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Effects of Gonadal and Adrenal Androgens in a Novel Androgen-Responsive Human Osteoblastic Cell Line

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Abstract While androgens have important skeletal effects, the mechanism(s) of androgen action on bone remain unclear. Current osteoblast models to study androgen effects have several limitations, including the presence of heterogeneous cell populations. In this study, we examined the effects of androgens on the proliferation and differentiation of a novel human fetal osteoblastic cell line (hFOB/AR-6) that expresses a mature osteoblast phenotype and a physiological number (~4,000/nucleus) of androgen receptors (AR). Treatment with 5α-dihydrotestosterone (5α-DHT) inhibited the proliferation of hFOB/AR-6 cells in a dose-dependent fashion, while it had no effect on the proliferation of hFOB cells, which express low levels of AR (<200/nucleus). In hFOB/AR-6 cells, co-treatment with the specific AR antagonist, hydroxyflutamide abolished 5α-DHT-induced growth inhibition. Steady-state levels of transforming growth factor-β_1 (TGF-β_1) and TGF-β-induced early gene (TIEG) mRNA decreased after treatment of hFOB/AR-6 cells with 5α-DHT, suggesting a role for the TGF-β_1-TIEG pathway in mediating 5α-DHT-induced growth inhibition of hFOB/AR-6 cells. In support of this, co-treatment of hFOB/AR-6 cells with TGF-β_1 (40 pg/ml) reversed the 5α-DHT-induced growth inhibition, whereas TGF-β_1 alone at this dose had no effect on hFOB/AR-6 cell proliferation. Furthermore, treatment of hFOB/AR-6 cells with 5α-DHT and testosterone (10^{-8} M) inhibited basal and 1,25-$(OH)_2D_3$-induced alkaline phosphatase (ALP) activity and type I collagen synthesis without affecting osteocalcin production. Thus, in this fetal osteoblast cell line expressing a physiological number of AR, androgens decrease proliferation and the expression of markers associated with osteoblast differentiation. These studies suggest that the potential anabolic effect of androgens on bone may not be mediated at the level of the mature osteoblast. J. Cell. Biochem. 71:96–108, 1998.

Key words: androgens; androgen receptor; antiandrogens; differentiation; osteoblasts; proliferation

Androgens are of profound importance for the development and maintenance of the skeleton in both women and men [Bagatell and Bremner, 1996; Orwoll and Klein, 1995]. Androgen deficiency is a recognized risk factor in the development of osteoporosis in men [Finkelstein et al., 1992] and may also contribute to age-related bone loss [Ongphiphadhanakul et al., 1995; Wishart et al., 1995]. While androgens are administered to reverse the consequences of androgen deficiency on the skeleton [Behre et al., 1997], the cellular and molecular mechanisms of androgen action on bone cells remain unclear [Vanderschueren and Bouillon, 1995]. After cloning and characterization of the human androgen receptor (AR) gene [Chang et al., 1988; Lubahn et al., 1988], the detection of functional AR in human osteoblasts has implicated these cells as direct targets for androgens [Colvard et al., 1989]. Since then, other investigators have confirmed the presence of AR in various bone cells, including osteoblasts [Liesegang et al., 1994; Nakano et al., 1994; Orwoll et al., 1991; Takeuchi et al., 1992; Zhuang et al., 1992], osteoclasts [Mizuno et al., 1994], and marrow-derived stromal cells [Bellido et al., 1995].

While the effects of androgens on osteoblast proliferation and differentiation have been subsequently investigated, the results of these studies have been inconsistent [Vanderschueren and Bouillon, 1995]. Androgens have been shown to have mitogenic effects on normal and transformed osteoblast-like cells in most [Gray et al.,

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1992; Kasperk et al., 1989; Masuyama et al., 1992; Nakano et al., 1994; Somjen et al., 1989; Weisman et al., 1993], but not all systems [Benz et al., 1991]. Moreover, the effects of testosterone and the adrenal androgen dehydroepiandrosterone (DHEA) may or may not be identical to the effects of 5α-DHT, depending on the conversion of these androgens to 5α-DHT or to estrogens [Bodine et al., 1995; Vanderschueren and Bouillon, 1995]. In addition, other factors, such as 1,25-$(OH)_2D_3$ may modulate androgen effects on the proliferation of osteoblast cells [Gray et al., 1992; Somjen et al., 1989].

With respect to osteoblast differentiation, androgens have been shown to increase alkaline phosphatase (ALP) activity in osteoblast-like cells and the percentage of ALP-positive cells, suggesting a shift toward a more differentiated phenotype [Kasperk et al., 1989, 1996, 1997b]. Androgen effects on type I collagen synthesis have been variable, with some studies showing a stimulatory effect [Benz et al., 1991; Gray et al., 1992; Kasperk et al., 1996], and others finding no effect [Canalis and Raisz, 1978; Nakano et al., 1994; Pilbeam and Raisz, 1990]. Several studies have previously suggested a potential role for transforming growth factor-β (TGF-β) as a local mediator of androgen action in adult human osteoblasts [Bodine et al., 1995; Kasperk et al., 1989].

At least part of the variability in androgen effects on bone cells may be due to the different model systems used [Vanderschueren and Bouillon, 1995], including differences in species (rodent vs human), developmental stage (fetal, neonatal, adult) or age (young vs elderly adults), as well as the site (calvaria, spine, femoral head) [Kasperk et al., 1997a], culture techniques (primary vs permanent cell line), and the phenotype and stage of differentiation of the osteoblast-like cells (normal vs. osteosarcoma-derived osteoblasts) [Marie, 1994]. In addition, commonly used osteosarcoma cell lines have recently been demonstrated to lack androgen transcriptional activity despite the presence of AR [Czerwiec et al., 1997]. Moreover, the AR content in these various osteoblast models is subject to large intra- and intersystem variation, which may limit direct comparison between different studies [Vanderschueren and Bouillon, 1995].

