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Common Mechanism for the Estrogen Agonist and Antagonist Activities of Droloxifene

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Abstract The incidence of postmenopausal osteoporosis is increasing as the population ages. Even though estrogen replacement therapy has proven beneficial in reducing the number of skeletal fractures, the known risks and associated side-effects of estrogen replacement therapy make compliance poor. Recent research has focused on the development of tissue specific estrogen agonist/antagonists such as droloxifene which can prevent estrogen deficiency-induced bone loss without causing uterine hypertrophy. Furthermore, droloxifene acts as a full estrogen antagonist on breast tissue and is being evaluated for treatment of advanced breast cancer. In this report we propose a common mechanism of action for droloxifene that underlies its estrogen agonist and antagonist effects in different tissues. Droloxifene and estrogen, which have identical effects on bone *in vivo,* both induced p53 expression and apoptosis in cells of *in vitro* rat bone marrow cultures resulting in a decrease in the number of bone-resorbing osteoclasts. Droloxifene is growth inhibitory in MCF-7 human breast cancer cells and therefore acts as an antagonist, whereas estrogen is mitogenic to these cells and acts as an agonist. Droloxifene, but not estrogen, induced p53 expression and apoptosis in MCF-7 cells. These results indicate that the induction of apoptosis by droloxifene may be the common mechanism for both its estrogen agonist effects in bone and its antagonist effects in breast tissue. J. Cell. Biochem. 65:159–171.

Key words: breast cancer; droloxifene; estrogen replacement therapy; apoptosis; osteoclasts

Postmenopausal osteoporosis is a poorly treated disease that results from the decline in circulating estrogen at menopause and affects a large percentage of women. The incidence of osteoporosis is increasing as the population ages [Riggs and Melton, 1992]. Estrogen replacement therapy has proven beneficial in reducing the number of skeletal fractures [Riggs and Melton, 1992; Turner et al., 1994; Lobo, 1995; Paganini-Hill and Henderson, 1994]. In spite of estrogen's efficacy in preventing postmenopausal bone loss, widespread and long-term acceptance of estrogen therapy is limited because of side effects on reproductive tissues and increased risks of breast and uterine cancer [Riggs and Melton, 1992; Turner et al., 1994; Lobo, 1995].

Droloxifene is a member of a new class of nonsteroidal estrogen agonists/antagonists in clinical development which possess the desirable bone-protective and lipid-lowering activities of estrogen [Riggs and Melton, 1992; Lobo, 1995; Paganini-Hill and Henderson, 1994] without the side effects profile associated with conventional hormone replacement therapy. Droloxifene prevents ovariectomy-induced bone loss in rats, and is in concurrent clinical trials for the prevention and treatment of osteoporosis and treatment of advanced breast cancer [Chen et al., 1995; Ke et al., 1995; Eppenberger et al., 1991; Roos et al., 1983]. The mechanism by which droloxifene is able to exert an agonist effect on bone and an antagonist effect in breast tissue remains unknown. Furthermore, the molecular mechanism by which estrogen exerts its bone protective effects is not clearly understood [Riggs and Melton, 1992; Turner et al., 1994; Lobo, 1995; Lindsay, 1991; Ericksen et al., 1988; Komm et al., 1988].

Recent evidence suggests that 17β-estradiol decreases the levels of IL-6 mRNA and suppresses osteoclast development in primary murine bone cells [Girasole et al., 1992]. In vitro cultures of bone marrow from ovariectomized mice show increased numbers of osteoclasts

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and this increase can be prevented by the addition of either 17β-estradiol or antibodies against IL-6 [Jilka et al., 1992]. Droloxifene or estrogen treatment in rats results in a decrease in bone resorption which correlates well with the reduced number of osteoclasts seen *in vivo* in treated animals [Chen et al., 1995; Ke et al., 1995].

Tamoxifen, an estrogen agonist in breast tissue, has been shown to induce apoptosis in human MCF-7 breast cancer cells [Chen et al., 1996]. Apoptosis or programmed cell death serves as a physiological suicide mechanism that preserves tissue homeostasis [Wyllie, 1980; Kerr et al., 1994]. Unlike necrotic cell death, apoptosis is characterized by chromatin condensation, cytoplasmic blebbing, and DNA fragmentation [Wyllie, 1980]. Numerous studies have shown that while the tumor suppressor gene p53 is a negative regulator of cell proliferation, it can also play an important role in programmed cell death or apoptosis [Oren, 1994].

The purpose of this study was to understand the tissue-specific effects of droloxifene and compare the mechanism of action of droloxifene and estrogen in two clinically important target tissues. First, we established an *in vitro* osteoclast differentiation system. Second, we showed that both droloxifene and estrogen decrease the number of differentiated osteoclasts that form *in vitro,* in parallel with the decrease in bone resorption observed *in vivo.* Finally, we provide evidence for specific induction of p53-mediated apoptosis upon estrogen or droloxifene treatment in a subpopulation of CD61 (+) rat bone marrow cells. In contrast, droloxifene but not estrogen can inhibit growth and induce apoptosis of the estrogen receptor-positive human MCF-7 breast cancer cell line.

METHODS

Ovariectomy, Isolation, and Culture of Rat Bone Marrow Cells

The ovariectomized (OVX) rat is a widely accepted preclinical model for estrogen deficiency-induced bone loss. Following surgical induction of estrogen deficiency, rat skeletons exhibit an increase in osteoclast number that is coupled with the eventual loss of trabecular bone [Kalu, 1991; Wronski et al., 1989; Kalu et al., 1991]. In order to investigate the mechanism of action of droloxifene and estrogen on osteoclast formation, we developed a rat marrow culture system to parallel the *in vivo* rat OVX model. Female Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 240 to 280 g were allowed to acclimate to vivarium conditions for 1 week before surgery. Groups of 10 rats each were sham-operated or bilaterally ovariectomized according to institutionally approved protocols. Bone marrow samples for *in vitro* culture were collected 2 weeks after surgery.

