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Effects of Estrogen on Collagen Synthesis by Cultured Human Osteoblasts Depend on the Rate of Cellular Differentiation

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Abstract Estrogen is known to act on osteoblasts according to their stage of differentiation and estrogen receptor (ER) isoform expression. The aim of this study was to determine when type I collagen (COL1) synthesis by cultured low-passage, human bone-derived osteoblasts (hOBs) is upregulated in response to estrogen. Cell lines from female donors aged 1 and 66 years were cultured for 11 days on collagen in growth medium supplemented with human serum, hydrocortisone, and β-glycerophosphate. Young-donor hOBs grew more quickly than old-donor hOBs and did not mineralize. Old-donor hOBs formed mineralized nodules 5 days after reaching confluence. Changes in mRNA levels with time for ERs, type I collagen, and alkaline phosphatase reflected the faster differentiation of the old-donor cells. The ERβ/ERα ratio fell threefold in young-donor hOBs but rose 300-fold in old-donor hOBs. Increased ERβ/ERα ratios prevented ligand-dependent downregulation of ERα transcription, resulting in reduced proliferation in old-donor hOBs. Upregulation of COL1 mRNA expression in response to estrogen was confined to intermediate stages of differentiation, resulting in significant increases in COL1 mRNA by estradiol only in young-donor cells. Since the young and old-donor hOBs were cultured under identical conditions, our results indicate that the response of hOBs to estrogen is largely dependent on intracellular mechanisms that control the timing of cellular differentiation. J. Cell. Biochem. 86: 251–257, 2002.

Key words: ER alpha and beta; bone cells; proliferation; mineralization; gene expression

Estrogen plays an essential role in the regulation of bone modeling and remodeling. It is necessary for the normal closure of growth plates and for normal skeletal development in adolescent boys and girls. Estrogen deficiency is a major pathogenic factor in menopause-associated bone loss and the development of postmenopausal osteoporosis [Compston, 2001]. Estrogen-replacement therapy (ERT) prevents postmenopausal bone loss predominately by reducing activation frequency [Vedi et al., 1996], but studies of long-term high-dose ERT have demonstrated that estrogen also increases osteoblastic activity [Wahab et al., 1997; Vedi et al., 1999] including collagen synthesis [Khastgir et al., 2001].

Oursler [1998] reviewed the apparently contradictory results of the many studies of the effects of estrogen on proliferation, matrix protein production and cytokine, and growth factor expression of cultured osteoblasts. Although the models of osteoblastic growth used in these experiments varied considerably, both transformed and non-transformed cells from various species being cultured under differing conditions, it was possible to suggest a model in which estrogen acted on osteoblasts according to their stage of differentiation. Arts et al. [1997] had shown that the α and β forms of the classical estrogen receptor (ER) were differentially expressed during differentiation of an immortalized human fetal osteoblastic cell line that mineralized when cultured on collagen films in medium containing dexamethasone and β-glycerophosphate. The ratio ERβ/ERα began to increase when the cells became confluent and

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continued to increase during mineralization. The authors suggested that the two isoforms act in conjunction with each other via heterodimerization [shown in vitro by Petterson et al., 1997]. Since the isoforms can exert different transcriptional regulation at estrogen-responsive elements, ERβ could thus modulate the activity of ERα as the cells progressed along the differentiation pathway. Hall and McDonnell [1999], using transfected non-osteoblastic cells, confirmed that ERα and ERβ heterodimerize in vivo and that ERβ modulates ERα transcriptional activity at subsaturating levels of hormone, decreasing the sensitivity of ERα-expressing cells to estradiol.

Recent evidence suggests that estrogen regulation of human osteoblast function is determined both by stage of differentiation and ER isoform expression [Waters et al., 2001]. Stably-transfected fetal osteoblast cell lines expressing only one or other isoform were used to measure responses to estrogen at early, middle, and late stages of differentiation. Estrogen effects on alkaline phosphatase (ALP) activity, type I collagen (COL1) production, and mineralization showed different patterns in ERα and ERβ expressing cells. COL1 production was increased by estrogen in ERα—but not in ERβ—expressing cells with the increase being restricted to the middle stage of differentiation (matrix maturation/early mineralization).

