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2-Methoxyestradiol Inhibits Differentiation and Is Cytotoxic to Osteoclasts

A. Maran,[1]* G. Gorny,[3] M.J. Oursler,[2] M. Zhang,[1] K.L. Shogren,[1] M.J. Yaszemski,[1] and R.T. Turner[4]

[1]Department of Orthopedics, Mayo Clinic, Rochester, Minnesota 55905
[2]Endocrine Research Unit, Mayo Clinic, Rochester, Minnesota 55905
[3]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905
[4]Department of Nutrition and Exercise Sciences, Oregon State University, Corvallis, Oregon 97330

Abstract 2-Methoxyestradiol (2-ME), a naturally occurring metabolite of 17β-estradiol, is highly cytotoxic to a wide range of tumor cells but is harmless to most normal cells. However, 2-ME prevented bone loss in ovariectomized rats, suggesting it inhibits bone resorption. These studies were performed to determine the direct effects of 2-ME on cultured osteoclasts. 2-ME (2 μM) reduced osteoclast number by more than 95% and induced apoptosis in three cultured osteoclast model systems (RAW 264.7 cells cultured with RANKL, marrow cells co-cultured with stromal support cells, and spleen cells cultured without support cells in media supplemented with RANKL and macrophage colony stimulating factor (M-CSF)). The 2-ME-mediated effect was ligand specific; 2-hydroxyestradiol (2-OHE), the immediate precursor to 2-ME, exhibited less cytotoxicity; and 2-methoxyestrone (2-MEOE$_1$) the estrone analog of 2-ME, was not cytotoxic. Co-treatment with ICI 182,780 did not antagonize 2-ME, suggesting that the cytotoxicity was not estrogen receptor-dependent. 2-ME-induced cell death in RAW 264.7 cells coincided with an increase in gene expression of cytokines implicated in inhibition of differentiation and induction of apoptosis. In addition, the 2-ME-mediated decrease in cell survival was partially inhibited by anti-lymphotoxin(LT)β antibodies, suggesting that 2-ME-dependent effects involve LTβ. These results suggest that 2-ME could be useful for treating skeletal diseases in which bone resorption is increased, such as postmenopausal osteoporosis and cancer metastasis to bone. J. Cell. Biochem. 99: 425–434, 2006. © 2006 Wiley-Liss, Inc.

Key words: estrogen metabolite; apoptosis; bone resorption; cytokines

Homeostasis of bone mass is maintained via bone remodeling by ongoing actions of osteoblasts and osteoclasts. The ability of osteoclasts to efficiently resorb bone distinguishes these cells. A shift in the remodeling balance in favor of resorption leads to bone loss in disorders such as autoimmune arthritis, periodontitis, postmenopausal osteoporosis, bone tumors, and Paget's disease. Hence, restoration of normal bone resorption in these conditions is essential to normalize bone turnover and prevent bone loss.

Estrogen replacement therapy is effective in preventing increased bone resorption in postmenopausal women as well as in ovariectomized rats [Aitken et al., 1973; Heaney et al., 1978; Richelson et al., 1984]. However, the risks of hormone replacement to other organ systems outweigh the benefits to the skeleton [Fleming, 2003; Suparto et al., 2003; Hulley and Grady, 2004]. 17β-Estradiol, the most potent natural estrogen, is metabolized by several hydroxylation pathways resulting in the formation of 2-, 4-, and 16α-hydroxylated derivatives [Zhu and Conney, 1998]. 16α-Hydroxy derivatives are estrogen agonists on osteoblasts in culture and in ovariectomized rats [Robinson et al., 2000; Lotinun et al., 2001]. In contrast, 2-hydroxylated metabolites are neither estrogen agonists nor antagonists on bone cells [Robinson et al., 2000; Lotinun et al., 2001].

2-Hydroxyestradiol (2-OHE) undergoes further metabolism prior to clearance. 2-OHE is

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*Correspondence to: A. Maran, PhD, 3-69 Medical Sciences Building, Department of Orthopedics, Mayo Clinic College of Medicine, Rochester, MN 55905.
E-mail: maran@mayo.edu

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O-methylated at peripheral sites to form 2-methoxyestradiol (2-ME) [Gelbke and Knuppen, 1976], which is present in human blood and urine [Gelbke and Knuppen, 1976; Fotsis et al., 1994]. 2-ME has been reported to exhibit anticancer properties [Mukhopadhyay and Roth, 1997; Seegers et al., 1997; Schumacher et al., 1999]. 2-ME has little effect on most normal cells. However, it inhibits endothelial cell proliferation and angiogenesis [Fotsis et al., 1994; Yue et al., 1997; Zhu and Conney, 1998], as well as vascular invasion of growth plate cartilage [Sibonga et al., 2002].

Two general mechanisms of action for the cytotoxic activity of 2-ME have been proposed: (1) disruption of the cytoskeleton during cell division and (2) induction of apoptosis. 2-ME inhibits tubulin formation which antagonizes mitosis [D'Amato et al., 1994]. Additionally, 2-ME stimulates the expression of p53 gene, which leads to the induction of apoptosis [Mukhopadhyay and Roth, 1997, 1998]. 2-ME can induce apoptosis in the absence of p53 in a pancreatic tumor cell line [Schumacher et al., 1999; Pribluda et al., 2000], suggesting that it can act through redundant pathways. This conclusion is supported by the observation that 2-ME activated several caspases in pancreatic cells through the upregulation of DR5 protein.

2-ME is a potential therapeutic agent for treatment of bone cancer. It is well tolerated by mice at blood concentrations active on cultured tumor cells. 2-ME induces cell death in osteosarcoma cells and not in normal osteoblasts [Maran et al., 2002]. Administration of high doses of 2-ME to growing female rats did not disturb normal bone turnover [Turner and Evans, 2000]. 2-ME antagonized bone loss in ovariectomized rats by preventing the expected increase in bone turnover [Sibonga et al., 2003]. This unexpected finding suggests that the osteoclast is a target for 2-ME. The direct effects of 2-ME on cultured osteoclasts have not been investigated. Based on these intriguing findings, we determined the direct effects of 2-ME on in vitro osteoclast differentiation and survival.