The bone marrow culture system was adapted from methods previously described for the mouse [Hata, et al., 1992; McSheehy and Chambers, 1987; Takahashi et al., 1988b and c]. Briefly, diaphyseal bone marrow from the tibiae of OVX or sham-operated Sprague-Dawley rats was collected 14 days post-surgery in complete medium consisting of phenol red-free α-MEM (Gibco, Grand Island, NY) with 15% fetal bovine serum (FBS, Gemini, Calabasas, CA) and antibiotics. After gentle trituration of the marrow fragments to produce a single cell suspension, the suspension was filtered through 100-μm nylon mesh to remove bone fragments. Cells were plated at 5×10^5 cells/cm^2 in complete medium with 10^{-8} M 1α,25-dihydroxyvitamin D_3 (Biomol, Plymouth Meeting, PA). After overnight culture, the cells were separated into adherent and nonadherent cell populations. Nonadherent cells were pipeted off the dish, the dish rinsed several times with calcium-magnesium free phosphate buffered saline (CMF/PBS), pelleted, and counted. The remaining adherent cells were trypsinized off the dish, pelleted, and counted. Adherent marrow cells were plated at 1,000 cells/cm^2 in 24-well plates with complete medium containing 5 ng/ml platelet-derived growth factor (Human PDGF-BB, Collaborative Research, Bedford, MA) and cultured for 2 days, while nonadherent cells were plated at 5×10^5 cells/cm^2 in complete medium without PDGF and cultured for 2 days. Nonadherent cells were then added back to the adherent cell cultures in complete medium with 10^{-8} M 1α,25-dihydroxyvitamin D_3. Droloxifene and 17β-estradiol were added from 10^{-2} M stocks and diluted to the indicated final concentrations. The cultures were maintained for 6 more days with one-half the medium containing fresh compounds and 10^{-8} M 1α,25-dihydroxyvitamin D_3 being replaced every 3 days. To visualize the osteoclasts formed in culture, histochemical staining for tartrate-resistant acid phosphatase (TRAP) was carried out using the substrate napthol AS-BI phosphate with Fast Garnet as the color development reagent (Sigma, St. Louis, MO).

Pit Formation on Bone Slices

To confirm that the multinucleated cells formed in culture had the capability to resorb bone, we used the resorption pit assay as described previously [Sato and Grasser, 1990]. Briefly, rat bone marrow cells (5×10^5 cells/cm^2) were plated onto 100 µm thick sterilized bovine bone slices (4.5 mm^2) in 96-well plates and cultured as described above with one-half the culture medium being replaced every 3 days. Bone slices were collected at 8, 10, 12, and 14 days of culture, placed in distilled water, and sonicated for 3×30 sec each at room temperature (RT). Bone slices were then rinsed briefly ethanol, air dried, stained with 0.1% toluidine blue, and examined for the presence of resorption pits under reflected light.

Calcitonin Autoradiography

To further confirm the osteoclastic phenotype, autoradiography of [^{125}I]-calcitonin binding was performed as described [Takahashi et al., 1988a]. Briefly, rat bone marrow cells were cultured as described above in four-well chamber culture slides (Nunc, Inc., Naperville, IL) for 8 days. Before incubation with [^{125}I]-calcitonin (2,000 Ci/mmol; Amersham, Arlington Heights, IL), cells were washed with α-MEM containing 0.1% bovine serum albumin (BSA) for 10 min at RT. Cells were then incubated with 0.2 nM [^{125}I]-calcitonin in α-MEM containing 0.1% BSA plus or minus 200 nM salmon calcitonin (Bachem California, Torrance, CA) for 1 h at RT. After labelling, the cells were rinsed 2×5 min with cold α-MEM, fixed with 2% paraformaldehyde in 0.1 M sodium cacodylate for 10 min, washed with α-MEM (2×5 min), and TRAP stained. Slides were then dipped into Kodak NTB-2 autoradiography emulsion, exposed in the dark for 2 weeks at 4°C, developed in D-19 developer diluted 1:2 with water for 4 min at 16°C, fixed and mounted with 90% glycerol:10% PBS for viewing under an Olympus BH-2 microscope.

Immunolocalization of Estrogen Receptor (ER)

Biological responses to droloxifene and estradiol are mediated by ER [Eppenberger et al., 1991]. To confirm the ability of bone marrow cells to respond to estradiol, we showed the presence of ER in cultured bone marrow cells by immunohistochemistry using an antibody raised against the first 21 amino acids in the estrogen receptor by G. Greene, University of Chicago. Bone marrow cells were cultured on glass chamber slides for 8 days, fixed in 4% paraformaldehyde in calcium and magnesium free phosphate buffered saline (CMF/PBS), pH = 7.4 for 20 min at RT after 2 h of pretreatment with 10^{-8} M 17β-estradiol at 37°C. Cells were pretreated with 17β-estradiol to maximize the nuclear localization of ER. Cultures were then rinsed with CMF/PBS, 0.1% BSA (2×5 min), followed by 0.1% saponin in CMF/PBS for 5 min at RT. Treatment with 0.1% saponin, as opposed to a harsher detergent like Triton X-100, resulted in better ER immunostaining [Grasser, unpublished observations]. Non-specific binding was blocked by incubating in 1.5% donkey serum in CMF/PBS for 30 min at RT. Cells were then incubated with polyclonal ER antibody or antibody that had been neutralized (bound to a 60-fold weight excess of immunizing peptide for 2 h at RT) overnight at 4°C in a humid chamber. Cells were rinsed in CMF/PBS ($3 \times$ 5 min) and then incubated with Cy3-conjugated-donkey anti-rabbit $F(ab')_2$ fragments (Jackson Immunochemicals, West Grove, PA) for 60 min at RT. Slides were rinsed in CMF/PBS (3×5 min) and mounted in Fluorosav (Testog, Chicago, IL) and viewed under epifluorescent illumination.

Cell Cycle Analysis of MCF-7 Cells

To study the effect of droloxifene and estradiol on cell growth, cell cycle analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using propidium iodide (PI) to stain nuclear DNA as described [Taylor, 1980]. MCF-7 cells were plated in six-well plates (Costar, Cambridge, MA) at 3×10^6 cells/well in DMEM/F12 media with 10% FBS and cultured for 48 h at 37°C in 5% CO_2. Media was replaced with fresh media containing 0.2% FBS and either 10^{-8} M droloxifene, 10^{-8} M 17β-estradiol, or vehicle (0.01% ethanol), and cultured overnight. For cell cycle analysis, cell cultures were stained by adding 0.2 ml/ml of stain concentrate (5X stock: 0.25 mg/ml PI, 0.01 mg/ml Triton-X 100; Sigma, St. Louis, MO) and rocking cultures for 5 min at RT to remove cells from substrate. Cells were carefully disaggregated by pipetting up and down, incubated 20 min and then subjected to flow cytometry. Data were then analyzed using Modfit (Verity Software House, Inc., Topsham, ME).

For TUNEL labelling, 10^6 cells were plated on each 22 mm^2 glass coverslip, cultured, and labelled as described below.