The aim of this study was to determine whether cultured human bone-derived osteoblasts (hOBs) produce more COL1 in response to estrogen only during intermediate stages of differentiation. Since differentiation limits cell proliferation, total COL1 production by a given number of proliferative hOBs would then depend on the rate of cellular differentiation. We hypothesized that rate of differentiation and change in ERβ/ERα ratio would affect total COL1 synthesis by hOBs cultured with estrogen. Hence, we measured the short-term response to 17β-estradiol of two low-passage human hOB lines that showed very different differentiation patterns when cultured under identical conditions.

MATERIALS AND METHODS

Cells

Two lines of hOBs were used in these experiments. Young-donor hOBs from a 1-year-old female donor were supplied by BioWhittaker UK Ltd. (Wokingham) and old-donor hOBs from a 66-year-old female donor were supplied by Promocell GmbH (Heidelberg, Germany). Both cell lines were used at passage 7.

Culture Conditions

Preliminary experiments to determine the effects of collagen on cell proliferation were done using proliferation medium (McCoy's 5A (modified) medium containing 2.8 μM ascorbic acid and supplemented with 10% pooled male human AB serum, 200 mM glutamine and antibiotics). Thereafter, culture conditions were chosen to mimic more closely those found in vivo. Cells were cultured on collagen in differentiation medium (medium as above supplemented with 200 nM hydrocortisone and 7.5 mM β-glycerophosphate) for 2 days before addition of carrier or 17β-estradiol. On day 0 of the experiment, β-cyclodextrin (carrier) was added at 10^{-7} M for control cells and cyclodextrin-encapsulated 17β-estradiol was added at 10^{-10} M (low-dose) and 10^{-8} M (high-dose). The medium was changed every 2 days.

Cell Proliferation Assay

Cell proliferation was measured using the ViaLight™ HS kit from LumiTech (BioWhittaker UK Ltd., Wokingham, UK). Cells were seeded into 96-well plates with and without collagen coating (BD Biosciences, Oxford, UK). The initial cell density was 6,400 cells/well. ATP was measured in eight replicate wells at 2-day intervals after a lag period of 5 days.

RNA Extraction

Poly(A) RNA was extracted from cultured cells using the MicroPoly(A)Pure™ kit (Ambion (Europe) Ltd., Huntingdon, UK) according to the manufacturer's instructions.

Preparation of RNA Standards

PCR products for ERα, ERβ, COL1A1, ALP, OC, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were made by RT-PCR of human endometrial, OB, or tendon RNA. Primer sequences are shown in Table I. The PCR products were cloned into the T-tailed vector pCR II-TOPO (Invitrogen Ltd., Paisley, UK), which has opposing SP6 and T7 RNA polymerase promotor sites. BamHl restriction sites were added to the forward primers so that the orientation of each insert could be determined by cutting the plasmids with BamHl. Plasmids

