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Aromatase in Bone Cell: Association with Osteoporosis in Postmenopausal Women

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To clarify the possible action of adrenal androgen on bone cell, the existence, characteristics and regulation of aromatase in human osteoblast-like osteosarcoma cells (HOS) and primary cultured osteoblast-like cells from normal human bones (HO) were examined in this study. Significant positive correlation between bone mineral density (BMD) and serum dehydroepiandrosterone sulfate (DHEA-S) was found in 120 postmenopausal women (51–99 years old) but no correlation was seen between BMD and serum estradiol (E_2). In subset analysis, strongly positive correlation of serum DHEA-S and estrone (E_1) with BMD was observed in postmenopausal women aged less than 69 years old. Administration of DHEA to ovariectomized rat significantly increased BMD and decreased relative osteoid volume in femur. These *in vivo* findings strongly suggested that serum adrenal androgen may be converted to estrogen in peripheral organ, especially, osteoblast and be important steroids to maintain BMD. [^{3}H]DHEA was converted to [^{3}H]androstenedione and [^{3}H]androstenedione to [^{3}H]estrone in primary cultured human osteoblast. Osteoblast-like cells showed aromatase activity, and an apparent K_m and the V_{max} were 4.74 ± 0.78 nM (mean $\pm$ SD, $n = 3$) and 0.83 ± 0.79 fmol/mg protein/h for HOS, and 4.6 ± 2.9 nM and 279 ± 299 fmol/mg protein/h (mean $\pm$ SD, $n = 19$) for HO, respectively. The aromatase activity was significantly increased by dexamethasone in a dose-dependent manner. Reverse transcription-polymerase chain reaction analysis revealed that dexamethasone increased the transcript of $P450_{AROM}$ gene. Osteoblast-specific promoters were also determined. Dexamethasone and 1α,25-dihydroxyvitamin D_3 synergistically enhanced aromatase activity and $P450_{AROM}$ mRNA expression. These results demonstrate that adrenal androgen, DHEA, is converted to E_1 in osteoblast by $P450_{AROM}$ which is positively regulated by glucocorticoid and 1α,25-dihydroxyvitamin D_3 and important to maintain BMD in the 6 to 7th decade, after menopause.

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INTRODUCTION

Many studies have shown that estrogen deficiency is one of the factors in developing osteoporosis in postmenopausal women [1–3]. However, all postmenopausal women do not have problems with osteoporosis in spite of decreased estrogen production. Serum testosterone (T) and adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), were suggested to be other factors which may affect the bone mineral density (BMD) or fracture rate in postmenopausal women [4, 5]. Aromatase belongs to a cytochrome $P450$ enzyme which converts androgens to estrogens. Cytochrome $P450$ aromatase ($P450_{AROM}$) gene has been cloned [6] and tissue-specific and hormonally controlled utilization of the alternative promoters has recently been reported [7, 8]. Aromatase activity and $P450_{AROM}$ mRNA were found in a variety of tissues, including placenta, ovary, testis, brain, and adipose tissue [6–8]. In the present study, to clarify the possible androgen actions on normal human osteoblast, we have examined the existence and characteristics of

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aromatase activity and $P450_{AROM}$ mRNA in primary cultured osteoblast-like cells prepared from normal human bones.

EXPERIMENTAL

Subjects

One hundred and twenty postmenopausal women (51–99 years old, mean 76.3 years old) in whom at least 2 years had passed after menopause were examined. They have no severe liver disease, renal insufficiency, or bone disease except osteoporosis. To control for the effect of age, we divided them into three groups by age: Group A, 51–69 ($n = 28$); group B, 70–79 ($n = 41$); and group C, 80–99 years old ($n = 51$).

Animals

Female rats (40–43 weeks old) were divided into 4 groups: sham group ($n = 4$), ovariectomized (OVX) group ($n = 8$), OVX + DHEA group ($n = 5$); and OVX + testosterone (T) group ($n = 5$). Oriental chow containing 0.3% DHEA or 0.3% T were administered *ad libitum*. Twelve weeks after treatment, they were killed by decapitation, and their plasma and femurs were collected.

Bone mineral density

Bone mineral density (BMD) of lumbar vertebrae and femur of rat was measured by the method of dual energy X-ray absorptiometry (DEXA), using QDR-1000 (Hologic, MA, U.S.A.). The score was noted by mean BMD of vertebrae from the first (L1) to fourth (L4) lumbar spine except fractured spine.

Radioimmunoassay

Serum E_1, E_2, T and DHEA-S were measured by their specific radioimmunoassays (RIA).

Cell culture

The fragments of femur necks were obtained at surgery from 12 males and 7 females. Osteoblasts were prepared from the bone fragments [9] and grown in Eagle's minimum essential medium alpha (MEM-α, Gibco, NY, U.S.A.) and 10% fetal calf serum. The integrity of osteoblasts was examined by the ability to produce osteoblast-specific substances, namely, alkaline phosphatase, osteocalcine, type I collagen and TGF-β1. The cells were harvested using 0.05% trypsin and 0.1% collagenase at 37°C and cultured serially in 75 cm² tissue culture flasks.

Aromatase assay

Aromatase activity was determined by measuring $[^3H]H_2O$ released upon the conversion of $[1\beta\text{-}^3H]$androstenedione [A] to estrone as previously described [10].

