CLINICAL STUDY

Regulation of CYP19 gene expression in primary human osteoblasts: effects of vitamin D and other treatments

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Abstract

Objective: Extragonadal estrogen biosynthesis is relevant for the regulation of bone metabolism. The aims of this paper were: (i) to examine CYP19 (aromatase) gene expression in primary cultures of osteoblasts under several hormone and cytokine treatments and (ii) to study the promoter usage of CYP19 in these cells.

Methods: Primary cultures of osteoblasts were obtained from healthy donors. The effects of vitamin D and other factors on CYP19 expression were analysed by semiquantitative RT-PCR. Furthermore, CYP19 alternative promoter usage under the different treatments was characterized by RT-PCR.

Results: CYP19 transcripts were detected in cultured human osteoblasts in serum-free conditions. Vitamin D, dexamethasone, 17β-estradiol and testosterone increased transcript levels of CYP19, whereas interleukin-1β or tumor necrosis factor α decreased them. Aromatase mRNA produced under treatment with vitamin D was transcribed from promoters I.4 and I.3, while stimulation with dexamethasone or dexamethasone plus vitamin D also involved promoter I.2. Testosterone activated promoters I.2 and I.4.

Conclusions: Our results suggested that vitamin D, testosterone, estrogens and glucocorticoids regulate CYP19 gene expression in human primary osteoblasts and the main promoter used appears to be promoter I.4. Promoters II and I.3 seem to be related to basal transcription and may mediate estrogen stimulation, while promoter I.2 seems to play a role in the effect of glucocorticoids. These findings indicate that vitamin D and several hormones regulate local estrogen synthesis in human osteoblasts mainly through usage of promoters I.4 and I.3.

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Introduction

Non-ovarian estrogen synthesis has proven relevant to bone metabolism in postmenopausal women, as well as in men (1–3). Aromatase, the cytochrome P450 enzyme involved in the conversion of androgenic steroids into estrogens, has been documented in bone (4–6), strongly suggesting that aromatase expression in this tissue plays an important role in both postmenopausal and male osteoporosis.

Cytochrome P450 aromatase (hereafter called aromatase), together with NADPH-cytochrome P450 reductase, forms the so-called aromatization complex (7), found exclusively in estrogen-producing cells and catalyses the conversion of androgens (C19 steroids) into estrogens (C18 steroids) (8). Aromatase activity has been detected in several human adult and fetal tissues, and pathologic samples (9, 10). With regard to bone, aromatase activity and/or aromatase mRNA have been detected in tissue samples (11–13), in human cultured osteoblasts (14, 15), in human osteosarcoma cell lines HOS, U2OS and MG63 (4–6, 14, 16), in differentiating human osteoblastic cells such as SV-HPO (17), and in SV40-immortalized human osteoblasts (5, 6). A fairly recent study (15) has shown that promoter usage and regulation of aromatase gene expression in osteoblast-like cells may be specific and different from those of other tissues such as placenta, ovary and adipose tissue.

CYP19, the gene for human aromatase, maps to chromosome 15p21.1, consists of an open reading frame of 1509 bp and codes for a 503 amino acid polypeptide (18–20), in differentiating human osteoblastic cells such as SV-HPO (17), and in SV40-immortalized human osteoblasts (5, 6). A fairly recent study (15) has shown that promoter usage and regulation of aromatase gene expression in osteoblast-like cells may be specific and different from those of other tissues such as placenta, ovary and adipose tissue.
expression is achieved through usage of alternative tissue-specific promoters, each of which is linked to a specific downstream untranslated first exon. Differential splicing produces specific transcripts bearing different 5' ends (9, 10).

In recent years, the understanding of the role of estrogens in both females and males has expanded greatly and considerable emphasis has been focused on the regulation of extragonadal estrogen biosynthesis. The observation of an osteoporotic phenotype in a man with a mutation in the estrogen receptor α (ERα) gene (1) and in several men with mutations in the CYP19 gene (3, 24, 25) has suggested an important role for non-ovarian estrogen production in the maintenance of bone mineralization, the pubertal growth spurt, epiphyseal fusion, skeletal maturation and the prevention of osteoporosis, since these men exhibit failure of epiphyseal closure, osteopenia and delayed bone age. The same conclusion may be drawn from the observed phenotypes of several knockout mice, namely, the ERα-knockout (aERKO), the ERβ-knockout (βERKO), the double-mutant aβERKO (26–29) and the aromatase-knockout (30, 31) mice.

