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DIFFERENTIAL EXPRESSION OF ESTROGEN RECEPTORS α AND β mRNA DURING DIFFERENTIATION OF HUMAN OSTEOBLAST SV-HFO CELLS

JANINE ARTS, GEORGE G.J.M. KUIPER[#], JOSINE M.M.F. JANSSEN, JAN-ÅKE GUSTAFSSON[#], CLEMENS W.G.M. LÖWIK[*], HUIBERT A.P. POLS and JOHANNES P.T.M. VAN LEEUWEN.

*Department of Internal Medicine III, Erasmus University Medical School, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, part of Postgraduate School Molecular Medicine: Pathophysiology of Growth and Differentiation; # Center for Biotechnology, Department of Biosciences, Karolinska Institute, Huddinge, Sweden; *Department of Endocrinology, Academic Hospital Leiden, The Netherlands.*

ABSTRACT: Estrogens have been shown to be essential for maintaining a sufficiently high bone mineral density and ERα expression has been demonstrated in bone cells. Recently, a novel estrogen receptor, estrogen receptor β (ERβ) has been identified. Here we demonstrate that also ERβ is expressed in human osteoblasts, and that ERα and ERβ are differentially expressed during human osteoblast differentiation. ERβ mRNA expression increased gradually during osteoblast culture, resulting in an average increase of 9.9±5.3 fold (mean±S.D., n=3) at day 21 (mineralization phase) as compared to day 6 (proliferation phase). In contrast, ERα mRNA expression levels increased only slightly until day 10 (2.3±1.7 fold) and then remained constant. The observed differential regulation of ERα and β is suggestive for an additional functional role of ERβ to ERα in bone metabolism.

Osteoporosis is a major health problem that affects half of the elderly female population. An important cause of postmenopausal osteoporosis is the decline in estrogen levels after menopause. However, the exact mechanisms underlying the positive effects of estrogen on bone mass are still unknown. Several studies have reported direct effects of estrogens on the bone forming cells, the osteoblasts, *in vitro* (1-3). A low level of estrogen receptor α (ERα) expression has been demonstrated in osteoblasts, and their estrogen responsiveness is related to the ER expression level (4-6). An important role for ERα in human bone metabolism is also supported by the report of a man with estrogen resistance due to a mutation in the ERα gene suffering from, amongst others, a decreased bone mineral density (7). The ERα knock out mouse, however, showed only a modest decrease in bone density (8).

Recently, a novel estrogen receptor subtype was cloned, estrogen receptor β (ERβ) (9, 10). The presence, and role of, this novel estrogen receptor in bone is presently unknown. We investigated the expression of both ERα and ERβ mRNA during human osteoblast differentiation. Here we show that the novel estrogen receptor ERβ is indeed expressed in human osteoblasts, and that ERα and β are differentially expressed during human osteoblast differentiation.

Materials and Methods

Cell culture

Early passages (up to passage 16) of the human fetal osteoblastic cell line (SV-HFO) were used. This cell line, which is immortalized with SV-40 virus, differentiates in the presence of dexamethasone (11). Cells were maintained in α-MEM medium supplemented with 20 mM HEPES (Sigma, St.Louis, MI), 9 % (v/v) heat- inactivated FCS (Whittaker, Verviers, Belgium), 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco, Paisley, UK), at 37°C under a humidified 5% CO_2 / 95% air atmosphere, and the medium was replaced every 2-3 days. For experiments, cells were seeded in collagen coated dishes (0.3μg/cm^2 collagen S type I; Boehringer Mannheim, Mannheim, Germany) at a density of 1×10^4 cells per cm^2. After 3 days the cells were refed with culture medium supplemented with 10 mM β-glycerophosphate (Sigma, St.Louis, MI) and 10^{-6} M dexamethasone (9α-Fluoro-16α-methylprednisolone; Sigma, St.Louis, MI) and cultured for a 21 day period.