MATERIALS AND METHODS

Cell Culture, Metabolite Treatment, and Cell Growth

2-Methoxyestradiol, 2-OHE, 2-methoxyestrone (2-MEOE$_1$), and E$_2$ were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of the metabolites at their respective concentrations were made in 95% ethanol. ICI 182,780 was kindly provided by Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK).

RAW 264.7 Cells

RAW 264.7 cells were plated at 5×10^4 cells per well into 24-well plates containing 1 ml/well medium. After allowing the cells to attach overnight, media were replaced and the cells were maintained in 20 ng/ml of RANKL (Chemicon, Temucula, CA) and 25 ng/ml of macrophage colony stimulating factor (M-CSF) (R&D, Minneapolis, MN) for 48 h. Metabolites were then added into each well, diluted 1,000-fold to give the final required concentrations and maintained for 72 h. Cell growth was measured by taking the viable cell count. After 72 h of metabolite treatment, cells were harvested, stained with trypan blue, and counted with the aid of light microscopy.

Osteoclast Culture and Purification

The culture and purification of osteoclasts were carried out as described [Gingery et al., 2003]. Mouse marrow and spleen containing osteoclast precursors were obtained from female BalB/c mice (Taconic, Germantown, NY). Four- to six-week-old mice were sacrificed and long bones of the hind limbs and spleen were aseptically removed. The distal ends of bones were clipped and the marrow was flushed out by injecting sterile Mosconas buffer (8% NaCl, 0.2% KCl, 0.06% $NaH_2PO_4 + H_2O$, 2% glucose, 0.02% bicarbonate) into the marrow cavity with a 27-gauge needle. Marrow cells were counted and stored at 2.4×10^6 cells/tube in liquid nitrogen until used.

To generate marrow-derived osteoclasts, precursors were cultured with ST-2 cells (Riken Cell Bank, Tsukuba, Japan) during differentiation as described [Gingery et al., 2003]. ST2 cells were plated in alpha-modified minimal essential media (αMEM, Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT) and antibiotic/antimycotic in 48-well plates (for differentiation studies) at 0.8×10^5 cell/well or in 24-well plates (for apoptosis assay) on glass coverslips at a density of 1.6×10^5 cells/well.

For generating spleen-derived osteoclasts, spleen cells were plated at 4×10^6 cells per well

in a 24-well plate as detailed for this model system [Sells Galvin et al., 1999]. Base medium was supplemented with 7×10^{-3} M ascorbic acid prior to plating and cultures were supplemented with 30 ng/ml of RANKL (Chemicon) and 25 ng/ml of M-CSF. For both marrow- and spleen-derived cultures, media were changed every 3 days and cells were either fixed in 1% paraformaldehyde in phosphate-buffered saline to terminate culture and processed for differentiation studies or processed for apoptosis assay as described below. For performing apoptosis assay, the spleen cells were plated at 2×10^6 spleen cells per well in 24-well plates.

Differentiation Assay

Cells were cultured and treated with Veh or 2-ME for 13 days and then tartrate resistant acid phosphatase (TRAP) staining was used as described [Gingery et al., 2003] to visualize differentiated cells according to manufacturer's directions (Sigma Chemical Co.). The numbers of mononuclear and multinucleated TRAP-positive cells were counted using an Olympus inverted microscope at 200× magnification.

Apoptosis Assay

The apoptosis assays to detect chromatin condensation were performed using Hoechst 33258 dye in cultured osteoclasts as described [Gingery et al., 2003]. Briefly, osteoclasts in culture were treated with vehicle and 2-ME and were fixed with 1% paraformaldehyde. Fixed osteoclasts were stained for 60 min with Hoechst 33258 diluted to 5 μg/ml in PBS containing 0.01% Tween 20 [Gingery et al., 2003]. The cells were then TRAP stained using a kit from Sigma Chemical Company.

RNA Isolation

RAW 264.7 cells were plated at 10^6 cells per flask in T75 culture flasks a day prior to treatment. The next day, cells were incubated with fresh medium containing 2 μM of 2-ME. 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 [Chomczynski and Sacchi, 1987].

RNase Protection Assay for Cytokines

Total RNA isolated was analyzed by RNase protection assay using anti-sense RNA probes [Evans et al., 1998; Maran et al., 2002] and synthesized using cDNA templates as described (Pharmingen, San Diego, CA). mRNA concentration of the following cytokines was evaluated: transforming growth factor (TGF)-β 1, 2, 3; tumor necrosis factor (TNF) α and β; interleukin (IL) 1α, 1β, 1Ra, 6, 10, 12 (p35 and p40); interferon (IFN) β and γ; and lymphotoxin (LT)-β. Quantitation of protected RNA fragments was performed by PhosphorImager analysis and normalized to ribosomal structural protein L32 [Evans et al., 1998; Maran et al., 2002].

Antibody Treatment

RAW 264.7 cells cultured as described above in the presence of RANKL and M-CSF and treated with vehicle and 2-ME in the presence and absence of anti-LTβ or anti-IFNβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The cell growth was measured by taking the viable cell count at the end of 72 h.

Statistical Analysis

All values are expressed as mean ± standard error. 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 ANOVA. Significance was considered at P values <0.05.

RESULTS

Effect of Estrogen Metabolites on RAW 264.7 Cell Survival

RAW cells were examined after 72 h in the presence of 2-ME (Fig. 1A). Compared to vehicle control, 60% of the plated cells were killed at 1 μM, 97% of cells were killed at 2 μM and 5 μM, and 99% at 10 μM and 20 μM. 2-OHE (2 μM) reduced RAW cell survival by 56% and the estrone derivative 2-MEOE$_1$ by 6% (Fig. 1B) compared to vehicle control.

