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Estrogen Modulates Estrogen Receptor α and β Expression, Osteogenic Activity, and Apoptosis in Mesenchymal Stem Cells (MSCs) of Osteoporotic Mice

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Abstract In the mouse, ovariectomy (OVX) leads to significant reductions in cancellous bone volume while estrogen (17β-estradiol, E2) replacement not only prevents bone loss but can increase bone formation. As the E2-dependent increase in bone formation would require the proliferation and differentiation of osteoblast precursors, we hypothesized that E2 regulates mesenchymal stem cells (MSCs) activity in mouse bone marrow. We therefore investigated proliferation, differentiation, apoptosis, and estrogen receptor (ER) α and β expression of primary culture MSCs isolated from OVX and sham-operated mice. MSCs, treated in vitro with 10^{-7} M E2, displayed a significant increase in ERα mRNA and protein expression as well as alkaline phosphatase (ALP) activity and proliferation rate. In contrast, E2 treatment resulted in a decrease in ERβ mRNA and protein expression as well as apoptosis in both OVX and sham mice. E2 up-regulated the mRNA expression of osteogenic genes for ALP, collagen I, TGF-β1, BMP-2, and cbfa1 in MSCs. In a comparison of the relative mRNA expression and protein levels for two ER isoforms, ERα was the predominant form expressed in MSCs obtained from both OVX and sham-operated mice. Cumulatively, these results indicate that estrogen in vitro directly augments the proliferation and differentiation, ERα expression, osteogenic gene expression and, inhibits apoptosis and ERβ expression in MSCs obtained from OVX and sham-operated mice. Co-expression of ERα, but not ERβ, and osteogenic differentiation markers might indicate that ERα function as an activator and ERβ function as a repressor in the osteogenic differentiation in MSCs. These results suggest that mouse MSCs are anabolic targets of estrogen action, via ERα activation. J. Cell. Biochem. Suppl. 36:144–155, 2001.

Key words: estrogen; estrogen receptor; mesenchymal stem cells (MSCs); osteogenesis; apoptosis; osteoporosis

Estrogen deprivation results in postmenopausal osteoporosis; long-term treatment with estrogen in appropriate doses reduces the risk of hip fractures by 50 to 60% and the risk of vertebral deformation by 90% [Notelovitz, 1997]. Bone loss in ovariectomized (OVX) animals is similar to that in postmenopausal women [Frost and Jee, 1992]. Estrogen maintains bone mass by preserving the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption [Turner et al., 1994]. The protective effect of estrogen on the skeleton of postmenopausal women appears to be mediated by suppression of bone resorption, with little evidence to suggest that conventional doses of estrogen also stimulate osteoblast activity. However, recent studies demonstrate that prolonged exposure of postmenopausal women to relatively high doses of estrogen results in sustained stimulation of osteoblast function [Tobias and Compston, 1999]. Systemically administered 17β-estradiol (E2) enhanced bone formation in animals [Takano-Yamamoto and Rodan, 1990; Chow

Abbreviations used: MSCs, mesenchymal stem cells; ER, estrogen receptor; E2, 17β-estradiol; ALP, alkaline phosphatase; BrdU, bromodeoxyuridine; OVX, ovariectomy.

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et al., 1992a, 1992b; Bain et al., 1993; Samuels et al., 1999], but the precise mechanism of E2 induced bone formation is not clear [Turner, 1999].

Estrogen exerts its major long-term effects on cell growth, cell differentiation, and cell function via intracellular estrogen receptors and eventually by activating particular genes [Beato, 1989; Carson-Jurica et al., 1990; Glass, 1994; Beato et al., 1995; Rickard et al., 1999]. The concentration of ER is an important determinant of cellular responsiveness to estrogen [Santagati et al., 1997]. In addition to ERα, a newly identified estrogen receptor (ER) subtype, ERβ was isolated from rat tissues [Kuiper et al., 1996]. The existence of ERβ and the fact that it dimerizes with ERα [Pettersson et al., 1997] adds to the complexity of gene activation in response to estrogen [Pace et al., 1997]. ERα and ERβ may play different roles in gene regulation [Paech et al., 1997]. Though ER α and β have been identified in osteoblasts as well as in their precursors [Eriksen et al., 1988; Komm et al., 1988; Pensler et al., 1990; Ohashi and Kusuhara, 1993; Turner et al., 1994; Lim et al., 1999; Vidal et al., 1999; Windahl et al., 2000], the precise role that estrogen plays in the regulation of osteoblastic function is not clear. It is uncertain whether osteoprogenitor cells including bone mesenchymal stem cells (MSCs) are targets of estrogen action [Rickard et al., 1999].

