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Estrogen and the selective estrogen receptor modulator (SERM) protection against cell death in estrogen receptor alpha and beta expressing U2OS cells

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ABSTRACT

In the current work we compared the ability of 17 β -estradiol (E₂) and the selective estrogen receptor modulators (SERMs) tamoxifen (Tam), raloxifene (Ral) and ospemifene (Osp) to promote the survival of osteoblast-derived cells against etoposide-induced apoptosis. In order to compare the roles of the two estrogen receptor (ER) isotypes, we created a U2OS human osteosarcoma cell line stably expressing either ER α (ER α) or ER β (ER β). Transfection with either of the ERs was able to render the U2OS cells sensitive to E₂. We show that E₂ opposed etoposide-induced apoptosis and that the effect was mediated via both ER isotypes. The ER isotype selective agonists propyl-pyrazole-triol (PPT) and diarylpropionitrile (DPN) had the same effect in U2OS/ER α and U2OS/ER β cells, respectively. Osp also opposed apoptosis at least in U2OS/ER α cells. Tam and Ral were not able to protect against etoposide-induced cell death. In order to evaluate the protective effects of E₂ and Osp upon etoposide challenge we studied the expression of two E₂-regulated, osteoblast-produced cytokines, IL-6 and OPG in E₂ and SERM-treated U2OS/ER α and U2OS/ER β cells. Etoposide strongly increased expression of IL-6 and decreased that of OPG. E₂ opposed IL-6 increase only in U2OS/ER α cells and OPG decrease primarily in ER β cells. Osp opposed the effect of etoposide on OPG primarily in U2OS/ER β cells but interestingly, it had little effect on IL-6 expression. E₂, PPT, DNP and Osp also inhibited etoposide-induced death and cytokine changes in SAOS-2 osteosarcoma cells expressing endogenous ER α and ER β . Collectively, our results suggest that the osteoblast protective antiapoptotic effects of E₂ are mediated by both ER α and ER β but those of Osp primarily by ER α . In addition, E₂ and Osp opposed the etoposide-induced increase of IL-6 and decrease of OPG which changes would increase osteoclastic activity. These antiresorptive effects of E₂ and

Osp upon etoposide challenge differed from each other and they seemed to be differentially mediated in ER α and ER β expressing osteoblast-derived U2OS cells.

INTRODUCTION

Estrogen has recently been demonstrated to protect different types of cells from apoptosis induced by various substances (Bynoe *et al.*, 2000; Haynes *et al.*, 2000; Perillo *et al.*, 2000; Choi *et al.*, 2001; Zhang *et al.*, 2001; Gu *et al.*, 2005; Zheng *et al.*, 2007). The functions of 17 β -estradiol (E₂) are largely mediated through two distinct nuclear estrogen receptor (ER) isoforms, ER α (ER α) and ER β (ER β). Moreover, recent work has supported that, in addition to nuclear events, E₂ is capable of bringing about rapid membrane-initiated signaling events in a variety of cell types (Edwards 2005).

Selective estrogen receptor modulators (SERMs) are compounds that have both estrogen agonistic and estrogen antagonistic properties. They can function in the same way as estrogen in some tissues (e.g. bone) and more like antiestrogen in some other tissues (e.g. breast) and have important clinical applications. For example, tamoxifen (Tam) is widely used in chemotherapy and prevention of breast cancer (Fisher *et al.*, 1998, Radmacher and Simon 2000) and it has also been shown to have a beneficial effect on bone mineral density (BMD) in postmenopausal women (Love *et al.*, 1994, Powles *et al.*, 1996). Raloxifene (Ral) is used in the treatment and prevention of osteoporosis (Jordan 1998; Ettinger *et al.*, 1999; Gradishar *et al.*, 2000; Cicek and Oursler 2006). It also has a strong anticancer effect in breast. Ospemifene (Osp) is a novel SERM that is being developed for the treatment of vaginal

atrophy in postmenopausal women. It has also shown promise in the prevention and treatment of osteoporosis (Taras *et al.*, 2001, Qu *et al.*, 2000, Komi *et al.*, 2006).

Estrogen has an important role in the development and growth of bones and later in the maintenance of bone mass. It is believed that the major action of estrogen on the skeleton in vivo is through the inhibition of bone resorption (Riggs *et al.*, 2002). Although some of the anti-resorptive effects of estrogen are via direct actions on bone-resorbing osteoclasts, estrogen has also been shown to have indirect effects by regulating bone-forming osteoblasts and bone marrow stromal cells (Zallone 2006). As one of these effects, E₂ has been suggested to protect osteoblasts from apoptosis (Kousteni *et al.*, 2001; Chen *et al.*, 2006; Zallone 2006)

Estrogen modulation of osteoclastic bone resorption is mediated, at least in part, by regulating the production of several pro-resorptive factors such as the TNF ligand family member named the receptor activator of nuclear factor κ B ligand (RANKL), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) by the cells of osteoblastic lineage. Estrogen has also been shown to regulate the synthesis of secreted TNF superfamily member osteoprotegerin (OPG). It acts as a non-signaling decoy receptor for RANKL. OPG regulates bone turnover by preventing RANKL from binding to and activating the signaling receptor RANK on the osteoclast surface. It thus functions as an antiresorptive factor.

The role of osteoblastic/osteocytic apoptosis in the regulation of bone metabolism has been demonstrated by several investigators (Urayama *et al.*, 2000, Kameda *et al.*, 1995, Kawakami *et al.*, 1997, Nakashima *et al.*, 1998, Kawakami *et al.*, 1998, Weinstein *et al.*, 1998). Especially apoptosis of osteoblasts is currently getting more attention since it has been considered to be an important determinant of bone formation and, therefore, of skeletal

integrity (Manolagas, 2000; Weinstein and Manolagas, 2000). Disorders that promote osteoblastic apoptosis are associated with increased bone fragility (Weinstein *et al.*, 1998), and treatments that inhibit it are associated with anti-fracture efficacy (Jilka *et al.*, 1999).