Effect of Anti-Estrogen ICI 182,780 on 2-ME-Mediated Cell Killing

To determine whether 2-ME-mediated cell death in osteoclast cell culture requires binding to either isoform of the estrogen receptor (ER-α or ER-β), the cytotoxic effect of 2-ME on RAW 264.7 cells was determined in the presence and absence of ICI 182,780 (Fig. 2). 2-ME at 2 μM

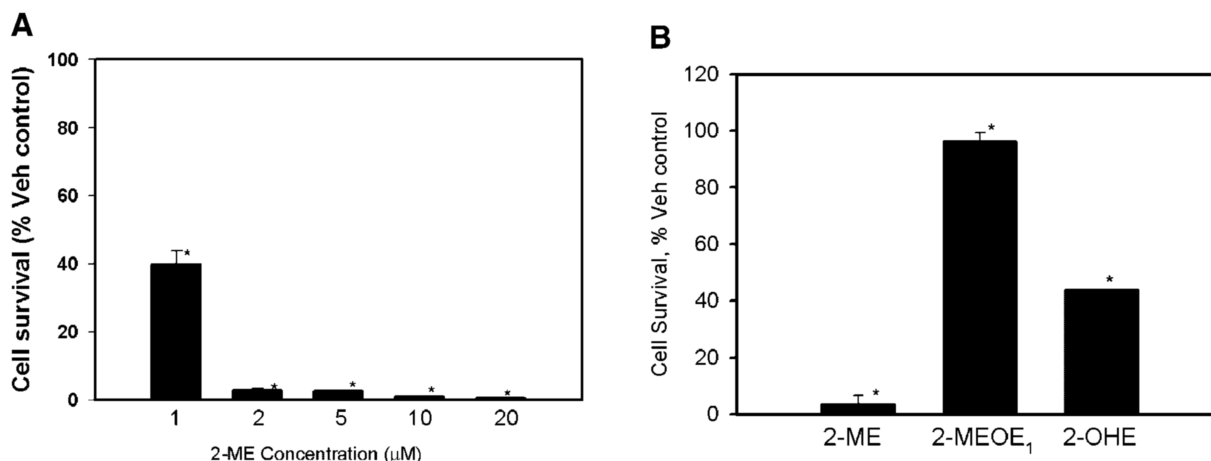

Fig. 1. Dose-dependent and ligand-specific effects of 2-ME on RAW 264.7 cells. **A**: RAW 264.7 cells were treated with vehicle (Veh) or 2-methoxyestradiol (2-ME) (1–20 μM) for 72 h; (**B**) cells were treated with Veh or 2 μM 2-ME, 2-hydroxyestradiol (2-OHE) and 2-methoxyestrone (2-MEOE$_1$). Values are mean ± SE (N = 3 replicate cultures). *$P < 0.05$ (compared with Veh). The experiment shown is representative of six independent experiments.

decreased RAW 264.7 cell survival by 89% compared to vehicle controls. Co-treatment with ICI 182,780 at 2 μM and 20 μM did not block cell killing by 2-ME and ICI 182,780. ICI 182,780 alone at 2 and 20 μM reduced cell survival by 28% and 25%, respectively.

Effect of 2-ME on Osteoclast Differentiation

2-Methoxyestradiol treatment prevented osteoclast differentiation in RAW 264.7 cells, co-cultured marrow-derived osteoclasts, and in spleen-derived osteoclasts (Fig. 3), as TRAP-positive cells could not be detected, whereas numerous TRAP-positive cells were detected in no addition and vehicle controls (Fig. 3).

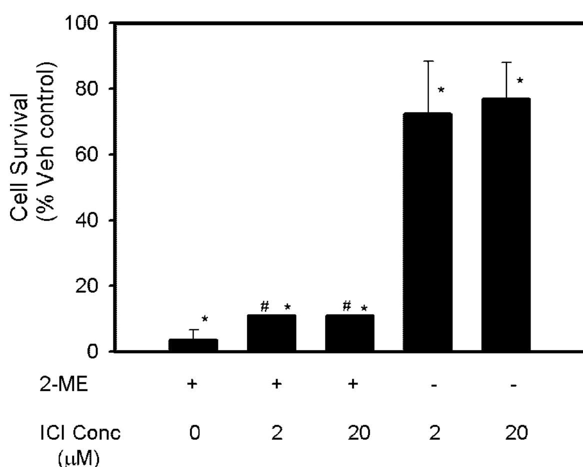

Fig. 2. Effect of ICI 182,780 on 2-ME-induced killing of RAW 264.7 cells. RAW 264.7 cells were treated with Veh or 2 μM 2-ME in the presence and absence of 2 and 20 μM ICI 182,780 (ICI) for 72 h. Values are mean ± SE (N = 3 replicate cultures). *$P < 0.05$ (compared with Veh); $^{\#}P < 0.05$ (compared with ICI). The experiment shown is representative of three independent experiments.