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Nested RT-PCR

Total RNA was isolated as described previously by Chomczynski and Sacchi (12) and quantified spectrophotometrically at 260 nm. cDNA was synthesized by reverse transcription of equal amounts of total RNA (0.5 μg) in 25 μl containing 20 pmol of reverse ERα primer, 20 pmol of reverse ERβ primer and 100 pmol of 3' 28S primer, 25 nmol of each dNTP (Sphaero Q, Leiden, The Netherlands), 1 x reverse transcriptase buffer (Gibco, Paisley, U.K.), 10 mM DTT (Gibco, Paisley, U.K.), 5×10^{-2} U RNAse inhibitor (RNAGuard, Pharmacia Biotech, Woerden, The Netherlands) and 25 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase (Gibco, Paisley, U.K.). In order to check whether the amount of PCR product increased linearly as a function of the amount of total RNA used, a concentration curve of template RNA was made, supplemented with the appropriate amount of tRNA (Boehringer Mannheim, Mannheim, Germany) to maintain a total amount of 0.5 μg of RNA. Following incubation for 1 h at 37°C, the cDNA was heated to 94°C for 10 min, snap cooled on ice and stored at

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-20°C. PCR amplification was performed in 50 μl containing 50 ng of cDNA template, 5 pmol of the forward and reverse ERα primers or 5 pmol of the forward and reverse ERβ primers, 10 nmol of each dNTP, 1x Taq buffer (Sphaero Q, Leiden., The Netherlands) and 2.5 U of Taq polymerase (Sphaero Q, Leiden, The Netherlands) . Twenty cycles of PCR (30 sec 94°C, 1 min 53°C and 1 min 72 °C) were performed on a Perkin Elmer Thermal Cycler 480. To minimize the risk of contamination, the second PCR was performed within the same tube. 50 μl nested primer mix was added, containing 100 pmol of the nested forward and reverse ERα or ERβ primers, 10 nmol of each dNTP, 1x Taq buffer and 2.5 U of Taq polymerase. Amplification was performed for 14 to 16 cycles (30 sec 94°C, 1 min 53°C and 1 min 72 °C). Since the expression of normal housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase have been reported to change during cell differentiation (13), we used the level of 28S RNA to quantitate ERα and ERβ mRNA expression. In short, 28S PCR amplification was performed in 50 μl containing 50 ng of cDNA template, 20 pmol of the forward and reverse 28S primers, 10 nmol of each dNTP, 1x Taq buffer and 2.5 U of Taq polymerase. PCR amplification was performed for twelve to fourteen cycles (30 sec 94°C, 1 min 53°C and 1 min 72 °C). All PCR products were electrophoresed on a 1.5% agarose (Ultrapure, Gibco, Paisley, U.K.) gel containing 0.2 μg/ml ethidium bromide and photographed under UV light using a Polaroid ISO 3000/36° film. The intensities of the bands present on the photo were read on a scanning densitometer.

All primers used were from Pharmacia Biotech (Woerden, The Netherlands) and had the following sequences:

ERα forward : 5'-AATTCAGATAATCGACGCCAG-3'
ERα reverse : 5'-GTGTTTCAACATTCTCCCTCCTC-3'
ERα forward nested: 5'-GACAAGGGAAGTATGGCTATGGA-3'
ERα reverse nested: 5'-TTCATCATTCCCACTTCGTAGC-3'
ERβ forward: 5'-TAGTGGTCCATCGCCAGTTAT-3'
ERβ reverse: 5'-GGGAGCCACACTTCACCAT-3'
ERβ forward nested: 5'-CGGAACCTCAAAAGAGTCCCTGG-3'
ERβ reverse nested: 5'-CCGAAGTCGGCAGGCCTGGCAGC-3'
28S forward: 5'-GTGCAGATCTTGGTGGTAGTAGC-3'
28S reverse: 5'-AGAGCCAATCCTTATCCCGAAGTT-3'
ERα internal: 5'-GGGCTGCAAGGCCTTCTTCA-3'
ERβ internal: 5'-GCCAGCCCTGTTACTGGTCCAG-3'

Statisticals

Statistical significance of differences was calculated using Student's t-test for paired data with the level of significance selected to be $p \leq 0.05$.

Results

Long term culture of the SV-HFO cells resulted in differentiation which was characterized by extracellular matrix synthesis and finally mineralization. The cells reached confluency after about 10 days which appeared to coincide with maximal alkaline phosphatase mRNA expression. Collagen type I mRNA expression on the other hand was maximal after 6 days of culture and mineralization of the extracellular matrix was detectable after 21 days (data not shown).