To circumvent some of these inherent limitations, we recently generated conditionally immortalized human fetal osteoblastic cell lines stably transfected with the human wild-type AR cDNA (hFOB/AR) [Hofbauer et al., 1997]. The parent cell line (hFOB) expresses a differentiated osteoblast phenotype, including markers of osteoblast differentiation (ALP, type I collagen, and osteocalcin), and these cells form mineralized nudules in vitro under appropriate conditions [Harris et al., 1995a]. However, hFOB cells lack detectable levels of functional AR. By contrast, hFOB/AR-6 cells, which are transfected with the human AR, constitutively express 3,987 ± 823 (mean ± SEM) functional AR per nucleus [Hofbauer et al., 1997], which is in the physiological range of AR present in human reproductive and nonreproductive tissues, including bone. Treatment of hFOB/AR-6 cells with the nonaromatizable gonadal androgen 5α-DHT increased the expression of a transiently transfected androgen-responsive element-chloramphenicol transferase (ARE-CAT) reporter construct in a dose- and time-dependent fashion, and this was inhibited by the specific AR antagonist, hydroxyflutamide [Hofbauer et al., 1997]. These cells thus provide a useful model of a mature osteoblast phenotype that is androgen responsive. In this study, we examine the effects of gonadal and adrenal androgens on the proliferation and differentiation of this androgen-responsive osteoblast cell line, as well as the potential role of TGF-β in mediating the effects of androgens in this system. Moreover, since at least part of the actions of TGF-β in bone cells may be mediated by a recently identified TGF-β-induced early gene (TIEG) [Subramaniam et al., 1995], we also assessed TIEG expression in response to androgens in these cells.

MATERIALS AND METHODS

Materials

Culture flasks and dishes were purchased from Corning (Corning, NY), cell culture media and supplements were from Sigma Chemical Co. (St. Louis, MO). Unless otherwise stated, all other chemical reagents were of molecular biology grade and were obtained from Sigma. The random primer labeling kit (Decaprime II) was from Ambion (Austin, TX) and the radiolabeled nucleotides [α-^{32}P]-dATP, [α-^{32}P]-dCTP, and [^{3}H]thymidine were from DuPont-NEN (Boston, MA). The Bradford protein reagent was from BioRad (Hercules, CA) and the 1,25-$(OH)_2D_3$ from Biomol (Plymouth Meeting, PA). The human transforming growth factor-β_1

(TGF-β_1) DNA probe was a gift from Dr. S.A. Harris (Rhone-Poulenc Rorer, Collegeville, PA), and the pRSVβ-GAL plasmid containing the β-galactosidase gene (β-GAL) and the human TIEG cDNA probe were a gift from Dr. Thomas C. Spelsberg (Mayo Clinic, Rochester, MN). Recombinant human (rh)TGF-β_1 was from R&D Systems (Minneapolis, MN). Hydroxyflutamide (OHF) was kindly provided by Dr. Rudolph Neri (Schering-Plough, Klenisworth, NJ).

Cell Cultures

The hFOB/AR cell lines were established by stably transfecting the hFOB cells with the AR expression vector pAR-HYG encoding the wild-type human AR cDNA [Hofbauer et al., 1997]. This vector contains a CMV promotor, the SV40 polyadenylation signal, and the hygromycin B resistance gene controlled by the thymidine kinase promotor. The hFOB/AR cell lines were maintained in phenol-free Dulbecco's Modified Eagle Medium/Ham's F12 medium (DMEM/HF12) containing 10% (v/v) charcoal-stripped FBS (csFBS) alternately supplemented with either geneticin (300 µg/ml) or hygromycin B (100 µg/ml) to select for hygromycin B resistance conferred by the pAR-HYG vector. Of the 16 hFOB/AR subclones obtained, three (hFOB/AR-2, -6, and -16) have been found to have similar characteristics [Hofbauer et al., 1997]. The temperature-sensitive mutant SV40 large T antigen is most active at the permissive temperature (33.5°C), which was the temperature used to maintain the cells. The parental hFOB cells were maintained in DMEM/HF12 containing 10% (v/v) csFBS and geneticin (300 µg/ml). Both the hFOB/AR-6 and the hFOB cells differentiate and display a mature, stable osteoblastic phenotype when cultured at 39.5°C [Hofbauer et al., 1997].

Primary human osteoblast (hOB) cultures were obtained using trabecular bone explants from 6 men (mean age: 73 ± 3 years) without evidence of metabolic bone disease undergoing corrective orthopedic procedures following approval by the Mayo Institutional Review Board. Bone fragments were digested with 200 U/ml of type II collagenase (Worthington, Freehold, NJ) for 2 h at 37°C followed by long-term culture over 4–6 weeks in α-modified essential medium (α-MEM) supplemented with 10% (v/v) csFBS and 1% (v/v) penicillin-streptomycin in the absence of calcium at 37°C as described previously [Robey and Termine, 1985]. To minimize heterogeneity between the different donors, individual hOB cells were pooled following trypsinization, expanded and used for experiments between passages 4 and 6.

Cell Proliferation

Cellular DNA synthesis was assessed using a [^{3}H]thymidine incorporation assay [Benz et al., 1977]. The cells were plated into 24-well plates at a density of 2×10^4 cells per well in DMEM/HF12 (1:1) supplemented with 10% (v/v) of csFBS and incubated for 24 h. For synchronization, the cells were washed twice with phosphate-buffered saline (PBS) and cultured in serum-free DMEM/HF12 (1:1) supplemented with 0.25 % (w/v) bovine serum albumin (BSA) for 48 h at 33.5°C. In some experiments (as indicated), cells were synchronized using the aphidicolin method [Fedarko et al., 1990]. Briefly, the cells were grown in DMEM/HF12 supplemented with 1% (v/v) csFBS and 2 mM thymidine for 24–33 h. Cells were rinsed three times with PBS and cultured in DMEM/HF12 supplemented with 1% (v/v) csFBS, 24 µM deoxycytidine and 24 µM thymidine for 24 h and 5 µg/ml aphidicolin for the last 12 h before treatment. The cells were washed twice with PBS and cultured in DMEM/HF12 containing 1% (v/v) csFBS and either steroid or vehicle (ethanol) at 33.5°C for the time indicated. At 24 h before the assay, [^{3}H]thymidine (0.5 µCi/well) was added. The cells were rinsed once with 100% (w/v) trichloracetic acid (TCA), then with 5% (w/v) TCA and resuspended in 0.1 M NaOH; 10 ml scintillation fluid was added for quantitation of [^{3}H] activity in precipitable cellular DNA.

