EXPERIMENTAL STUDY

Functional analysis of the I.3, I.6, pII and I.4 promoters of CYP19 (aromatase) gene in human osteoblasts and their role in vitamin D and dexamethasone stimulation

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Abstract

Objective: Current evidence suggests that extragonadal estrogens play an important role in bone metabolism. Estrogen biosynthesis is catalyzed by P450aromatase, encoded by the CYP19 gene. The aims of this paper were to study CYP19 gene expression in human osteoblasts under several hormone and cytokine treatments and to define promoter regions involved in this regulation.

Methods: CYP19 transcript levels were measured from primary human osteoblasts and MG-63 cells by real-time PCR in basal conditions, and in response to seven different hormones and cytokines. Four promoters of CYP19 gene were cloned upstream of the luciferase gene and transfected into MG-63 cells. The effect of vitamin D and dexamethasone in these promoter activities was evaluated.

Results: Vitamin D and dexamethasone were potent stimulators of CYP19 transcription, while testosterone and 17β-estradiol stimulated moderately. Promoter pII proved the most potent in driving transient luciferase expression. Promoter I.4 displayed moderate activity, while promoters I.3 and I.6 were weak. A region upstream of exon I.3, including exon I.6, was identified as containing repressor elements of promoter pII. Promoter I.3 activity was modulated by repressors located within exon I.3, while an enhancer of promoter I.4 was detected within exon I.4. In the absence of fetal calf serum, dexamethasone stimulation was observed on promoters I.3 and I.4, while vitamin D stimulation acted only on promoter I.3.

Conclusions: Four regulatory regions of promoters pII, I.3 and I.4 are relevant to CYP19 expression in human osteoblasts. Vitamin D and dexamethasone modulate transcription through these regions.

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Introduction

Osteoporosis, the most common metabolic bone disease in Western society, constitutes an imbalance of the bone remodeling process in favor of bone resorption, leading to decreased bone mineral density and bone quality with an increased propensity to fracture.

The bone phenotypes of several knockout mice, namely, estrogen receptor-α (ERα)-knockout, ERβ-knockout, double-mutant αβ-knockout and aromatase-knockout mice (1–4) show that estrogens play an important role in bone tissue. In recent years, the discovery in men of an osteoporotic phenotype due to a mutation in the ERα gene, or to mutations in the CYP19 gene, has suggested that non-ovarian estrogen production is relevant in the maintenance of bone mineralization, pubertal growth spurt, epiphyseal fusion, skeletal maturation, and the prevention of osteoporosis, since these men exhibited failed epiphyseal closure, osteopenia and delayed bone age (5–7). Therefore, the understanding of the role estrogens play in both females and males has grown significantly and considerable emphasis has been placed on the regulation of extragonadal estrogen biosynthesis, including bone estrogen production.

Cytochrome P450 aromatase is the key enzyme for estrogen biosynthesis. Aromatase catalyzes the conversion of testosterone to estradiol, of androstenedione to estrone, and of 16-hydroxylated dehydroepiandrosterone to estriol (8). Aromatase is encoded by a single gene, CYP19, localized on 15q21.2. The human CYP19 gene spans about 123 kb with nine coding exons (exons II to X) and several alternative non-translated 5′ exons (9). To date, ten such exons have been described: I.1, I.2, I.3, I.4, I.5, I.6, I.7, 2a, 1f and pII.

(10–13). Regulation of CYP19 expression is achieved by use 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 (10, 14). Aromatase activity and CYP19 gene expression have been detected in human cultured osteoblasts (15, 16), as well as in the human osteosarcoma cell lines HOS, U2OS and MG-63 (17–21). Several studies have shown that promoter usage and regulation of aromatase gene expression in osteoblasts is specific and different from those of other tissues such as placenta, ovary and adipose tissue (22–25).

In the present study we have investigated the regulation of CYP19 gene expression in primary human osteoblasts (hOBs) and MG-63 cells in response to 1,25 vitamin D3 (Vit D), dexamethasone (DEX), testosterone, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and leptin. Moreover, we have examined the use of CYP19 promoters in response to such treatments. We have performed a luciferase reporter assay in the MG-63 cell line using plasmid constructs with several promoters of the CYP19 gene to define regulatory regions relevant to its expression in hOBs.