TdT-Mediated dUTP Nick End Labelling (TUNEL), p53, and CD61 Localization

To identify apoptotic bone marrow or MCF-7 cells, fragmented nuclear DNA was labelled by TUNEL followed by co-localization with p53 or CD61 antibodies to further confirm apoptosis (p53) and cell identity (CD61). A fluorescent TUNEL kit was used according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN). On day 3 of culture, cells were treated with 10^{-8} M droloxifene, 10^{-8} M 17β-estradiol, or vehicle (0.01% ethanol) for 3 h (bone marrow) or 12–16 h (MCF-7) at 37°C. Cells were then fixed in 4% paraformaldehyde in CMF/PBS for 30 min at RT, rinsed with CMF/PBS (2 × 10 min), and permeabilised with 0.1% Triton X-100, 0.1% sodium citrate for 2 min at 4°C. Cells were rinsed 2 × 10 min with CMF/PBS and then incubated in a humidified chamber with the TUNEL reaction mixture for 60 min at 37°C in the dark. The cells were then rinsed 3 × 5 min with CMF/PBS and then incubated with 5 μg/ml anti-p53 monoclonal antibody, (Ab1; Oncogene Sciences, Cambridge, MA) or 5 μg/ml anti-CD61 monoclonal antibody (Clone F11; Pharmingen, San Diego, CA) diluted in CMF/PBS containing 0.1% BSA for 60 min at RT. After incubation with primary antibody, cells were rinsed 2 × 10 min with CMF/PBS and incubated in a 1:2000 dilution of Cy3-conjugated donkey anti-mouse $F(ab')_2$ secondary antibody in CMF/PBS containing 0.1% BSA (Jackson Immunochemicals, West Grove, PA) for 60 min at RT. Cells were rinsed 2 × 10 min with CMF/PBS and mounted in Fluorosav. Labelled cells were viewed under an Olympus BH-2 microscope equipped for fluorescent microscopy.

Statistical Analysis

Data are expressed as mean ± SEM. Statistics were calculated using StatView 4.0 software (Abacus Concepts, Inc., Berkeley, CA). The analysis of variance (ANOVA) test followed by Fisher's protected least significant difference was used to compare differences between groups [Neter et al., 1982].

RESULTS

Inhibition of Osteoclast Number

The OVX rat is a widely accepted preclinical model for estrogen deficiency-induced bone loss. Following surgically-induced estrogen deficiency, rat skeletons exhibit an increase in osteoclast number that is coupled to the eventual loss of trabecular bone [Kalu, 1991; Wronski et al., 1989; Kalu et al., 1991]. In order to investigate the mechanism of action of droloxifene and estrogen on osteoclast formation, we developed an in vitro rat bone marrow culture system to parallel this aspect of the *in vivo* rat OVX model. This rat bone marrow culture system produced multinucleated cells which were osteoclast-like based on: 1) histochemical staining for TRAP (Fig. 1a); 2) their ability to excavate resorption lacunae on bovine bone slices (Fig. 1b); and 3) calcitonin receptor expression (Fig. 1c,d). Multinucleated cells possessing these osteoclastic characteristics were first noted after 5–6 days in culture.

Cells with nuclear localization of ER could be demonstrated throughout the 8-day culture period (Fig. 1e). Nuclear staining was completely absent using ER antibodies that were neutralized with ER peptide (Fig. 1f). This confirmed that cells in the rat bone marrow culture system possessed ER and therefore, had the ability to respond to droloxifene and estradiol by a receptor-mediated mechanism.

As previously reported, OVX increased the numbers of osteoclasts *in vivo* [Chen et al., 1995]. The potential of rat bone marrow cultures to form osteoclast-like cells in the presence of 10^{-8} M 1α,25-dihydroxyvitamin D_3 was similarly increased 2 weeks after OVX. (Fig. 2). Treatment of OVX bone marrow cultures for eight days with droloxifene (10^{-8} M to 10^{-12} M) or 17β-estradiol (10^{-8} M to 10^{-11} M) significantly reduced the number of multinucleated TRAP (+) osteoclast-like cells in a dose dependent manner (Fig. 2). The reduction in osteoclast number *in vitro* was nearly identical for both droloxifene and estradiol and closely paralleled *in vivo* results obtained with these compounds [Chen et al., 1995].

Droloxifene Treatment Increased the Number of Apoptotic Cells in Marrow

The change in osteoclast cell number seen after OVX could be caused by decreasing the rate of cell proliferation or by increasing the rate of apoptosis [Wyllie, 1980]. In the OVX rat, the observed increase in the number of differentiated osteoclasts presumably results from an increased recruitment of pre-osteoclastic cells into the osteoclast lineage. Since droloxifene or 17β-estradiol treatment suppressed the OVX-associated increase in functional osteoclasts, we asked whether droloxifene or estradiol increased the rate of apoptosis of pre-osteoclastic