Northern Blot Analysis

Total RNA was isolated using a commercially available RNeasy kit (Qiagen, Hilden, Germany). A total of 10 µg of total RNA was fractionated under denaturing conditions on a 1% (w/v) agarose gel containing 2.2 M formaldehyde [Lehrach et al., 1977]. RNA was transferred to a positively charged nylon membrane (Hybond N^+; Amersham, Arlington Heights, IL) by capillary blotting [Thomas, 1980], using 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). Methylene blue staining (15 min in 5% acetic acid, followed by 5 min in 0.04% (w/v) methylene blue and 0.8 M sodium acetate, pH 4.0) [Herrin and Schmidt, 1988] of the membrane, ensured equal loading and transfer. Hybridization was carried out overnight at 42°C in 5× SSC, 10%

(w/v) dextrane sulfate, 50% (v/v) formamide, 1% (w/v) N-lauroyl-sarcosine, and 0.25 mg/ml herring sperm DNA. The cDNA inserts were radiolabeled using 4 µl [α-^{32}P]dATP and 4 µl [α-^{32}P]dCTP (spec acti $>10^9$ cpm/µg DNA) with a random primer DNA labeling kit [Feinberg and Vogelstein, 1983]. After stringent washing for 3 × 5 min at room temperature [2× SSC, 0.5% (w/v) N-lauroyl-sarcosine] and for 3 × 20 min at 42°C [0.1× SSC, 0.1% (w/v) N-lauroyl-sarcosine], autoradiography on an intensifying screen at −80°C was performed. For quantitation, laser scanning densitometry (Pharmacia LKB Biotechnology, Piscataway, NJ) of the autoradiograms and the respective methylene blue-stained 28S rRNA bands on the membranes was performed. Representative blots of a total of three independent experiments were shown.

ALP, Osteocalcin, and Type I Collagen Assays

The hFOB/AR-6 and hFOB cells were plated in 24-well plates at a density of 40,000 cells per well in DMEM/HF12 supplemented with 10% csFBS and incubated at 33.5°C for 2 days. Then, the cells were washed twice with PBS, and the medium was changed to DMEM/HF12 supplemented with 1% (v/v) csFBS containing 1,25-$(OH)_2D_3$ (10^{-8} M), ascorbic acid (50 µg/ml), and menadione sodium bisulfite (vitamin K_3; 2 ng/ml). At that time, cells were switched to 39.5°C, the nonpermissive temperature. The cells were then treated with either vehicle or steroid (as indicated). The conditioned cell medium was collected, centrifuged to remove cell debris, and stored at −80°C until used for the osteocalcin and C-terminal propeptide of type I collagen (PICP) assays. ALP activity and total protein content were measured in osteoblast extracts. ALP activity was assessed as a function of p-nitrophenol phosphate conversion into p-nitrophenol, according to the method of Boyan et al. [1989]. Briefly, cells were rinsed twice in PBS, lysed in 250 µl of alkaline buffer (0.75 M 2-amino-2-methyl-1-propanol, pH 10.3) containing p-nitrophenol phosphate (2 mg/ml), and incubated for 2 h at 37°C. The reaction was terminated by adding 300 µl of NaOH; absorbance was measured at 410 nm. Total protein content per well was determined in 30 µl of cell lysate, using the Bradford protein assay. Coefficients of variance derived from intra-assay and inter-assay precision analyses were <7% for the ALP activity assay and <9% for the Bradford protein assay, respectively.

OC production was assessed using a commercially available competitive immunoassay with a monoclonal mouse anti-osteocalcin antibody (Novocalcin, Metra Biosystems, Mountain View, CA). Assays were performed at room temperature using 25 µl of undiluted conditioned medium, followed by measurement of the absorbance at 405 nm, according to the manufacturer's instructions. Coefficient of variance for OC measurements was <5%. The C-terminal PICP was assessed with a commercial sandwich immunoassay using a monoclonal mouse anti-PICP antibody and a rabbit anti-PICP antiserum (Prolagen-C, Metra Biosystems, Mountain View, CA). A total of 100 µl of diluted (1:50) conditioned medium was incubated at room temperature, following the manufacturer's instructions, and absorbance was measured at 405 nm. Coefficient of variance for PICP measurements was <3%. ALP activity of cell lysates as well as OC and PICP concentrations in conditioned medium were corrected for total protein content in cell extracts.

Statistical Analysis

All numerical values represent the mean ± SEM. Treatment effects were compared to the vehicle control using a two-sided Student's *t*-test. To evaluate differences between multiple treatment groups, one-way analysis of variance (ANOVA), followed by Fisher's least significant difference analysis was used. Statistical analyses were performed using Statview II software (Abacus Concepts, Cupertino, CA). A *P*-value of <0.05 was considered significant.

RESULTS

Effects of Androgens on the Proliferation of Human Osteoblastic Cells

To demonstrate that proliferation of hFOB/AR-6 cells at the permissive temperature (33.5°C) is regulated, the cells were grown in 0.1%, 1%, and 10% csFCS. The proliferation rate with 1% and 10% csFCS was 121 ± 9% and 179 ± 12%, respectively, of the rate with 0.1% csFCS ($P < 0.001$ by ANOVA). By contrast, treatment with 5α-DHT inhibited the proliferation of hFOB/AR-6 cells (grown at 33.5°C) by 25–35% in a dose-dependent fashion ($P < 0.001$) (Fig. 1). Growth inhibition was also observed after 2 days of treatment (data not shown). This

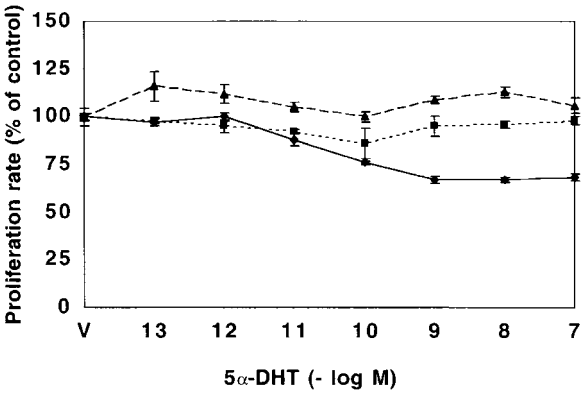


Fig. 1. Effect of 5α-DHT on the proliferation of hFOBAR-6, hFOB, and hOB cells as assessed by [^{3}H]thymidine incorporation. Synchronized (by serum depletion) cells (2×10^4 cells) were cultured in 1% (v/v) charcoal-stripped fetal bovine serum (csFBS) and either 5α-DHT (as indicated) or vehicle (ethanol) (hFOB/AR-6, diamonds, solid line; hFOB, rectangles, dotted line; hOB, triangles, dashed line) for 6 days in the presence of [^{3}H]thymidine (0.5 μCi/well) for the last 24 h. [^{3}H]thymidine incorporation was determined in a scintillation counter. The values indicate means ± SEM from a total of 7 (hFOB/AR-6), 4 (hFOB), and 3 (hOB) experiments. *P*-values by ANOVA for the effects of 5α-DHT: hFOB/AR-6, $P < 0.001$; hFOB, $P = 0.22$; hOB, $P = 0.38$.