TABLE I. Cloning Primers and GeneAMP 5700 SDS Primers and Probes

Gene	Forward primer	Reverse primer	Probe
Cloning primers			
GAPDH	TGAAGGTCGGAGTCAACGGATTTG	GTTGGTGGTGCAGGAGGCATTGCT	
ERα[a]	AATTCAGATAATCGACGCCAG	GTGTTTCAACATTCTCCCTCCTC	
ERβ[a]	TAGTGGTCCATCGCCAGTTAT	GGGAGCCACACTTCACCAT	
COL1A1	CCCCCTCCCCAGCCACAAAGA	TCTTGGTCGGTGGGTGACTCT	
ALP	GTACTGGCCATTGGCACCTG	GGCCTTCACCCCACACAG	
OC	CTCGCCCTATTGGCCCTG	GATAGGCCTCCTGAAAGCCAT	
GeneAMP 5700 SDS primers and probes			
GAPDH	TTTTAACTCTGGTAAAGTGGATATTGTTG	TGACGGTGCCATGGAATTT	ATTGACCTCAACTACATGGTTTACATGTTCCAATATG
ERα	TGCTTCAGGCTACCATTATGGA	GTTTTTATCAATGGTGCACTCGTA	ACACATATAGTCGTTATGTCCTTGAATACTTCTCTTGAAGAA
ERβ	TCAAAAGAGTCCCTGGTGTGAAG	CTCTTTGAACCTGGACCAGTAACAG	CGGTTCCCACTAACCTTCCTTTTCAGTGTCTCT
COL1A1	CGCACGGCCAAGAGGAA	CATGGTACCTGAGGCCGTTCT	CCAAGACGAAGACATCCCACCAATCACC
ALP	CAGGCTGGAGATCGACAAGTTC	GGACCTGGGCATTTGGTGTT	CCTTCGTGGCCCTCTCCAAGACGT
OC	AGCAAGGTGCAGCCTTTGT	ACAGGTAGCGCCTGGGTCT	CTTCACTACCTCGCTGCCCTCCTGCTT

[a]Arts et al. [1997].

that contained inserts, suitable for transcription with SP6 RNA polymerase, were selected and cut with Not1. RNA transcripts were purified using Microspin S-300 columns (Amersham Biosciences UK Ltd., Little Chalfont, UK) after treatment with DNase1 to remove plasmid DNA.

RT-PCR Using GeneAmp 5700 SDS

One-step RT-PCR reagents (Applied Biosystems, Warrington, UK) were used in the Gene Amp 5700 SDS. Primer Express™ software was used to design the primers and probes. All primer pairs were chosen to include introns in the gene sequences. Probes were chosen to span intron-exon boundaries so that they were unable to hybridize to genomic DNA at the annealing temperature of the PCRs. The sequences are shown in Table I. A standard curve was included in each assay so that the overall efficiency of the assay could be calculated.

Relative Quantification of Gene Expression

mRNA levels were determined using the comparative threshold-cycle (C_T) method [Fink et al., 1998]. First, the amount of target mRNA in each sample was normalized to the amount of housekeeper mRNA (GAPDH), designated as calibrator, to give ΔC_T (C_T target $- C_{TGAPDH}$). Secondly, the amounts of target mRNA in the samples were compared using the formula:

$$\text{Amount of target mRNA} = 2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T = \Delta C_{T\,\text{sample 1}} - \Delta C_{T\,\text{sample 2 (calibrator)}}$ assuming that the efficiencies of the PCR reactions were close to 1. The efficiency of each assay was calculated using the formula:

$$E = 10^{-1/S} - 1$$

where S = slope of the standard curve.

RESULTS

Proliferation of Young- and Old-Donor hOBs on Plastic and Collagen

Young-donor hOBs grew more quickly than old-donor hOBs on plastic in proliferation medium. Maximal doubling times for young- and old-donor hOBs were 25 and 48 h, respectively. Old-donor hOBs tended to remain in clumps rather than spreading over the surface. However, old-donor hOBs grew faster on collagen than on plastic and formed confluent cell layers.

Changes in Growth and Gene Expression With Time in Young- and Old-Donor hOBs Cultured With no Added Estradiol

Young- and old-donor hOBs cultured in differentiation medium on collagen, formed confluent monolayers after 4 days. Old-donor hOBs formed mineralized nodules 5 days after reaching confluence (Fig. 1), but there was no sign of nodule formation and mineralization by young-donor hOBs during the 11-day culture period.