Thin layer chromatographic analysis

The osteoblastic cells were treated with 10^{-7} M dexamethasone for 24 h, and then further incubated with 25 nM $[^3H]$DHEA or 25 nM $[1,2,6,7^3H]$A (3.52 TBq/mmol) for 12 h. The media (1.5 ml) were extracted with 5 ml of chloroform–ethyl acetate (1:1, v/v). The extracts were dried, dissolved in 50 μl of ethanol containing non-radioactive androstenedione and estrone for markers, and applied to thin layer plates (Silica Gel 60 F254, Merck, Darmstadt, Germany), then chromatographed twice in chloroform–ethyl acetate (1:1, v/v). The marker steroids were located by spraying with methanol–sulfuric acid (1:1) followed by heating and the amounts of radioactivity of the spots of each steroid were measured.

Determination of mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

For first strand cDNA synthesis, 1–2 μg of total RNA and 100 pmol of random oligodeoxyhexamer were incubated at 42°C for 60 min in 20 μl of a RT buffer containing 40 units of RNAaseH⁻ reverse transcriptase (GIBCO BRL) and 10 units of RNAsin. The cDNA was amplified by PCR as previously described [11]. One PCR cycle consists of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 2 min. PCR products were electrophoresed on a 1.0% agarose gel, visualized by ethidium bromide (0.5 μg/ml) staining and photographed under UV light using a positive/negative instant film (Polaroid 665, Nippon-Polaroid, Tokyo, Japan). Relative intensity resulting from each amplified product on the negative films was then determined by a densitometric scanner (RF-5000, Shimadzu, Kyoto, Japan), and the results were expressed as arbitrary units [12]. Standardization of the amplification efficiency of mRNA between samples was carried out using the co-amplified β-action sequence as an internal standard as described by Kinoshita *et al.* [13]. The sequences of sense and antisense primers to amplify the open reading frame of cDNAs were 5′- CCGGCCTT-GTTCGTATGGTCA-3′ (exon 5) and 5′-GTCT-CATCTGGGTGCAAGGA-3′ (exon 10), respectively, for human $P450_{AROM}$ [6], 5′-GAAGT-CAAGTGCCATTGAGG-3′ and 5′-CGTTAG CTTCATGCTGCACT-3′, respectively, for human vitamin D receptor [14], and 5′CCCAGCA CAATGAAGATCAA-3′ and 5′TTTCTGCGCA AGTTAGGTTTTGACAA-3′, respectively, for β-actin [15]. The expected size of the PCR products for $P450_{AROM}$, vitamin D receptor and β-actin are 987, 457 and 224 bp, respectively. The sequences of sense primers to amplify various 5′-untranslated regions transcribed from different exons by alternative promoters for $P450_{AROM}$ gene were 5′-CTGGAGGGCT-GAACACGTGG-3′, 5′-GACCAACTGGAGCCTG ACAG-3′, 5′-CCTTGTTTTGACTTGTAACCA-

3′, and 5′-AACAGGAGCTATAGATGAAC-3′ for exons I.1, I.4, I.3 and II, respectively [7, 8]. The antisense primer used for the 5′-untranslated regions was common, and its sequence was 5′-GTGCCCT-CATAATTCCACAC-3′, which corresponds to a part of exon 2 [6].

Statistics

Statistical analysis of the results was performed by using analysis of variance, followed by Student's *t*-test.

RESULTS AND DISCUSSION

Correlation between BMD and sex steroid hormones

An age-associated significant decrease in BMD was observed in postmenopausal women (51–99 years old) ($n = 127$, $r = 0.335$, $P < 0.005$) (Fig. 1). Significant positive correlation between BMD and serum DHEA-S was found in 127 postmenopausal women (51–99 years old) ($r = 0.224$, $P < 0.05$) (Fig. 2), but no correlation was seen between BMD and serum E_2. In subset analysis, serum DHEA-S ($n = 28$, $r = 0.39$, $P < 0.05$) and E_1 ($n = 28$, $r = 0.44$, $P < 0.05$) showed positive correlation with BMD in postmenopausal women of group A (51–69 years old) (Fig. 3). But there was no significant correlation between them in group B or C. Significant positive correlation between BMD and serum androstenedione or DHEA-S in postmenopausal women has been also demonstrated by other studies [4, 5]. However, no difference in serum sex steroid hormones including DHEA-S between osteoporotic and normal postmenopausal women was observed by Davidson *et al.* [16]. This discrepancy may be caused by the age and race of the subjects, especially because the age of their subjects were about 75 years old in mean age. We grouped the postmenopausal women according to age. Strongly positive correlation between BMD and DHEA or E_1 was observed in group A at an age of 51–69 yr. No correlation between them above 70 years old was found.

Effect of DHEA or T in ovariectomized rat

Ovariectomy significantly decreased BMD in femur of rat. Administration of DHEA or T to ovariectomized rat for 12 weeks significantly increased BMD and decreased relative osteoid volume in femur of rat (Fig. 4). These *in vivo* findings strongly suggested that serum DHEA-S may be converted to estrogen in peripheral organs, especially osteoblast, or directly act on bone tissue and be important steroids to maintain BMD.

Aromatase activity in human osteoblast-like osteosarcoma cell (HOS) and primary cultured human osteoblast (HO)

Aromatase is a cytochrome *P*450 enzyme that converts androgen to estrogen. Aromatase activity was determined in HOS cells by measuring $[^3H]H_2O$ released from $[1\beta\text{-}^3H]$androstenedione. An apparent

