2-Methoxyestradiol Suppresses Osteolytic Breast Cancer Tumor Progression In vivo

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Abstract

2-Methoxyestradiol (2ME2), a physiologic metabolite of 17β-estradiol (estrogen), has emerged as a promising cancer therapy because of its potent growth-inhibitory and proapoptotic effects on both endothelial and tumor cells. 2ME2 also suppresses osteoclast differentiation and induces apoptosis of mature osteoclasts, and has been shown to effectively repress bone loss in an animal model of postmenopausal osteoporosis. Given these observations, we have examined whether 2ME2 could effectively target metastasis to bone, osteolytic tumors, and soft tissue tumors. A 4T1 murine metastatic breast cancer cell line was generated that stably expressed Far Red fluorescence protein (4T1/Red) to visualize tumor development and metastasis to bone. In an intervention study, 4T1/Red cells were injected into bone marrow of the left femur and the mammary pad. In the latter study, 2ME2 (10, 25, and 50 mg/kg/d) treatment began on the same day as surgery and was continued for the 16-day duration of study. Tumor cell growth and metastasis to bone were monitored and bone volume was determined by micro-computed tomography. 2ME2 inhibited tumor growth in soft tissue, metastasis to bone, osteolysis, and tumor growth in bone, with maximum effects at 50 mg/kg/d. Furthermore, tumor-induced osteolysis was significantly reduced in mice receiving 2ME2. In vitro, 2ME2 repressed osteoclast number by inducing apoptosis of osteoclast precursors as well as mature osteoclasts. Our data support the conclusion that 2ME2 could be an important new therapy in the arsenal to fight metastatic breast cancer. [Cancer Res 2007;67(21):10106–11]

Introduction

Eighty percent of breast cancer patients develop bone metastasis as the disease advances. Bone marrow is a primary site of metastasis, most likely due to its extensive blood supply, available growth factors, and other aspects of the microenvironment. Once tumors lodge in bone, progressive tumor growth leads to osteoclast-mediated bone loss (osteolysis), which causes debilitating pain and pathologic fracture (1). Bisphosphonates antagonize metastatic tumor progression and decrease the number of bone-resorbing osteoclasts at sites of osteolysis (2). However, bisphosphonate therapy does not impede growth of tumors in soft tissues and, as a consequence, tumors at these sites continue to grow and metastasize (3). Therapies that target both osteolysis and tumor growth would be an improvement in the battle against breast cancer. 2-Methoxyestradiol (2ME2), a physiologic metabolite of 17β-estradiol that does not bind to the estrogen receptor (ER), has recently emerged as a promising anticancer agent because of its potent growth-inhibitory and proapoptotic effects on both endothelial and tumor cells. In vivo studies of several tumor types, including sarcoma, lung, and breast cancer cells, have documented potent inhibitory effects on tumor cells and angiogenesis while showing no clinical signs of toxicity (4–10). Recently, in a rat orthotopic brain tumor model, 2ME2 significantly inhibited growth of malignant gliosarcoma (11). Investigation of the effect of 2ME2 on bone metabolism in normal and ovariectomized rats revealed that 2ME2 suppressed bone loss in the ovariectomized rats in the absence of ER binding without causing uterotrophic effects (12, 13). In further studies, it was documented that 2ME2 repressed osteoclast differentiation while inducing mature osteoclast apoptosis (14). These combined data raise the strong possibility that 2ME2 treatment may decrease tumor burden while blocking tumor-induced osteolysis in vivo. In this report, we document that 2ME2 effectively represses tumor progression in a model that examines both tumor metastasis and tumor growth. Furthermore, we also found that 2ME2 induces osteoclast precursor apoptosis, revealing a mechanism by which 2ME2 reduces tumor-induced osteolysis.

Materials and Methods

Cell Culture, Reagents, and Animals

Cell lines were cultured in standard growth medium [α-MEM, 10% bovine serum albumin (BSA), 500 μg/mL G418]. 2ME2 was purchased from Calbiochem and dissolved in 95% ethanol. Recombinant receptor activator of nuclear factor-κB ligand (RANKL) was expressed in E. coli and purified using glutathione S-transferase (GST)-Sepharose columns. Macrophage colony-stimulating factor (M-CSF) was purchased from R&D Systems. α-MEM, DMEM, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA, used for cell culture, were purchased from Life Technologies, Inc. Reagents for tartrate-resistant acid phosphatase (TRAP) staining, bisbenzimide for staining nuclei, and all other chemicals were purchased from Sigma-Aldrich.