Effect of 2-ME on Osteoclast Apoptosis

We examined purified marrow-derived osteoclasts survival after 2-ME treatment (Fig. 4) by TRAP staining for osteoclast detection (Fig. 4A,B) and Hoechst staining for chromatin condensation (Fig. 4C,D). The condensed nuclei

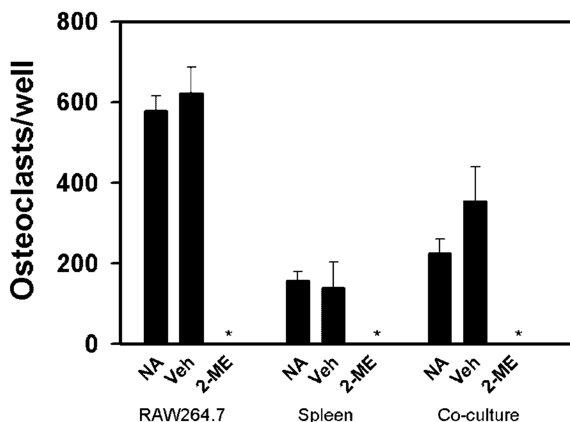

Fig. 3. Effect of 2-ME on osteoclast differentiation. Spleen- and marrow-derived co-cultured osteoclasts were subjected to no addition (NA), Veh or 2 μM 2-ME for 13 days. Then fixed and stained for TRAP. Values are mean ± SE (N = 3 replicate cultures). *$P < 0.05$ (compared with Veh). The experiment shown is representative of three independent experiments.

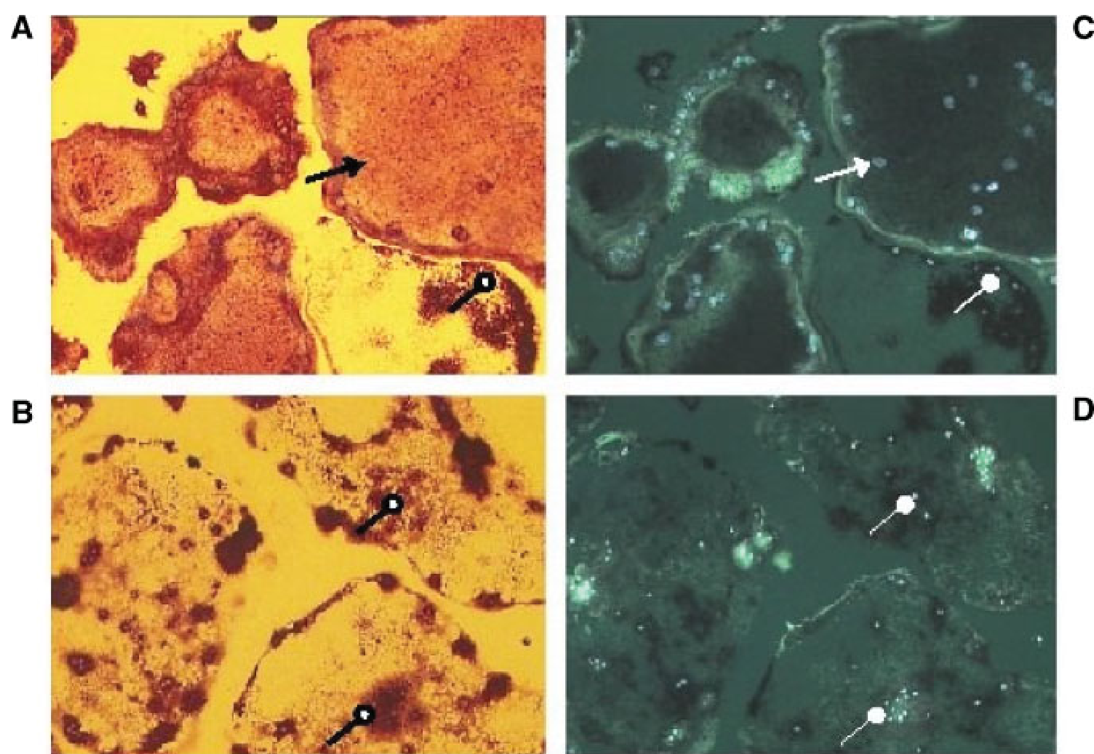

Fig. 4. Effect of 2-ME on osteoclast apoptosis. Purified marrow-derived osteoclasts were cultured and treated with Veh (**A** and **C**) or 2-ME (**B** and **D**), fixed and stained for TRAP (A and B) and chromatin condensation for apoptosis by Hoechst staining (C and D). Arrows indicate non-apoptotic cells. The wands point to selected apoptotic cells.

distinguish apoptotic osteoclasts in the presence of 2-ME treatment (Fig. 4D). The arrows indicate non-apoptotic cells and the wands point to selected apoptotic cells.

2-Methoxyestradiol treatment increased apoptosis in multiple osteoclast model systems (Fig. 5). Whereas ~15% of the control osteoclasts were apoptotic, 55% and 65% of osteoclasts became apoptotic following 2-ME treatment of spleen derived and RAW 264.7 cells, respectively (Fig. 5A).

2-Methoxyestradiol treatment resulted in a time-dependent increase in apoptosis in purified and co-culture derived osteoclasts that was significantly enhanced compared to vehicle within 3 h and reached a peak at 48 h of 2- and 2.5-fold, respectively (Fig. 5B). At 72 h there was a decline in number of apoptotic cells in 2-ME-treated cultures; however, the number of surviving cells was greatly reduced compared to the control (Fig. 5B).

Effect of 2-ME on Cytokines Related to Apoptosis

Many cytokine genes, including IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12, IFNγ, TGF-β2, TGFβ3, and TNFβ were not detected in either control or 2-ME-treated cultures. 2-ME treatment increased mRNA levels for TNFα, IFN-β, TGFβ1, and LTβ (Fig. 6). TNFα mRNA levels were increased 3-, 6-, 8-, and 9-fold at 12, 24, 48, and 72 h, respectively. IFNβ mRNA level was increased at 12 h (1.5-fold) and stayed elevated at 24 h (2.5-fold), 48 h (7-fold), and 72 h (6-fold). TGFβ1 mRNA level was increased by twofold at 48 and 72 h. LTβ mRNA level was increased by 4- and 3.5-fold at 48 and 72 h, respectively.