Since ER mRNA levels in human osteosarcoma cell lines are very low, we developed a semi-quantitative nested RT-PCR for both ERα and ERβ mRNA. The use of nested primers often improves quantitative RT-PCR, since fewer nonspecific amplification products are produced which otherwise interfere with quantification (14). Primers for RT-PCR were chosen in the N-terminal and the hinge region which are highly different in basepair sequence between ERα and ERβ. After 20 PCR cycles, a 20-fold excess of nested primers was added and amplification was continued for another 14 to 20 cycles. Theoretically four bands from each set of primers, forward and reverse, forward and nested reverse, nested forward and reverse, and nested forward and nested reverse, can be expected. However, due to the 20-fold excess of nested primers, the PCR product from the nested forward and nested reverse primer was preferentially formed, resulting in an RT-PCR product of 202 bp and 323 bp for ERα and ERβ, respectively (Fig.1A). Specificity of the RT-PCR product was further confirmed by hybridisation with a specific internal oligonucleotide probe (data not shown). Both the ERα and the ERβ RT-PCR product reached detectability on an agarose gel after 34 PCR cycles only. The amount of PCR product for both ERα and ERβ increased exponentially for up to 40 cycles, and increased linearly as a function of the amount of total RNA used (data not shown). Moreover, no PCR product was observed with a mock RT reaction containing all agents except reverse transcriptase, showing that the amplified product was mRNA specific (Fig. 1A, -RT).

As shown in Fig.1, ERβ mRNA expression gradually increases during SV-HFO culture, reaching maximal levels at day 21. In three independent experiments, the average induction of ERβ mRNA levels was 9.9±5.3 fold (mean±S.D., n=3, Fig.1B) compared to the expression level at day 6. The amount of RT-PCR product present at the different time points is expressed relative to that found at day 6, because in some experiments the ERα and ERβ RT-PCR products were undetectable at day 3. The increases in ERβ mRNA expression are significant between all different time-points ($p \leq 0.05$, n=3), except between day 6 and 10. This, however, can be attributed to the variation in mRNA increase between experiments. Within each separate experiment, the increase in ERβ mRNA expression between day 6 to 10 is also significant ($p \leq 0.05$, n=3). ERα mRNA levels slightly increased up to day 10 (maximally 3.3-fold) but, in contrast to ERβ, then remained constant, resulting in a not significant 2.3±1.7 fold increase at day 21 as compared to day 6.

Discussion

The present study demonstrates for the first time the mRNA expression of both ERα and of the novel estrogen receptor

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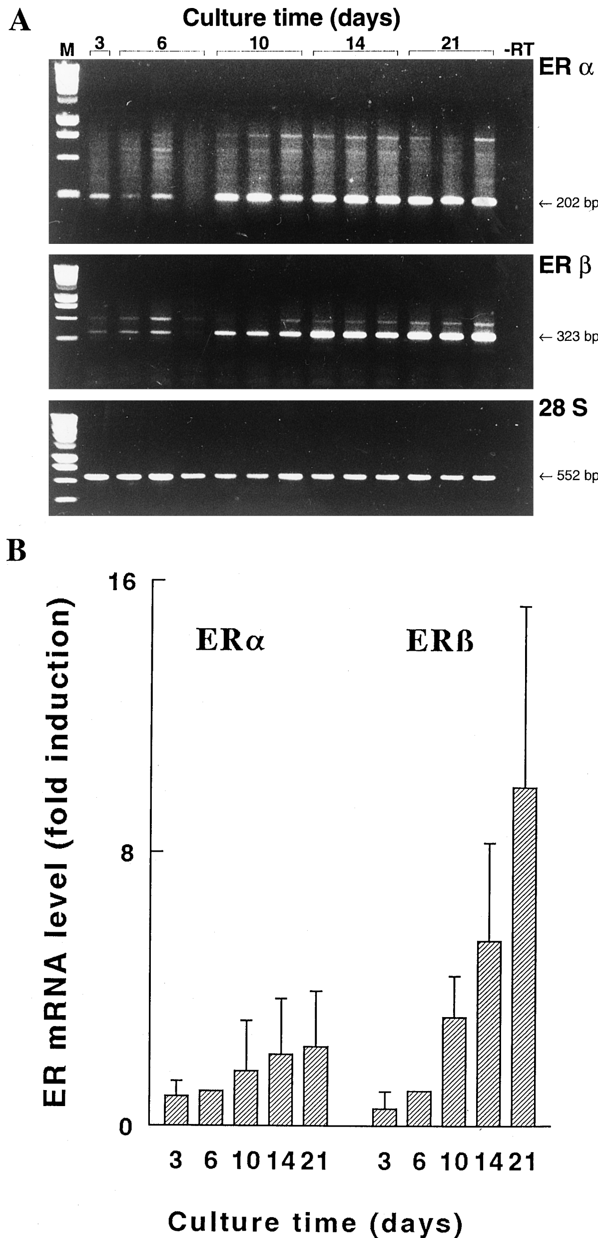