effect was observed in hFOB/AR-6 cells synchronized by serum depletion (as indicated) or after aphidicolin treatment (not shown). Thymidine incorporation at 39.5°C was 5–10% of that at 33.5°C, and not altered by 5α-DHT in hFOB/AR-6 cells. Treatment with 5α-DHT had no significant effect on the proliferation rate of hFOB or hOB cells, which express low (<200) or undetectable levels of AR (Fig. 1). Moreover, 5α-DHT also dose-dependently inhibited the proliferation of the cell line hFOB/AR-2, which, similar to hFOB/AR-6 cells, has approximately 4,000 AR per nucleus (data not shown) [Hofbauer et al., 1997]. To test the effects of other androgens on hFOB/AR-6 and hFOB cells, the cells were treated with testosterone or the adrenal androgen DHEA. Neither had any significant effects on thymidine incorporation in either hFOB/AR-6, hFOB, or hOB cells (Table I).

To test the specificity of the effects of 5α-DHT on hFOB/AR-6 proliferation, the cells were co-treated with 5α-DHT and varying doses of the androgen antagonist, OHF (10^{-9}–10^{-7} M) (Fig. 2). As shown, OHF completely prevented the 5α-DHT-induced growth inhibition of hFOB/AR-6 cells, while OHF, when administered alone (10^{-13}–10^{-7} M), resulted only in a slight (10%) increase of thymidine incorporation ($P = 0.08$ by ANOVA) (data not shown).

TABLE I. Effect of Testosterone and DHEA on the Proliferation of hFOBAR-6, hFOB, and FOB Cells as Assessed by [^{3}H]Thymidine Incorporation*

	hFOB/AR-6	hFOB	hOB
Testosterone			
Vehicle	100 ± 3.1	100 ± 11	100 ± 6.6
10^{-13} M	110 ± 5.7	99 ± 9	110 ± 4.2
10^{-12} M	97 ± 4.3	105 ± 13	115 ± 5.3
10^{-11} M	108 ± 8.2	90 ± 5.7	95 ± 6.9
10^{-10} M	93 ± 1.1	109 ± 8.4	102 ± 4.8
10^{-9} M	117 ± 11	102 ± 12	103 ± 5.1
10^{-8} M	92 ± 9.8	94 ± 7	107 ± 12
10^{-7} M	107 ± 6.4	104 ± 13	97 ± 5.3
DHEA			
Vehicle	100 ± 4.9	100 ± 11	100 ± 4.2
10^{-13} M	91 ± 2.9	112 ± 12	101 ± 7.3
10^{-12} M	91 ± 1.7	99 ± 11	106 ± 7.6
10^{-11} M	95 ± 4.3	97 ± 15	113 ± 6.3
10^{-10} M	99 ± 1.9	103 ± 7.1	93 ± 7.4
10^{-9} M	98 ± 4.3	104 ± 6.3	108 ± 10
10^{-8} M	95 ± 1.8	112 ± 5.1	104 ± 0.4
10^{-7} M	101 ± 3.4	116 ± 7.1	97 ± 7.5

*Synchronized (by serum depletion) hFOB/AR-6, hFOB, and hOB cells (2×10^4 cells) were grown in 1% (v/v) csFBS in the presence of steroid (as indicated) or vehicle (ethanol) for 6 days with [^{3}H]thymidine (0.5 μCi/well) added for the last 24 h. Values represent means ±SEM of the [^{3}H]thymidine incorporation as measured by scintillation counting (n = 6), expressed as a percentage of vehicle alone.

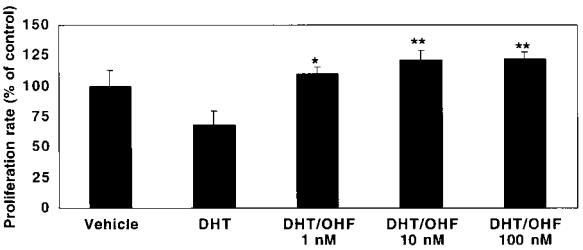
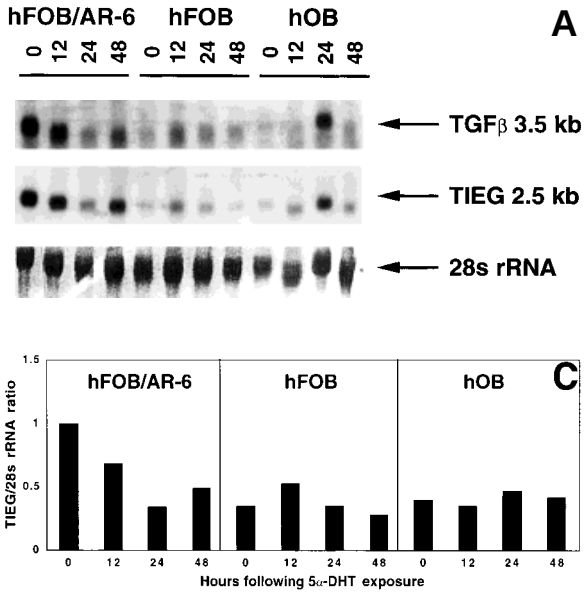


Fig. 2. Antagonism of 5α-DHT-induced growth inhibition by co-treatment with OHF in hFOB/AR-6 cells as assessed by [^{3}H]thymidine incorporation. Synchronized hFOB/AR-6 cells (2×10^4 cells) were treated with either vehicle (ethanol), 5α-DHT (10^{-10} M), or 5α-DHT (10^{-10} M) and varying concentrations of OHF (10^{-9}–10^{-7} M); n = 6, *$P < 0.01$; **$P < 0.005$ versus 5α-DHT alone (vehicle vs 5α-DHT: $P < 0.05$).