The aim of the present work was to compare the ability of E_2 and the osteoprotective SERMs Tam, Ral and Osp to have antiapoptotic effects in osteoblast-derived cells and to evaluate the role of the ER subtypes ER α (ER α) and ER β (ER β) in their cell survival effect. As a model we used the human U2OS osteosarcoma cell line stably expressing either ER α or ER β . The effects of the SERMs were also studied in another osteoblast-like cell line, SaOS-2 that expresses endogenous ER α and ER β . We demonstrate that E_2 and Osp are able to oppose etoposide-induced apoptosis, and that the effect of E_2 is mediated via both ER isotypes. In addition, the ER isotype selective agonists PPT and DPN selectively opposed apoptosis in U2OS/ER α and U2OS/ER β cells, respectively. We also studied the effect of E_2 and Osp on the expression of the osteoblast-produced bone regulatory cytokines IL-6 and OPG in etoposide-treated U2OS/ER α and U2OS/ER β cells. We observed that etoposide treatment caused changes in IL-6 and OPG that would increase osteoclastic activity and bone resorption while the pretreatment of the cells with E_2 or Osp opposed these changes in a receptor subtype and ligand specific manner.

MATERIALS AND METHODS

Cell Culture and Preparation of U2OS-ER α and ER β Stable Cell Lines

The U2OS (human osteosarcoma) cells were stably transfected with human ER α or ER β .

The following constructs were transfected to U2OS cells together with pcDNA3.1 expression vector which contains a neomycin resistance gene:

1) pSG5-hER α :

Human estrogen receptor α coding sequence: 1,8 kb in pSG5-vector (=HEO), cloned at the EcoR1 site. In HEO the ER α sequence contains the artificial Gly400Val mutation (GGG to GTG), which destabilizes it in the absence of a ligand. Thus, it gives a lower background and clearer ligand-induced transactivation data than that with wild-type ER α (=HEGO) (Bruder *et al.*, 1997).

2) pSG5-hER β

Human estrogen receptor β coding sequence: 1,6 kb in pSG5-vector, cloned at the BAMH1 site encoding a full length ER β (Fuqua *et al.*, 1999). ER β cDNA was PCR-cloned from human ovary mRNA.

Clones were selected with the antibiotic G418 (0,5 mg/ml). Expression of the ER constructs on U2OS cells was confirmed by RT-PCR, western blotting and immunofluorescence microscopy (data not shown). The trans-activation ability of transfected ER α and ER β was detected with an ERE-containing reporter gene. These assays indicated high activity for ER α and moderate activity for ER β (data not shown).

Parental U2OS cells were maintained in McCoy's 5A culture medium supplemented (10%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. For ER α -, ER β - and vector transfected U2OS cell medium was supplemented with G-418 (0,25 mg/ml). SaOS-2 cells were cultured in McCoy's 5A culture

medium supplemented (15%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For the experiments, cells were grown overnight in phenol-free DMEM/F-12 media containing 0,1% BSA supplemented with E₂, ER ligands or SERMs. According to the cell death analyses, the lowest concentrations to give statistically significant protection against etoposide were 1 µM for Osp and 10 nM for E₂, PPT and DPN. Thus, we chose to use these drug concentrations also on the following experiments. The pre-incubation time of 24 hrs with SERMs has been previously used also by others (Monroe *et al.*, 2006; Kian Tee *et al.*, 2004, Brady *et al.*, 2002). The incubation times for OPG and IL-6 assays were chosen on the basis of cell death analyses. Treatment times for TNFα and etoposide were one hour and six hours, respectively.

The estrogen-sensitive MCF-7 human breast tumor cell line was originally obtained from the laboratory of Dr. C. K. Osborne (University of Texas Health Science Center, San Antonio, Texas). The cells were maintained in RPMI 1640 culture medium supplemented (10%) with heat-inactivated fetal bovine serum (iFBS), 2 mM L-glutamine, insulin (4 µg/ml) and 1 nM 17β-estradiol (E₂). McCoy's 5A, RPMI, DMEM/F-12, L-glutamine, Tam and insulin were purchased from Sigma (St. Louis, MO) and FBS was purchased from Life Technologies, Inc. (Paisley, Scotland, U.K.). Ral and Osp were from Hormos Medical Oy (Turku, Finland). All drugs were dissolved in DMSO (Sigma, St. Louis, MO).

Confirmation of expression of human ERα in U2OS/ERα cells and human ERβ in U2OS/ERβ cells

The expression of transfected ERα and ERβ in U2OS cells was verified by RT-PCR, Western blotting, immunofluorescence microscopy and reporter gene assays. For RT-PCR analysis the RNAs were isolated from MCF-7, parental U2OS and ERα or ERβ transfected U2OS cells

by using the Trizol reagent (Invitrogen) and purified according to the manufacturer's instructions. cDNA was synthesized from 2 µg of total RNA in a 20 µl reaction containing random hexanucleotides and AMV reverse transcriptase enzyme (Finnzymes, Espoo, Finland). RT-PCR was performed using the Eppendorf Mastercycler. Amplification reactions for ER α were performed for 35 cycles. Successful cDNA synthesis was verified by PCR for GAPDH. The following primers were used:

ER α (forward)	5'- ACA AGC GCC AGA GAG ATG AT -3'
ER α (reverse)	5'- AGG ATC TCT AGC CAG GCA CA -3'
ER β (forward)	5'- TGA AAA GGA AGG TTA GTG GGA ACC -3'
ER β (reverse)	5'- TGG TCA GGG ACA TCA TCA TGG -3'
GAPDH (forward)	5'-AGC CAC ATC GCT CAG ACA C-3'
GAPDH (reverse)	5'-GCC CAA TAC GAC CAA ATC C-3'

For analysis of expression of ER α and ER β by Western blotting, aliquots of whole cell lysate protein (50 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). The membranes were blocked with 8% skimmed milk in TBS/0.05% Tween-20 and incubated with the primary antibodies for ER α and ER β (Santa Cruz Biotechnology). The proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) with colored markers (BioRad, Hercules, CA) as size standards.