Fig. 1. Time profile of ERα and ERβ mRNA expression in SV-HFO cells analyzed with semi-quantitative RT-PCR.
Panel A: SV-HFO cells were cultured for up to 21 days as described in the methods section. The cells reached confluency after 10 days and mineralization of the extracellular matrix was detectable after 21 days (data not shown). At the indicated times, RNA was isolated and used for quantitative RT-PCR of ERα mRNA (ERα) , ERβ mRNA (ERβ) and 28S RNA (28S). M: marker, -RT: no reverse transcriptase was added.The experiment shown is representative for three independent experiments. Panel B: Quantitative representation of ERα and ERβ mRNA expression during osteoblast differentiation. The amounts of ERα and ERβ RT-PCR product were quantified by densitometry and adjusted for the corresponding 28S RT-PCR values. The results shown are means ± S.D. of 3 different experiments.

ERβ in *in vitro* differentiating human osteoblasts. The human osteoblast cell line used in the present study proved to be a good *in vitro* osteoblast differentiation and mineralization model (present study, 11, 15) resembling the well described rat osteoblast differentiation model (16). A most intriguing observation was that ERα and ERβ mRNAs are differentially expressed during osteoblast differentiation. Until reaching confluence, i.e. the moment of maximal expression of alkaline phosphatase mRNA (day 10) the mRNA expression of both ERα and ERβ increased. However, in the following period the mRNA expression of ERα did not change whereas the mRNA expression of ERβ further increased up to the mineralization period resulting in a final 10 fold increase compared to the proliferative phase. On the basis of the current study no comparison of the absolute amounts of ERα and ERβ mRNA levels can be made. Studies to address this issue as well as experiments with a recently developed ERβ-specific antibody to analyse protein levels are in progress.

For ERβ so far no data are available on expression in osteoblasts or changes during osteoblast differentiation. The higher expression of ERα mRNA during the post-proliferative state (day 10-21) as compared to proliferating cells seems to be in line with previous data. Osteosarcoma cell lines with a more differentiated phenotype expressed higher levels of ERα than osteosarcoma cells with characteristics of non-differentiated osteoblasts (4). However, these osteosarcoma cells should be considered to be representative of only a specific phase during osteoblast differentiation. They do not proceed through the whole osteoblast differentiation process as the currently used human osteoblasts do. Therefore, like for ERβ, so far the expression profile of ERα mRNA during differentiation and mineralization of human osteoblasts was unknown.

The significance of ERα for bone metabolism has recently been demonstrated by the description of the phenotype of a man lacking ERα and the ERα knock-out mouse (7, 8). The functional role of ERβ in general and in bone metabolism in particular is unknown. Some preliminary studies show differences between ERα and ERβ in transactivation of transiently transfected estrogen response element containing reporter constructs (10, 17, 18). The coordinate expression of ERα and ERβ mRNA in osteoblasts also opens the possibility that ERα and ERβ act in conjunction with each other, via heterodimerization. The ERα and ERβ proteins are indeed able to form DNA binding heterodimers (18) and it might be speculated that ERα/ERβ heterodimerisation is involved in the tissue specific action of certain anti-estrogens (19). In conclusion, the presence of both ER subtypes in osteoblasts further supports the involvement of the estrogen endocrine system in bone. In addition, the observed differential regulation of ERα and ERβ mRNA expression during osteoblast differentiation and mineralization is suggestive for an additional functional role of ERβ to ERα in bone metabolism. Obviously, the next essential step is functional analysis of ERβ in bone which is currently under investigation

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