The effects of estrogen on bone cell function have been studied in numerous cell models, but the results have been conflicting [Sutherland et al., 1996; Rickard et al., 1999]. Several factors may contribute to these divergent and conflicting findings, among them are the wide heterogeneity of the model systems used and the fact that most in vitro cell culture systems do not accurately mimic the in vivo situation [Turner et al., 1994]. Unlike cell lines derived from long-term marrow cell cultures which may lose or acquire phenotypes depending on their state of differentiation, primary cultures of mesenchymal stem cells (MSCs) provide a better representation of the bone marrow cells as they are found in vivo [Zhang et al., 1995]. Because they adhere to plastic, MSCs can be separated from other marrow cells [Prockop, 1997]. It is generally believed that osteoblast precursors are derived from MSCs [Owen and Fridenstein, 1988; Kahn et al., 1995; Bruder et al., 1997; Jaiswal et al., 1997; Krebsbach et al., 1997; Prockop, 1997; Pittenger et al., 1999]. Since one mechanism by which the E2-dependent increase in bone formation would involve the proliferation and differentiation of osteoblast precursors, we hypothesized that E2 regulates mesenchymal stem cells (MSCs) activity in mouse bone marrow. We therefore investigated proliferation, differentiation, apoptosis, and ER α and β expression in primary culture MSCs isolated from OVX and sham-operated mice.

MATERIALS AND METHODS

Animals

Two months old Swiss-Webster female mice (ICR) were subjected to either ovariectomy (OVX) or sham operation (Sham). After five months period, uterus, tibias, and femurs were subjected to experiment in vivo and in vitro. Histomorphometry of femora and tibiae was performed as described previously [Gazit et al., 1999].

Cell Isolation and Primary Cell Culture

For in vitro experiments, the bone marrow was isolated from femurs and tibias of OVX and sham-operated (Sham) ICR mice (5 months after operation), as modified from a procedure described previously [Gazit et al., 1999]. The bone marrow cells were maintained in DMEM (Phenol red free, 1.0 g/l glucose, Biological Industries, Israel) with 15% FCS (Charcoal stripped, heat-inactivated), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and supplemented with 50 μg/ml ascorbic acid, 10 mM β-Glycerophosphate, and 10^{-8} M dexamethasone. Each time the medium was replaced, the cultures were treated with 17β-estradiol (E2) (Sigma), ICI182,780 (Zeneca Pharmaceuticals, UK).

RNA Isolation and RT-PCR

Total RNA was isolated from MSCs, by using RNAzol B (Biotecx Lab. Inc., Texas) or RNeasy Mini Kit (QIAGEN Inc., CA), according to the manufacturer's protocols. RT-PCR was modified from a procedure described previously [Orly et al., 1994], here we used 2μg total RNA. The primers of ERα were designed based on known cDNA sequences with modification as described previously [Bellido et al., 1993], forward primer: 5′-TCTGCCAAGGAGACTCGCTACTGT-3′ and reverse primer: 5′-GCTTGGCCAAAGGTTGG-CAG-3′ flank the 350-base pair of the ERα

fragment. The primers of 226 bp mouse L/B/K-ALP fragment were designed by modifying primers described previously [Gu et al., 1997] and the known mouse ALP sequence [Terao and Mintz, 1987], forward: 5′-CATGACATCCCA-GAAAGAC-3′ and reverse: 5′-GTTGTGAGCG-TAATCTACC-3′. Mouse BMP-2 primers (a kind gift from Dr. B. Sibley, Genetics Institute) were designed according to mouse BMP-2 cDNA sequence [Feng et al., 1994], forward: 5′-CAT-CCAGCCGACCCTTG-3′ and reverse: 5′-CT-CTCCCACTGACTTGTG-3′ amplified a 505 bp fragment. The primers of the internal controls: RPL19 (190 bp) [Orly et al., 1994], ERβ (260 bp) [Iafrati et al., 1997; Tremblay et al., 1997], TGF-β1 (401 bp) [Watabe et al., 1995], Collagen I (225 bp) [Pereira et al., 1995] and Cbfa1 (289 bp) [Komori et al., 1997] as described previously. The PCR reactions were performed by using specific conditions for each gene in a MJ Minicycler (MJ Research Inc., MA).

MSCs Differentiation Detected by Alkaline Phosphatase Activity

Histochemical staining for alkaline phosphatase (ALP) activity of MSCs colonies was performed by using Sigma Kit No. 86R. For the quantitative analysis of ALP positive cells, we classified each group of ten cells or more as a colony. The number of colonies per dish was counted microscopically, and the percent area of ALP positive colonies was determined in 35 mm dishes by automatic image morphometrical analysis.

MSCs Proliferation Detected by BrdU

MSCs were cultured on chamber slides. The cell culture medium was removed and replaced with diluted BrdU labeling solution. After we incubated the cells at 37°C for 2 h, we stained them immunohistochemically using the Zymed BrdU staining kit according to manufacturer's directions (Zymed Laboratories Inc., South San Francisco). All the slides were counterstained with hematoxylin solution. The results were expressed as the percent of positive cells (brown nuclei) among total cells.

MSCs Apoptosis

After the removal of culture medium, cells were washed once for 5 min with DAPI (4′, 6′-diamido-2-phenylindole hydrochloride) solution, 1 μg/ml in methanol, and incubated with DAPI solution at 37°C for 15 min. After incubation, cells were washed by methanol, mounted in fluoregel, and inspected using a microscope in the fluorescent mode. In parallel experiments, cells were also stained with 10 μg/ml PI (propidium iodide) in PBS [Pandey and Wang, 1995]. Cells that contained highly dense and irregular nuclear chromatin inclusions were defined as apoptotic, in comparison with non-apoptotic cells in which the DNA staining throughout the nucleus was more moderate and homogeneous [Keren-Tal et al., 1995]. For a positive control, the MSCs in one of the wells were treated with 100 μg/ml etoposide for 6 h [Smeyne et al., 1993]. MSCs in both the early and late stages of apoptosis were detected using ApoAlert Annexin V Apoptosis Kit of Clontech, and inspected by using a Zeiss 410 LSM confocal microscopy. Early and late stage apoptosis were detected by incubating cultured cells with Annexin V-FITC 1 μg/ml and 2.5 μg/ml PI. Early stage apototic cells which have bound Annexin V-FITC show green staining in the plasma membrane. Late stage apoptotic cells which have lost membrane integrity show yellow–orange staining (PI) throughout the cytoplasm and a green staining (FITC) on the cell surface [Martin et al., 1995].