Role of TGF-β in Mediating Androgen Effects in hFOB/AR-6 Cells

As noted earlier, previous studies [Bodine et al., 1995; Kasperk et al., 1989] with adult human osteoblast-like cells have suggested that TGF-β may mediate some of the effects of gonadal [Bodine et al., 1995; Kasperk et al., 1989] and adrenal [Bodine et al., 1995] androgens in



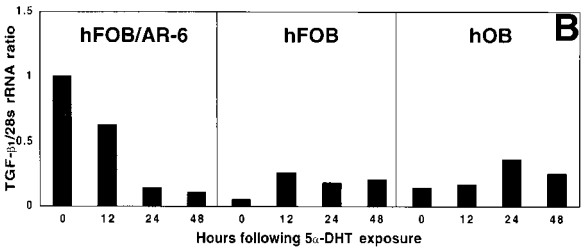


Fig. 3. Northern blot analysis of TGF-β_1 and TIEG mRNA expression in hFOB/AR-6 (~ 4,000 AR per nucleus), hFOB and hOB cells (both <200 AR/nucleus) after treatment with 5α-DHT. After incubation in serum-free medium in the presence of 5α-DHT (10^{-8} M) for the time indicated, total RNA was isolated and 10 µg of total RNA was subjected to gel electrophoresis under denaturing conditions. Membranes were hybridized to a radiolabeled cDNA for TGF-β_1 and TIEG and exposed onto autoradiography films. **A:** Northern blot analysis for TGF-β_1 mRNA (2.5 kb), TIEG mRNA (3.5 kb) and 28S rRNA expression. **B,C:** Gene/28S rRNA ratios, as determined by laser scanning densitometry of the Northern blot shown in (A) (vehicle-treated hFOB/AR-6 normalized to 100%).

that system. Thus, we analyzed mRNA expression for TGF-β_1 and TIEG, a transcription factor that mediates some effects of TGF-β_1 [Subramaniam et al., 1995], in response to 5α-DHT in hFOB/AR-6, hFOB and primary human osteoblastic (hOB) cells. 5α-DHT led to a decrease of TGF-β_1 steady-state mRNA levels (2.5 kb) in hFOB/AR-6 cells at 33.5°C, but a variable and slight increase or no consistent change in hFOB and hOB cells, both of which have <200 AR/nucleus (Fig. 3A,B). TIEG mRNA expression (3.5 kb) was also decreased by 5α-DHT in hFOB/AR-6 cells but was not altered in hFOB and hOB cells (Fig. 3A,C). Similar results were also observed in hFOB/AR-6 cells grown at 39.5°C (data not shown). TGF-β_1 and TIEG mRNA steady state levels did not change over time in the absence of 5α-DHT. Treatment of the hFOB/AR-2 cell line with 5α-DHT also resulted in a similar decrease in both TGF-β_1 and TIEG mRNA expression (data not shown).

Thus, we further hypothesized that the growth-inhibiting effect of 5α-DHT in hFOB/AR-6 cells may be due to a decrease in de novo TGF-β_1 production, as reflected by a decrease of the mRNA expression of TGF-β_1 and its mediator, TIEG, after treatment with 5α-DHT. To test this hypothesis, we treated hFOB/AR-6 cells with 5α-DHT (10^{-8} M) and varying doses of rhTGF-β_1. Recombinant hTGF-β_1 dose-dependently reversed the growth inhibition with a minimum effective dose of 40 pg/ml ($P < 0.005$; Fig. 4A), whereas rhTGF-β_1 alone at this dose had no significant effect of hFOB/AR-6 cell proliferation (Fig. 4B). Co-treatment with 5α-DHT and rhTGF-β_1 or treatment with rhTGF-β_1 alone (at 40 pg/ml) had no significant effect on the proliferation rate of hFOB or hOB cells (data not shown). TGF-β_1 at a concentration of 1–10 ng/ml had potent mitogenic effects on hFOB/AR-6 and hFOB proliferation (data not shown).

Regulation of Bone Cell Differentiation of hFOB/AR-6 Cells by Androgens

To assess the effects of androgens on osteoblast differentiation, hFOB/AR-6 and hFOB cells were incubated at 39.5°C in the presence of 1% (v/v) csFBS, 1,25-$(OH)_2D_3$ (10^{-8} M), ascorbic acid (50 µg/ml), and vitamin K_3 (2 ng/ml). Without additional treatment, the ALP activity (corrected for total protein) in hFOB/AR cells increased progressively over time by threefold at days 4–5 and then decreased. Compared with the vehicle-treated control at day 4, where the peak of ALP activity occurred, treatment with 5α-DHT (10^{-8} M) significantly decreased ALP activity, while co-treatment with 5α-DHT (10^{-8} M) and OHF (10^{-6} M) or OHF (10^{-6} M) alone led to intermediate ALP activities (Fig. 5A). Testosterone treatment decreased ALP activity in a manner similar to 5α-DHT. Treatment with DHEA showed a similar trend, although this was not statistically significant.

PICP synthesis (corrected for differences in total protein content) showed a time course similar to that for ALP activity in untreated hFOB/AR-6 cells (increase by 2.7-fold at days