Materials and methods

Cell culture

Human bone cells were obtained from specimens of healthy individuals without remodeling bone disease who had undergone surgery for acute traumatic conditions. The protocol used for primary hOB culture has been previously described (22). Osteoblasts were characterized by alkaline phosphatase activity and osteocalcin synthesis in response to stimulation with Vit D (Roche, Basel, Switzerland), MG-63 cells were purchased from ATCC (http://www.atcc.org). Experimental cultures were grown with Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Grand Island, NY, USA), supplemented with 20% fetal calf serum (FCS) (Biological Industries, Kibbutz Beth Haemek, NY, USA), supplemented with 20% fetal calf serum (FCS) (Biological Industries, Kibbutz Beth Haemek, Israel) in primary hOB cultures and with 10% FCS in MG-63 cultures. In order to synchronize cells, at confluence, culture medium was replaced with DMEM containing 0.1% BSA (Sigma-Aldrich Quimica S.A., Madrid, Spain) for 48 h. Subsequently, osteoblast and MG-63 cells were incubated in DMEM for 6 h with or without FCS and alternative treatments: DEX 10^{-7} mol/l (Sigma), Vit D 10^{-7} mol/l, E2 10^{-8} mol/l (Sigma), testosterone 10^{-6} mol/l (Sigma), recombinant human IL-1β 10^{-5} mol/l (R&D Systems, Inc., Minneapolis, MN, USA), recombinant human TNF-α 10^{-7} mol/l (R&D Systems) and recombinant human leptin (10 100 and 1000 ng/ml) (R&D Systems).

Extraction of total RNA and reverse transcription

Total RNA was prepared from cultured hOBs using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer’s protocols. Reverse transcription of RNA, for first-strand cDNA synthesis was performed using 1 μg total RNA and 25 ng/ml oligo (dT)12–18 primer (Invitrogen) 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 following addition of reaction buffer containing 50 mmol/l Tris–HCl (pH 8.3), 75 mmol/l KCl and 3 mmol/l MgCl2, 10 mmol/l dithiothreitol, 1 mmol/l of each dNTP and 100 U SuperScript II Rnase H−Reverse Transcriptase (Invitrogen) 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 real-time PCR

Real-time PCR for CYP19 was conducted in a volume of 20 μl containing 2 μl cDNA (1/10 dilution of reverse transcriptase mixture), 900 nmol/l each primer, 50 nmol/l TaqMan-MGB probe (Applied Biosystems, Foster City, CA, USA) and 10 μl TaqMan Universal PCR Master Mix 2X (Applied Biosystems) in the following sequence: 2 min at 50°C, followed by 50 cycles at 95°C for 15 s, and then at 60°C for 60 s in 384-well plates with the ABI PRISM 7900 HT Detection System (Applied Biosystems). The sequences of forward and reverse primers were: 5’-ACCCCTCTGCTGCCTGTGTCAT-3’ (exon V) and 5’-TTGCCATGCATCAAAATTAACCT-3’ (exon VI) respectively, and FAM-TaqMan-MGB probe was 5’-TTGGACGAAAATGTGCTATC-3’ (between exon V and exon VI). Primers and the TaqMan-MGB probe were designed using the Primer Express software Ver. 1.2 (Applied Biosystems). Real-time PCR for β-actin was carried out under the same conditions, using a β-actin Assay on Demand (Applied Biosystems) containing primers and a VIC-TaqMan-MGB probe. A standard curve for each gene was constructed using multiple dilutions of an external cDNA sample. Results were been analyzed using the SDS TM Applied Biosystems Software Ver. 1.0 and expression levels calculated from a linear regression of the standard curve. Results are given as CYP19 expression vs β-actin expression (CYP19 relative expression) using arbitrary units. All real-time PCR reactions for each sample were performed in duplicate.

Promoter constructs

The 5’-flanking region of CYP19 was amplified by PCR from human genomic DNA in a reaction mixture of 50 μl containing 100 ng genomic DNA, 60 mmol/l
Synthesis, and then lysis was performed with Cell in order to ensure promoter activation and luciferase addition. MG-63 cells were incubated for 24 h used as a control (with and without FCS). After treat-
or with 10% FCS. MG-63 cells with no treatment were 2
(pSV-
sense (pSV-βGal vector) (Promega). After transfection, MG-
were named pGL3/CYP19 (A to H) (Fig. 3).

Transfection and luciferase assays

MG-63 cells were grown in DMEM with 10% FCS at 50–80% confluency and transient transfection was carried out using the FuGene 6 Reagent (Roche). Cells were co-transfected with 2
b-galactosidase driven by the SV40 early promoter, MG-63 cells transfected with a pGL3 vector, without insert, were used as a basal expression control. Luciferase and β-galactosidase assays were performed using the Luciferase Assay System Kit (Promega) and the β-Gal Reporter Gene Assay Kit (Roche) respectively. Firefly luciferase activities were normalized to β-galactosidase activities to cor-
rect for differences in transfection efficiency. Transfection experiments were replicated four or more times for each construct, and all chemiluminescent assays were performed in duplicate.