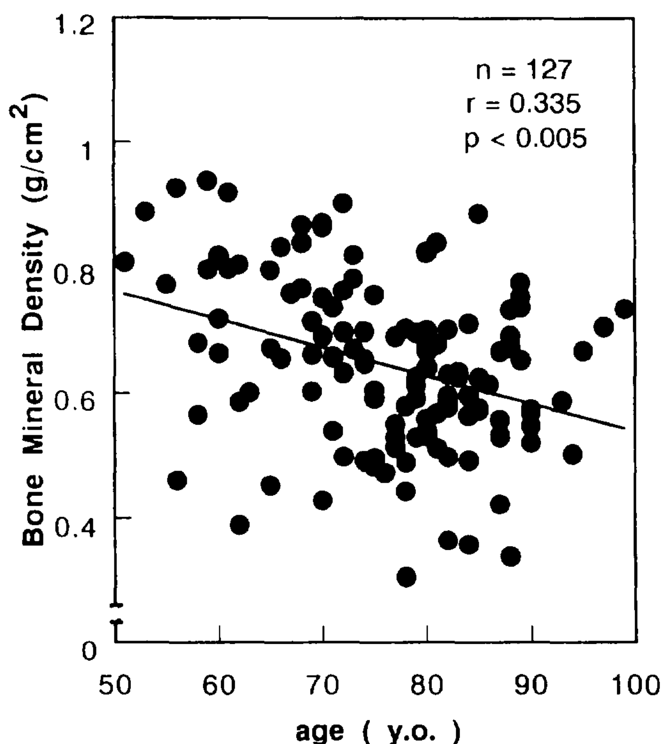

Fig. 1. Age-associated change in bone mineral density of postmenopausal women (51–99 years old). Bone mineral density (g/cm²) of lumbar vertebrae was determined by DEXA as described in Experimental and plotted against age of 127 postmenopaused women.

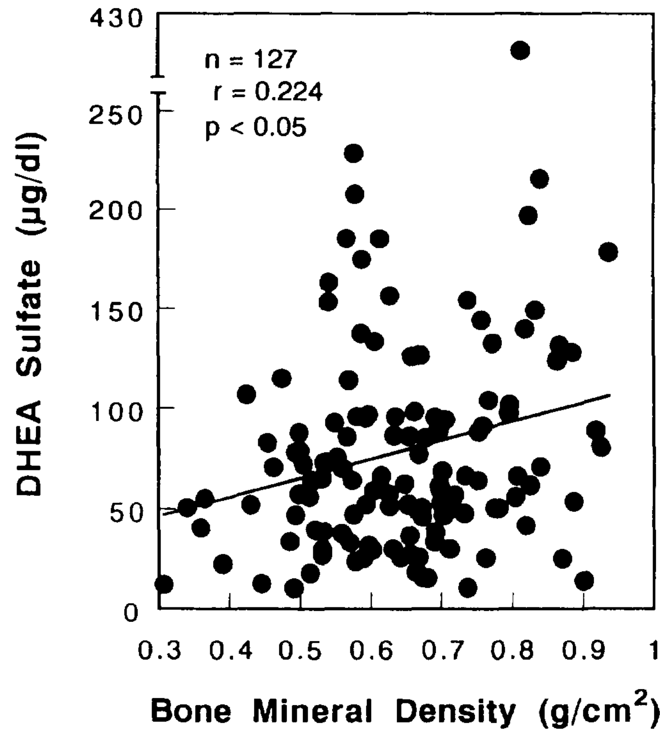

Fig. 2. Correlation between serum DHEA-S and bone mineral density in postmenopausal women (51–99 years old). Bone mineral density (g/cm^2) of lumbar vertebrae of 127 postmenopausal women determined by DEXA was plotted against serum DHEA-S.

Michaelis constant (K_m) of 4.7 ± 0.8 nM (mean $\pm$ SD, $n = 3$) was obtained by Lineweaver–Burk plots and the V_{max} was 0.83 ± 0.79 fmol/mg protein/h [7]. Pretreatment with 10^{-7} M dexamethasone for 72 h increased the aromatase activity. Dexamethasone increased the V_{max} but did not affect the K_m value compared with those in untreated HOS cells (Fig. 5). Aromatase activity in primary cultured HO also showed typical Michaelis–Menten type kinetics, and the apparent K_m and the V_{max} values were 4.6 ± 2.9 nM and 279 ± 299 fmol/mg protein/h (mean $\pm$ SD, $n = 19$), respectively (Fig. 6). The K_m and V_{max} obtained in 19

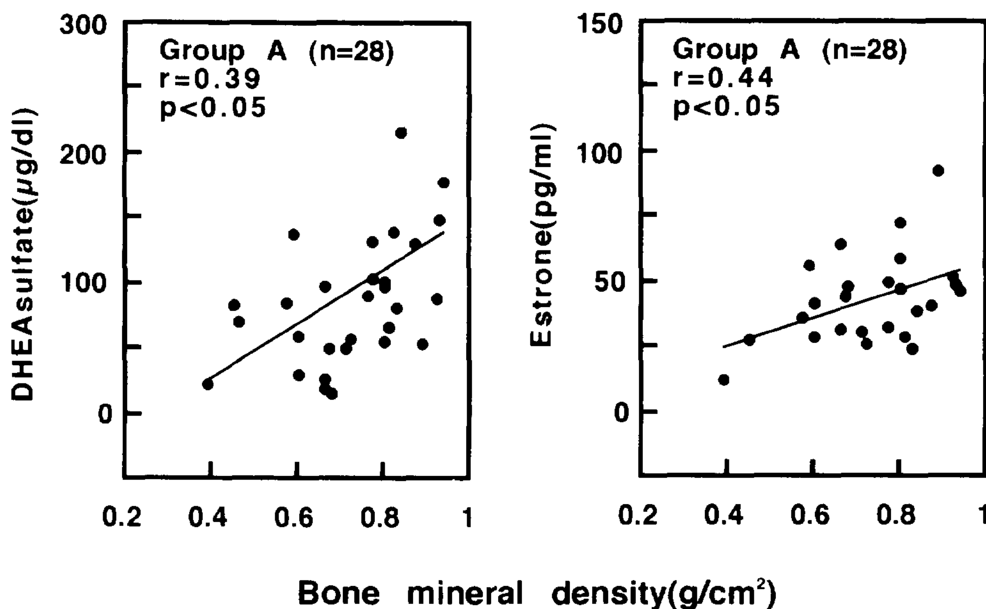

Fig. 3. Correlation between bone mineral density and serum DHEA-S in group A of postmenopausal women. Bone mineral density (g/cm^2) of lumbar vertebrae of postmenopausal women at 51–69 years old (group A) determined by DEXA was plotted against serum DHEA-S or serum E_1.