Immunofluorescence microscopy was used for the localization of transfected ERs in U2OS/ER α and U2OS/ER β cells. The cells were incubated for 2 hours with ER α or ER β specific monoclonal antibodies (Santa Cruz Biotechnology) after which the media were

replaced with media containing Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) and the cells were further incubated for 45 minutes. The cells were then incubated with DAPI nuclear dye for 10 minutes. Microscopic imaging was carried out on a Zeiss Wide-Field microscope (Zeiss, Jena, Germany).

Luciferase reporter gene assays were used to characterize the functionality of transfected ERs. The U2OS/ER α and U2OS/ER β cells were transfected with only a luciferase reporter plasmid under the control of two ER response elements or a human ER α or ER β plasmid and the same luciferase reporter plasmid by using Fugene transfection reagent (Roche, Madison, WI). Twenty-four hours after transfection the cells were treated for 24 hours with 10 nM E₂ and the luciferase activity was measured using a dual-luciferase reporter assay according to the manufacturer's instructions (Promega, Madison, WI). For quantitation, in the absence of hormone, the luciferase activity was set to 1.

IL-6 and OPG mRNA expression

As previously, total cellular RNA was extracted from the U2OS and SaOS-2 cells using the Trizol reagent and purified according to the manufacturer's instructions. cDNA was synthesized from 2 μ g of total RNA in a 20 μ l reaction containing random hexanucleotides and AMV reverse transcriptase enzyme.

Semiquantitative RT-PCR Analysis

PCR was performed using the Eppendorf Mastercycler. The following primers were used:

TNF α (forward)	5'-TGC TTG TTC CTC AGC CTC TT-3'
TNF α (reverse)	5'-TGG GCT ACA GGC TTG TCA CT-3'
IL-6 (forward)	5'-CCT TCC AAA GAT GGC TGA AA-3'

IL-6 (reverse) 5'-AGC TCT GGC TTG TTC CTC AC-3'
 GAPDH (forward) 5'-AGC CAC ATC GCT CAG ACA C-3'
 GAPDH (reverse) 5'-GCC CAA TAC GAC CAA ATC C-3'

After an initial denaturation at 94°C for 3 minutes 25 cycles of PCR amplification were performed, each consisting of a denaturing step of 94 °C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds, followed by a final step at 72 °C for 10 minutes. The number of 25 cycles was selected because in preliminary experiments we verified that with this number of cycles the reaction was still in a linear range for all three genes (data not shown). The amplified fragments were detected by 1,5% agarose gel electrophoresis and ethidium bromide staining.

Quantification of apoptotic cells

Apoptotic cells were quantified by visualization of changes in nuclear morphology by DAPI staining. Cells (1×10^5) were grown in 3.5-cm diameter Petri dishes with coverslips overnight. Culture media were replaced with DMEM/F12 phenol-red-free media containing 0,1% BSA and either E₂, PPT, DPN or SERMs at the concentrations indicated in the results. Non-treated cells served as controls and these cultures had equivalent volumes of DMSO solvent. After 24 hours, etoposide was added to half of the plates for 6 hours. After treatment, the cells were fixed with 3% paraformaldehyde and the coverslips were mounted on the slides with Vectashield mounting medium with DAPI (Vector Laboratories Inc. Burlingame, CA). Microscopic imaging was carried out on a Zeiss Wide-Field microscope (Zeiss, Jena, Germany). Fifteen areas of 1300×1000 pixels in one sample were randomly selected from the image for quantification of apoptotic cells. Data of three independent experiments are presented as the percentage of dead cells (means $\pm$ SE) for each treatment.

Quantitative real-time PCR

Real-time quantitative PCR analysis was carried out using a 5700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA). Amplification reactions were set up in 25 μ l reaction volumes containing amplification primers and SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA). The primer concentrations and cDNA sample volumes were optimized for each primer set. Primer sequences for IL-6, OPG and β -actin were as follows:

IL-6 (forward) 5'-TACCCCCAGGAGAAGATTCC-3'

IL-6 (reverse) 5'-AAAGAGGCACTGGCAGAAAA-3'

OPG (forward) 5'-TGCAGTACGTCAAGCAGGAG-3'

OPG (reverse) 5'-TGTATTTCGCTCTGGGGTTC-3'

β -actin (forward) 5'-CGTGGGCCGCCCTAGGCACCA-3'

β -actin (reverse) 5'-TTGGCCTTAGGGTTCAGGGGG-3'

Amplifications were performed in 96-well reaction plates. Calibration curves were derived for each gene following serial dilutions of a stock cDNA which were then used as standards. Calibration curves were run in parallel in triplicates for each analysis. Each sample was analyzed three times during each experiment. The experiments were carried out three times on each cell line. Amplification data were analysed using the Sequence Detector System Software (PE Applied Biosystems). The results were normalized to β -actin and expressed as a percentage of controls.

OPG assay

Cell culture media were collected, and OPG was determined with ELISA according to the manufacturer's instructions (Biomedica, Wien, Austria). Briefly, standards, samples, positive

control, assay buffer, and detection antibody were pipetted into a 96-well plate precoated with monoclonal anti-OPG antibody and mixed. After incubation for 24 h at +4°C, the plate was washed five times with washing buffer, streptavidin peroxidase conjugate was added to the wells, and the cells were incubated for 1 h at room temperature. The plate was washed five times, and tetramethylbenzidine was added as a substrate to develop the color reaction. After incubation for 20 minutes at room temperature, stop solution was added, and OPG was measured with a Victor model 2 instrument (EG & G Wallac) at 450-nm absorbance.