For the quantitative analysis of apoptotic cells, we examined four to seven random fields of each well of chamber slides. Apoptotic and total cells were counted. The percentage of apoptotic nuclei was calculated for each field and the data were expressed as the mean for each chamber slide [Keren-Tal et al., 1995].

Immunohistochemistry of ER α and β in MSCs

Immunohistochemical staining was performed using the Zymed HISTOSTAIN SP kit according to manufacturer's directions (Zymed Laboratories Inc., South San Francisco). For ERα, the MSCs were stained by incubating them with 1 μg/ml rabbit polyclonal anti-ERα antibody MC-20 at room temperature for 1 h. Rabbit polyclonal anti-ERα antibody MC-20 (Santa Cruz Biotechnology, Inc., CA) is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 580–599 at the carboxy-terminus of the mouse ER α it does not cross-react with steroid receptors other than ERα. The ERβ was detected by incubating MSCs 1 h at 37°C with 4 μg/ml goat polyclonal anti-ERβ antibody (Y-19, Santa Cruz Biotechnology, Inc., CA).

Statistical Analysis

Quantitative data were analyzed by using either the non-parameteric Mann-Whitney test or the ANOVA test. All experiments were performed three to five times independently. Data are presented as mean values ± the standard error of the mean (SEM).

RESULTS

The Skeletons of OVX Mice are Osteopenic (Osteoporotic)

The osteopenic/osteoporotic nature of the skeletons of OVX mice is readily apparent by a marked reduction in the metaphyseal femoral and tibial trabecular bone volume (TBV) (Table I); a reduction in uterus weight, indicating the effectiveness of the OVX procedure, is also seen (Table I). The bones of the OVX mice contain significantly fewer trabeculae than do their sham-operated counterparts. In the latter, the trabeculae have a nearly plate-like morphology, reflecting not only a larger amount of bone, but also suggesting greater mechanical strength. In addition, in OVX mice the number of osteoblasts involved in matrix synthesis is reduced as reflected by the significant reduction in the extent of single and double-labeled surfaces in the tibial metaphyseal area (Table I). Moreover, in such OVX mice the number of osteoblasts involved in matrix calcification is also reduced as reflected by the reduction in the endosteal mineral apposition rates [Roberts et al., 1988; Hock et al., 1990; Rosen et al., 1994; Dallas et al., 1995; Gazit et al., 1998]. Together, these results suggest that in OVX mice there are reduced rates of both matrix synthesis and mineralization in trabecular and endosteal bone.

The Differentiation Effect of E2 on MSCs

The effect of E2 on differentiation of MSCs was studied by using alkaline phosphatase (ALP) as a marker for the phenotype of mature osteoblasts. Compared to the sham-operated group, in the OVX group the number and area of ALP positive colonies were significantly decreased (Fig. 1A and B), OVX control vs. sham control, $P < 0.05$ in colony area and $P < 0.001$ in colony number. In vitro, the E2 enhanced expression of ALP in MSCs was dose dependent (Fig. 1). The most pronounced effect was achieved by a concentration of 10^{-7} M E2 (Fig. 1A and B). Anti-estrogen ICI 182, 780 specifically inhibited the dose-dependent stimulatory effect of E2 on ALP expression (Fig. 1C and D) suggesting that the effect of E2 on MSCs differentiation mediated via the ER.

E2 Increases Proliferation Rate of MSCs In Vitro

The effect of E2 on MSCs proliferation was assayed by BrdU immunochemical staining. Proliferative activity was significantly reduced in MSCs obtained from OVX mice vs. that obtained from sham-operated animals, $P < 0.001$ (ANOVA) (Fig. 2). When 10^{-7} M E2 was added to the cultures, the proliferative activity increased in MSCs obtained from both the above groups (Fig. 2).

E2 Reduces Apoptosis in MSCs In Vitro

Apoptosis of MSCs was assayed by using Annexin V-FITC/PI staining, which detects early and late stage apoptosis [Martin et al., 1995] and DNA-based DAPI staining [Keren-Tal et al., 1995]. In in vitro studies, early and late stages of apoptosis were about 50% higher in MSCs obtained from OVX mice than from sham-operated mice (Fig. 3A). When 10^{-7} M E2 was added to MSCs in vitro, the baseline levels of apoptosis are reduced in similar proportions by both Annexin-V (Fig. 3A) and DAPI assays (Fig. 3B).