In this context, it is interesting to evaluate the contribution of local estrogen production and, to date, no detailed study of CYP19 expression in primary human osteoblasts has been carried out. Here we present a transcription study in primary normal human osteoblasts including assessment of alternative promoter usage. We have evaluated the influence of calcitriol (1,25 dihydroxyvitamin D₃; Vit D), dexamethasone (DEX), estrogens (17β-estradiol; E₂), testosterone, tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) on CYP19 gene expression in these cells. Our results have shown, for the first time, that Vit D stimulates aromatase transcription in human osteoblasts.

**Materials and methods**

**Osteoblast cell culture and treatments**

Human bone cells were obtained from surgical specimens of healthy people, undergoing surgery for acute traumatic conditions and without remodeling bone disease. The protocol used was based on a method described by Marie et al. (32) with some modifications (33). Osteoblasts were characterized by alkaline phosphatase activity and osteocalcin synthesis in response to stimulation with Vit D (Roche, Basel, Switzerland). In all cases, cells stained intensely using a histochemical reaction for alkaline phosphatase (33).

Experimental cultures were grown in Petri dishes with Dulbecco’s modified Eagle medium (DMEM) (GibcoBRL Life Technologies S.A., Grand Island, NY, USA) supplemented with 20% fetal calf serum (FCS) (Biological industries, Kibbutz Beth Haemek, Israel). In order to synchronize osteoblasts, at confluency culture media were replaced with DMEM containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich Quimica S.A., Madrid, Spain) for 48 h. Subsequently, osteoblast cells were incubated in DMEM for 6 h in the absence of FCS with alternative treatments: (1) 0.1% BSA, (2) 10% FCS, (3) 100 nM DEX (Sigma), (4) 100 nM Vit D, (5) 100 nM Vit D+100 nM DEX, (6) 100 nM E₂ (Sigma), (7) 100 nM testosterone (Sigma), (8) 0.2 nM human recombinant IL-1β (R&D Systems Inc., Minneapolis, MN, USA) and (9) 0.3 nM TNFα (R&D Systems Inc.).

**Extraction of total RNA and reverse transcription**

Total RNA was prepared from cultured human osteoblasts by a guanidium thiocyanate–phenol–chloroform method using Tri Reagent (Molecular Research Centre, Inc. Cincinnati, OH, USA) according to the manufacturer’s protocols. Reverse transcription of RNA, for first strand cDNA synthesis, was performed using 0.5 μg total RNA and 25 ng/ml oligo (dT)₁₂–₁₈ primer (GibcoBRL) in a final volume of 6 μl. The reaction was incubated at 70°C for 10 min and immediately chilled on ice. Primer extension was then performed at 42°C for 50 min after addition of reaction buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM of each dNTPs and 100 U SuperScript II Rnase H—reverse transcriptase (GibcoBRL) in a final volume of 10 μl. The reaction was inactivated by heating at 70°C for 10 min. cDNA was stored at −20°C.

**Study of aromatase mRNA expression by PCR**

PCR amplification of aromatase cDNA was performed in a 50 μl reaction mixture containing 2 μl cDNA (1/10 dilution of RT mixture), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each deoxy-NTP, 0.3 μM of each sense and antisense primer and 1.5 U Taq DNA polymerase (GibcoBRL), using an initial denaturation step of 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and polymerization at 72°C for 90 s, with a final elongation step of 10 min at 72°C. The sequences of sense and antisense primers were 5’-ACACCTCTAAACAGCTCTTC-3’ (exon V) and 5’-GCTCTCAACACTGTTCTT-3’ (exon X) respectively. As a control, PCR for β-actin cDNA was carried out under the same conditions, except for 35 cycles and an annealing temperature of 55°C. β-Actin primers were: sense 5’-TCATGAACTGGTACGTTGACATCCGT-3’ and antisense 5’-CCTAGAAGCATTGTCGTTTAG-3’. The expected sizes of the PCR products for aromatase and β-actin are 821 bp and 285 bp respectively.