IL-6 assay

Similarly to the OPG assay, cell culture media were collected, and IL-6 was determined with ELISA according to the manufacturer's instructions (Sanquin, Amsterdam, The Netherlands). Briefly, standards, samples, positive control, assay buffer, and detection antibody were pipetted into a 96-well plate precoated with monoclonal anti-IL-6 antibody and mixed. After incubation for 24 h at room temperature, the plate was washed five times with washing buffer; standards and samples were added to the wells and incubated for 1 h at room temperature. Wells were washed again as previously after which biotinylated antibody was added and the plate was incubated for 1 hour at room temperature. After a third wash streptavidin-HRP conjugate was added to the wells, and the plate was incubated for 30 min at room temperature. The plate was washed, and tetramethylbenzidine was added as a substrate to develop the color reaction. After incubation for 30 minutes in the dark at room temperature, stop solution was added, and IL-6 was measured with a Victor model 2 instrument (EG & G Wallac) at 450-nm absorbance.

Statistical analyses

The statistical significance of differences between the vehicle control and the etoposide-treated sample (+), vehicle control and E₂, Osp, PPT and DPN treated samples (#) or etoposide-treated cells and cells treated with a combination of etoposide and ER ligands (*) were determined by one-way analysis of variance (ANOVA). The critical value for significance was $P < 0,05$ (a, b). Values are given as means $\pm$ SE.

RESULTS

Expression of estrogen receptors α and β in U2OS human osteosarcoma cells

U2OS human osteosarcoma cells do not endogenously express ERs at a detectable level. However, the U2OS cells stably transfected with expression constructs for the human ER α and ER β full length sequences expressed the receptors at RNA and protein level as demonstrated by RT-PCR and Western blotting (Fig. 1A and B). Translocation of ER α and ER β to the nuclei was observed in E₂-treated transfectants but not in U2OS parent cells in immunofluorescence microscopy (Fig. 1C). The transactivation capacity of transfected ERs was confirmed by ERE-luciferase reporter gene assays (Fig. 1D). In the presence of 10nM E₂, there was a seventy-fold increase in luciferase activity in ER α transfected cells, which indicates a high level of receptor activity (Fig. 1D). Transient expression of ER α in U2OS/ER α cells further increased luciferase expression. In the case of U2OS/ER β , E₂-treatment resulted in a five-fold increase in luciferase activity thus indicating a moderate level of receptor activity (Fig. 1D). Luciferase expression was not increased by transient transfection of ER β to U2OS/ER β cells.

Estrogen inhibits TNF α -induction of IL-6 and TNF α in U2OS/ER α and U2OS/ER β cells

In order to further confirm that transfected ERs are functional in U2OS cells, we studied whether ER α and ER β are able to restore the effects of E₂ on TNF α –induced production of IL-6 and TNF α itself in U2OS/ER α cells and U2OS/ER β cells, respectively. The ability of estrogen to decrease the expression of these cytokines in osteoblastic cells has previously been shown by several researchers (Spelsberg *et al.*, 1999; Brady *et al.*, 2002; Khosla *et al.*, 2002). Transfected U2OS cells were incubated overnight with 0,1nM or 1 nM E₂, after which cells were either treated for one hour with 5ng/ml TNF α or left untreated. Control cells received vehicle (0.2% DMSO) only. Total RNAs were then isolated and TNF α -induced production of IL-6 and TNF α was studied by semi-quantitative RT-PCR. In ER α -transfected U2OS cells E₂ at a concentration of 0,1 nMol/L was able to oppose TNF α –induced production of the TNF α and IL-6 (Fig. 2). In the case of U2OS/ER β , a higher concentration of 1 nM E₂ was required to inhibit production of either of the two cytokines studied. However, both U2OS/ER α cells and U2OS/ER β cells were found to respond to estrogen treatment. In parental U2OS cells E₂ had no effect on production of either TNF α or IL-6.

E2 and ER isotype selective agonists PPT and DPN oppose apoptosis in U2OS/ER α and U2OS/ER β cells

Next we studied the ability of E₂ to protect against etoposide-induced cell death in parental and ER-transfected cells. A 6-h treatment of U2OS parental cells with etoposide markedly increased the relative number of dead cells (data not shown). Pretreatment of the parental U2OS cells with E₂ for 24 h did not affect the etoposide effect. Similar results were obtained in U2OS/mock cells pretreated with E₂ or with the ER α and ER β agonists PPT and DPN (Fig. 3A). However, in U2OS/ER α and U2OS/ER β cells E₂ was able to protect against etoposide-induced cell death (Fig. 3B and C). Addition of E₂ together with etoposide resulted

in almost 40% and 50% decrease in the number of dead cells, respectively, when compared to treatment with etoposide only. Similarly, the U2OS/ER α specific agonist PPT was also able to decrease the number of dead cells close to the level observed in E₂-treated U2OS/ER α cells, whereas the decreasing effect of DPN in U2OS/ER β cells was not statistically significant. E₂, PPT and DPN alone did not increase the rate of cell death compared with the vehicle treatment.

Ospemifene prevents etoposide-induced cell death in U2OS/ER α cells

In order to study whether the bone sparing selective estrogen receptor modulators (SERMs) Tam, Ral and Osp have estrogen-like antiapoptotic or proapoptotic effect in the ER α - and ER β - transfected U2OS cells, we pre-incubated the cells with 1 μ M of these SERMs for 24 hours before 6 hours of 100 μ M etoposide treatment. We observed that only E₂ and Osp were able to oppose etoposide –induced apoptotic cell death in U2OS/ER α cells in a statistically significant way (Fig. 4A). Treatment of ER α –transfected cells with both etoposide and E₂ resulted in 38% less cell death than treatment with etoposide only. In the case of Osp instead of E₂, this level was 41%.