Modulation of Expression of ER α and β in MSCs Obtained From OVX and Sham Operated Mice

ERα and ERβ were immunohistochemically detected in vitro with specific anti-ERα and ERβ

TABLE I. Effect of Ovariectomy on Mouse Uteri and Bones (5 Months Post Operation)

	Uterus weight	Femoral TBV (%)	Tibial TBV (%)	Tibial labelling (%)	Tibial double labelling (%)	MARt (μm/day)	MARc (μm/day)
Sham (n = 4)	207 ± 30	6.6 ± 0.7	18.3 ± 0.6	6.6 ± 0.5	2.3 ± 0.5	1.2 ± 0.5	3.6 ± 0.7
OVX (n = 5)	45 ± 11	3.2 ± 0.2	7.8 ± 1.2	3.1 ± 0.8	0.5 ± 0.2	0.8 ± 0.2	1.3 ± 0.4
P	< 0.001	< 0.001	< 0.05	< 0.05	< 0.01	NS	<0.05

TBV, Trabecular bone volume; % Labelling, % Fluorescent labeling in proximal tibial metaphysis; MARt, Trabecular mineral apposition rate; MARc, Cortical mineral apposition rate (endosteal); *P*, Nonparametric Mann-Whitney test; NS, Nonsignificant.

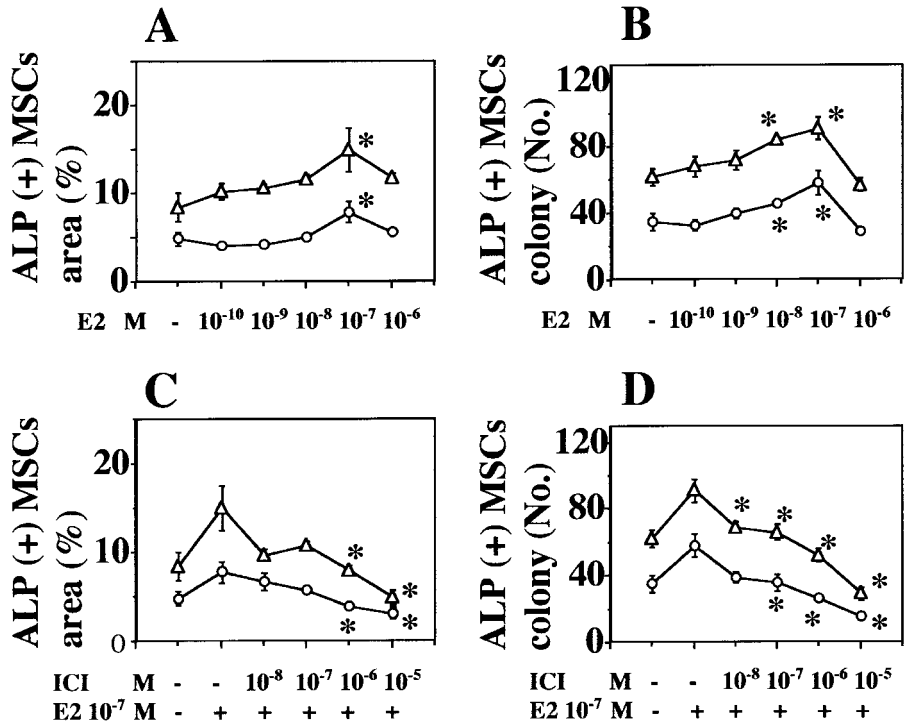

Fig. 1. The in vitro effect of E2 and anti-estrogen ICI 182, 780 (8 days treatment) on the differentiation of MSCs obtained from OVX (O) and Sham-operated (Δ) mice. **A:** The effect on ALP (+) MSCs colony size of E2 is dose-dependent. **B:** The effect of E2 on the number of ALP (+) MSCs colony is dose-dependent. For both A and B, $*P < 0.05$, E2-treated vs. non treated values. **C:** ICI reduces ALP (+) MSCs colony size. **D:** ICI reduces the number of ALP(+) MSCs colony, $*P < 0.05$, ICI treated vs. non-treated values. The data were analyzed by the Nonparametric Mann-Whitney test. The results given are mean ± SE for four experiments (n = 4), four dishes in each group of each experiment.

polyclonal antibody, ERα and ERβ positive cells have dark brown cytoplasm and nuclei. In MSCs obtained from sham-operated mice, the number of ERα positive cells was significantly higher than in MSCs obtained from OVX mice (Fig. 4A). E2 treatment (10^{-7} M) in vitro significantly enhanced ERα (Fig. 4A) and reduced ERβ (Fig. 4B) expression in MSCs obtained from OVX and sham operated mice.

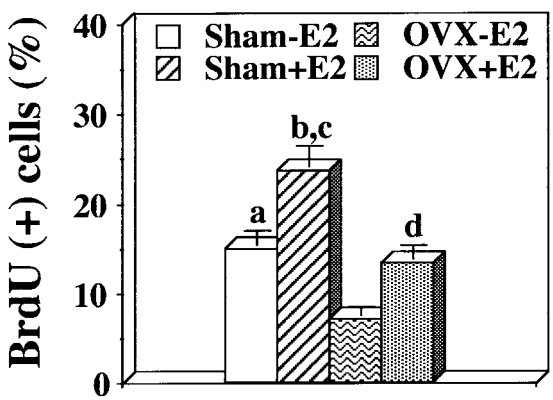

Fig. 2. The in vitro effect of E2 (8 days treatment) on the proliferation of MSCs from OVX and Sham-operated mice. Bar graph representing the percent of BrdU positive cells in MSCs cultures (n = 3; a, c, d: $P < 0.001$; b: $P < 0.005$). a: Sham-E2 vs. OVX-E2, (b) Sham + E2 vs. Sham-E2, (c) Sham + E2 vs. OVX + E2, and (d) OVX + E2 vs. OVX-E2 (ANOVA). The experiments are performed three times, two 4 wells chamber slides in each group of each experiment.