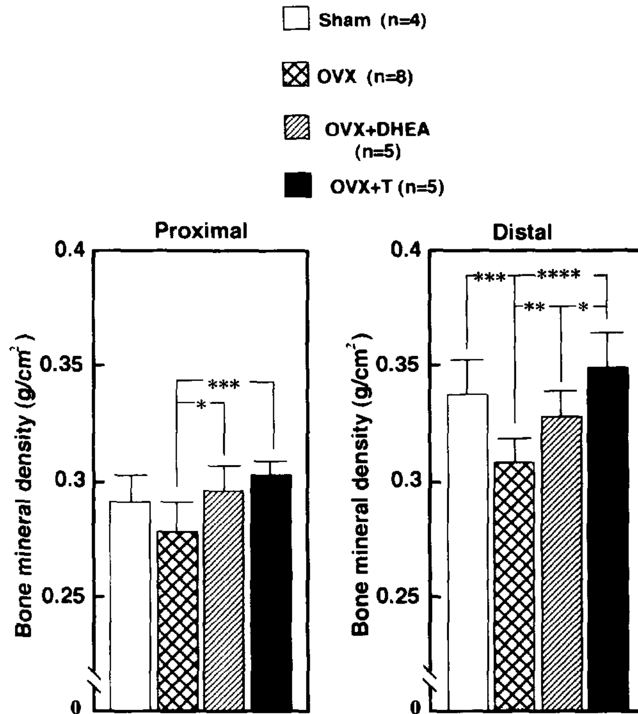

Fig. 4. Effect of DHEA and T on bone mineral density in OVX rat. Bone mineral density (g/cm^2) of proximal and distal portion of femur of OVX rat was determined 12 weeks after 0.3% DHEA- or 0.3% T-treatment.

subjects showed no significant correlation with age, sex or menopause. The K_m values of HOS and HO cells are in the same range of affinity, and much higher than those in human adipocyte and fibroblast (19–51 nM). Aromatase activity in HO cells was about 100 times higher than that in HOS cells. HO cells are thus superior to study the regulation of the $P450_{AROM}$ gene expression. Thin layer chromatographic analyses further revealed that [^{3}H]DHEA was converted to [^{3}H]androstenedione and [^{3}H]androstenedione to [^{3}H]estrone in cultured bone cells. Cleland *et al.* [18] described the increased aromatase activity of adipose

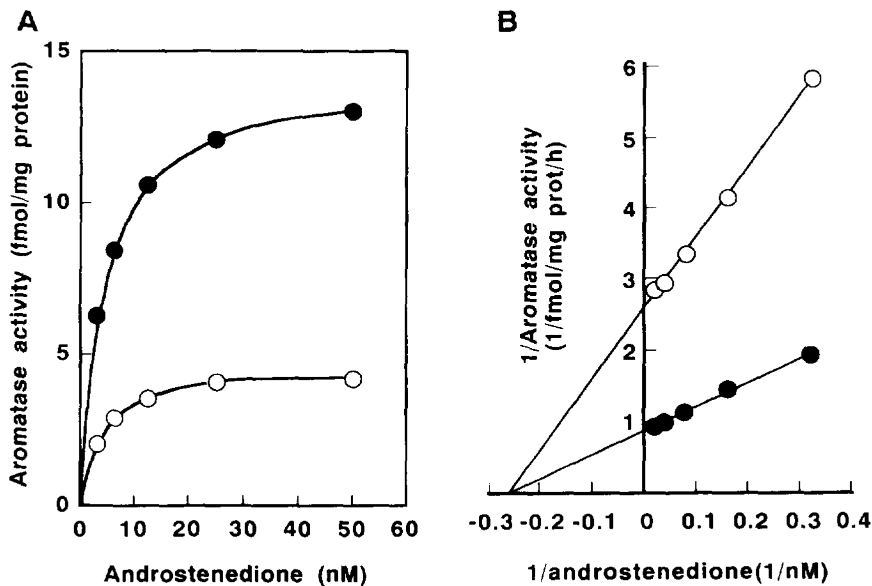

Fig. 5. Aromatase activity in human osteoblast-like osteosarcoma (HOS) cells. (A) Effect of substrate concentration on aromatase activity in HOS cells. HOS cells were incubated with (●) or without (○) 10^{-7} M dexamethasone for 72 h and aromatase activity was assayed in the presence of 6.25 to 100 nM [1β-^{3}H]androstenedione as described in Experimental. (B) Lineweaver–Burk plot of initial velocity of [^{3}H]H_2O formed versus concentration of androstenedione.