In U2OS/ER β cells Osp was not able to oppose etoposide –induced cell death as well as in U2OS/ER α cells, since treatment with both Osp and etoposide together resulted in only approximately 15% decrease in the number of dead cells when compared with treatment with etoposide only (Fig. 4B). Tam and Ral were not able to significantly prevent apoptosis in the U2OS/ER α and U2OS/ER β –transfected cells. None of the SERMs studied was able to oppose etoposide –induced death of mock -transfected U2OS cells (data not shown).

E₂ and Osp oppose etoposide-induced decrease of OPG expression

In order to study how the antiapoptotic E₂ and Osp influenced the characteristic function of osteoblastic cells exposed to apoptosis inducing factor we examined the expression of OPG and IL-6 in etoposide-treated ER α and ER β containing U2OS cells. U2OS ER α , U2OS ER β and control cells were first incubated overnight with either 1 μ M Osp or 10 nM concentration of E₂, PPT or DPN after which 100 μ M etoposide was added to cultures for six hours, after which OPG expression was detected with real-time quantitative PCR. We found that, while etoposide-treatment significantly decreased OPG production in both ER α and ER β - transfected cells, all ligands showed a tendency to oppose the effects of etoposide on OPG expression (Fig. 5A and 5B). In ER β -transfected cells E₂ did not induce expression of OPG as efficiently as Osp and DPN, since the mRNA expression level in the samples treated with a combination of E₂, Osp or DPN and etoposide was 20%, 55% and 65%, higher, respectively, when compared with the sample treated with etoposide only. In the case of vector-transfected U2OS/mock cells, E₂, Osp did not oppose etoposide-induced decrease of OPG expression (data not shown).

Next we used a sensitive sandwich ELISA method to measure OPG protein concentrations in the conditioned media collected after treating the cells as above for QRT-PCR. As seen in Fig.6A, in ER α -transfected U2OS cells treated with a combination of etoposide and PPT, the OPG concentration was approximately 5 pM, whereas in the sample treated with etoposide alone the OPG concentration was 3 pM. The effect of PPT was also statistically significant. In the case of ER β -transfected U2OS cells (Fig. 6B) the OPG concentrations in the samples treated with combinations of etoposide and either E₂, Osp or DPN were 13 pM, 9 pM and 12 pM, respectively, while in the sample treated with etoposide solely, the OPG concentration

was approximately 2,5 pM. This indicated that all the ligands tested were able to prevent the effects of etoposide on the production of OPG.

The effects of E₂, PPT, DPN and Osp on IL-6 expression in ER-transfected U2OS cells

We also studied the effect of E₂, PPT, DNP and Osp on the expression and production of IL-6 in etoposide-treated U2OS cells containing ER α and ER β . IL-6 has been demonstrated to increase osteoclastic activity (Manolagas 1995, Manolagas *et al.*, 2002) and E₂ has previously been shown to downregulate cytokine-induced expression of IL-6 in osteoblasts (Manolagas *et al.*, 2002). U2OS/ER α , U2OS/ER β and mock-transfected cells were first incubated for 24 hours with either 10 nM E₂, 10 nM PPT, 10 nM DPN or 1 μ M Osp after which 100 μ M etoposide was added to cultures for six hours after which IL-6 expression was determined with real-time quantitative PCR. Control cells were treated with vehicle (0.2% DMSO) only. Fig 7A shows that in U2OS /ER α cells etoposide strongly induced IL-6 expression (approximately two-fold) when compared to vehicle-treated cells and after addition of E₂ or PPT the level of IL-6 expression was even lower than in control cells. However, Osp could not oppose the effect of etoposide on IL-6 expression. In U2OS ER β -cells (Fig. 7B), addition of E₂, Osp or DPN together with etoposide opposed etoposide-induced increase in IL-6 expression to some extent, but this effect was not statistically significant.

To study the effects of ER ligands on IL-6 production, we used ELISA to measure protein concentrations in the media collected from the cells treated as for RNA measurements. In ER α -transfected U2OS cells etoposide significantly increased the level of IL-6 protein secretion when compared to the vehicle (DMSO) –treated sample. All ER-ligands tested opposed the effect of etoposide since after addition of E₂, Osp or PPT the IL-6 protein levels were approximately 57%, 30% and 45% lower when compared to the sample treated with

etoposide only. The effects of E₂ and PPT were statistically significant. However, in the case of U2OS/ER β cells, none of the ER ligands tested opposed etoposide-induced IL-6 secretion at a statistically significant level (Fig.8B).

The effects of E₂, PPT, DPN and Osp on SaOS-2 cells

In addition to U2OS cells, we also studied the effects of different ER ligands on another osteoblast-like cell line, SaOS-2, which, unlike the parent U2OS cells, expresses both ER α and ER β . We found that all ER ligands tested (E₂, Osp, PPT and DPN) significantly decreased etoposide-induced apoptosis (Fig. 9A). In addition, all ER ligands used opposed etoposide-induced decrease of OPG expression to some extent, but only the effect of DPN was statistically significant (Fig. 9B). Furthermore, we observed that E₂, Osp, PPT and DPN also opposed the effect of etoposide on IL-6 mRNA expression (Fig. 9C).