E2 Modulates ERα and ERβ mRNA in MSCs

To test the regulation of estrogen on ER mRNA, we examined the expression of ERα and ERβ by using RT-PCR. In MSCs obtained from both OVX and sham-operated mice treatment with 10^{-7} M E2 resulted in the up-regulation of ERα mRNA (Fig. 5A) and the down-regulation of ERβ mRNA (Fig. 5B). In a comparison of the relative mRNA expression levels for two ER isoforms, ERα was the predominant form expressed in MSCs obtained from both OVX and sham-operated mice. This is in contrast with the results of Qu et al. [1998] in which ER expression was not affected by E2 in mouse MSCs and similar with the results of Weihua et al. [2000] in uterus. Our results indicate that ERα and ERβ differ in their responses to estrogen in autologous ligand-dependent regulation.

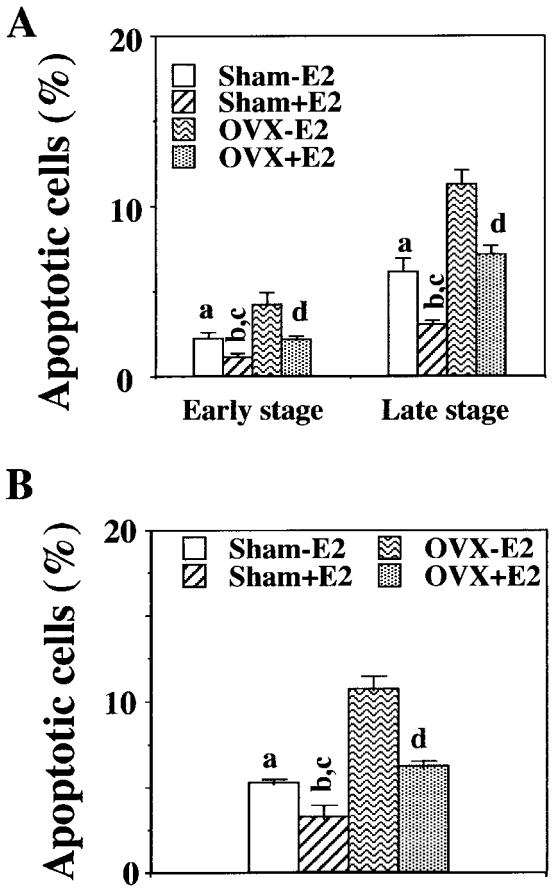

Fig. 3. The in vitro effect of E2 (8 days treatment) on the apoptosis of MSCs from OVX and Sham-operated mice. **A:** Determination of early stage apoptosis (Annexin V-FITC) and late stage apoptosis (Annexin V-FITC and Propidium Iodide staining), bar graph representing the percent of early ($n = 3$; a, b, c, d: $P < 0.05$), late ($n = 3$; a, c: $P < 0.001$; b, d: $P < 0.05$) apoptotic cells in MSCs cultures. One 4 wells chamber slide in each group of each experiment. **B:** Bar graph representing the percent of apoptotic cells in MSCs by DAPI staining ($n = 3$; a, c, d: $P < 0.001$; b: $P < 0.01$). Two 4 wells chamber slides in each group of each experiment. (a) Sham-E2 vs. OVX-E2, (b) Sham+E2 vs. Sham-E2, (c) Sham + E2 vs. OVX + E2, and (d) OVX + E2 vs. OVX-E2 (ANOVA).