Statistical analysis

All data analyses were performed using the SPSS 11.0 statistical package. Significant differences between any two groups were determined by Student’s t-test or a Mann–Whitney U-test. When multiple groups were compared, one-, two- or three-way ANOVA was utilized, followed by a Student–Newman–Keuls or Tukey mul-
tiple contrast test, when applicable. A value of P < 0.05 was considered significant.

Results

Effects of Vit D, DEX, E2, testosterone, TNF-α, IL-1β or leptin on CYP19 gene expression

CYP19 transcript levels were measured by real-time PCR in cultures of primary hOBs (three men and a woman aged 60, 67, 72 and 67 years respectively) and MG-63 cells treated with Vit D, DEX, E2, testosterone, TNF-α, IL-1β or leptin, with or without 10% FCS (Fig. 1). Basal expression was detected in both cell types, but was lower in MG-63 cells and was not affected by FCS. Treatments with either 10⁻⁷ mol/l DEX or 10⁻⁷ mol/l

<table>
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<tr>
<th>Inserts</th>
<th>Primer sequence</th>
<th>MgSO₄ (mmol/l)</th>
<th>T°C</th>
<th>PCR product (bp)</th>
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<td>59</td>
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<tr>
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<td>59</td>
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<tr>
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<td>2</td>
<td>59</td>
<td>831</td>
</tr>
<tr>
<td>Ins C</td>
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<td>59</td>
<td>523</td>
</tr>
<tr>
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<td>59</td>
<td>330</td>
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<tr>
<td>Ins D</td>
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<tr>
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<tr>
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<td>2</td>
<td>59</td>
<td>831</td>
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</table>

S: sense (kpnI restriction site: 5'-GGTACC-3').
A: antisense (XhoI restriction site: 5'-CTCGAG-3').
MgSO₄: PCR MgSO₄ concentration.
T°C: PCR annealing temperature.

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Vit D resulted in significant stimulation of CYP19 expression (4.8-fold in primary osteoblasts for DEX; and 4.5-fold in primary osteoblasts and 10.5-fold in MG-63 for Vit D). However, while DEX stimulation was enhanced by 10% FCS, Vit D stimulation was inhibited by it. Similar results were obtained with 10^{-8} mol/l of each treatment (data not shown).

Testosterone or E2 also stimulated CYP19 transcription, but to a much lesser extent (3.1-fold and 1.6-fold respectively) and only in primary osteoblasts in the absence of FCS (Fig. 1B). Control cultures for the steroid hormone experiments were carried out by adding ethanol and the results were the same as those of the treatment-free experiments (data not shown). Similarly, treatment with TNF-α resulted in reduced CYP19 transcript levels, but only in primary osteoblasts in the absence of FCS (not shown). Treatments with IL-1α or leptin proved ineffective in this system.

**Functional analysis of different CYP19 upstream regions**

Several CYP19 promoters were cloned upstream of the luciferase reporter gene of the pGL3Basic vector and were transiently transfected in MG-63 cells to assay functionality. Eight different constructs (named A to H) were obtained (Fig. 2), three of which (A–C) contained promoter pII, two (D, E) promoter I.3 alone, one (F) promoter I.6, and two (G, H) promoter I.4. Luciferase assays were performed from cultures maintained with or without 10% FCS. The pII promoter, up to exon I.3 (construct A), displayed the highest activity of all: more than 2.8 times higher than promoter I.4 (construct G), the second most active. When 600 bp of upstream sequence were added to promoter pII (construct B), a drastic reduction (8.5-fold) in activity was observed, suggesting the presence of one or more repressor elements. The activity of promoter I.3 was assayed singly with construct D, in which exon I.3 was directly fused to the luciferase gene.

This construct displayed the least activity of all. Indeed, by removing 137 bp of exon I.3 (positions −207 to −71; construct E) a 3-fold increase in promoter I.3 activity was observed, suggesting the presence of
specific exon I.3 repressors. Promoter I.6 (included in construct F, together with 20 bp of exon I.6) displayed moderate activity. Finally, promoter I.4 was the second most active (construct G). However, deletion of 257 bp of exon I.4, as well as part of the following intron (positions +71 to +328; construct H), resulted in a 2-fold reduction in activity, indicating the possible presence of enhancer elements(s). When the eight constructs were assayed in the presence of 10% FCS, the same relative activities were observed, although the absolute values were reduced by half (Fig. 2).