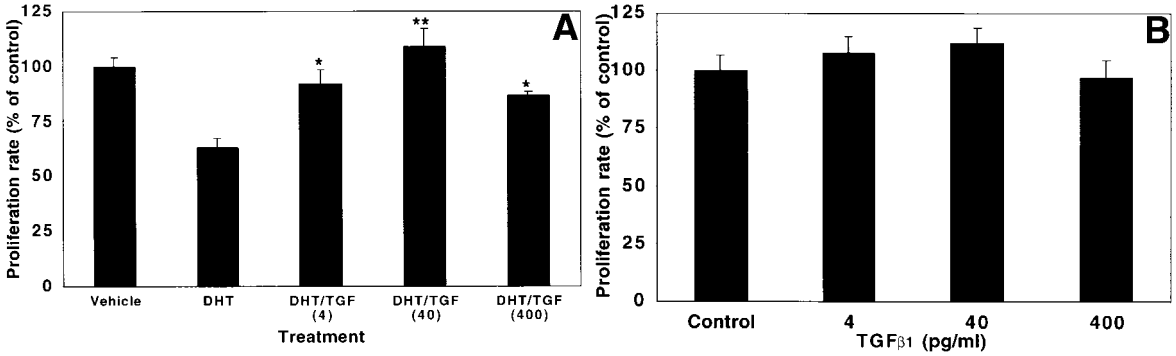


Fig. 4. Antagonism of 5α-DHT-induced proliferation by co-treatment with rhTGF-β_1. Synchronized hFOB/AR-6 cells (2×10^4 cells) were treated with either vehicle (ethanol), 5α-DHT (10^{-10} M), 5α-DHT (10^{-10} M), and varying concentrations of rhTGF-β_1 (4–400 pg/ml) **(A)** or rhTGF-β_1 (4–400 pg/ml) alone **(B)**. The values represent means ± SEM of the [^{3}H] activity in precipitated DNA (n = 6). A: *P*-value compared with 5α-DHT-treated replicates: $*P < 0.01$; $**P < 0.005$ (vehicle vs 5α-DHT: $P < 0.05$).

4–5). Compared with the vehicle-treated control on day 4, PICP synthesis decreased significantly after treatment with 5α-DHT (10^{-8} M). Again, co-treatment with 5α-DHT (10^{-8} M) and OHF (10^{-6} M) or OHF (10^{-6} M) alone led to intermediate PICP values (Fig. 5B). Furthermore, compared to the control, PICP synthesis also decreased after testosterone treatment and, to a lesser extent, after DHEA treatment. 5α-DHT decreased ALP activity (Fig. 6A) and PICP synthesis (Fig. 6B) in a dose-dependent fashion after 4 days of treatment. Since all the above studies assessed androgen effects in the setting of 1,25-$(OH)_2D_3$ stimulation of osteoblast markers, we also assessed 5α-DHT effects on these cells under basal conditions, in the absence of 1,25-$(OH)_2D_3$ stimulation. While treatment with 5α-DHT (10^{-8} M) in the absence of 1,25-$(OH)_2D_3$ had no effect on ALP activity in hFOB cells, which lack sufficient numbers of AR (Fig. 7A), it significantly decreased both ALP activity (Fig. 7B) and PICP synthesis (Fig. 7C) in hFOB/AR-6 cells ($P < 0.001$). Similar results were found for treatment of testosterone (data not shown). By contrast, treatment with 1,25-$(OH)_2D_3$ (10^{-8} M), as expected, increased ALP activity and PICP synthesis in both hFOB and hFOB/AR-6 cells (Fig. 7A–C).

Osteocalcin levels did not differ significantly in untreated hFOB/AR cells between day 0 (100 ± 17.8%), day 2 (57 ± 7.1%), day 4 (129 ± 25.1%), and day 7 (68 ± 25.0%). Measurement of osteocalcin levels in conditioned medium at 4 days revealed no significant differences between different treatment groups (Table II). Furthermore, co-treatment with androgens and rhTGF-β_1 had no effect on ALP activity, PICP synthesis, or osteocalcin production (data not shown). Treatment with gonadal and adrenal androgens or antiandrogens had no statistically significant effect on ALP activity or PICP synthesis in hFOB or hOB cells (data not shown).

DISCUSSION

Androgens have a major effect on the achievement and maintenance of bone mass in women and men, and largely account for sexual dimorphism of the skeletal structure between women and men [Orwoll and Klein, 1995]. Clinically, reduction of androgen levels following the development of hypogonadism or during aging is associated with an accelerated loss of bone mass and increased fracture risk [Finkelstein et al., 1992; Ongphiphadhanakul et al., 1995; Orwoll and Klein, 1995; Wishart et al., 1995], while the exogenous administration of androgens in this situation is osteoprotective and, in part, reverses these abnormalities [Behre et al., 1997; Greenspan et al., 1989]. Studies on the effects of androgens on osteoblasts are limited by the absence of appropriate model systems that combine completeness of the osteoblast phenotype and an appropriate growth rate with the stable expression of physiologically relevant levels of the AR [Boyan et al., 1989; Vanderschueren and Bouillon, 1995]. To circumvent these obstacles, we recently developed a conditionally immortalized human fetal osteoblastic cell line stably transfected with the human wild-type AR cDNA (hFOB/AR-6) [Hofbauer et al., 1997]. These cells express approximately 4,000 functional AR per nucleus, which is in the range of the number of AR present in reproductive and nonreproduc-