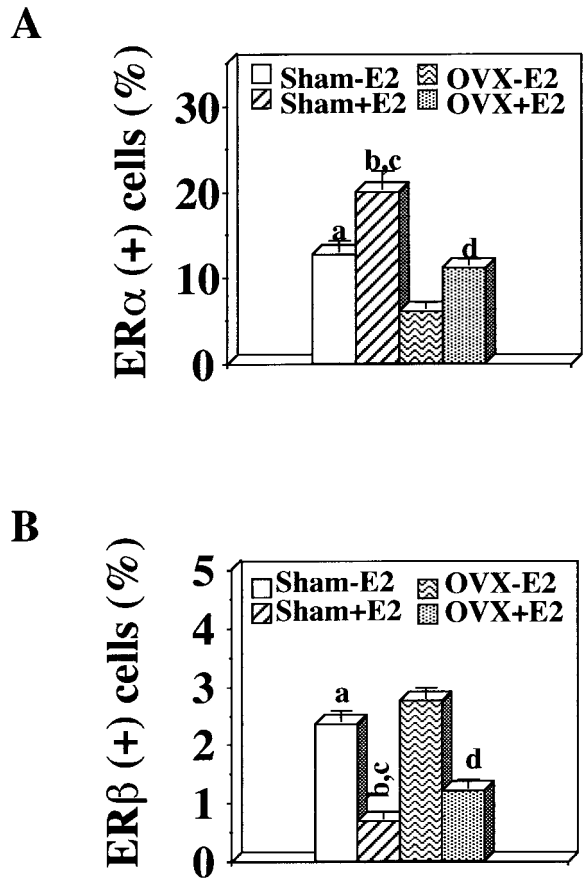

Fig. 4. The in vitro effect of E2 (8 days treatment) on ER α and β expression detected by immunohistochemical staining in MSCs from OVX and Sham-operated mice. Bar graphs representing the percent of ER α (**A**) and β (**B**) positive cells in MSCs cultures (ERα: $n = 3$; a, b, c, d: $P < 0.001$; ERβ: $n = 3$; a, b, c, d: $P < 0.05$). (a) Sham-E2 vs. OVX-E2, (b) Sham + E2 vs. Sham-E2, (c) Sham + E2 vs. OVX + E2, and (d) OVX + E2 vs. OVX-E2 (ANOVA). Two 4 wells chamber slides in each group of each experiment for ERα, one 4 wells chamber slide in each group of each experiment for ERβ.

E2 Modulates ALP, COL1A1, cbfa1, TGF-β1, and BMP-2 mRNA in MSCs

We used RT-PCR to measure the mRNA expression of osteogenic genes for ALP, type I collagen (COL1A1), cbfa1, TGF-β1, and BMP-2. The ribosomal protein L19 (RPL19) mRNA served as an internal control [Orly et al., 1994]. MSCs from OVX mice had a lower osteogenic gene expression than did MSCs from Sham mice, suggesting that estrogen depletion in vivo reduced the osteogenic potential of MSCs at the gene level. Compared with MSCs not treated with E2, the mRNA levels are higher in MSCs treated with E2 from both OVX and sham-operated mice, as seen on ethidium bromide-stained 5% polyacrylamide gels (Fig. 6A). The signal intensities from the gels were quantified by densitometry and normalized to the internal control RPL19, as shown in Figure 6B. The results imply that E2 augments the osteogenic potential of MSCs by regulation of their osteogenic gene expression in vitro as well as in vivo.

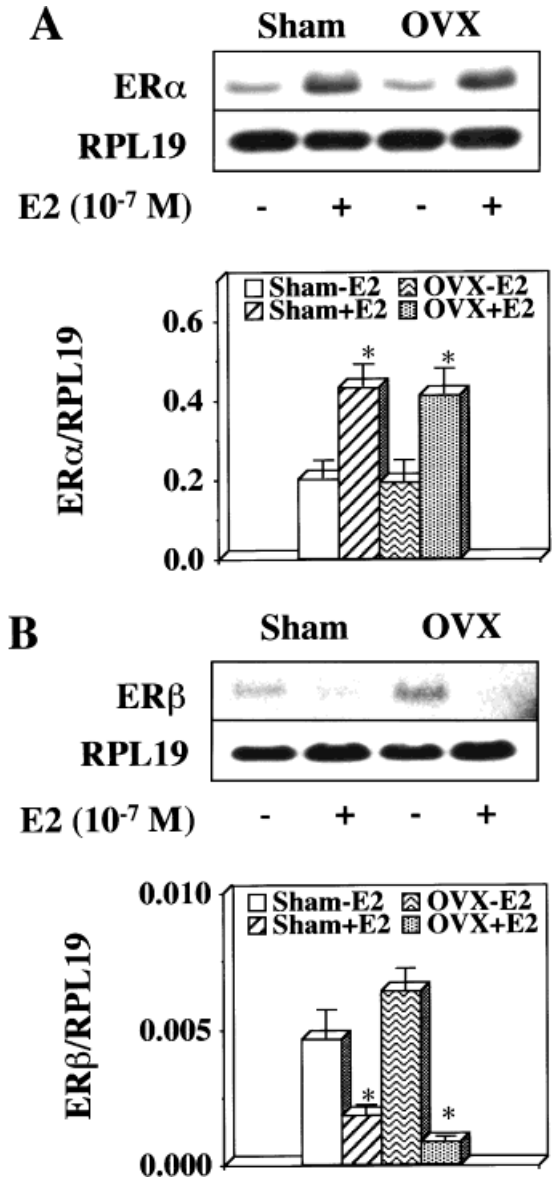

Fig. 5. The in vitro effect of E2 (8 days treatment) on the levels of ERα (**A**) and ERβ (**B**) mRNA detected by RT-PCR in MSCs obtained from OVX and Sham-operated mice. An autoradiograph (top) and quantitative results of the radioactive bands by phosphoimager (bottom). Quantitative results were expressed by normalizing the densitometry units of ER to those of RPL19 (internal control). The data were analyzed by Nonparametric Mann-Whitney test: $^*P < 0.001$ (A) and $^*P < 0.05$ (B), E2 treated vs. nontreated. The RT-PCR was performed five times for ERα and three times for ERβ in independent experiments by using total RNA that was isolated from 3–6 animals each time.