**Effects of Vit D and DEX on the different constructs**

Luciferase assays of the eight constructs were also performed under treatment with either DEX, Vit D or DEX + Vit D (Fig. 3). In cultures without FCS, DEX significantly enhanced expression by constructs containing either promoter I.3 (B–D) or I.4 (G), while Vit D only enhanced activity from promoter I.3 (B–D). In the presence of 10% FCS, DEX stimulation occurred in promoters I.3 and I.4, and was noticeably stronger. By contrast, Vit D + 10% FCS resulted in cancellation of Vit D stimulation.

**Discussion**

In this study we have shown that out of seven different hormone and cytokines, only Vit D and DEX were potent stimulators of CYP19 transcription, while testosterone and E2 were moderate stimulators. Moreover, we have defined promoter regions of CYP19 relevant to its expression in hOBs, in basal conditions and in response to Vit D and DEX treatments.

Consistently with results obtained in our previous study using primary hOBs (22), luciferase assays showed that pII, I.3 and I.4 promoters were active in MG-63 cells. Here we report that the sequence containing promoter pII up to exon I.3 (−221 to +1) displayed high activity in MG-63 cells. A similar region (−278 and −43) has been shown to mediate human CYP19 gene expression in the ovary. This region is known to contain two regulatory elements, a cAMP-responsive element-like sequence (−208/−199) and a steroidogenic factor-1 site (−132/−125) (26). In addition, our results showed that the region located between −867 and −211 bp contains repressor element(s) for this promoter activity in MG-63 cells. An inhibitory effect of 5′ sequences, located between −2700 and −278 bp on pII activity, has similarly been described.
in ovarian tissue (27). Transcriptional activity of pII or I.3 promoters was partly recovered when exon I.6 was removed, indicating that this exon contains some of the repressor elements. Our results therefore underscore the importance of exon I.6 in the regulation of pII or I.3 promoter activity in osteoblasts. Regarding promoter I.4, an important activity was observed in MG-63 cells, consistent with our previous findings in hOBs and those of other groups (15, 16, 22). Additionally the presence of an enhancer element in exon I.4 (+71 to +328) was demonstrated. This region is known to contain an Sp1 site (28). Regarding promoter I.3, we observed the presence of specific repressors within exon I.3 (−207 to −71).

In this study, we observed that both Vit D and DEX treatments increased the transcriptional levels of CYP19 in cultured normal hOBs and in MG-63 cells. Moreover, FCS exerted an inhibitory effect on Vit D stimulation of CYP19 gene expression. The stimulation of CYP19 gene by Vit D alone had been described previously by our group and by others (15, 16, 22). This serum effect might explain the lack of CYP19 stimulation by Vit D observed in other studies (15, 20, 24). The effects of treatments with testosterone or E2 confirm previously reported findings (22). Both treatments increased CYP19 gene expression in hOBs, and FCS showed an inhibitory effect on this stimulation. We have also observed that TNF-α decreased transcriptional levels of CYP19 in primary hOBs but had no effect in MG-63 cells, while IL-1β did not exert any effect. These results differ from other published works (32, 33), where stimulation or no effect in aromatase mRNA expression by IL-1β and TNF-α was described in HOS cells and osteoblast-like cells. The TNF-α inhibitory effect in CYP19 gene expression might be in agreement with its local resorptive action in bone.

Previous studies have demonstrated that leptin stimulates estrogen production by increasing aromatase expression and activity in human luteinized granulosa cells (34), adipose stromal cells (35) and the MCF-7 cell line (36). We and others have observed that hOBs do express the leptin receptor mRNA (37–39). However, leptin had no effect in our experiments, either in primary hOBs or in MG-63 cells. These results suggest leptin has no effect on CYP19 gene expression in hOBs, and whether leptin acts on other aspects of osteoblast function remains an open issue.

Figure 3  Luciferase assays of different CYP19 promoter constructs under different treatments. MG-63 cells, co-transfected with one pGL3-CYP19 construct (A, B, C, D, E, F, G or H; left) and the pSV-bGal plasmid, were treated with vitamin D (Vit D), dexamethasone (DEX), or Dex + Vit D in either the absence or presence of 10% FCS. For a given treatment and culture medium, relative luciferase activity for each construct was measured and compared with the same construct activity in the absence of treatment (Mann–Whitney U-test; *marginal P-value; n.s.: not significant).
This study is a first step to explore promoter regions involved in CYP19 gene expression in hOBs. However, further work is needed to define the mechanisms of transcription regulation in more detail and to identify the nuclear factors involved.

In conclusion, we show a stimulatory effect of Vit D and DEX on CYP19 gene expression in primary hOBs and MG-63 cells, and demonstrate the functional relevance of several upstream regions in this hormonal regulation. These results contribute to unraveling the complexity of CYP19 expression in hOBs.

Acknowledgements

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References


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