2-Methoxyestradiol-Mediated Osteoclast Cell Death Is Reduced by Antagonizing LTβ

To determine whether the cytokines induced by 2-ME contribute to its cell killing effect, we studied the cell survival in the presence of blocking antibodies to cytokines LTβ and IFNβ. We investigated the cell survival in 2-ME-treated RAW 264.7 cells in the presence of anti-LTβ antibody treatment (Fig. 7). Anti-LTβ treatment increased osteoclast survival in 2-ME-treated cultures by 21% and 58% after 48 h and 72 h, respectively. Identical experiments carried out in the presence of anti-IFNβ antibody

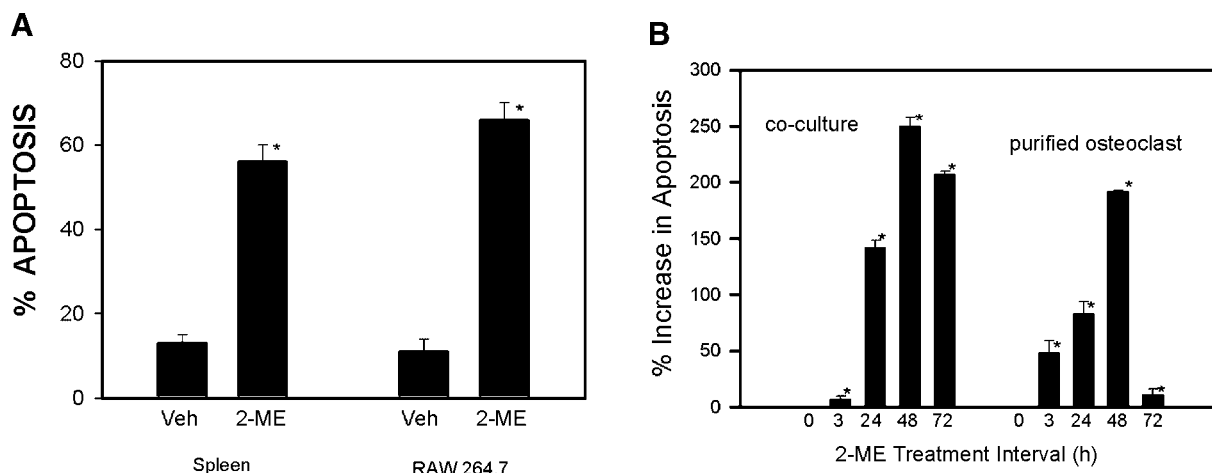

Fig. 5. Pattern of 2-ME-induced apoptosis. **A**: Spleen-derived osteoclasts and RAW 264.7 cells at 72 h; (**B**) time course of marrow-derived co-cultured and purified osteoclasts. Osteoclasts in culture were treated with Veh or 2 μM 2-ME. The cells were then stained for TRAP and apoptosis by Hoechst staining. Apoptotic osteoclasts were identified as TRAP-stained cells having highly condensed nuclei. The percentage of increase in apoptotic osteoclasts is shown. Values are mean ± SE (N = 3 replicate cultures). *$P < 0.05$ (compared with Veh). The experiment shown is representative of three independent experiments.

did not have any effect on 2-ME-mediated cell death in osteoclasts (data not shown).

DISCUSSION

Bone loss is often accompanied by elevated osteoclast number. Osteoclast differentiation and/or survival regulate the number of osteoclasts. We have examined the effect of 2-ME on differentiation and survival of osteoclasts using three different cell culture systems. These studies demonstrate that 2-ME inhibits osteoclast differentiation and is toxic to mature osteoclasts.

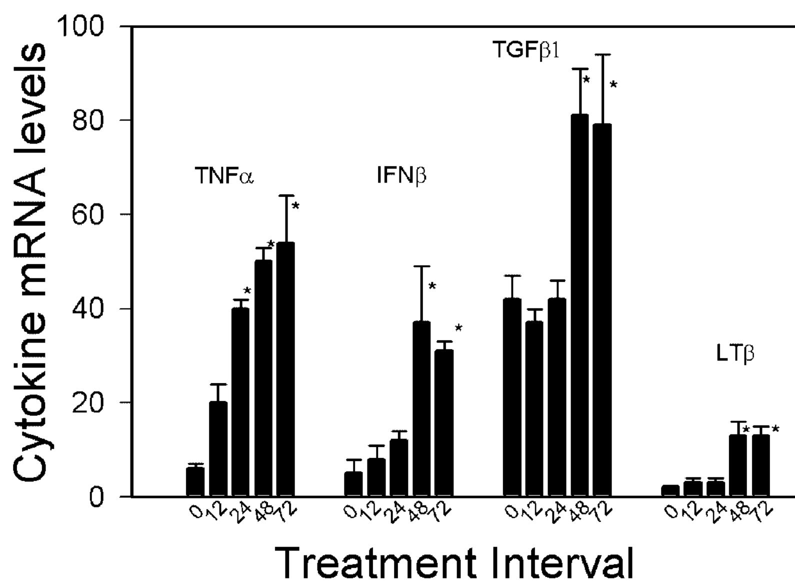

Fig. 6. Time course effect of 2-ME on mRNA levels for cytokines and growth factors in osteoclasts. RAW 264.7 cells were treated with Veh or 2 μM 2-ME for up to 72 h. Total RNA isolated was analyzed by RNase Protection assay and normalized to L32 mRNA levels. Values are mean ± SE (N = 3 replicate cultures). *$P < 0.05$ (compared with Veh). The experiment shown is representative of three independent experiments. TNFα, tumor necrosis factor α; IFNβ, interferon β; TGFβ1, transforming growth factor β1; LTβ, lymphotoxin β.