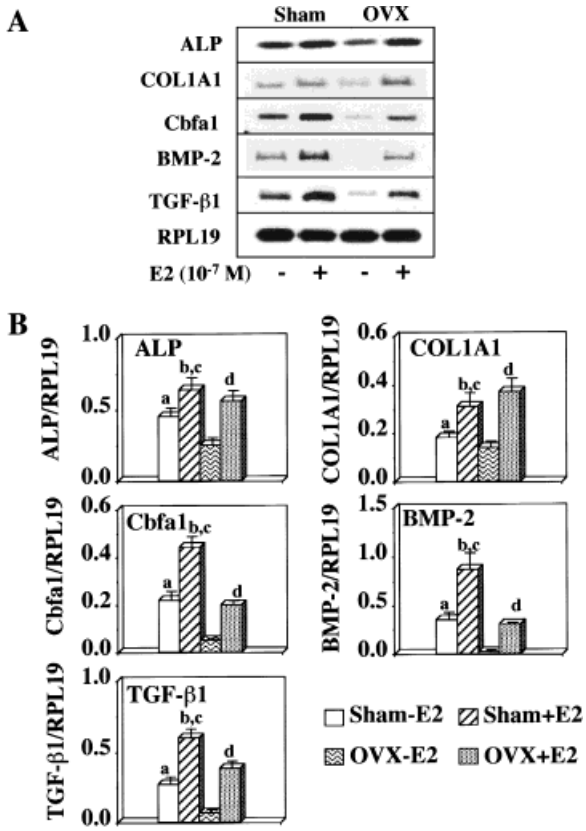

Fig. 6. The in vitro effect of E2 (8 days treatment) on the levels of ALP, COL1A1, cbfa1, TGF-β1, and BMP-2 mRNA in MSCs obtained from OVX and Sham-operated mice. **A:** Negative photos of ethidium bromide staining RT-PCR products of 5% non-denaturing polyacrylamide gel. **B:** Quantitative results were expressed by normalizing the densitometry units to those of RPL19 (internal control). The data were analyzed by ANOVA: (a) Sham-E2 vs. OVX-E2, (b) Sham + E2 vs. Sham-E2, (c) Sham + E2 vs. OVX + E2, and (d) OVX + E2 vs. OVX-E2. ALP: a, d: $P < 0.01$, b: $P < 0.05$, c: non-significant; COL1A1: a, c: non-significant, b: $P < 0.05$, d: $P < 0.01$; Cbfa1: a, b, c, d: $P < 0.05$; TGF-β1: a, b, c, d: $P < 0.01$; BMP-2: a, b, c, d: $P < 0.05$. The RT-PCR was performed 3–4 times in independent experiments for each gene described by using total RNA that was isolated from 3–6 animals each time.

DISCUSSION

Estrogen plays an important role in maintaining bone mass, and postmenopausal osteoporosis is clearly linked to its deprivation. The precise role that estrogen plays in the regulation of osteoblastic function is not clear. The MSCs present in bone marrow include a stem cell population with intrinsic osteogenic potential. This potential was demonstrated most comprehensively in several studies [Krebsbach et al., 1997; Kuznetsov et al., 1997; Bruder et al., 1998], which showed that MSCs transplanted to ectopic sites in suitable host animals developed into bone, and this differentiation into bone take place without an externally applied bone-inducing stimulus. This finding led to the conclusion that some of the MSCs are progenitor cells that, in a suitable environment, are intrinsically

capable of differentiating into chondrocytes and osteoblasts [Aubin et al., 1995; Krebsbach et al., 1999]. Since such progenitors appear to represent a target that is responsive to E2, we hypothesized that in post-menopausal osteoporosis E2 plays an important role in MSCs osteogenesis.

To characterize the effect of E2 on MSCs, we conducted a series of experiments to assay the in vitro effects of E2 on MSCs proliferation, differentiation, and apoptosis, as well as the regulation of ER expression. Our results show that ERα and ERβ were expressed in a certain fraction of MSCs, and that their expressions were regulated by E2. In a comparison of the relative mRNA expression and protein levels for the two ER isoforms, ERα was the predominant form expressed in MSCs, similar findings have been reported in osteoblast precursors and trabecular bone [Lim et al., 1999].

Reports on the effect of E2 on osteoblastic cell proliferation are contradictory and might be ascribed to differences in the phenotype of the transformed osteoblastic cells [Ikegami et al., 1993; Robinson et al., 1997; Rickard et al., 1999; Spelsberg et al., 1999]. Qu et al. [1998] found that cell proliferation in the early phase of the bone marrow culture was stimulated by E2; this stimulation could be blocked by pure anti-estrogen ICI 182, 780. Similar to our data, Ikegami et al. [1994] showed qualitatively that the ER-positive cells (%) are strongly correlated with BrdU positive cells (%) in human osteoblast-like osteosarcoma cells. In our study, using BrdU immunohistochemical staining, we found that E2 stimulates MSCs proliferation and ERα expression in both Sham and OVX mice. These results indicate that the effect of E2 on ERα expression may play an important role in MSCs proliferation. ERβ might directly inhibit cell proliferation or alternatively, indirectly inhibit cell proliferation via ERα modulation, as described in uterus [Weihua et al., 2000]. Whether ERβ has the same function in MSCs need be elucidated.