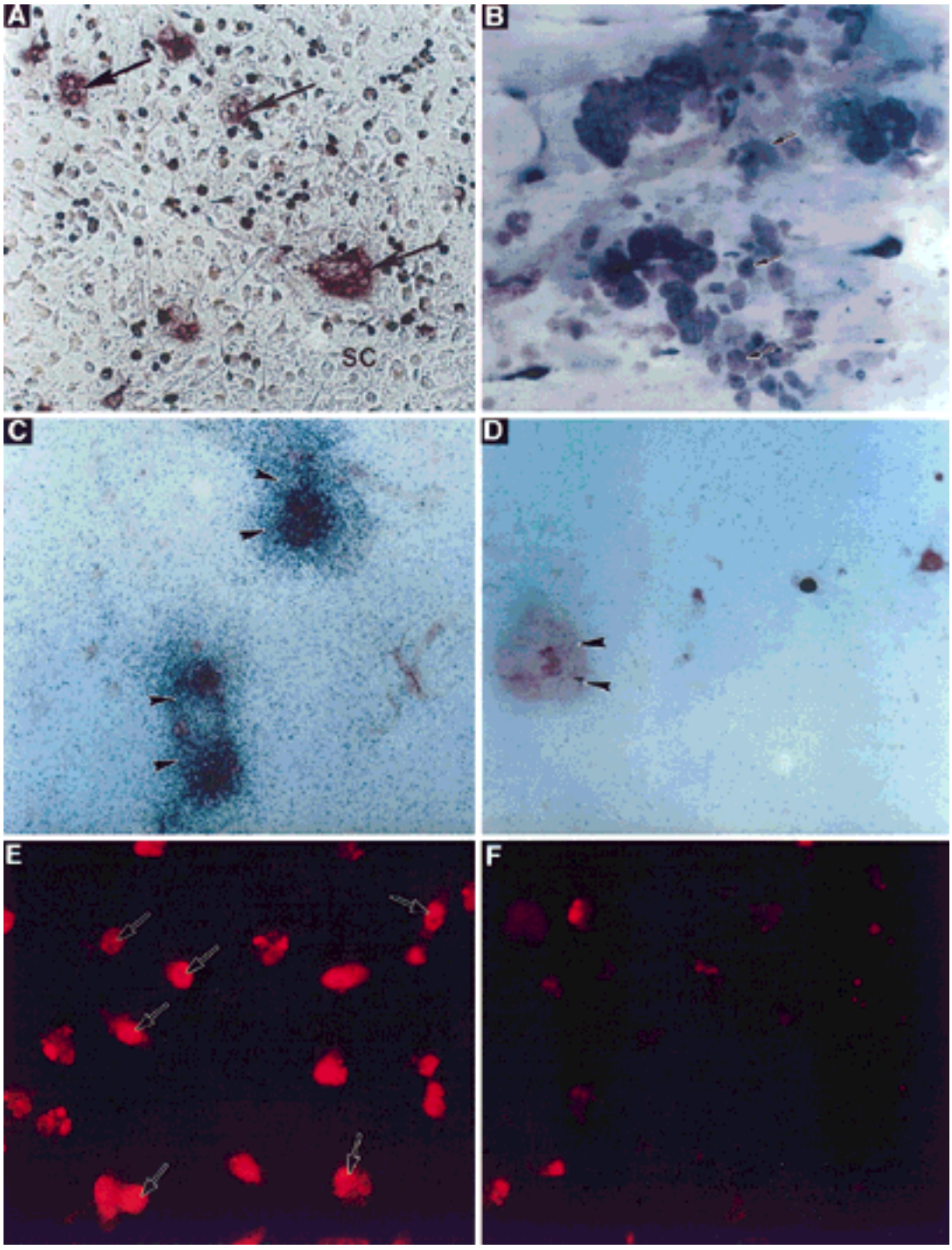

Fig. 1. Osteoclast differentiation in cultures of rat bone marrow cells. The differentiation of rat marrow cells into functional osteoclasts was confirmed by using TRAP histochemical localization, resorption pit formation, and calcitonin receptor binding as markers of osteoclasts. **A:** TRAP staining of 8-day cultures. TRAP (+) cells with three or more nuclei were considered to be osteoclasts (examples indicated by arrows). Stromal cells (SC) are TRAP (−). **B:** Resorption lacunae on bovine bone slices. The development of resorption lacunae (several are indicated by arrows) show that functional osteoclasts were produced. **C:** Calcitonin receptor co-localization with TRAP. Silver grains can be seen over TRAP (+) multinucleated cells in 8-day rat bone marrow cultures as indicated by arrowheads. **D:** No silver grains can be observed over the TRAP (+) multinucleated cells after incubation with an excess of unlabelled calcitonin. **E:** Immunolocalization of ER in Day 3 cultures. Nuclear localization of the ER was observed. (examples indicated by arrows) and was also observed throughout the 8-day culture period in rat bone marrow cultures (data not shown). **F:** Incubation of the cultures with neutralized ER antibody resulted in the lack of nuclear staining. Magnifications are 200× in (A); 250× in (B,C,D), and 200× in (E,F).