**Semiquantitiation of CYP19 gene expression**

For semiquantitiation, amplifications of aromatase and β-actin cDNAs were performed under the conditions
described above, except for the number of cycles (25 for aromatase and 17 for β-actin).

Subsequently, 15 μl of the amplified products were subjected to electrophoresis on 1.5% agarose gels and blotted to Hybond-N+ nylon filters (Amersham Ibérica S.A., Madrid, Spain) according to an alkaline transfer standard protocol. Filters were hybridized with the corresponding cDNA probes. The cDNA fragments produced in the RT-PCR reactions described above were purified from agarose gels and labeled with digoxigenin (DIG)-11-dUTP using the random priming method (Boehringer Mannheim GmbH, Biochemicals, Mannheim, Germany). DIG-labeled DNA was quantitated by enzyme-linked immunoassay using an antibody conjugate (anti-DIG alkaline phosphatase conjugate) and the colorimetric DIG nucleic acid detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocols.

Prehybridization, hybridization and chemiluminescent detection were performed essentially as described by Engler-Blum et al. (34), using 30 ng/ml DIG DNA probe. The CDP-Start (Boehringer Mannheim GmbH) ultra-sensitive fast reagent was used as chemiluminescent substrate for alkaline phosphatase. Densitometry of bands was performed using a Bio-Rad image analysis system and the corresponding Macintosh software.

**Results**

**Detection of CYP19 gene expression in non-stimulated primary human osteoblasts in vitro**

To assay the presence of aromatase mRNA in primary human osteoblast cell cultures, we applied the RT-PCR technique (Fig. 1). Aromatase transcripts were amplified from total RNA of non-stimulated cells (0.1% BSA) as well as of FCS- or DEX-treated cells. Moreover, CYP19 gene expression was detected both in first passage and sixth passage cultures, with or without stimulators. RNA from a pancreatic cell line, used as a negative control, failed to yield CYP19 transcripts. These results were replicated four times using primary cultured osteoblasts from four different individuals (see Materials and methods).

**Semiquantitation of aromatase and β-actin mRNAs in primary human osteoblasts in vitro**

In order to compare the effects of different treatments on aromatase mRNA levels, we set up a semi-quantitative method. It consisted of a low cycle RT-PCR followed by electrophoresis, Southern blotting and densitometric analysis of the bands. By testing different amounts of cDNA and PCR cycles we were able to obtain autoradiographic signals proportional to the amount of cDNA used in the low cycle PCR reaction. As seen in Fig. 2A, by using 1 or 2 μl of a 1/10 dilution of the cDNA in a 25 cycle PCR reaction we obtained increasing signals within a linear range, without reaching saturation. Figure 2B shows that at 25 cycles the PCR reaction was below the saturation level. In order to use β-actin expression as an internal control, we determined that a 17 cycle PCR using 1 μl of a 1/10 dilution of the cDNA was the optimal condition.

Results shown in Fig. 2A correspond to osteoblasts incubated in either 0.1% BSA or 0.2 nM IL-1β. The same results were obtained when applying these conditions (1 μl of a 1/10 dilution of the cDNA in a

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences</th>
<th>Expected sizes of PCR products</th>
<th>T° annealing</th>
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<tr>
<td><strong>Forward primers</strong></td>
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<tr>
<td>AroEIII</td>
<td>5’-CGG TTG TAG TAG TTG CAG GCA C-3’</td>
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T° annealing: annealing temperature in a PCR reaction.
25 cycle PCR reaction) to all the other culture treatments (not shown). This semiquantitative method was then used to determine if repeated cell passage resulted in variation of aromatase expression in non-stimulated conditions. Figure 2C shows that there were no major differences in mRNA levels when first and sixth passage cells were compared.