Generation of Far Red–Fluorescing 4T1 Cells

4T1 cells were grown in α-MEM supplemented with 10% BSA, 1% L-glutamine, and 1% penicillin and streptomycin in 5% CO2 and 95% air at 37°C. The cells were passaged at 80% to 90% confluence using a solution of 0.125% trypsin and 2 mmol/L EDTA in Ca2+/-Mg2+-free DMEM. The pRRES2-DsRed2 vector (BD Biosciences/Clontech) coding for Far Red protein was transfected stably into 4T1 cells by Lipofectamine (Invitrogen) according to the vendor’s instruction. The stable transfectants were selected in medium containing 500 μg/mL geneticin (Invitrogen) and cloned by limiting dilution. The stable clones expressing the Far Red protein were characterized under fluorescence microscopy.

In vivo Metastasis

Mice were maintained according to the National Cancer Institute Animal Care and Use Committee guidelines under approved animal study protocols.
Animals were anesthetized with ketamine/xylazine (0.65 mL ketamine + 0.25 mL xylazine + 9.1 mL sterile saline) before surgery for injection of tumor cells. The dose was determined on the basis of the mouse weight (17 μL/g of body weight).

**Experiment 1.** Mice (6–7 weeks old) were randomized into two groups (n = 3 per group) and inoculated with 10³ 4T1 cells expressing fluorescent FarRed protein (4T1/Red) into the left thoracic mammary pad (group 1) or the left femur marrow cavity (group 2). Sixteen days after implantation of tumor cells, mice were euthanized by carbon dioxide narcosis and femurs and tibias were excised and examined using a Kodak IS4000 imaging system.

**Experiment 2.** Mice (6–7 weeks old) were randomized into four treatment groups (n = 4 per group). Four of the groups were inoculated with 10³ 4T1/Red cells into the mammary pad and left femur. The other four groups were operated but did not receive tumor cells. 2ME2 treatment began on the same day as surgery and was administered to the tumor-inoculated and sham-operated groups by s.c. injection at 0 (ethanol vehicle), 10, 25, and 50 mg/kg 2ME2 doses daily. Mice were euthanized by carbon dioxide narcosis on day 16 and mammary pads, femurs, and tibias removed and fixed in 75% ethanol. Quantification of tumor cell burden in mammary pad and bone was done by calculating the fluorescence index density at 565 nm.

### Micro-Computed Tomography

A micro-computed tomography (micro-CT) was used for nondestructive three-dimensional evaluation of bone volume. Femora were scanned using a Scanco μCT40 scanner (Scanco Medical AG) at a voxel size of 12 × 12 × 12 μm and a threshold of 265 (gray scale, 0–1,000). Bone volume (mm³) was determined in 100 slices (1.2 mm) at a distance of 0.6 to 1.8 mm proximal to the highest point of the growth plate of the femur.

### Bone Histology

Tumor-injected vehicle (ethanol)- and 2ME2-treated bones were processed for glycol-methacrylate (JB4, Polysciences) embedding following fixation and dehydration in alcohols. Two-micrometer sections were stained for TRAP to localize osteoclasts and their immediate precursors as previously described (15).

### In vitro Osteoclastogenesis Assay

Freshly isolated bone marrow cells from BALB/c mice (6–7 weeks old) were cultured in α-MEM plus 10% FBS containing M-CSF (50 ng/mL) for 24 h. Nonadherent cells were collected, seeded at an initial density of 4.5 × 10⁵ per well in 24-well plates, and cultured in the presence of recombinant RANKL (100 ng/mL) and M-CSF (30 ng/mL). Media were changed on day 3. To evaluate the effects of 2ME2 on osteoclast precursor apoptosis, day 3 adherent precursors were treated with 2 μmol/L 2ME2 or
vehicle for 12 and 24 h and the cells were fixed in 1% buffered neutral paraformaldehyde. Hoechst and TRAP staining was carried out as previously described (16). Cells with condensed chromatin were considered apoptotic. The percentage of apoptotic cells was determined as the number of apoptotic mononuclear or multinucleated (>3 nucleated cells) cells versus the total number of mononuclear or multinucleated cells with the use of a fluorescence microscope.

Statistics
The results are calculated as mean ± SD. Statistical significance was determined by Student’s t test.

