANKL activation through releasing OPG in the tumor microenvironment in vivo. OPG effects appear to require the bone microenvironment as it does not have any direct effect on osteosarcoma in vitro [Lamoureux et al., 2007]. This possibility is supported by our co-culture studies involving osteoclasts where 2-ME treatment blocks osteoclast-specific TRAP activity. OPG has been previously shown to block tumor-induced osteoclastogenesis, bone destruction and bone pain in vivo [Clohisy et al., 2000; Honore et al., 2000]. In addition, our studies show that 2-ME could have a beneficial effect on osteoblast activity, as OPG containing conditioned media from 2-ME-treated

osteosarcoma cultures increased the alkaline phosphatase activity in human fetal osteoblasts. These results are in agreement with the earlier in vitro and in vivo studies, which show that 2-ME inhibits osteoclast growth and activity [Maran et al., 2006; Cicek et al., 2007].

In conclusion, the present study shows that OPG is activated by the anti-cancer agent, 2-ME, and may have a therapeutic function in osteosarcoma. Further work involving the in vivo tumor model will be necessary to better elucidate the therapeutic role of OPG in tumor microenvironment in the presence of 2-ME treatment.

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