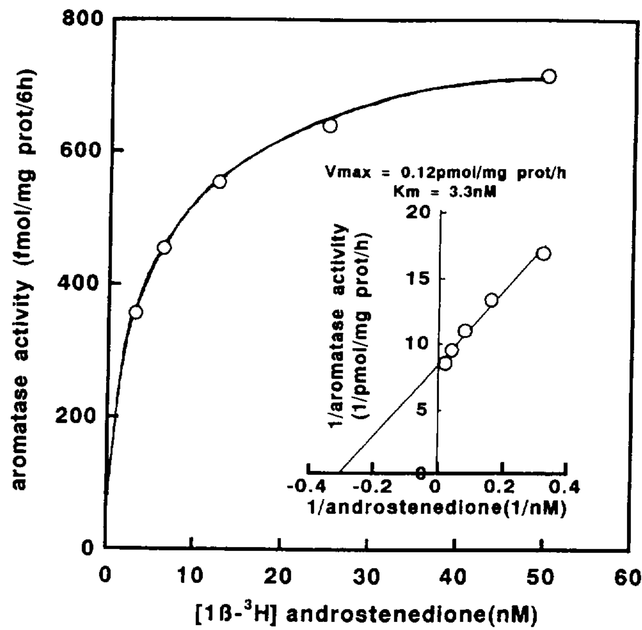

Fig. 6. Effect of androstenedione concentration on aromatase activity in human osteoblast. Aromatase activity was assayed in the presence of 6.25–100 nM [1β-^{3}H]androstenedione after 10^{-7} M dexamethasone-treatment.

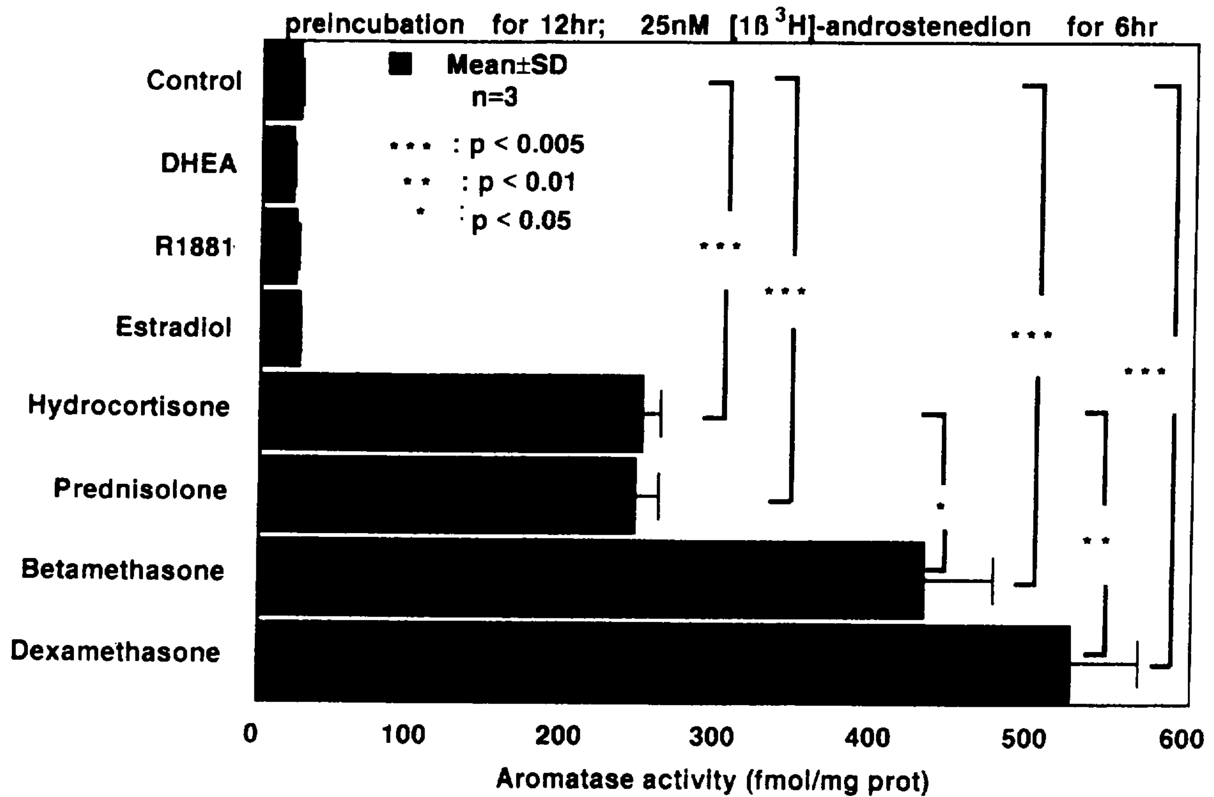

Fig. 7. Effects of various steroid hormones on aromatase activity in HO. Cells were preincubated with 10^{-7} M DHEA, R1881, estradiol, hydrocortisone, prednisolone, betamethasone or dexamethasone for 12 h and the aromatase activity was assayed as described in Experimental. Each value represents the mean $\pm$ SD of triplicate determinations.

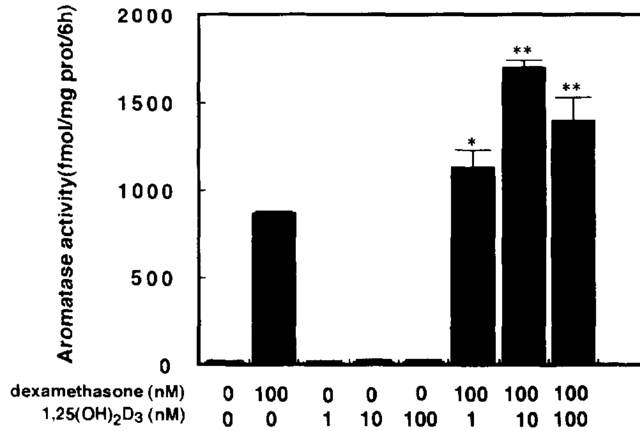

Fig. 8. Effect of 1,25VitD$_3$ on aromatase activity in HO. Cells were preincubated for 12 h with 100 nM dexamethasone in the presence and absence of increased concentrations of 1,25VitD$_3$. Each value represents the mean ± SD of triplicate determinations.

tissue stromal cells with advanced age, but we could not find any age- or sex-associated change of aromatase activity.