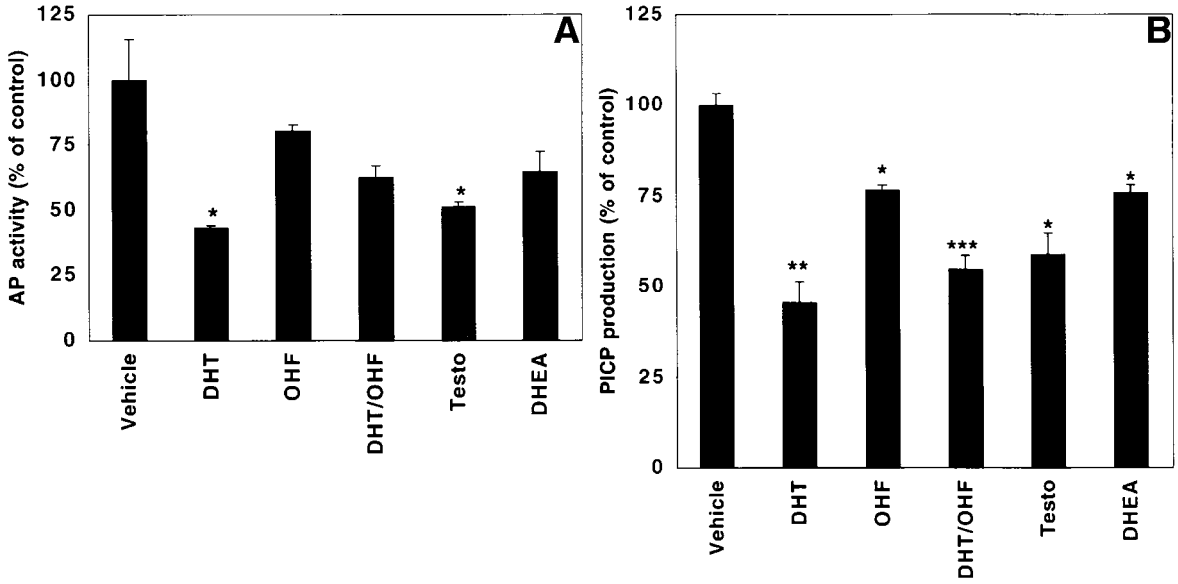


Fig. 5. Effects of androgens and antiandrogens on alkaline phosphatase (ALP) activity **(A)** and type I collagen secretion **(B)** in hFOB/AR-6 cells. Human FOB/AR-6 cells (4×10^4 cells) were cultured for 4 days at the restrictive temperature (39.5°C) in the presence of 1% (v/v) charcoal-stripped fetal bovine serum (csFBS), 1,25-$(OH)_2D_3$ (10^{-8} M), vitamin C (50 μg/ml), and vitamin K_3 (2 ng/ml). In addition, cells were treated with androgens (as indicated at 10^{-8} M: DHT, 5α-dihydrotestosterone; Testo, testosterone; DHEA, dehydroepiandrosterone), the antiandrogen hydroxyflutamide (OHF) (10^{-6} M), or vehicle (ethanol). The mean ± SEM (n = 3) measurements of ALP activity (A) and type I collagen secretion (B) corrected for total protein are shown. A: *P* compared to vehicle-treated replicates: $^*P < 0.05$. B: *P* compared with vehicle-treated replicates: $^*P < 0.005$, $^{**}P = 0.001$, $^{***}P < 0.001$.

tive tissues, including primary human osteoblasts after short-term culture. Using a reporter gene, hFOB/AR-6 cells also respond to transcriptional transactivation following treatment with 5α-DHT in a specific, dose- and time-dependent fashion [Hofbauer et al., 1997]. Moreover, like the parental hFOB cell lines [Harris et al., 1995a], hFOB/AR-6 cells express a mature osteoblast phenotype, including markers of osteoblast differentiation (ALP, type I collagen, osteocalcin), and these cells form mineralized nodules in vitro under appropriate conditions.

In the present study, we report on effects of gonadal and adrenal androgens on the proliferation and differentiation of these hFOB/AR-6 cells. Treatment with 5α-DHT inhibited the proliferation of synchronized hFOB/AR-6 cells as assessed by [^{3}H]thymidine incorporation in a dose-dependent fashion. Testosterone and the adrenal androgen DHEA had no significant effect on hFOB/AR-6 cell proliferation. Androgens had no effect on cell proliferation in the parental cell line hFOB [Harris et al., 1995a] and hOB cells, suggesting that hFOB and hOB cells, which have about 200 AR per nucleus [Hofbauer et al., 1997], lack an appropriate number of functional AR to confer androgen responsiveness. Moreover, when hFOB/AR-6 cells were co-treated with both OHF and 5α-DHT, OHF abrogated the growth-inhibitory effects of 5α-DHT, suggesting that the observed effects were directly mediated by the AR. Our results that 5α-DHT inhibits the proliferation of normal human osteoblasts are in contrast to some [Gray et al., 1992; Kasperk et al., 1989; Masuyama et al., 1992; Nakano et al., 1994; Somjen et al., 1989; Weisman et al., 1993], but not all studies [Benz et al., 1991]. In fact, Benz et al. [1991] showed that 10 nM of both 5α-DHT and testosterone inhibited the proliferation of the osteosarcoma cell line, TE-85, by 18–25%. These cells express a similar number of AR (2,800/nucleus) as the hFOB/AR-6 cells [Benz et al., 1991]. Thus, as noted earlier, the effect of androgens on osteoblasts may be variable depending on the cell system and culture techniques used. This may explain contradictory results between different cell systems and, even within one cell system in which the presence of calcitropic hormones (1,25-$(OH)_2D_3$) may modulate androgen effects on the proliferation of osteoblast cells [Gray et al., 1992; Somjen et al., 1989]. Moreover, the differential effects of andro-

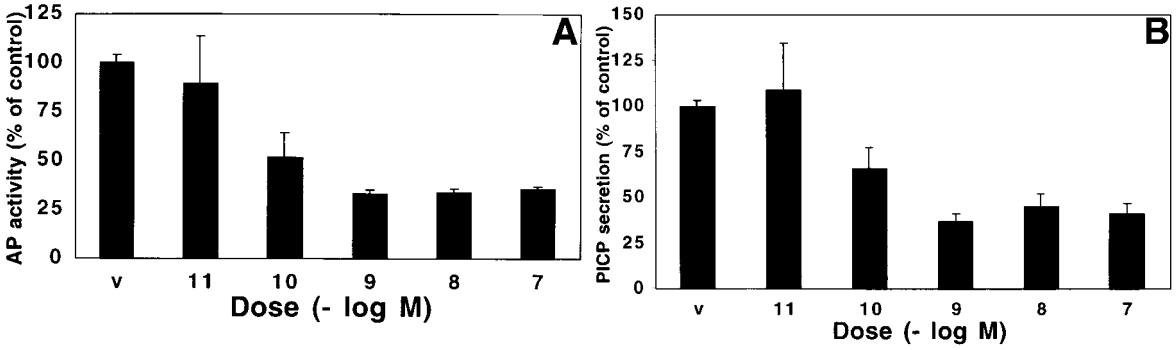


Fig. 6. Dose response of 5α-DHT on alkaline phosphatase activity **(A)** and type I collagen secretion **(B)** in hFOB/AR-6 cells. Human FOB/AR-6 cells (4×10^4 cells) were cultured for 4 days in the presence of 1% (v/v) csFBS, 1,25-$(OH)_2D_3$ (10^{-8} M), vitamin C (50 μg/ml), and vitamin K_3 (2 ng/ml). In addition, cells were treated with 5α-DHT at the doses indicated or vehicle (v). The mean ± SEM (n = 4) measurements of ALP activity (A) and type I collagen secretion (B) (corrected for total protein) are shown. A: $P < 0.001$ (by ANOVA). B: $P < 0.001$ (by ANOVA).

gens other than 5α-DHT on the proliferation of human osteoblast-like cells may be due in part to cell model-inherent variations in the presence or absence of enzymes involved in androgen metabolism (5α-reductase, 3β-hydroxysteroid dehydrogenase) [Bodine et al., 1995; Kasperk et al., 1997b; Vanderschueren and Bouillon, 1995]. Data derived from primary human osteoblastic cell cultures from healthy adults, although physiologically relevant, should be carefully interpreted, given certain inherent limitations [Vanderschueren and Bouillon, 1995], including their heterogeneous cell population with the potential presence of contaminating cells and their variation of AR content per cell [Bodine et al., 1995; Kasperk et al., 1997b].