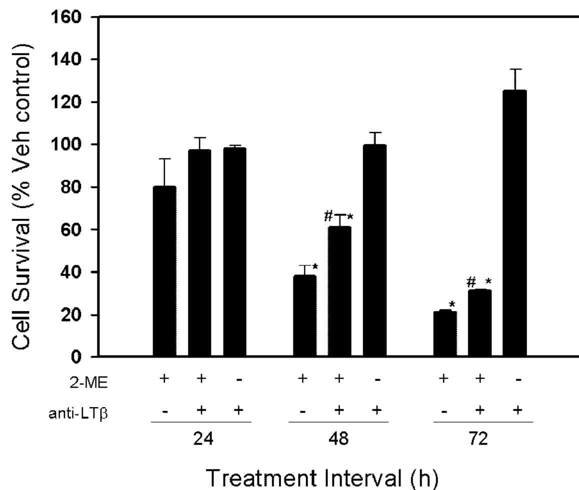

Fig. 7. Anti-LTβ antibody reverses 2-ME-mediated cell death. RAW 264.7 cells in culture were treated with Veh or 2 μM 2-ME for 72 h in the presence and absence of anti-LTβ antibody. Values are mean ± SE (N = 3 replicate cultures). $^{*}P < 0.05$ (compared with Veh); $^{\#}P < 0.05$ (compared with 2-ME). The experiment shown is representative of three independent experiments.

The 2-ME-mediated action on osteoclasts is not antagonized by the potent estrogen receptor antagonist ICI 182,780 and, therefore, is unlikely to be estrogen receptor dependent. This is in agreement with studies reporting that ICI 182,780 does not block 2-ME-mediated inhibition of bone turnover in rats [Sibonga et al., 2003] or inhibition of proliferation of cultured osteosarcoma cells [Maran et al., 2002]. Also, 2-ME-mediated inhibition of proliferation and induction of apoptosis in breast cancer cells has been shown to involve estrogen receptor-independent pathways [LaVallee et al., 2002].

2-Methoxyestradiol-mediated growth arrest and cell death is not completely understood although one of the mechanisms it acts through is the induction of apoptosis. 2-ME-induced programmed cell death may be mediated in some cells by known regulators of apoptosis such as BCL-2 or p53 [Mukhopadhyay and Roth, 1997; Attalla et al., 1998]. BCL-2 suppresses apoptosis, and phosphorylation of BCL-2 in 2-ME-treated cells results in the inactivation of BCL-2 [Attalla et al., 1998]. In lung cancer cells, 2-ME induces apoptosis through the activation of p53 [Mukhopadhyay and Roth, 1997]. In another report, however, 2-ME-mediated induction of apoptosis did not require p53 [Pribluda et al., 2000]. In gastric carcinoma cells, 2-ME induced apoptosis by activating caspase 3 [Lin et al., 2001]. Taken as a whole, these observations suggest that 2-ME can induce apoptosis by multiple pathways that may be cell specific.

2-Methoxyestradiol prevents osteoclast differentiation, a complex process involving multiple growth factors and cytokines, many of which also play a role in regulating apoptosis [Manolagas, 1995; Mundy et al., 1995; Kayagaki et al., 1999; Roodman, 1999; Chambers, 2000; Takayanagi et al., 2000, 2002; Katagiri and Takahashi, 2002]. 2-ME-mediated cell death in osteoclasts is preceded by an increase in expression of proapoptotic cytokine genes TNF-α, IFN-β, TGF-β, and LT-β. In a recent report, IFN-β has been shown to block osteoclast differentiation [Takayanagi et al., 2002]. However, we failed to detect an effect of incubating osteoclast cultures with an antibody to IFN-β on 2-ME-induced cell death. In contrast, blocking LTβ activity partially reduced cell death. LTβ, a member of TNF family, has been implicated in apoptosis in a variety of types of normal and tumor cells [Ware et al., 1996; Kashii et al., 1999; Lu et al., 2002]. The present studies demonstrate a protective effect of LTβ antibodies on osteoclast survival. Antagonizing LTβ failed to completely block the toxic effects of 2-ME on osteoclasts, suggesting that additional cytokines are involved. Furthermore, the cytokines involved may be cell specific. 2-ME acts through an increase in vascular endothelial growth factor (VEGF) in breast cancer cells [Klauber et al., 1997], whereas in head and neck squamous cell carcinoma and breast cancer, it blocks angiogenesis through the inhibition of hypoxia-inducible factor (HIF)-1α and VEGF [Mabjeesh et al., 2003; Ricker et al., 2004]. In some models, 2-ME-mediated anti-proliferative activities require TNF-related apoptosis inducing ligand (TRAIL)-dependent death receptor (DR)-5 proteins [LaVallee et al., 2003]. TNF-α has also been shown to cooperate with 2-ME and induce cell death in Ewing tumor [Djavaheri-Mergny et al., 2003]. In osteosarcoma cells, 2-ME-mediated cell death is accompanied by a large increase in IFN-β mRNA expression [Maran et al., 2002]. In contrast, 2-ME-mediated cell death in chondrosarcoma cells does not involve IFN gene induction but accompanied by an increase in TNF-α mRNA levels (Maran, A., Turner, R.T., and Scully, S. unpublished observations). We observed an increase in TNF-α gene expression in 2-ME-treated osteoclasts. However, TNF-α is reported

to promote osteoclast survival [Lee et al., 2001]. It is clear that further investigation is necessary to resolve the role of specific cytokines in the molecular pathways involved in LTβ-dependent and LTβ-independent cytotoxic and anti-proliferative effects of 2-ME on osteoclasts.

2-Methoxyestradiol acts through the regulation of transcription factor nuclear factor κ-B (NFκ-B) in neuroectodermal brain tumors [Kumar et al., 2003]. NFκ-B is a transcription factor that plays a major role in osteoclast regulation and mediating cytokine responses in several systems. But it remains to be determined whether the cytokines that respond to 2-ME-mediated regulation in osteoclasts act through NFκ-B.