E2, in parallel, up-regulated both ERα expression and ALP expression in MSCs. Our findings are supported by the fact that in an SAOS cell line, E2 enhanced ALP activity and increased ERα expression [Sutherland et al., 1996], and the studies of differentiating osteoblasts showed a progressive increase in expression from very low levels for ERα with differentiation [Bodine et al., 1998]. The cascade mechanism between ERα expression and differentiation of MSCs is not clear, but the correlation between ERα expression and MSCs differentiation imply that E2 exerts its osteogenic effect through ERα. This notion is supported by the finding that the inhibition of the effect of E2 on ALP expression by ICI 182, 780 was dose-dependent. The precise role of ERβ in MSCs differentiation remains unknown. Recent in vivo data of ERα, β and double knockout female mice demonstrated that ERβ plays a negative role in trabecular and cortical bone, at least in the presence of ERα [Windahl et al., 1999; Gentile et al., 2000; Sims et al., 2000] and a repressive role in regulating osteoblast function [Windahl et al., 1999]. The mechanisms of bone loss in OVX ERβ knockout mice and the protective effects of estrogen are independent from ERβ [Ke et al., 2000], suggesting that estradiol effects on bone are mainly mediated through ERα [Ederveen et al., 2000]. These in vivo data support our in vitro data, implying that co-expression of ERα, but not ERβ, and osteogenic differentiation markers might indicate that ERα function as an activator and ERβ function as a repressor in the osteogenic differentiation of MSCs.

There is an increasing evidence that estrogen interacts with a membrane receptor and affects cells by a non-genomic mechanism [Somjen et al., 1997; Revelli et al., 1998; Rickard et al., 1999]. The non-classic ER pathways response to rapid estrogen actions which are too rapid to be compatible with the involvement of changes in mRNA and protein synthesis [Revelli et al., 1998]. Although there are membrane binding sites of estrogen in osteoblast-like cells [Endoh et al., 1997; Somjen et al., 1997], the role of non-genomic pathways in osteoblastic differentiation and proliferation has not yet been elucidated. Estrogen exerts its major long-term effects on cell growth, differentiation, and function via its intracellular receptors that activate target genes [Glass, 1994; Beato et al., 1995; Rickard et al., 1999; Spelsberg et al., 1999]. Our results imply that in MSCs, estrogen effects are mainly through estrogen receptors; nevertheless, it will be important to elucidate any non-genomic effects of estrogen on MSCs proliferation and differentiation.

A considerable body of evidence accumulated during the last decade has shown that the rate of genesis of osteoblasts and osteoclasts, as well as the prevalence of their apoptosis, is essential for

the maintenance of bone homeostasis; and that common metabolic bone disorders such as osteoporosis result largely from a derangement in the birth or death of these cells [Manolagas, 2000]. As far as we know, we are the first to demonstrate that apoptosis in MSCs is regulated by E2 and might be an important mechanism in the pathophysiology of postmenopausal osteoporosis. Here we have shown that among the MSCs obtained from OVX mice there were significantly more apoptotic cells than there were among MSCs obtained from Sham mice. This increase in the number of apoptotic cells among the MSCs from OVX mice could be the basis for an explanation for the development of osteopenia. In addition to increased apoptosis in MSCs from OVX mice, we also found decreased proliferation and differentiation. Thus, there seems to be a functional "triad" of increased apoptosis and decreased proliferation and differentiation in the pathophysiology of osteoporosis. It is known that E2 and growth factors regulate the apoptosis of osteoclasts [Hughes et al., 1996] and osteoblasts [Kitajima et al., 1996a, 1996b; Jilka et al., 1998; Meleti et al., 2000]. It is known that apoptosis in MCF-7 cells is regulated via estrogen receptors [Wang and Phang, 1995]. However, our data link apoptosis in the osteoblastic lineage to post-menopausal osteoporosis via MSCs, and were supported by the results of osteocyte apoptosis in vivo [Tomkinson et al., 1998]. We have shown here that treatment by E2 attenuates both the early and the late progress of apoptosis in MSCs obtained from both OVX and sham-operated mice.

In MSCs obtained from OVX mice, we found that E2 up-regulated osteoblastic marker genes including the genes for ALP and COL1A1, growth factor TGF-β1 and BMP-2, and transcriptional factor cbfa1 (Fig. 6). In general, there were lower osteogenic gene mRNA levels in MSCs obtained from OVX mice than from sham-operated mice, suggesting that estrogen depletion reduced the osteogenic potential of MSCs at the level of the genes. The cascade mechanism of E2 regulating ER and osteogenic gene expression in MSCs is not clear. TGF-β1 and BMP-2 are known to induce osteoblastic marker genes [Linkhart et al., 1996], and cbfa1 plays an essential role in osteogenesis [Komori et al., 1997]. In MSCs obtained from both OVX and Sham-operated mice, TGF-β1, BMP-2, cbfa1, and ERα are upregulated by E2, indicating that both in vitro and probably in vivo, E2 augments the osteogenic potential of MSCs by regulating their osteogenic gene and ERα expression.

Cumulatively, our results indicate that estrogen in vitro directly augments the proliferation and differentiation, ERα expression, osteogenic gene expression and inhibits apoptosis and ERβ expression in MSCs obtained from OVX and Sham-operated mice. Co-expression of ERα, but not ERβ, and osteogenic differentiation markers might indicate that ERα function as an activator and ERβ function as a repressor in the osteogenic differentiation in MSCs. These results suggest that mouse MSCs are anabolic targets of estrogen action, via ERα activation.

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