DISCUSSION

Recent data from our laboratory indicates that E₂ is able to protect breast cancer cells against tamoxifen-induced cell death (Zheng *et al.*, 2007). Other reports have shown evidence for estrogen inhibition of apoptosis induced by various substances in other cell types (Bynoe *et al.*, 2000; Haynes *et al.*, 2000; Perillo *et al.*, 2000; Choi *et al.*, 2001; Zhang *et al.*, 2001). In addition to osteoclast-directed actions, inhibition of osteoblast and osteocyte apoptosis has been proposed as one potential mechanism in estrogen maintenance of bone mass (Kousteni *et al.*, 2001; Gu *et al.*, 2005; Chen *et al.*, 2006; Zallone 2006; Jilka *et al.*, 2007). E₂ has been shown to protect calvaria-derived osteoblastic cells from etoposide-induced apoptosis (Kousteni *et al.*, 2001; Zallone 2006) as well as to protect osteoblasts from ethanol-induced

bone loss (Chen *et al.*, 2006). We have recently demonstrated that E₂ opposes glucocorticoid-induced apoptosis of primary osteocytes (Gu *et al.*, 2005).

In this work we compared the ability of Tam, Ral and Osp, which are known to have bone-sparing effects, with E₂ and synthetic ER isoform specific ligands PPT and DNP to promote the survival of osteoblast-derived osteosarcoma cells. In addition, we asked which of the two ER isotypes plays the predominant role in mediating the effects of E₂ and SERMs. To examine the individual actions of ER α and ER β we used the U2OS human osteosarcoma cell line that lacks detectable endogenous ER expression to produce cell lines that stably express ER α and ER β . The transfected ERs seemed to be functional in terms of nuclear translocation and activated transcription of a reporter gene upon E₂ stimulation. The obtained cell lines also responded appropriately to TNF α stimulation by decreased TNF α and IL-6 expression. Corresponding cell lines have also been created and used by others for various purposes (Monroe *et al.*, 2003; Stossi, *et al.*, 2004 and Kian Tee *et al.*, 2004). To examine whether the results obtained with U2OS cells also apply to other osteoblastic cells, we studied the effects of E₂ and other ER ligands on apoptosis of another osteoblast-derived osteosarcoma cell line SaOS-2, which is known to express both ER α and ER β (Sutherland *et al.*, 1996, Vidal *et al.*, 1999). In addition to ER expression, there are differences between the U2OS and SaOS-2 cell lines that potentially influence the antiapoptotic actions of ER ligands in these cells. For example, U2OS cells are known to be p53 positive, whereas SaOS-2 cells lack p53, which may affect their apoptotic responses (Sandoval *et al.*, 2004; Olijslagers *et al.*, 2007). Our results demonstrate, however, that ER ligands were able to protect both of these cell lines against drug-induced apoptosis.

Apoptosis of osteoblasts can be induced by various substances including for example cancer chemotherapy drugs and oxidative stress. We used etoposide at an osteoblast apoptosis inducing concentration (Urayama *et al.*, 2000). Etoposide is a chemotherapy drug that is given as a treatment for some types of cancer (osteosarcoma included) and it has previously been used to study apoptosis of osteoblastic cells (Li *et al.*, 2001; Kousteni *et al.*, 2003; Plotkin *et al.*, 2006). Etoposide binds to the enzyme topoisomerase II and inhibits its function of ligating cleaved DNA molecules, which ultimately leads to apoptotic cell death. Our hypothesis was that, despite the nature of the apoptotic stimulus, E₂ and some other ER ligands such as SERMs can induce activation of an independent survival-signaling pathway in the cells. Indeed, existence of such ER-mediated antiapoptotic pathways has been described in osteoblasts (Kousteni *et al.*, 2001; Kousteni *et al.*, 2003).

Our findings suggest that both estrogen receptor isotypes are able to mediate the pro-survival effects of E₂. The protective effect of E₂ was not observed in ER-negative parental (data not shown) or vector-transfected U2OS cells, which supports the conclusion that the anti-apoptotic effect is mediated mainly via estrogen receptors. In addition, in SaOS-2 cells both PPT and DPN were able to oppose etoposide-induced effects, indicating the involvement of both ER α and ER β . ER α has been demonstrated to play the primary role in the protective effects of E₂ on bone structure (McDougall *et al.*, 2003; McCauley *et al.*, 2003; Vidal *et al.*, 2000). However, earlier *in vivo* and *in vitro* results from our laboratory (Parikka *et al.* 2005; Michael *et al.*, 2007) as well as studies by others (Sims *et al.* 2003) also suggest a role for ER β in maintaining bone mass at least in females. We hypothesize that the mechanisms observed in the present study may contribute to these effects.

The bone sparing SERMs Tam, Ral and Osp differed in their ability of protecting the osteoblastic cells from threatening death. The SERM Osp showed anti-apoptotic capacity but

Tam and Ral did not have corresponding effect. Ral has been reported to decrease sodium nitroprusside-induced apoptosis of osteoblasts (Olivier *et al.*, 2005) but in our experimental setup Ral did not protect the osteoblastic cells against etoposide-induced apoptosis. Corresponding finding has previously been done by Kousteni *et al.* (Kousteni *et al.*, 2003). Furthermore, van Essen *et al.* (van Essen *et al.*, 2007) did not find any evidence for of Ral effect on osteocyte apoptosis, either. These results suggest that the anti-osteoporotic actions of Ral are obtained by other means than opposing osteoblast apoptosis. Such effects could be Ral inhibition of osteoclast formation (Taranta *et al.*, 2002, Ramalho *et al.*, 2002; Manolagas *et al.* 2000) and downregulation of the production of bone resorption enhancing cytokines by osteoblastic cells (Taranta *et al.*, 2002, Cheung *et al.*, 2003). In addition, Ral enhances OPG production and suppresses IL-6 secretion in normal human osteoblastic cells that express predominantly ER α (Viereck *et al.*, 2003).

In contrast to Ral, Osp was found to partly share the anti-apoptotic capacity of E₂. The ability of Osp to protect osteoblastic cells from apoptosis has not been demonstrated previously. Unlike E₂, Osp seemed to signal anti-apoptotic effects mainly via ER α . Like Ral, also Osp has been shown to decrease bone resorption (Komi *et al.*, 2004). However, Osp seems to enhance osteoblastic differentiation with a mechanism that differs from that of Ral (Qu *et al.* 1999). Komi *et al.* (2006) have recently reported that Osp and Ral differentially affect markers for osteoblastic and osteoclastic activities in postmenopausal women.