Reduction of bone resorption is a well-established strategy to prevent pathological bone loss. Several classes of bone resorption inhibitors with very different mechanisms of action are in use or are in the process of development. Some (estrogens and SERMs) act as estrogen receptor agonists while others (RANKL antagonists) block RANKL–RANK interactions [Chambers, 2000; Riggs, 2000; Katagiri and Takahashi, 2002]. Several small molecules (Src kinase inhibitor, cathepsin K inhibitor, and calcitonin) function as direct osteoclast inhibitors through effector-specific mechanisms. Nitrogen-containing bisphosphonates reduce osteoclastic bone resorption by interfering with protein prenylation and the HMG-CoA reductase pathway [Luckman et al., 1998] while non-nitrogen-containing bisphosphanates reduce resorption by producing toxic analogs of ATP and activation of caspases which cause osteoclast death [Frith et al., 1997; Rogers et al., 2000; Benford et al., 2001].

2-Methoxyestradiol appears to act by novel and multiple distinct molecular mechanisms because it antagonizes osteoclast differentiation and reduces osteoclast lifespan. Currently being evaluated in phase I and phase II clinical trials for breast cancer and multiple myeloma, 2-ME differs from SERMs and estradiol in that its effects on tumor cells and normal osteoclasts do not depend on ER. Preliminary results suggest that it is far less toxic to normal cells than other anti-tumor drugs, presumably because it is exquisitely sensitive to transformed cells. But it is not entirely without effect on normal cells, as evidenced by its effects on normal osteoclasts. Inability to distinguish between target and non-target cells frequently produces unacceptable side effects for anti-cancer treatments. Ironically, in the case of 2-ME, by extending its repertoire to non-transformed osteoclasts, it may become a therapeutic agent for postmenopausal osteoporosis.

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REFERENCES

Aitken JM, Hart DM, Lindsay R. 1973. Oestrogen replacement therapy for prevention of osteoporosis after oophorectomy. Br Med J 3:515–518.

Attalla H, Westberg JA, Andersson LC, Adlercreutz H, Makela TP. 1998. 2-Methoxyestradiol-induced phosphorylation of Bcl-2: Uncoupling from JNK/SAPK activation. Biochem Biophys Res Commun 247:616–619.

Benford HL, McGowan NW, Helfrich MH, Nuttall ME, Rogers MJ. 2001. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. Bone 28:465–473.

Chambers TJ. 2000. Regulation of the differentiation and function of osteoclasts. J Pathol 192:4–13.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

D'Amato RJ, Lin CM, Flynn E, Folkman J, Hamel E. 1994. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. Proc Natl Acad Sci USA 91:3964–3968.

Djavaheri-Mergny M, Wietzerbin J, Rouillard D, Besancon F. 2003. TNFalpha potentiates 2-methoxyestradiol-induced mitochondrial death pathway. Ann NY Acad Sci 1010:159–162.

Evans GL, Morey-Holton E, Turner RT. 1998. Spaceflight has compartment- and gene-specific effects on mRNA levels for bone matrix proteins in rat femur. J Appl Physiol 84:2132–2137.

Fleming RM. 2003. Do women taking hormone replacement therapy (HRT) have a higher incidence of breast cancer than women who do not? Integr Cancer Ther 2:235–237.

Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP, Schweigerer L. 1994. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. Nature 368:237–239.

Frith JC, Monkkonen J, Blackburn GM, Russell RG, Rogers MJ. 1997. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5′-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. J Bone Miner Res 12:1358–1367.