Effects of various hormones on aromatase activity in HO

The effects of various steroid hormones at 10^{-7} M on the aromatase activity are shown in Fig. 7. The aromatase activity was significantly increased in order of dexamethasone > betamethasone > predonisolone = hydrocortisone, whereas methyltrienolone (R1881), 17β-estradiol, and DHEA had no effect on aromatase activity. Preincubation with 1α,25(OH)$_2$ vitamin D$_3$ (1,25VitD$_3$) alone had no effect on aromatase activity. When the vitamin D$_3$ was added in the cultured medium with dexamethasone, the increase of the aromatase activity was 1.5–2.0 times higher than that observed with dexamethasone alone (Fig. 8). This synergistic effect is specific for 1,25VitD$_3$.

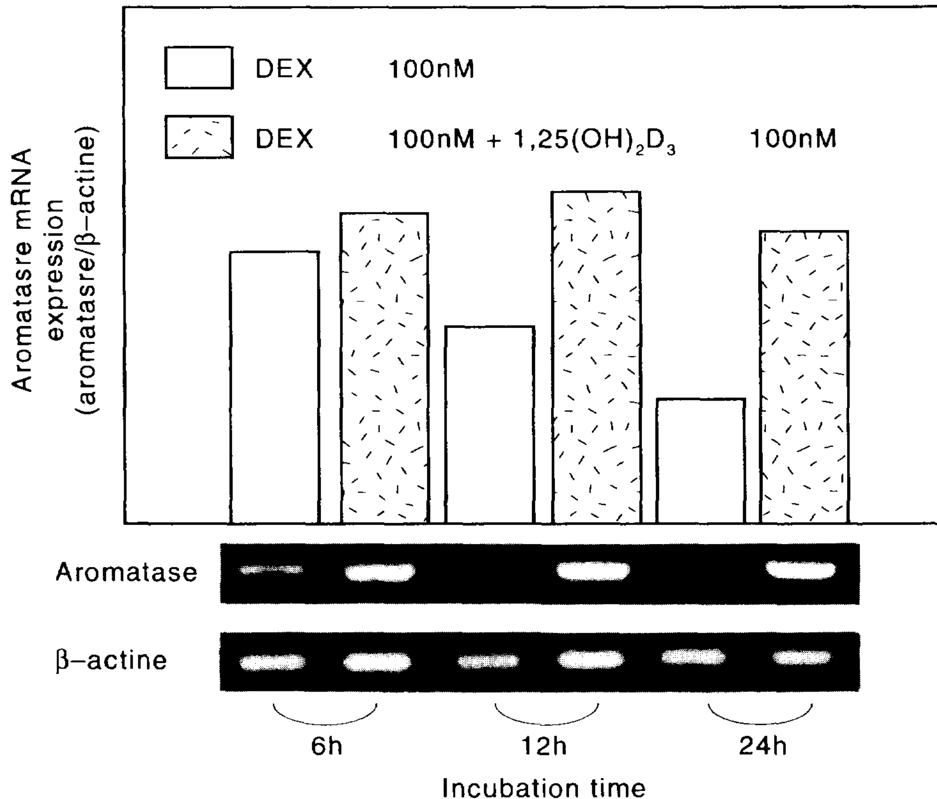

Fig. 9. Induction of $P450_{AROM}$ gene expression in HO by dexamethasone and 1,25VitD$_3$. Cells were incubated with 10^{-7} M dexamethasone in the presence and absence of 10^{-7} M 1,25VitD$_3$, and harvested at the time indicated. The mRNA levels were quantified as described in Experimental and expressed as relative intensity to that of β-actin.

Table 1. Tissue specific utilization of the promoter of $P450_{\mathrm{AROM}}$ gene

	Placenta Ex. I.1 (Ex.1a)	Fetal Liver Skin Fibroblast Ex. I.4 (Ex.1b)	Ovary Ex. I.3 (Ex.1c)	Prostate Ovary Ex. II (Ex.1d)
Fetal liver	+	+ + +	+	+
Ovary	−	−	+ + +	+ + +
Skin fibroblast	−	+ + +	+	+
Placenta	+ + + +	+	+	+
Prostate	−	−	−	+
Testis	−	−	−	+
Brain	−	+	−	−
Adipose tissue	−	+	+	−
Osteoblast	+	+ +	−	+

Induction of $P450_{AROM}$ gene expression in human osteoblast by dexamethasone and $1{,}25VitD_3$

The RT-PCR product for $P450_{\mathrm{AROM}}$ mRNA increased exponentially upto 35 cycles. Accordingly, with 30 cycles of amplification, the PCR product increased linearly as a function of the amount of RT product, indicating that PCR in the present conditions can be used in a semi-quantitative manner to evaluate the mRNA levels for $P450_{\mathrm{AROM}}$. The mRNA levels for vitamin D receptor and β-actin were also similarly quantified.

The level of $P450_{\mathrm{AROM}}$ mRNA in osteoblasts was negligible in the absence of dexamethasone, but increased with time of incubation with 10^{-7} M dexamethasone, reached the maximum at 6–12 h, and then decreased at 24 h (Fig. 9). The addition of $1{,}25\mathrm{VitD}_3$ during the incubation with dexamethasone further enhanced the $P450_{\mathrm{AROM}}$ mRNA level, moreover, the enhanced mRNA level was maintained up to 24 h of incubation. The enzyme activity of aromatase showed a similar time-course with a time lag of 6–12 h, probably due to protein synthesis.