To further compare the response of ER α and ER β -transfected cells to E₂ and other ER ligands, we studied the ability of these compounds to modulate etoposide-induced effects on the expression of IL-6 and OPG, which are two important bone regulatory cytokines. OPG acts as a decoy receptor of osteoblast and stromal cell –produced RANKL and contributes to

decreased osteoclastic activity and bone resorption (Syed and Khosla 2005). E_2 has previously been demonstrated to increase osteoblastic OPG mRNA and protein levels in a dose dependent way (Saika *et al.*, 2001, Lindberg *et al.*, 2001, Bord *et al.*, 2003; Hofbauer *et al.*, 1999). Our results demonstrate that in U2OS/ER β cells Osp and DPN opposed the effects of etoposide on the expression of OPG, while in ER α -transfected cells the etoposide opposing effect of the ligands was less pronounced. This suggests that the regulation of OPG is primarily mediated by ER β . The role of ER β in OPG regulation was further supported by the finding that in SaOS-2 cells DPN presented the most marked etoposide-opposing effect. Not just OPG, but also RANKL and the ratio of RANKL/OPG are important in the modulation of bone homeostasis by estrogen (Bord *et al.*, 2003; Bashir *et al.*, 2005). However, our U2OS cell lines appeared to produce RANKL at a very low level and we were unable to assess the RANKL/OPG ratio in a reliable way. Low RANKL expression by osteoblastic cell lines has also been previously reported by others (Hofbauer *et al.*, 1999, Cheung *et al.*, 2003).

IL-6 is a regulator of immune and inflammatory responses that strongly stimulates osteoclasts and bone resorption (Compston 2001; Manolagas *et al.*, 2002). It may also affect osteoblastic cells. The stimulatory effect of etoposide on IL-6 production has previously been demonstrated (Verdenqh *et al.*, 2002, Wood *et al.*, 2006, Tozava *et al.*, 2002), although there are also conflicting reports (Verdenqh *et al.*, 2003, De Vita *et al.*, 1998). Estrogens have previously been shown to regulate the induction of IL-6 by various cytokines that modulate osteoclastic bone resorption (Messalli *et al.*, 2007, Compston *et al.*, 2001). We found that etoposide strongly induced expression of IL-6 in osteoblastic cells and in the case of ER α both E_2 and PPT opposed this effect. In U2OS ER β cells, none of the ER ligands inhibited the etoposide-increased IL-6 expression in a statistically significant way. These results

suggest that E_2 regulates IL-6 expression primarily via $ER\alpha$. Interestingly, in contrast to E_2 , Osp did not significantly affect IL-6 expression in U2OS cells containing ER. It did, however, oppose the effect of etoposide on IL-6 expression in SaOS-2 cells, suggesting $ER\beta$ contribution and a cell-specific effect. Differences in the levels of ER expression in SaOS-2 cells and ER-transfected U2OS cells and in the ratio of $ER\alpha$ and $ER\beta$ as well as the relative binding affinities of E_2 and Osp to these receptors may explain various IL-6 responses of the two cell lines. Altogether, the results suggest that the bone-protective mechanisms of Osp (Qu *et al.*, 2000) differ from those of E_2 .

Different SERM responses of U2OS-based and Saos-2 cells also reflect the heterogeneity of the osteosarcoma cells lines. As transformed cells they can only partially represent osteoblasts, which fact limits the interpretation of the results considering normal bone function. The aim of this study was, however, to evaluate of the SERM actions and the roles of ER isoforms in osteoblast-derived cellular models that are available. Even with the above-mentioned limitations the results suggest that the anti-apoptotic and anti-resorptive responses of osteoblast-derived cells to SERMs are highly ligand and ER subtype-specific.

Cumulatively, the ability of E_2 and certain SERMs to oppose IL-6 increase and OPG decrease upon exposure to compounds that promote osteoblastic cell apoptosis primarily seem to contribute to the inhibition of bone resorption. Exploiting these mechanisms may help to maintain bone homeostasis and prevent osteoblastic death in association with various drug treatments. Understanding these mechanisms would also be needed when planning hormonal treatment for osteoporosis or chemotherapies for bone tumours or metastatic bone diseases that interfere with IL-6 and/or OPG-related systems.

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FIGURE LEGENDS

Fig.1. Confirmation of expression of human ER α in U2OS/ER α cells and human ER β in U2OS/ER β cells.

U2OS cells were stably transfected with either ER α or ER β and expression of ERs was detected by RT-PCR (A). Human ER α and ER β cDNA plasmids were used as a positive control and GADPH served as a loading control. Western blotting was used to detect the expression of ER α and ER β proteins (B). Antibody specificity was verified with recombinant human ER α or ER β and MCF-7 whole cell lysates. Localization of transfected ER α and ER β was detected by immunofluorescence microscopy (C). Reporter gene assays were used to measure the E₂-induced activity of transfected ERs (D). U2OS/ER α or U2OS/ER β cells were transfected with only a luciferase reporter plasmid under the control of two ER response elements, or a human ER α or ER β plasmid and the same luciferase reporter plasmid. In the absence of hormone, the luciferase activity was set to 1.

Fig.2. Estrogen inhibits TNF α -induction of IL-6 and TNF α in U2OS/ER α and U2OS/ER β cells

Parental U2OS cells and U2OS cells transfected with either ER α or ER β were incubated overnight with 0,1nM or 1 nM E₂, after which cells were either treated for one hour with 5ng/ml TNF α or left untreated. Control cells received vehicle (0.2% DMSO) only. Total RNAs were then isolated and TNF α -induced production of IL-6 and TNF α was studied by semi-quantitative RT-PCR. The experiment was carried out three times, with comparable results.