Results
In experiment 1, we injected buffer (sham) or 10³ 4T1/Red cells into the left femur marrow cavity or the mammary fat pad of BALB/c mice and monitored tumor growth and metastasis. Sixteen days after tumor implanting, the soft tissue tumor site and the left and right femurs and tibias were excised and examined by bioluminescence for tumor metastasis (Fig. 1). Mammary pad–injected tumor growth consistently metastasized to bones with a lower incidence of metastasis to soft tissues such as lung (data not shown). In addition to osteolytic tumor growth in the injected left limb from mice receiving tumor cells into the bone, metastases were also observed in the noninjected contralateral limb (Fig. 1, right limb). Thus, tumor injection into either site (mammary pad or bone) resulted in metastasis to bone.

In experiment 2, a dose response study of 2ME₂ effect on metastasis originating from simultaneous soft tumor and bone marrow injections was carried out (Fig. 2A–C). There was a significant inhibition of bone metastasis to the noninjected contralateral limb in mice receiving 25 mg/kg/d 2ME₂ (Fig. 2B) whereas 50 mg/kg/d 2ME₂ significantly inhibited tumor growth in both the injected and noninjected femurs (Fig. 2A and C). In the 50 mg/kg/d 2ME₂ treatment, there is variation of tumor suppression among injected bones (Fig. 2A). This could be the biological variability in responding to 2ME₂ treatment. However, overall there is significant inhibition observed in the 50 mg/kg/d 2ME₂ treatment group as compared with vehicle-treated group. Examination of the mammary fat pad tumor injection site

Figure 2. Fluorescence imaging of the in vivo dose-dependent effects of 2ME₂ on bone marrow– and mammary pad–injected 4T1/Red cells in BALB/c mice. A, 50 mg/kg/d 2ME₂ significantly inhibited tumor growth in bones. B, higher magnification of femur shows that metastatic tumor growth in the contralateral limb, into which no tumor cells had been injected, was also blocked by 50 mg/kg/d 2ME₂. Note that there was also significant inhibition of bone metastasis to the contralateral limb in mice receiving 25 mg/kg/d 2ME₂. C, fluorescence intensity analysis confirmed the inhibition of tumor growth by 2ME₂ in injected limbs and in the contralateral limbs. D, soft tissue tumor growth was significantly suppressed by 50 mg/kg/d 2ME₂.
revealed a significant suppression by the highest 2ME₂ dose, suggesting that 2ME₂ acts on primary tumor growth as well as metastasis (Fig. 2D).

Micro-CT analysis of the bones was used to quantify the effect of 2ME₂ on osteolysis (Fig. 3). Severe internal and external bone erosion in tumor-implanted vehicle-treated mouse bones was observed, indicating tumor expansion from medullary bone marrow to the external cortical bone (Fig. 3A, left images). Tumor-induced osteolysis was significantly reduced in the bones from mice receiving 2ME₂ (Fig. 3A, middle images). The bone volume of the distal femur from mice injected with 4T1/Red tumor cells was increased significantly in 50 mg/kg 2ME₂-treated mice, indicating that 2ME₂ clearly suppressed 4T1-induced osteolysis in tumor-injected mouse bones (Fig. 3B).

We have found that tumor cells secrete multiple cytokines and growth factors that simulate osteoclasts (17). We have also shown that 2ME₂ induces apoptosis of mature (multinucleated) osteoclasts and suppresses osteoclast differentiation (14). To examine 2ME₂ influences on osteoclasts and tumor cells in vivo, we examined the bone from vehicle- and 2ME₂-treated mice histologically (Fig. 3C). In vehicle-treated bones implanted with tumor cells, the bone surfaces adjacent to the marrow cavity had many acid phosphatase–positive osteoclasts whereas bones from 50 mg/kg 2ME₂–treated mice contained few, if any, osteoclasts (top left and right, respectively). To determine the mechanism by which 2ME₂ suppresses formation, mononucleated osteoclast precursors were treated with 2ME₂ during early differentiation (Fig. 4A). RANKL- and M-CSF–induced osteoclastogenesis was suppressed significantly when 2ME₂ (2 μmol/L) was added to culture media at the early stage of osteoclast differentiation (Fig. 4A). 2ME₂ treatment reduced the number of multinucleated osteoclasts and mononuclear TRAP-positive cells (Fig. 4B). 2ME₂ resulted in increased apoptosis of multinucleated osteoclasts and mononuclear TRAP-positive cells (Fig. 4C).