When the synergistic activating effect of $1{,}25\mathrm{VitD}_3$ on aromatase activity was examined in each sample from 15 cases, this synergistic effect of vitamin D_3 varied from 0.94 to 2.40-fold activation. The magnitude of vitamin D_3-induced enhancement was not correlated with sex or age, but significantly correlated with the mRNA level for vitamin D receptor ($r = 0.52$, $P < 0.05$).

Promoter specificity for $P450_{AROM}$ gene expression in HO

The $P450_{\mathrm{AROM}}$ gene has been shown to have multiple promoters [7, 8], and their utilization was tissue-

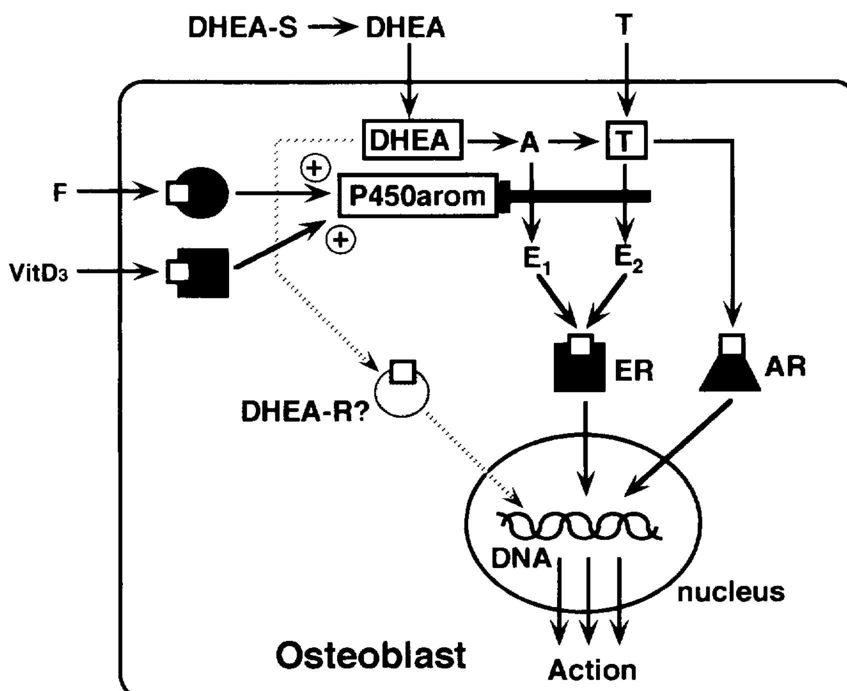

Fig. 10. Schematic representation of steroid hormone actions on osteoblasts.

specific, by which the gene expression would be regulated. When the promoter specificity was determined by RT-PCR using one common antisense primer and various sense primers corresponding to the initial exons specific for each of the alternatively used promoters, it was revealed that HOs activate predominantly exon I.4 and to a lesser extent exon I.1 and II. This pattern of expression has not been found in other reported tissues (Table 1) [7, 8], suggesting that HOs have a unique regulatory mechanism of $P450_{AROM}$ gene expression.

Possible actions of androgens on osteoblasts in post-menopausal women

Figure 10 summarizes schematically the osteoblastic aromatase characterized in the present study. DHEA, one of the adrenal androgens, is the major circulating androgen in postmenopausal women, which showed a positive correlation with bone mineral density in those women. There exist possible mechanisms for DHEA action on osteoblast. One is a direct action of DHEA, which remains to be clarified. Another more possible mechanism is the conversion of DHEA to estrogen by 3β-HSD and aromatase in peripheral tissues. A key enzyme for this pathway is $P450_{AROM}$, and osteoblast itself has a unique regulatory mechanism of this gene expression. Moreover, osteoblastic aromatase has a characteristically high affinity for androstenedione compared with the other tissue-specifically induced aromatase. Androgen receptor has been found in HO [19], therefore, circulating T or its reduced form, dihydrotestosterone, may also directly act on osteoblast. A circulating level of cortisol induces $P450_{AROM}$ transiently, however, 1,25VitD$_3$ can maintain its expression, dependent on a vitamin D receptor density of osteoblast. Interestingly, vitamin D receptor has recently been shown to be closely involved in the development of osteoporosis in postmenopausal women [20]. The mechanism of vitamin D$_3$-specific superinduction of $P450_{AROM}$ mRNA was not yet clarified in the present study. Berkovitz *et al.* [21] reported that cycloheximide enhances the dexamethasone-dependent induction level of $P450_{AROM}$ mRNA in human skin fibroblasts, and speculated the presence of negatively regulating transcription factor(s). Similar mechanisms might be involved in the superinduction by 1,25VitD$_3$ in osteoblast. The possibility of stabilization of $P450_{AROM}$ mRNA by 1,25VitD$_3$ may be also considered. There are multiple half-sequences corresponding to the vitamin D$_3$ responsive element in the 5′-upstream region of exon I.4 [7]. The possibility that 1,25VitD$_3$–vitamin D receptor complex binds to these sites and activates $P450_{AROM}$ gene expression should be considered, because it has been recently shown that multiple half-sites, not classical palindromic or direct repeat sequences, of steorid hormone responsive element are responsible for this type of mild enhancement of gene expression [22]. Further study is necessary for clarification of the mechanism of vitamin D$_3$-specific superinduction of osteoblastic aromatase.