Levels of COL1 and ALP mRNAs rose between day 2 and 9 in young-donor hOBs but fell in old-donor hOBs over the same period. On day 2, the old-donor hOBs were producing eightfold more COL1 mRNA than young-donor hobs, but on day 9 the situation was reversed with old-donor hOBs producing twofold less COL1 mRNA than young-donor hOBs. The level of ERβ mRNA decreased slightly in young-donor hOBs but rose substantially in old-donor hOBs. The total increase in ERβ mRNA in old-donor hOBs (day 0–9) was almost 300-fold. The ERβ/ERα ratio fell threefold in young-donor hOBs but rose 13-fold in old-donor hOBs between day 2 and 9. ERα and OC mRNAs increased between day 2 and 9 in both cells lines with smaller changes in the old-donor hOBs than in the young-donor hOBs (Fig. 2).

Changes in Gene Expression With Added Estradiol

The estradiol and cortisol levels in the human serum used for these experiments were 94 pM and 150 nM, respectively. Thus, our basic culture medium with 10% serum contained a sub-physiological level of estradiol, making pretreatment of cells with an estrogen antagonist unnecessary for demonstration of the effects of estradiol on gene expression.

After 2 days high-dose estradiol treatment, young-donor hOBs had significantly increased levels of ALP and COL1 mRNAs compared to untreated cells. On day 9, only COL1 mRNA was still higher in the treated cells than in the controls. At this time, the level of ER alpha mRNA was significantly lower in the treated cells than in the controls. Low-dose estradiol treatment of young-donor hOBs resulted on day 9 in significantly increased COL1 mRNA compared to controls.

In contrast, old-donor hOBs had similar levels on day 2 and 9 of all mRNAs measured, irrespective of estradiol treatment. However, at 6-h high-dose estradiol treatment significantly increased the levels of ERα mRNA compared to controls. The effects of estradiol on the cultured hOBs are summarized in Table II.

DISCUSSION

We have used two lines of bone-derived hOBs, cultured at passage 7 on collagen in growth medium supplemented with human serum, hydrocortisone, and β-glycerophosphate, to show that increased COL1 production in response to estrogen is restricted to intermediate stages of

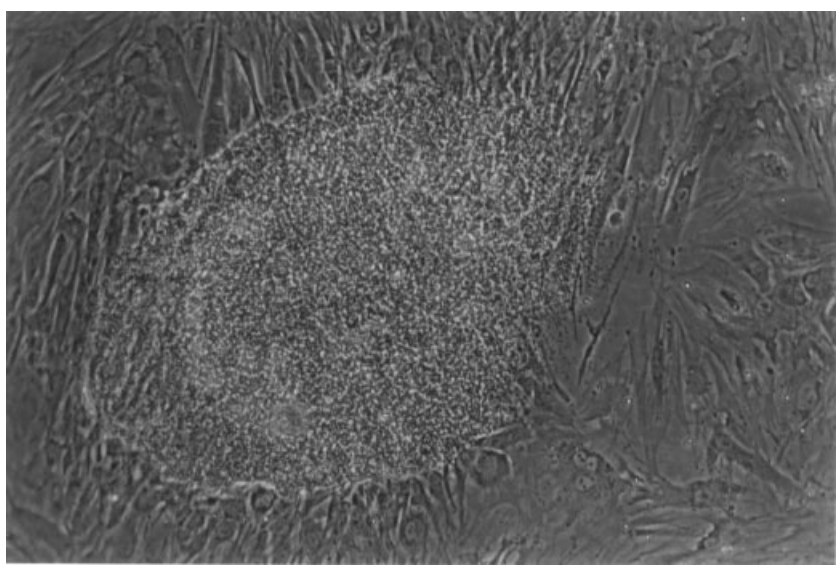

Fig. 1. Old-donor hOBs cultured in differentiation medium on collagen. Cells were photographed 5 days after reaching confluence, when von Kossa positive mineralized nodules were clearly visible.