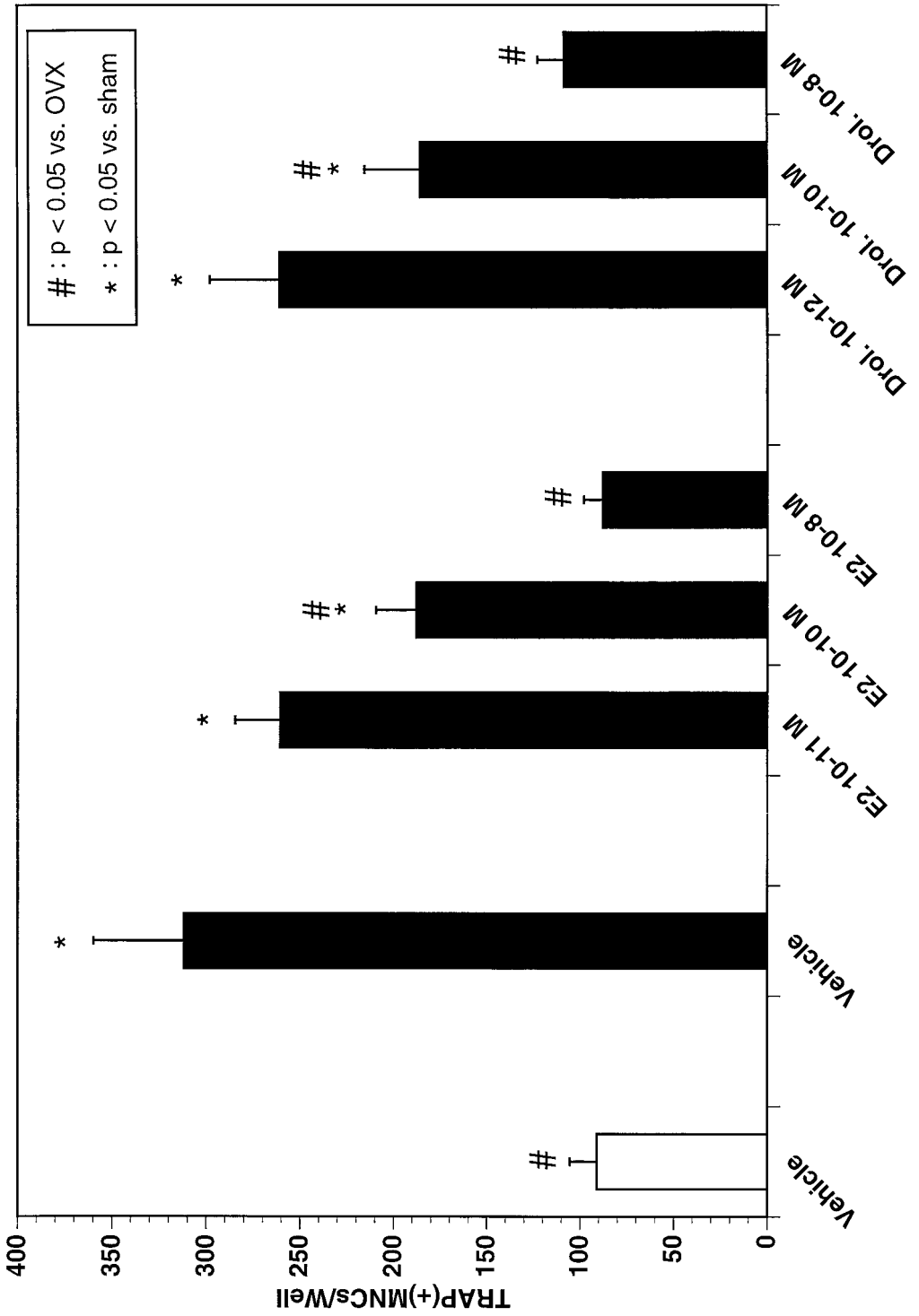

Fig. 2. Inhibition of osteoclastogenesis by droloxifene and estradiol treatment. Bone marrow cultures from OVX rats were treated with droloxifene (Drol.), 17β-estradiol (E2), or 0.01% ethanol vehicle (see Methods). Cultures from OVX or sham-operated rats were then stained for TRAP after 8 days of culture, and the number of stained multinucleated cells with three or more nuclei were scored. Data are expressed as mean ± SEM. #$P < 0.05$ vs. OVX; *$P < 0.05$ vs. sham.

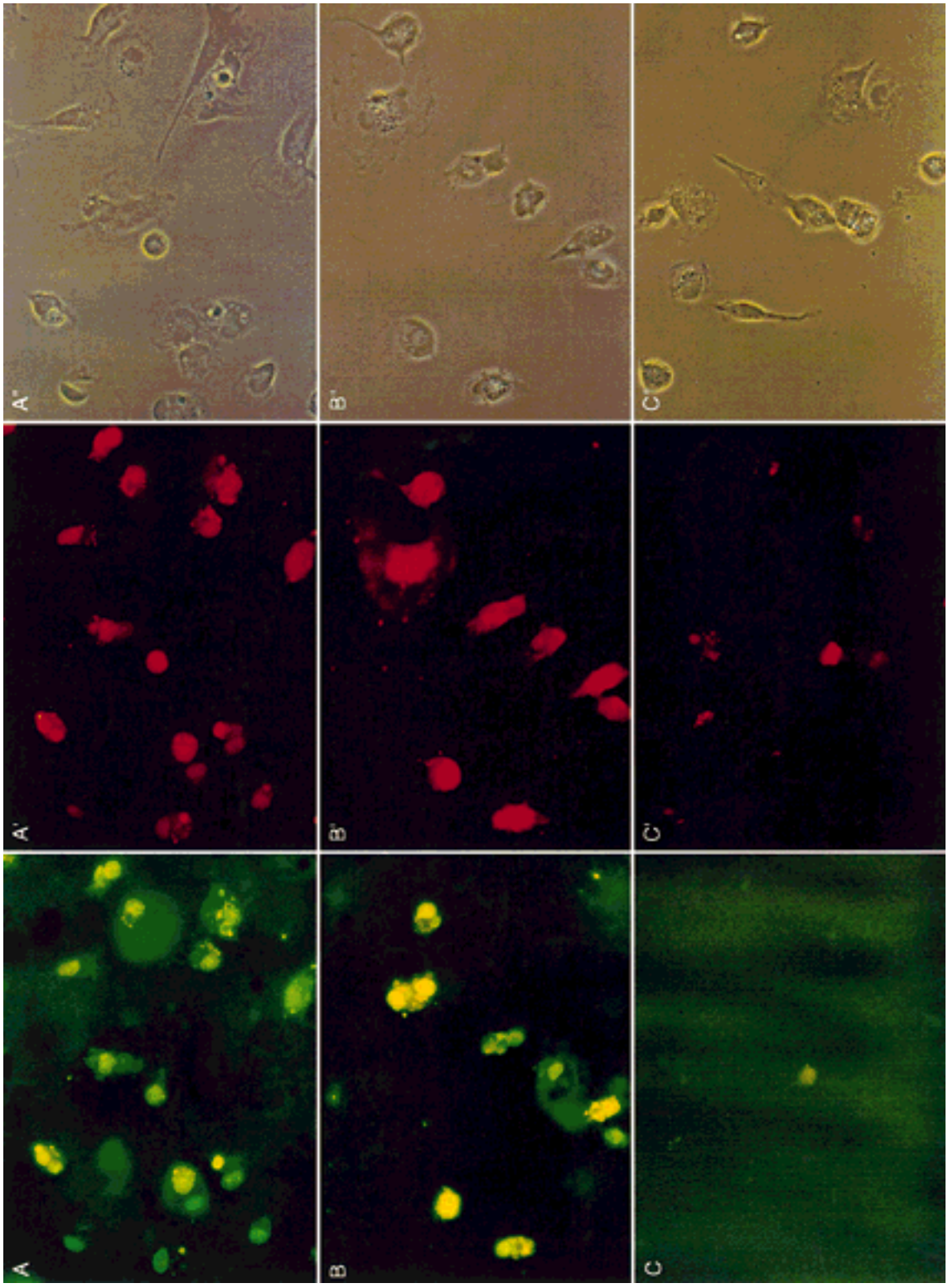

Fig. 3. Induction of apoptosis and p53 expression in bone marrow by droloxifene and estradiol. Fluorescent TUNEL labelling and p53 co-localization in 3-day bone marrow cultures from OVX rats treated for 3 h with (**A**) 10^{-8} M droloxifene, (**B**) 10^{-8} M 17β-estradiol, or (**C**) vehicle at 37°C. The same culture fields are shown for TUNEL (A–C), p53 (A′–C′) and phase contrast images (A″–C″). Magnification = 200×.

cells. Treatment of cultures with 10^{-8} M droloxifene or 17β-estradiol for 3 h resulted in a 2–3-fold increase in the number of apoptotic cells as identified by fluorescent TUNEL labelling when compared to control cultures (Fig. 3).

Colocalization of p53 in TUNEL Labelled Cells in Marrow

Increasing the level of the p53 tumor suppresser gene product can induce apoptosis and abundant expression of p53 is usually indicative of cells undergoing apoptosis [Wyllie, 1980; Kerr et al., 1994; Oren, 1994; Metz et al., 1995; Kroemer et al., 1995]. Using p53 specific antibodies, we co-localized high levels of p53 expression in TUNEL-positive bone marrow cells after treatment with 10^{-8} M droloxifene (Fig. 3a′) or 17β-estradiol (Fig. 3b′). In both cases, p53 expression was observed in all apoptotic cells suggesting that p53-mediated cell death may be a common pathway by which droloxifene and estradiol regulate the numbers of osteoclasts formed from bone marrow precursors.