**Effects of FCS, DEX, Vit D, testosterone, E2, TNFα and IL-1β on CYP19 expression in cultured human osteoblasts**

Effects of Vit D and different treatments on CYP19 gene expression were then assayed in first passage osteoblasts from two men, aged 47 and 50 years. As seen in Fig. 3, treatment with Vit D, DEX, Vit D+DEX, E2 or testosterone resulted in increased levels of CYP19 transcripts, relative to serum-free conditions, while treatment with either IL-1β or TNFα decreased them. Highest levels were obtained with Vit D, Vit D+DEX and testosterone. FCS seemed to have no stimulatory effect.

**Identification of promoter-specific CYP19 transcripts**

In order to characterize the promoter usage under different treatments, RT-PCR was performed using a common reverse primer and six different forward primers corresponding to six alternative exons I (Fig. 4A). Placental RNA was used as a control. All promoters tested seemed to be active in osteoblasts, at least under some conditions, except for promoter I.1, which was only active on placental control RNA (Fig. 4B). Promoter pII was mainly used in cells treated either with FCS or IL-1β. A slight band was also seen for E2-treated cells. Promoter I.3 transcripts were observed in several conditions: BSA, Vit D, DEX, DEX+Vit D and IL-1β. Again, a slight band was seen for E2-treated cells. The usage of promoter L2 was observed under DEX, DEX+Vit D and testosterone treatments. Transcripts corresponding to all these three promoters were also amplified on placental control RNA. Some extra bands were also detected. We performed a Southern blot analysis of the PCR products from the promoter experiments with a probe derived from exon II/III. This experiment showed that none of the byproducts corresponded to CYP19 transcripts except for one of the extra bands observed in lane P of promoter I.3. This extra band probably corresponds to an incompletely spliced transcript which retained the intron between exons I.3 and II. Promoter I.4 was used under all treatments. Aromatase mRNA produced under Vit D+DEX treatment was transcribed from promoters I.4, I.2 or I.3. The same pattern of promoter usage was observed when cells were treated only with DEX. Vit D stimulation involved only promoters I.4 and I.3. Testosterone activated promoters I.2 and I.4, the former not observed in the serum-free (BSA) lane. Under TNFα treatment, only transcription from the I.4 promoter was observed (lane 7). Finally, for IL-1β treatment a strong band was observed for the pII promoter. All these results were replicated in the four samples analysed.

**Discussion**

In this study, we have proved the expression of the CYP19 gene to be existent in primary cultured osteoblasts...
prepared from normal human bones. We have studied the effects of several treatments on this expression and we have focused on the use of the different alternative promoters under different conditions.

There are very few studies in cultured primary human osteoblasts from normal donors (6, 14). These studies have found CYP19 expression only when cells are treated, at least, with DEX. In contrast, we have observed CYP19 transcripts in serum-free cultures as well as under different treatments. The levels of CYP19 gene expression were maintained up to the sixth passage, consistent with the observations by Shozu & Simpson (15) on aromatase activity.

With regard to the different treatments, Vit D (with or without DEX), DEX, testosterone and E2 all increased transcriptional expression levels of CYP19 in osteoblasts in relation to serum-free conditions. The highest levels were obtained with Vit D+DEX. The effect of DEX is in agreement with those obtained in previous studies (4, 5, 15, 16), including the only one performed on primary human osteoblasts (14). We have observed an important inter-individual variability which should be further studied. However, the activation by Vit D and DEX and the inhibition by IL-1β and TNFα were consistently observed in samples from three other individuals (not shown).

In our study we have shown, for the first time, that Vit D alone induces a clear increase in CYP19 expression in osteoblasts. This increase is similar to, or even more relevant than, that obtained with DEX alone. Vit D stimulation of CYP19 gene expression has been recently documented in ovarian cells (35). Here, we have shown that this effect of Vit D also occurs in bone cells. In their study, Kinuta et al. (35) propose that the action of Vit D on estrogen biosynthesis may be due, in part, to its role in maintaining calcium homeostasis and, in part, to a direct regulation of the expression of the aromatase gene. As such, the stimulatory action of Vit D on CYP19 gene expression in osteoblasts may constitute an additional relevant effect on bone turnover regulation, beside its well-established stimulatory effect on genes such as osteocalcin (36) and osteopontin (37).