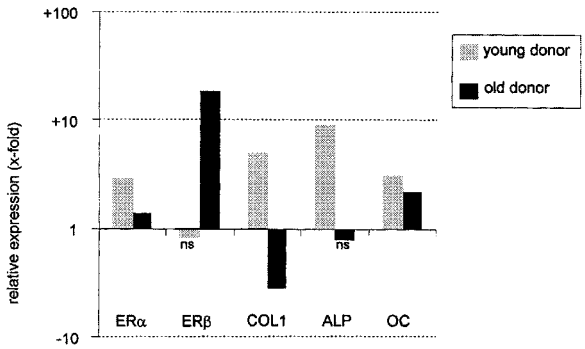

Fig. 2. Changes in mRNA levels with time in cultured hOBS. Values are for day 9 relative to day 2. Changes other than those indicated by ns (not significant) were statistically significant ($P < 0.05$). The experiment was repeated twice. Values shown are for five replicates in a representative experiment.

hOB differentiation. Estradiol treatment increased collagen mRNA synthesis in young-donor hOBs that showed no mineralization or increase in ERβ/ERα ratio but not in old-donor hOBs that mineralized with a marked increase in ERβ/ERα ratio.

The time course of proliferation, differentiation, and mineralization of cultured osteoblasts is affected by their substrate and culture medium. hOBs are generally cultured on plastic in basal medium containing fetal calf serum and ascorbate to promote growth and type I collagen production. Hydrocortisone and β-glycerophosphate are added to confluent cells to enhance differentiation and matrix mineralization. We modified these conditions to mimic more closely those found in vivo. Collagen appeared to increase cell proliferation by reducing the synthesis of ERα mRNA while having no effect on the other mRNAs measured. Studies of human breast cancer tissue have shown that ERα-negative cells are more proliferative and less differentiated than ERα-positive cells. ERβ mRNA levels in breast cancer tissues are not significantly related to proliferation and differentiation [Bieche et al., 2001].

The different growth rates of the two cell lines cultured on plastic in proliferation medium were in concordance with their donor ages (1 and 66 years). Normal osteoblast proliferation rates in basal medium have been shown to be age-dependent with the maximal growth rate falling sharply from age 1 to around 15 years and more slowly thereafter [Fedarko et al., 1995]. These authors demonstrated a direct relationship between collagen synthesis and cellular proliferation, since addition of a collagen-synthesis inhibitor to the culture medium was associated with a decrease in maximal cell proliferation rate.

Changes in mRNA levels from day 2 to 9 in young- and old-donor hOBs, cultured without added estradiol, reflected the faster differentiation, shown by the formation of mineralized nodules, of old donor cells. Levels of COL1 and ALP fall [Siggelkow et al., 1999] and ERβ mRNAs rise [Arts et al., 1997], when OBs mineralize. Consistent with these findings, young-donor hOBs showed increases in COL1 and ALP mRNAs and no increase in ERβ mRNA, whereas old-donor hOBs showed decreases in COL1 and ALP mRNAs and a sharp increase in ERβ mRNA during this period. The ERβ/ERα ratios in young- and old-donor hOBs decreased threefold and increased 13-fold, respectively. Thus, although old-donor hOBs produced more type I collagen mRNA on day 2 than young-donor hOBs, the level fell rapidly in the former cells, probably resulting in greater total collagen synthesis by young-donor hOBs over the culture period.

The increased rate of differentiation of old-donor hOBs appeared to make them less responsive than young-donor hOBs to estradiol. Although high-dose estradiol treatment for 6 h

TABLE II. mRNA Levels in Young- and Old-Donor hOBs Cultured With Low and High Dose Estradiol Compared to Control Cells With no Added Estradiol

			ER alpha	ER beta	Col1	ALP	OC
Young-donor hOBs	Day 2	Low E2	+1.1	+1.3	+1.3	+1.3	+1.1
		High E2	+1.1	+1.2	+1.9[a]	+1.8[a]	1.0
	Day 9	Low E2	1.0	+1.5	+1.8[a]	+1.3	+1.3
		High E2	−1.7[a]	1.0	+1.4	+1.0	+1.0
Old-donor hOBs	Day 2	Low E2	−1.1	+1.2	+1.2	+1.3	1.1
		High E2	1.0	nd	1.0	+1.2	1.0
	Day 9	Low E2	−1.1	−1.4	1.0	1.0	+1.1
		High E2	−1.2	−1.5	+1.1	+1.2	+1.2