Immunolocalization of CD61 in Apoptotic Cells in Marrow

Our studies show that treatment with droloxifene or estradiol produce an increase in apoptotic cells with a net decrease in osteoclast number at the end of the culture period. However, little is known about the ontogeny of the apoptotic cells in this system. The apoptotic cells could be osteoclast precursors, osteoblastic cells, stromal cells that support osteoclast differentiation via secretion of cytokines or some other cell type. In order to further characterize the apoptotic cell population, we tested the ability of a cell surface marker, CD61 (β_3 integrin) to co-localize to apoptotic cells. CD61 has been shown to be selectively expressed on cells of the osteoclastic lineage in multiple species [Athanasou et al., 1992; Helfrich et al., 1992]. Rat bone marrow cultures that were pre-treated for 3 h with 10^{-8} M droloxifene show increased numbers of TUNEL positive cells (Fig. 3). Co-localization with a monoclonal antibody specific for CD61 showed specific labelling of some (10–25%) of the apoptotic cells (Fig. 4A) with CD61 (Fig. 4B); however, not all of the cells that were positive for CD61 were undergoing apoptosis (data not shown). Since some of the cells undergoing apoptosis express CD61, it seems likely that a portion of the apoptotic cells belong to the osteoclast lineage.

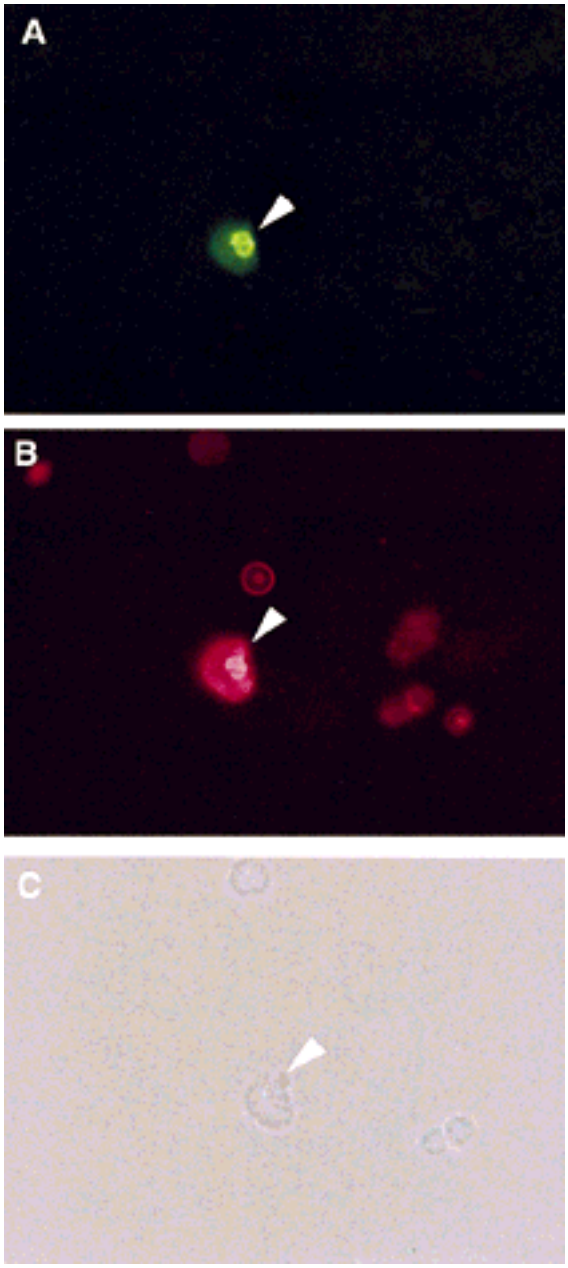

Fig. 4. Immunolocalization of CD61 in apoptotic cells. Three-day rat bone marrow cultures were TUNEL labelled followed by immunolocalization using a monoclonal antibody to CD61 after a 3 h 10^{-8} M droloxifene treatment. Three views of the same microscopic field show (**A**) TUNEL labelling, (**B**) CD61 immunofluorescence, and (**C**) phase contrast imaging. Magnification = 400×.

Treatment of MCF-7 Cells With Droloxifene and Estradiol

Like tamoxifen, droloxifene has antiestrogenic effects on MCF-7, an ER positive human mammary carcinoma cell line [Eppenberger et