Fig.3. ER isotype selective agonists PPT and DPN oppose apoptosis in U2OS/ER α and U2OS/ER β cells

U2OS cells transfected with either vector (A), ER α (B), or ER β (C) were first incubated overnight with either 10 nM PPT, 10 nM DPN or 10 nM E₂ after which 100 μ M etoposide was added to cultures for six hours to induce apoptosis. Control cells were treated with

vehicle (0.2% DMSO) only. Dead cells were visualized by means of nuclear staining under fluorescence microscope. Fifteen areas of 1300×1000 pixels in one sample were randomly selected from each plate for quantification of apoptotic cells. Data of three independent experiments are presented as the percentage of dead cells (means $\pm$ SE) for each treatment. The statistical significance of differences between vehicle control and etoposide-treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.4. Ospemifene prevents apoptosis of U2OS/ER α cells

ER α - (A) or ER β (B) -transfected cells were pre-incubated with either 10 nM E₂, 0,01 μ M or 1 μ M Tam, 0,01 μ M or 1 μ M Ral or 0,01 μ M or 1 μ M Osp for overnight before 6 hours of etoposide treatment, after which cell death was determined by microscopic visualization of apoptotic nuclei. Control cells were treated with vehicle (0.2% DMSO) only. Results are presented as the percentage of dead cells (means $\pm$ SE). The statistical significance of differences between vehicle control and etoposide-treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.5. The effects of E₂, PPT and Osp on etoposide-induced decrease of OPG expression

ER α - (A) or ER β (B) -transfected cells were pre-incubated with or without either 10 nM E₂, 1 μ M Osp or 10 nM PPT/DPN for overnight before 6 hours of etoposide treatment, after which OPG expression was detected by quantitative PCR. Control cells were treated with vehicle (0.2% DMSO) only. The columns represent values of three independent experiments (mean $\pm$ SE). The statistical significance of differences between either vehicle control and etoposide-

treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.6. The effects of E₂, PPT and Osp on etoposide-induced decrease of OPG protein secretion

ER α - (A) or ER β (B) -transfected U2OS cells were pre-incubated with either 10 nM E₂, 1 μ M Osp or 10 nM PPT/DPN for overnight before 6 hours of etoposide treatment, after which OPG expression was determined by ELISA. Control cells were treated with vehicle (0.2% DMSO) only. The columns represent values of three independent experiments (mean $\pm$ SE). The statistical significance of differences between either vehicle control and etoposide-treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.7. The effects of E₂, PPT and Osp on etoposide-induced increase of IL-6 expression

ER α - (A) or ER β (B) -transfected cells were pre-incubated with or without either 10 nM E₂, 1 μ M Osp or 10 nM PPT/DPN for overnight before 6 hours of etoposide treatment, after which IL-6 expression was detected by quantitative PCR. Control cells were treated with vehicle (0.2% DMSO) only. The columns represent values of three independent experiments (mean $\pm$ SE). The statistical significance of differences between either vehicle control and etoposide-treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.8. The effects of E₂, PPT and Osp on etoposide-induced decrease of IL-6 protein secretion.

ER α - (A) or ER β (B) -transfected U2OS cells were pre-incubated with either 10 nM E₂, 1 μ M Osp or 10 nM PPT/DPN for overnight before 6 hours of etoposide treatment, after which IL-6 expression was determined by ELISA. Control cells were treated with vehicle (0.2% DMSO) only. The columns represent values of three independent experiments (mean $\pm$ SE). The statistical significance of differences between vehicle control and etoposide-treated sample (+) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Fig.9. The effects of E₂, Osp, PPT and DPN on SaOS-2 cells

SaOS-2 cells were incubated with either 10 nM E₂, 1 μ M Osp, 10 nM PPT or 10 nM DPN for overnight and then treated with etoposide for six hours after which cell death was determined by microscopic visualization of apoptotic nuclei (A). Alternatively, cells were treated similarly, after which expression of OPG (B) or IL-6 (C) was detected by quantitative PCR. The statistical significance of differences between either vehicle control and etoposide-treated sample (+), vehicle control and E₂, Osp, PPT and DPN treated samples (#) or etoposide-treated cells and cells treated with combination of etoposide and ER ligands (*) were determined one-way analysis of variance (ANOVA).

Table 1. Summary of the effects of E₂, PPT, DNP and Osp on the etoposide-induced cell death and changes in expression of OPG and IL-6 in U2OS/ER α , U2OS/ER β and SaOS-2 cells.

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A)

B)

Figure 1.

C)

D)

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 9.

U2OS/ER α

	Cell Death	OPG mRNA	OPG protein	IL-6 mRNA	IL-6 protein
Etoposide	↑	↓	↓	↑	↑
+E2	↓	↑	↑	↓	↓
+PPT	↓	-	↑	↓	↓
+Osp	↓	↑	↑	-	↓

U2OS/ER β

	Cell Death	OPG mRNA	OPG protein	IL-6 mRNA	IL-6 protein
Etoposide	↑	↓	↓	↑	↑
+E2	↓	↑	↑	-	-
+DPN	↓	↑	↑	↓	-
+Osp	-	↑	↑	-	-

SaOS-2

	Cell Death	OPG mRNA	OPG protein	IL-6 mRNA	IL-6 protein
Etoposide	↑	↓	ND	↑	ND
+E2	↓	↑	ND	↓	ND
+PPT	↓	↑	ND	↓	ND
+DPN	↓	↑	ND	↓	ND
+Osp	↓	↑	ND	↓	ND

↑ = statistically significant increase ($p < 0,05$) or a trend to increase (increase more than 20% when compared to etoposide-treated cells)
 ↓ = statistically significant decrease ($p < 0,05$) or a trend to decrease (decrease more than 20% when compared to etoposide-treated cells)
 ND = not determined
 - = no effect