REFERENCES

1. Richelson L. S., Wahner H. W., Melton L. J. III. and Riggs B. L.: Relative contributions of aging and estrogen deficiency to postmenopausal bone loss. *N. Engl. J. Med.* **311** (1984) 1273–1275.
2. Kiel D. P., Felson D. T., Anderson J. J., Wilson P. W. F. and Moskowitz M. A.: Hip fracture and the use of estrogens in postmenopausal women: the Framingham Study. *N. Engl. J. Med.* **317** (1987) 1169–1174.
3. Felson D. T., Zhang Y., Hannan M. T., Kiel D. P., Wilson P. W. and Anderson J. J.: The effect of postmenopausal estrogen therapy on bone density in elderly women. *N. Engl. J. Med.* **329** (1993) 1141–1146.
4. Nordin B. E. C., Robertson A., Seamark R. F., Bridges A., Philcox J. C., Need A. G., Horowitz M., Morris H. A. and Deam S.: The relation between calcium absorption, serum dehydroepiandrosterone, and vertebral mineral density in postmenopausal women. *J. Clin. Endocr. Metab.* **60** (1985) 651–657.
5. Davidson B. J., Riggs B. L., Wahner H. W. and Jndo H. L.: Endogenous cortisol and sex steroids in patients with osteoporotic fracture. *Obstet. Gynec.* **61** (1983) 275–278.
6. Means G. D., Mahendroo M. S., Corbin C. J., Mathis M., Powell F. E., Mendelson C. R. and Simpson E. R.: Structural analysis of the gene encoding human aromatase cytochrome *P*-450, the enzyme responsible for estrogen biosynthesis. *J. Biol. Chem.* **264** (1989) 19,385–19,391.
7. Mahendroo M. S., Mendelson C. R. and Simpson E. R.: Tissue-specific and hormonally controlled alternative promoters regulate aromatase *P*450 gene expression in human adipose tissue. *J. Biol. Chem.* **268** (1993) 19,463–19,470.
8. Harada N., Utsumi T. and Katagi Y.: Tissue specific expression of the human aromatase cytochrome *P*-450 gene by alternative use of multiple exons 1 and promoters and switching of tissue-specific exons 1 in carcinogenesis. *Proc. Natn. Acad. Sci.* **90** (1993) 11,312–11,316.
9. Auf'mkolk B., Hauschka P. V. and Schwartz E. R.: Characterization of human bone cell in culture. *Calcif. Tissue. Int.* **37** (1985) 228–235.
10. Nawata H., Ono K., Ohashi M., Kato K. and Ibayashi H.: RU486 inhibits induction of aromatase by dexamethasone via glucocorticoid receptor in cultured human skin fibroblasts. *J. Steroid Biochem.* **29** (1988) 63–68.
11. Nakao R., Haji M., Yanase T., Ogo A., Takayanagi R., Katsube T., Fukumaki Y. and Nawata H.: A single amino acid substitution ($Met^{786} \rightarrow$ Val) in the steroid binding domain of human androgen receptor leads to complete androgen insensitivity syndrome. *J. Clin. Endocr. Metab.* **74** (1992) 1152–1157.
12. Nishi Y., Haji M., Takayanagi R., Iguchi H., Shimazoe T., Hirata J. and Nawata H.: Establishment and characterization of PTHrP-producing human pancreatic cancer cell line. *Int. J. Oncol.* **5** (1994) 33–39.
13. Kinoshita T., Imamura J., Nagai H. and Shimotohno K.: Quantification of gene expression over a wide range by the polymerase chain reaction. *Analyt. Biochem.* **206** (1992) 231–235.
14. Baker A. R., McDonnell D. P., Hughes M., Crisp T. M., Manglsdorf D. J., Haussler M. R., Pike J. W., Shine J. and O'Malley B. W.: Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 3294–3298.
15. Nakajima-Iijima S., Hamada H., Reddy P. and Kakunaga T.: Molecular structure of human cytoplasmic β-actin gene: Interspecies homology of sequences in introns. *Proc. Natn. Acad. Sci. U.S.A.* **82** (1985) 6133–6137.
16. Davidson B. J., Ross R. K., Paganini-Hill A., Hammond G. D., Siiteri P. K. and Judo H. L.: Total and free estrogen and androgens in postmenopausal woman with hip fractures. *J. Clin. Endocr. Metab.* **54** (1982) 115–120.
17. Tanaka S., Haji M., Nishi Y., Yanase T., Takayanagi R. and Nawata H.: Aromatase activity in human osteoblast-like osteosarcoma cell. *Calcif. Tissue. Int.* **52** (1993) 107–109.
18. Cleland W. H., Mendelson C. R. and Simpson E. R.: Effects of

aging and obesity on aromatase activity of human adipose cells. *J. Clin. Endocr. Metab.* **60** (1985) 174–177.

19. Colvard D. S., Eriksen E. F., Keeting P. E., Wilson E. M., Lubahn D. B., French F. S., Riggs B. L. and Spelsberg T. C.: Identification of androgen receptors in normal human osteoblast-like cells. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 854–857.
20. Morrison N. A., Qi J. C., Tokita A., Kelly P. J., Crofts L., Nguyen T. V., Sambrook P. N. and Eisman J. A.: Prediction of bone density from vitamin D receptor alleles. *Nature* **367** (1994) 284–287.
21. Berkovitz G. D., Chen S., Migeon C. J. and Levine M. A.: Induction and superinduction of mRNA specific for aromatase cytochrome *P*-450 in cultured human skin fibroblasts. *J. Clin. Endocr. Metab.* **74** (1992) 629–634.
22. Force W. R., Tillman J. B., Sprung C. N., and Spindler S. R.: Homodimer and heterodimer DNA binding and transcriptional responsiveness to triiodothyronine (T_3) and 9-*cis*-retinoic acid are determined by the number and order of high affinity half-sites in a T3 response element. *J. Biol. Chem.* **269** (1994) 8863–8871.