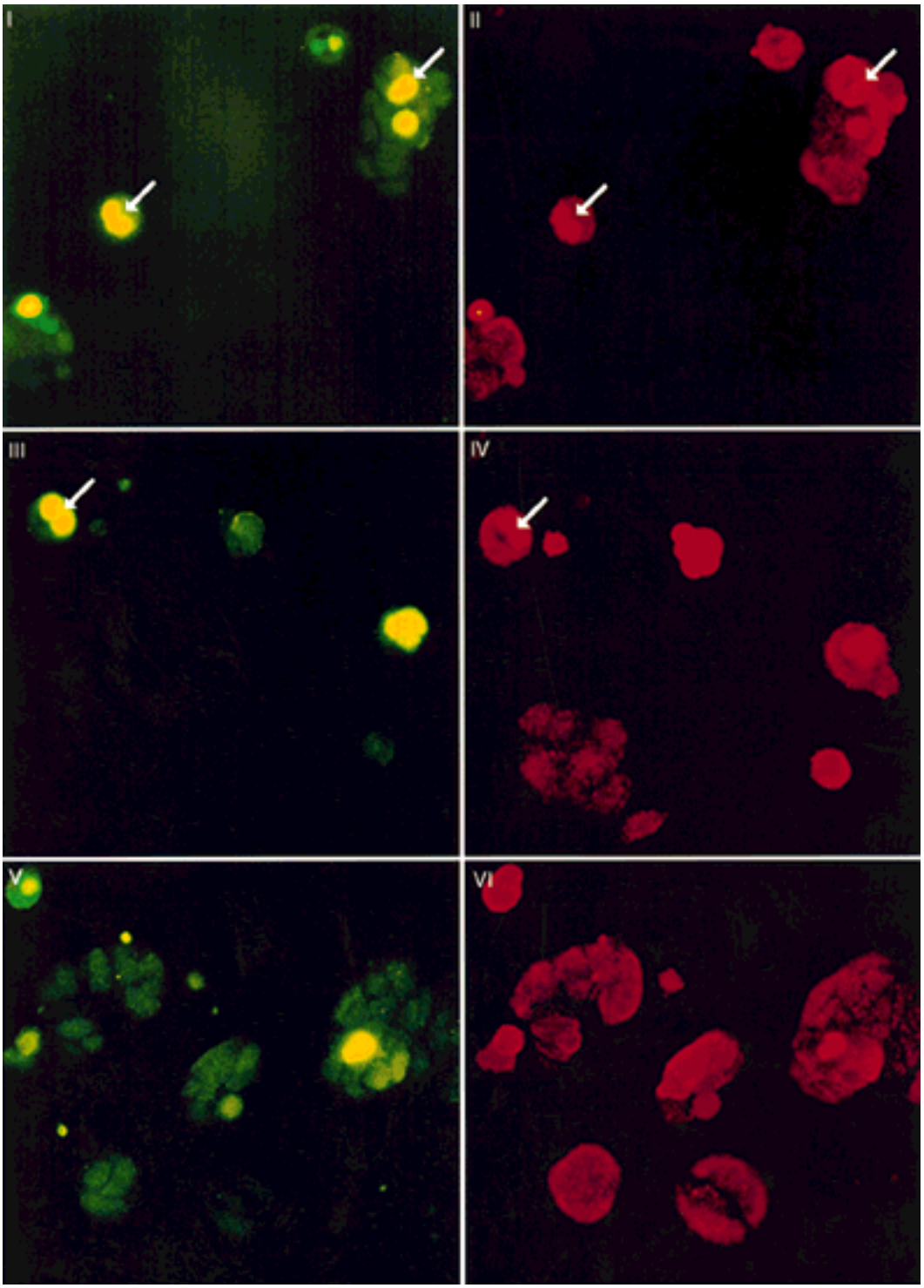

Fig. 5. Induction of apoptosis and p53 in MCF-7 cells by droloxifene. MCF-7 cells were treated with 10^{-8} M droloxifene, 10^{-8} M 17β-estradiol, or vehicle (0.01% ethanol) for 16–18 h at 37°C. After treatment, cells were TUNEL labelled and then p53 was immunolocalized in the TUNEL labelled cells as described in Methods section. Fluorescent images for TUNEL (**I,III,V**) and p53 (**II,IV,VI**) of the same microscopic field are shown for droloxifene (I,II), estradiol (III,IV) and vehicle (V,VI) treatments. Magnification = 400×.

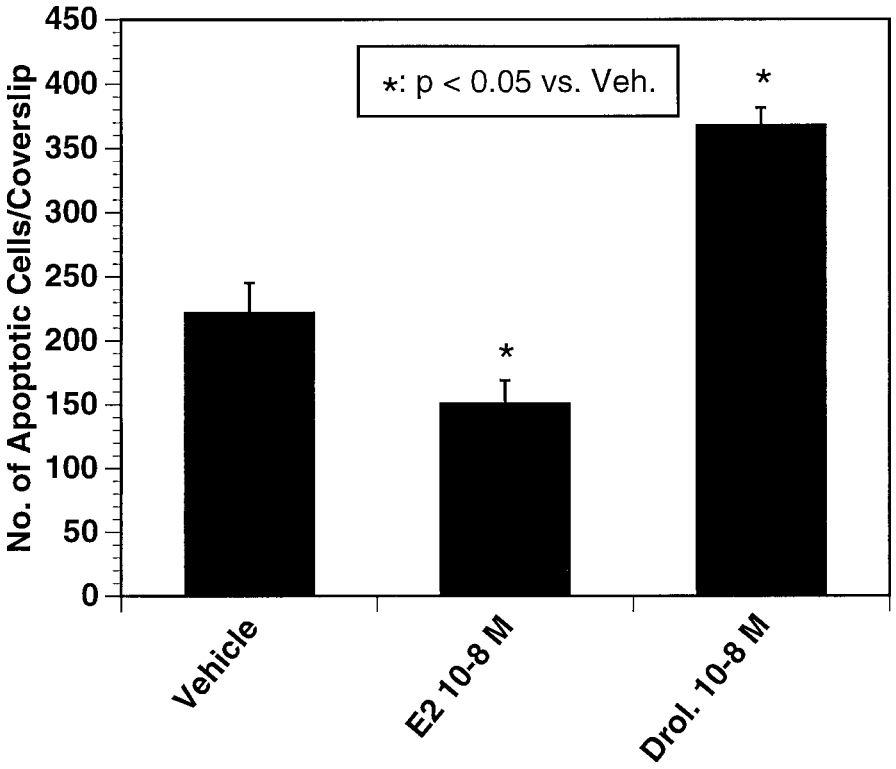

Fig. 6. Quantification of apoptotic MCF-7 cells. MCF-7 cells were treated with 10^{-8} M droloxifene, 10^{-8} M 17β-estradiol, or vehicle (0.01% ethanol) for 3 h at 37°C. Cells on coverslips were TUNEL labelled and stained with anti-p53 antibody. The total number of apoptotic cells (TUNEL and p53) was counted. Data are expressed as mean ± SEM, n = 3. *$P < 0.05$ vs. Veh. These results are representative of three replicate experiments.

al., 1991]. Droloxifene's antagonism of breast cancer cell growth might also be achieved through apoptosis as has been reported for tamoxifen [Chen et al., 1996]. MCF-7 cells treated with either droloxifene or 17β-estradiol (10^{-8} M) were assayed for cellular apoptosis using TUNEL. Apoptotic MCF-7 cells seen after droloxifene treatment showed p53 expression (Fig. 5), a result similar to that observed in apoptotic droloxifene-treated bone marrow cells. Droloxifene treatment also increased the number of apoptotic cells as compared to vehicle-treated cells (Fig. 6). In contrast, estrogen treatment of MCF-7 cell cultures stimulated cell growth, and decreased the number of apoptotic cells (Fig. 6). Analysis of cell cycle distribution by flow cytometry using PI staining showed that droloxifene increased the number of cells in the G_1 phase of the cell cycle (Fig. 7) indicating a reduction in cell proliferation. A similar reduction in cell proliferation using a ^{3}H-thymidine assay was observed (data not shown). The low frequency of apoptotic events (0.04%) determined by manually counting cells on coverslips (Fig. 6) was not detectable as a sub-G_1 peak of PI stained cells since our flow cytometry analysis utilized 10^4 cells per sample. Neither droloxifene nor estradiol had any effect on estrogen receptor-negative MDA-MB231 human breast cells (data not shown).