[a]Changes shown here are significant ($P < 0.05$).
nd, not done. The experiment was repeated twice. Values shown are for five replicates in a representative experiment.

resulted in significantly increased levels of ERα-protein or mRNA in both young- and old-donor hOBs compared to controls, ERα mRNA was significantly reduced after 9 days with high-dose estradiol only in young-donor hOBs. (Unfortunately, RNA was not extracted from estradiol-treated young donor hOBs after 6 h, but immunocytochemistry showed that ERα protein expression is upregulated in young-donor hOBs, 24 h after estradiol treatment [Bord et al., 2001]. The increased ERβ/ERα ratio in old-donor hOBs may have accounted for the lack of downregulation of ERα in these cells by high-dose estradiol.

In addition to lower ERα mRNA, young-donor hOBs treated for 9 days with high-dose estradiol had lower levels of ERβ, COL1, ALP, and OC mRNAs than cells treated for the same time with low-dose estradiol. It appears that increased differentiation of young-donor hOBs by high-dose estradiol was terminated by downregulation of ERα mRNA.

COL1 mRNA was significantly increased in young-donor hOBs on day 2 and 9 by high- and low-dose estradiol treatment, respectively. Old-donor hOBs showed no such increase in COL1 mRNA in spite of a ninefold greater level of ERα mRNA. After 9 days with high-dose estradiol, young-donor hOBs had a similar level of COL1 mRNA to untreated cells. Our findings are in agreement with the observations of Ankrom et al. [1998] who measured type I collagen production by osteoblasts taken from 12 female donors. These authors showed a significant lessening of estradiol responsiveness with increasing donor age in spite of increasing levels of ERα mRNA.1. Our results suggest that COL1 synthesis by hOBs cultured with estrogen depends on the cellular change in ERβ/ERα ratio with time. Increased collagen synthesis in these cells is restricted to that stage of the differentiation pathway, before mineralization is accompanied by increased ERβ expression. Estrogen concentration is clearly one of the extracellular factors that control the timing of differentiation of cultured hOBs but intracellular mechanisms, which may depend on donor age, are of greater importance.

An age-related decline in the biosynthetic ability of osteoblasts is only one of several possibilities that could explain changes in the rates of bone resorption and formation seen with aging. Impaired-osteoblast recruitment and differentiation from the bone marrow stem cells rather than decreased activity of mature osteoblasts is another. Stenderup et al. [2001] investigated the proliferative capacity of osteogenic stem cells during aging and in patients with osteoporosis. No significant differences were found between the groups of individuals in their study but all individuals were over 22 years of age. Several studies using younger donors [Majors et al., 1997; D'lppolito et al., 1999; Nishida et al., 1999] showed a decline in the number of fibroblastic cell colonies from patients up to about 30 years of age with little change after that. Apoptosis of osteoblasts and osteoclasts could also be important in altering the remodeling balance [Manolagas, 2000].

In summary, cultured hOBs produced more COL1 in response to estrogen only during intermediate stages of differentiation. These stages were prolonged in cultures of young-donor hOBs compared to old-donor hOBs. During the 11-day culture period, ERβ/ERα ratios decreased in young-donor hOBs but increased rapidly in old-donor hOBs. Increased ERβ/ERα ratios prevented ligand-dependent downregulation of ERα transcription, resulting in reduced proliferation in old-donor hOBs. Thus, cultures of old-donor hOBs generated altogether fewer cells that responded to estrogen by increased COL1 synthesis. The timing of cellular differentiation in the two hOBs lines depended mainly on intracellular mechanisms, which affected their responses to estrogen.

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