Testosterone might have a stimulatory effect in CYP19 gene expression in osteoblasts as well. Our results have shown a threefold increase for one of the donors but a very slight increase for the other.

Figure 3 Semiquantitative analysis of aromatase mRNA levels under different treatments. (A) Southern blots of aromatase and β-actin RT-PCR reactions using RNA from osteoblasts of a 47-year-old male. Treatments are indicated in the text. Comparison between different experiments showed that variation in β-actin mRNA levels were random and not associated with any particular treatment. (B) Histogram depicting aromatase optical density (OD) values, normalized by β-actin. For each treatment, the solid bars correspond to the sample shown in Fig. 3A, while the hatched bars correspond to a sample from the 50-year-old male. Test, testosterone.
A direct action of androgens on bone cells is firmly established and the presence of androgen receptors and 5α-reductase activity has been detected in osteoblasts (38, 39). Several studies support the fact that both androgens and estrogens regulate aromatization, and the stimulatory effect of testosterone on aromatase activity in brain has been demonstrated (40–43). Our results suggest the existence of a stimulatory effect of testosterone also in bone cells.

The action of estrogens in our experiments was comparable with that of testosterone. Again, there is a clear stimulation of CYP19 expression by E2 for only one of the donors. Although estrogen receptors are expressed in osteoblasts (44), E2 has not shown any effect on aromatization in human primary osteoblasts or osteoblast-like cells in previous works (14, 16). Further studies are necessary to confirm the putative stimulatory effect of estrogen, which, if true, would indicate an interesting positive feedback of estrogen on its own synthesis.

The IL-1β and TNFα treatments prove that these factors reduce the expression levels of CYP19 in human osteoblasts. These results differ from a recently published work (45), where stimulation of aromatase mRNA expression by IL-1β was found in HOS cells in the absence of DEX. While the role of IL-1β and TNFα on CYP19 expression is not clear, the inhibition that we observed may be consistent with their bone resorption-promoting action.

Several groups have shown that regulation of CYP19 gene expression is quite different in different tissues (12). The main promoters used are pII and I.3 in ovary, I.1 in placenta and I.4 in adipose stromal cells (10, 22, 23, 46). Studies of promoter usage in osteoblast cells are scarce and partial. Tanaka et al. (14) and Shozu & Simpson (15) both showed that, under treatment with DEX, promoter I.4 was the main promoter used in primary human osteoblasts and osteoblast-like cells respectively. We have carried out a comprehensive analysis of CYP19 alternative promoter usage in primary human osteoblasts. With regard to promoter I.4, our results indicated that this is the promoter of choice in osteoblasts not only under DEX but also under a variety of treatments, extending previous findings (14, 15). Also in agreement with Shozu & Simpson (15), we found the promoter pII responds to IL-1β. Our results differ from those of Tanaka et al. (14) since we detected a rather wide usage of promoter I.3 and we failed to find transcripts arising from promoter I.1. Finally, we have shown for the first time that promoter I.2 is utilized in osteoblast under some treatments, including DEX. In summary, promoter I.4 seems to play a main role in CYP19 expression in osteoblasts.

In conclusion, we have observed that normal human osteoblasts in primary culture exhibit expression of the CYP19 gene in serum-free conditions, and that it is regulated by Vit D, androgens, estrogens and glucocorticoids. Although many different treatments are able to stimulate aromatase expression, Vit D and testosterone are the single molecules that exert the highest effect. Promoter I.4 seems to be the main one in osteoblasts and may be the mediator of these two stimulations. On the other hand, pII and I.3 seem to be related to basal transcription and may mediate estrogen stimulation, while I.2 seems to play a role in the glucocorticoid effect. The results of this study contribute to the knowledge and comprehension of the local estrogen synthesis by bone cells.

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