Gelbke HP, Knuppen R. 1976. The excretion of five different 2-hydroxyoestrogen monomethyl ethers in human pregnancy urine. J Steroid Biochem 7:457–463.
Gingery A, Bradley E, Shaw A, Oursler MJ. 2003. Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. J Cell Biochem 89:165–179.
Heaney RP, Recker RR, Saville PD. 1978. Menopausal changes in bone remodeling. J Lab Clin Med 92:964–970.
Hulley SB, Grady D. 2004. The WHI estrogen-alone trial—Do things look any better? [comment]. JAMA 291:1769–1771.
Kashii Y, Giorda R, Herberman RB, Whiteside TL, Vujanovic NL. 1999. Constitutive expression and role of the TNF family ligands in apoptotic killing of tumor cells by human NK cells. J Immunol 163:5358–5366.
Katagiri T, Takahashi N. 2002. Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Dis 8:147–159.
Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K, Yagita H. 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs. J Exp Med 189:1451–1460.
Klauber N, Parangi S, Flynn E, Hamel E, D'Amato RJ. 1997. Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. Cancer Res 57:81–86.
Kumar AP, Garcia GE, Orsborn J, Levin VA, Slaga TJ. 2003. 2-Methoxyestradiol interferes with NF kappa B transcriptional activity in primitive neuroectodermal brain tumors: Implications for management. Carcinogenesis 24:209–216.
LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS. 2002. 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. Cancer Res 62:3691–3697.
LaVallee TM, Zhan XH, Johnson MS, Herbstritt CJ, Swartz G, Williams MS, Hembrough WA, Green SJ, Pribluda VS. 2003. 2-methoxyestradiol up-regulates death receptor 5 and induces apoptosis through activation of the extrinsic pathway. Cancer Res 63:468–475.
Lee SE, Chung WJ, Kwak HB, Chung CH, Kwack KB, Lee ZH, Kim HH. 2001. Tumor necrosis factor-alpha supports the survival of osteoclasts through the activation of Akt and ERK. J Biol Chem 276:49343–49349.
Lin HL, Liu TY, Wu CW, Chi CW. 2001. 2-Methoxyestradiol-induced caspase-3 activation and apoptosis occurs through G(2)/M arrest dependent and independent pathways in gastric carcinoma cells. Cancer 92:500–509.
Lotinun S, Westerlind KC, Turner RT. 2001. Tissue-selective effects of continuous release of 2-hydroxyestrone and 16alpha-hydroxyestrone on bone, uterus and mammary gland in ovariectomized growing rats. J Endocrinol 170:165–174.
Lu G, Janjic BM, Janjic J, Whiteside TL, Storkus WJ, Vujanovic NL. 2002. Innate direct anticancer effector function of human immature dendritic cells. II. Role of TNF, lymphotoxin-alpha(1)beta(2), Fas ligand, and TNF-related apoptosis-inducing ligand. J Immunol 168:1831–1839.
Luckman SP, Coxon FP, Ebetino FH, Russell RG, Rogers MJ. 1998. Heterocycle-containing bisphosphonates cause apoptosis and inhibit bone resorption by preventing protein prenylation: Evidence from structure-activity relationships in J774 macrophages. J Bone Miner Res 13:1668–1678.
Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Giannakakou P. 2003. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363–375.
Manolagas SC. 1995. Role of cytokines in bone resorption. Bone 17:63S–67S.
Maran A, Zhang M, Kennedy AM, Sibonga JD, Rickard DJ, Spelsberg TC, Turner RT. 2002. 2-Methoxyestradiol induces interferon gene expression and apoptosis in osteosarcoma cells. Bone 30:393–398.
Mukhopadhyay T, Roth JA. 1997. Induction of apoptosis in human lung cancer cells after wild-type p53 activation by methoxyestradiol. Oncogene 14:379–384.
Mukhopadhyay T, Roth JA. 1998. Superinduction of wild-type p53 protein after 2-methoxyestradiol treatment of Ad5p53-transduced cells induces tumor cell apoptosis. Oncogene 17:241–246.
Mundy GR, Boyce B, Hughes D, Wright K, Bonewald L, Dallas S, Harris S, Ghosh-Choudhury N, Chen D, Dunstan C. 1995. The effects of cytokines and growth factors on osteoblastic cells. Bone 17:71S–75S.
Pribluda VS, Gubish ER, Lavallee TM, Treston A, Swartz GM, Green SJ. 2000. 2-Methoxyestradiol: An endogenous antiangiogenic and antiproliferative drug candidate. Cancer Metastasis Rev 19:173–179.
Richelson LS, Wahner HW, Melton LJ III, Riggs BL. 1984. Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. N Engl J Med 311:1273–1275.
Ricker JL, Chen Z, Yang XP, Pribluda VS, Swartz GM, Van Waes C. 2004. 2-methoxyestradiol inhibits hypoxia-inducible factor 1alpha, tumor growth, and angiogenesis and augments paclitaxel efficacy in head and neck squamous cell carcinoma. Clin Cancer Res 10:8665–8673.
Riggs BL. 2000. The mechanisms of estrogen regulation of bone resorption [comment]. J Clin Invest 106:1203–1204.
Robinson JA, Waters KM, Turner RT, Spelsberg TC. 2000. Direct action of naturally occurring estrogen metabolites on human osteoblastic cells. J Bone Miner Res 15:499–506.
Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, Frith JC. 2000. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 88:2961–2978.
Roodman GD. 1999. Cell biology of the osteoclast. Exp Hematol 27:1229–1241.
Schumacher G, Kataoka M, Roth JA, Mukhopadhyay T. 1999. Potent antitumor activity of 2-methoxyestradiol in human pancreatic cancer cell lines. Clin Cancer Res 5:493–499.
Seegers JC, Lottering ML, Grobler CJ, van Papendorp DH, Habbersett RC, Shou Y, Lehnert BE. 1997. The mammalian metabolite, 2-methoxyestradiol, affects P53 levels and apoptosis induction in transformed cells but not in normal cells. J Steroid Biochem Mol Biol 62:253–267.
Sells Galvin RJ, Gatlin CL, Horn JW, Fuson TR. 1999. TGF-beta enhances osteoclast differentiation in hematopoietic cell cultures stimulated with RANKL and M-CSF. Biochem Biophys Res Commun 265:233–239.

Sibonga JD, Sommer U, Turner RT. 2002. Evidence that 2-methoxyestradiol suppresses proliferation and accelerates apoptosis in normal rat growth plate chondrocytes. J Cancer Res Clin Oncol 128:477–483.

Sibonga JD, Lotinun S, Evans GL, Pribluda VS, Green SJ, Turner RT. 2003. Dose-response effects of 2-methoxyestradiol on estrogen target tissues in the ovariectomized rat. Endocrinology 144:785–792.

Suparto IH, Williams JK, Cline JM, Anthony MS, Fox JL. 2003. Contrasting effects of two hormone replacement therapies on the cardiovascular and mammary gland outcomes in surgically postmenopausal monkeys. Am J Obstet Gynecol 188:1132–1140.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T. 2000. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma [comment]. Nature 408:600–605.

Takayanagi H, Kim S, Taniguchi T. 2002. Signaling crosstalk between RANKL and interferons in osteoclast differentiation. Arthritis Res 4 (Suppl 3):S227–S232.

Turner RT, Evans GL. 2000. 2-Methoxyestradiol inhibits longitudinal bone growth in normal female rats. Calcif Tissue Int 66:465–469.

Ware CF, Van Arsdale S, Van Arsdale TL. 1996. Apoptosis mediated by the TNF-related cytokine and receptor families. J Cell Biochem 60:47–55.

Yue TL, Wang X, Louden CS, Gupta S, Pillarisetti K, Gu JL, Hart TK, Lysko PG, Feuerstein GZ. 1997. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces apoptosis in endothelial cells and inhibits angiogenesis: Possible role for stress-activated protein kinase signaling pathway and Fas expression. Mol Pharmacol 51:951–962.

Zhu BT, Conney AH. 1998. Is 2-methoxyestradiol an endogenous estrogen metabolite that inhibits mammary carcinogenesis? Cancer Res 58:2269–2277.