DISCUSSION

The cellular and molecular mechanisms underlying the bone-protective activity of droloxifene or estradiol remain incompletely understood [Riggs and Melton, 1992; Turner et al., 1994; Ke et al., 1995; Eppenberger et al., 1991]. Recent *in vivo* studies have suggested that estradiol treatment of OVX rats increases the number of TRAP(+) multinucleated osteoclasts undergoing apoptosis [Hughes et al., 1995]. Our data suggest that an additional mechanism for estrogenic regulation of osteoclast number is the suppression of osteoclast production in the bone marrow via apoptosis of precursors. Using rat bone marrow cultures, both droloxifene and estradiol reduced the formation of osteoclasts *in vitro* to an extent that paralleled the *in vivo* effect of each compound on osteoclast number. *In vitro* treatment of rat bone marrow cultures

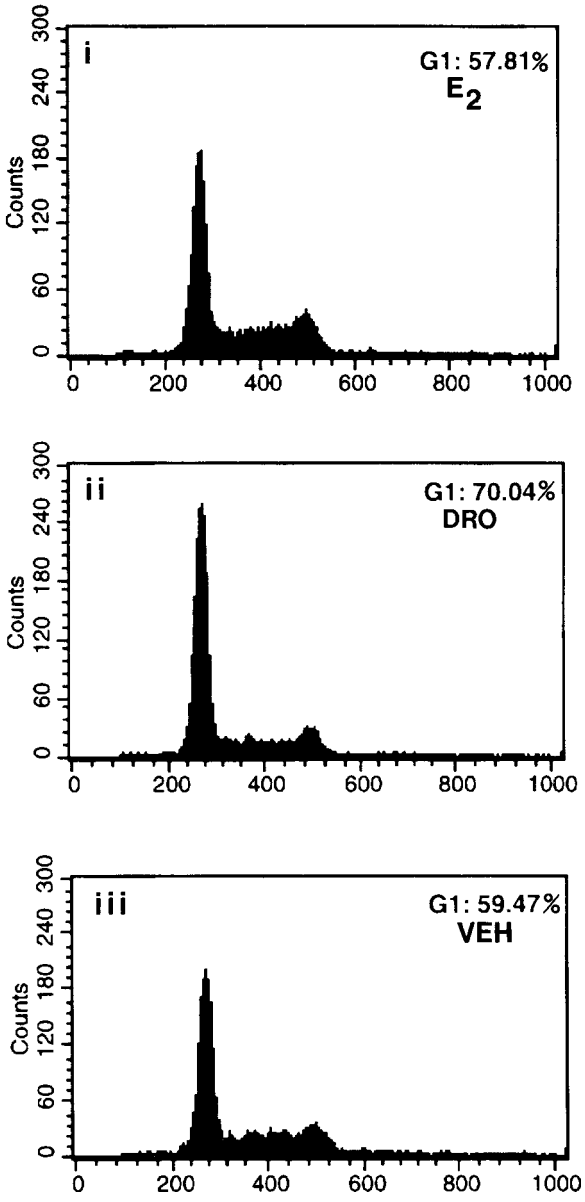

Fig. 7. Cell cycle analysis of MCF-7 cells. MCF-7 cells were stained with propidium iodide for cell cycle analysis using flow cytometry after (**i**) 10^{-8} M 17β-estradiol (E_2) treatment, (**ii**) 10^{-8} M droloxifene (DRO) treatment, or (**iii**) vehicle (VEH, 0.01% ethanol) treatment. Percent of cells in G_1 phase of the cell cycle is indicated in each figure after counting 1×10^4 cells. The data shown are from a single experiment; however, other experiments showed >20% increases in G_1.

with either droloxifene or estradiol showed p53 expression in the subset of mononucleated marrow cells which also showed TUNEL labelling. In addition, microscopic examination of TUNEL labelled cultures showed no increase in the number of cells undergoing necrosis after either droloxifene or estrogen treatment, confirming a lack of general toxicity of these treatments. Therefore, treatment with droloxifene or estradiol resulted in a decrease in differentiated osteoclasts with an increase in the number of mononucleated cells undergoing apoptosis.

The cells undergoing apoptosis could be precursors of osteoclasts responding directly to the droloxifene/estradiol treatment. Alternatively, the cells binding droloxifene/estradiol and undergoing apoptosis could be of the osteoblastic origin [Ericksen et al., 1988]. It is well known that osteoblastic signals are required for differentiation of osteoclasts [Takahashi et al., 1988b]; thus, a reduction in osteoblasts may result in a decreased number of differentiated osteoclasts. A third possibility is that the cells responding to droloxifene/estradiol are neither in the osteoblastic nor osteoclastic lineages but are secreting a cytokine such as TGF-β that induces apoptosis in pre-osteoclastic cells. A recent report showed that tamoxifen induced TGF-β1 activity and apoptosis in human MCF-7 breast cancer cells *in vitro* [Chen et al., 1996].

In order to further characterise the cells undergoing apoptosis, we used immunohistochemistry to co-localize a cell surface antigen on the apoptotic cells. These experiments showed that CD61 is detected on some apoptotic cells. Previous work has shown that CD61 is present on the cell surface of osteoclastic and pre-osteoclastic cells [Athansou et al., 1992; Helfrich et al., 1992]. Thus, it is reasonable to conclude that at least some of the cells undergoing apoptosis are of the osteoclast lineage. The observation that not all CD61 cells were undergoing apoptosis is consistent with our results since treatment with droloxifene or estrogen reduces but does not completely abolish the presence of TRAP positive cells or functional osteoclasts.

The present study cannot rule out the stimulation of apoptosis by a cytokine that is induced upon treatment with droloxifene or estrogen, a situation comparable to the induction of apoptosis via TGF-β1 expression stimulation by tamoxifen [Chen et al., 1996]. However, the induction of apoptosis in the bone marrow cultures is observed after only 3 h treatment with either droloxifene or estrogen suggesting a probable direct action of droloxifene or estrogen on the bone marrow cultures. Similarly, treatment of estrogen receptor positive human MCF-7 breast cancer cells with droloxifene inhibited growth and stimulated apoptosis. These apoptotic MCF-7 cells seen after droloxifene treat-

ment showed elevated p53 expression similar to droloxifene-treated bone marrow cells.

These observations provide insight into the mechanisms underlying the apparently paradoxical "agonist" activity of droloxifene in bone and "antagonist" activity in breast tissue. This mechanism of droloxifene action may be similar in breast and bone marrow cells since in both tissues, increased apoptosis is correlated with p53 expression. The ER bound to droloxifene may modulate some of the same growth regulatory target genes in these two tissues. In contrast, the ER/estrogen complex may have a different conformation and thus interact differentially with tissue-specific transcription factors that lead to the induction of p53 and apoptosis in bone, but not in the breast. A recent study showed that estrogen receptor ligands can be distinguished by their ability to activate gene transcription via different DNA response elements [Yang et al., 1996]. Therefore, these data suggest that tissue specific transcriptional regulation of cell cycle genes may be key to the agonist and antagonist actions of droloxifene.

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