Discussion

Breast cancer that metastasizes to bone leads to pathologic bone erosion, causing debilitating pain, fracture, and hypercalcemia. As a means to mimic the patient condition, we have examined therapeutic responses on bone tumor load in an animal model where tumor cells are directly deposited into the bone microenvironment (18). In our in vivo model, tumor cells injected directly into bone marrow metastasized to distant extremities. These data support the capability of tumor cells to disseminate from the bone marrow microenvironment to other bones or soft tissues. To our knowledge, this is the first report showing bone to bone metastasis of breast cancer in a mouse model and confirms that 4T1 breast cancer cells can metastasize from secondary sites to distant extremities and organs. This may, therefore, be an ideal model to study the mechanism of bone to bone metastasis in breast cancer.

Targeting metastatic tumors at sites of metastasis would be of great benefit for patients who have advanced cancer diseases. Destroying tumors in bone and also slowing the development of osteolytic lesions would be desirable therapies and would greatly improve the prognosis of patients who have bone micrometastasis. Our results showed that 2ME₂ inhibits growth of highly metastatic ER-negative 4T1 cells in vivo. Studies also indicated that 2ME₂ represses growth and induces apoptosis of a variety of cancer cell lines, including ER-positive and ER-negative breast cancers.
cancer cells (19). These data suggest that 2ME₂ has no differential effect on ER-positive and ER-negative breast cancer cells, which is not surprising given that 2ME₂ does not bind to and activate ERs.

A limited number of in vivo studies of 2ME₂ have investigated tumor development using sarcoma, lung, brain, and soft tissue transplanted breast cancer cells (6, 9–11, 13). To date, no studies have examined the effect of 2ME₂ on tumor progression in bone. In the present study, we showed that 2ME₂ inhibits tumor-induced osteolysis in vivo while blocking tumor metastasis. Frequently, tumor-injected bones seemed to be shorter when viewed with the digital images (Figs. 1 and 2, left limb of tumor-injected bones without 2ME₂ treatment). As can be seen with the micro-CT image (Fig. 3A), this is due to the sever erosion of the bone when tumor cells are not treated with 2ME₂.

The published literature and our data support that the estrogen metabolite 2ME₂ may be a potential therapy targeting tumor-induced osteolysis and tumor metastasis. However, clinical studies indicated that p.o. delivery of 2ME₂ as a primary tumor therapy showed significant limitations due to its apparent poor bioavailability (4). In contrast, we have found that 2ME₂ can be effectively delivered by s.c. injection in much lower doses than p.o. administration. Using this in vivo metastasis model, we have shown for the first time that the physiologic metabolite 2ME₂ has direct inhibitory effects on primary tumor growth, metastasis to bones, and tumor-driven osteolysis. 2ME₂ represses osteoclast differentiation when precursors are cocultured with stromal cells and treated with vitamin D and dexamethasone (14). However, growth factors have been shown to have opposing influences on osteoclast differentiation in coculture compared with direct effects when precursors are cultured without stromal cells and provided with M-CSF and RANKL (20). We therefore have used this latter model to determine the influences of 2ME₂ on osteoclast precursors. In this alternative model of osteoclast formation, 2ME₂ targeted osteoclast precursors

Figure 4. Effect of 2ME₂ treatment on the differentiation of TRAP-positive RANKL- and M-CSF–induced osteoclast cells. 2ME₂ (2 μmol/L) added to culture media in the presence of RANKL (100 ng/mL) and M-CSF (30 ng/mL) at day 3 and incubated for 12 and 24 h (A). Microscopically, the TRAP-positive cells containing three or more nuclei were counted as osteoclasts. 2ME₂ inhibited osteoclast-like cell formation and the ability of TRAP-positive precursor mononucleated cells to form osteoclasts (B). 2ME₂ also induced osteoclast precursor apoptosis and apoptosis of TRAP-positive mononucleated cells (C).
as well as mature osteoclasts by inducing apoptosis. Because of its low toxicity and inhibitory actions on osteoclasts as well as breast tumor cells, 2ME₂ is an attractive candidate for controlling metastasis to bone and bone disorders.

In conclusion, we have shown that 2ME₂ inhibits tumor metastasis and osteolysis in the bone microenvironment caused by metastatic breast cancer cells. The in vivo and in vitro data clearly indicated that 2ME₂ has direct effects on reducing tumor burden as well as inhibiting tumor-induced osteoclast-mediated osteolysis. Therefore, the reduction of osteoclast number in 2ME₂-treated mice prevents the tumor-induced bone loss. Based on these findings, we propose 2ME₂ as a therapeutic agent targeting primary tumor, metastasis to bone, and tumor-induced osteolysis.

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