Although the number of hAR in hFOB/AR-6 cells is on the order of other reproductive and nonreproductive androgen targets in vivo, including osteoblasts, inhibition of proliferation by 5α-DHT in hFOB/AR-6 cells may be attributable to the fact that hFOB/AR-6 cells were derived by transfecting the hAR into hFOB cells. Two recent studies using transfection of the hAR into tumor cell lines with a low number of hAR support this hypothesis: proliferation of both a human breast cancer cell line (MCF7) [Szelei et al., 1994] and an androgen-insensitive human prostate cancer cell line (PC-3) [Yuan et al., 1993] was inhibited by 5α-DHT after transfection with the hAR cDNA, while androgens have no effect on nontransfected PC-3 cells [Tilley et al., 1995]. Similar results have also been reported for the growth-inhibiting effects of estrogen on various osteosarcoma cell lines with low levels of endogenous ER after transfection with the ER cDNA [Migliaccio et al., 1992; Watts and King, 1994; Watts et al., 1989]. Moreover, treatment of hFOB cells stably transfected with the ER (hFOB/ER) [Harris et al., 1995b], with 17β-estradiol also inhibited osteoblast proliferation [Kassem et al., 1997; Robinson et al., 1997].

In hFOB/AR-6 cells, both the basal and 1,25-$(OH)_2D_3$-induced expression of ALP activity and PICP synthesis were inhibited androgens, whereas there was no effect on osteocalcin production. In contrast to our data, previous studies using primary osteoblast cultures have shown a stimulatory effect of androgens on ALP activity [Kasperk et al., 1989, 1996, 1997b], while androgen effects on type I collagen synthesis have been variable [Benz et al., 1991; Canalis and Raisz, 1978; Gray et al., 1992; Kasperk et al., 1996; Nakano et al., 1994; Pilbeam and Raisz, 1990]. Some of these differences may be related to the stage of osteoblast differentiation of the cells studied. Thus, hFOB and hFOB/AR-6 represent a homogenous population of relatively differentiated, mature osteoblasts [Harris et al., 1995a; Hofbauer et al., 1997]. By contrast, primary cultures from bone likely contain a heterogenous population of osteoblast-like cells at varying stages of osteoblast differentiation [Kasperk et al., 1997b]. It is possible that androgen effects on osteoblast differentiation markers vary depending on the relative osteoblast phenotype expressed by the cell. Clearly, more studies using homogenous cell populations at different stages of osteoblast differentiation are needed to address this issue.

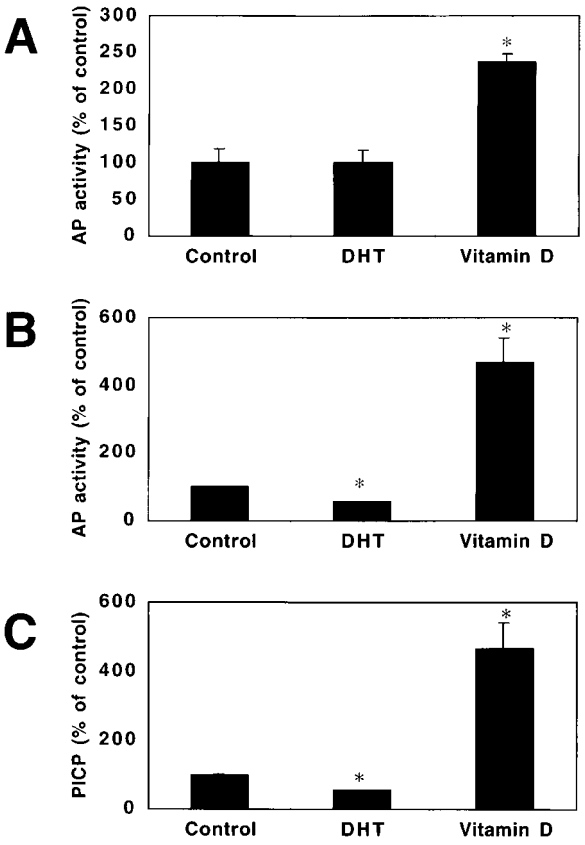


Fig. 7. Effect of 5α-DHT on alkaline phosphatase activity **(A,B)** and type I collagen secretion **(C)** in the absence of 1,25-$(OH)_2D_3$ in hFOB (A) and hFOB/AR-6 cells (B,C). Human FOB or FOB/AR-6 cells (4×10^4 cells) were cultured for 5 days in the presence of 1% (v/v) charcoal-stripped fetal bovine serum (csFBS), vitamin C (50 μg/ml), and vitamin K_3 (2 ng/ml). In addition, cells were treated with either vehicle (control), 5α-DHT (10^{-8} M) or 1,25-$(OH)_2D_3$ (10^{-8} M). The mean ± SEM (n = 4) measurements of ALP activity (A,B) and type I collagen secretion (C) (corrected for total protein) are shown. *$P < 0.001$.

While androgens are generally believed to have potential anabolic effects on bone [Bagatell and Bremner, 1996; Orwoll and Klein, 1995], studies directly addressing this issue have been inconclusive. Thus, while Baran et al. [1978] performed bone biopsies in a hypogonadal man both before and after 6 months of testosterone treatment and had noted increases in relative osteoid volume, total osteoid surface, linear extent of bone formation, and bone mineralization, Riggs et al. [1972] found that treatment of postmenopausal women with either estrogen or oxandrolone (a synthetic androgen) resulted in a decrease in both resorption and formation surfaces. Moreover, the recent description of estrogen receptor (ER)-negative [Smith et al., 1994] and aromatase-deficient males [Carani et al., 1997; Morishima et al., 1995] who, despite having normal testosterone levels, were osteopenic suggests that testosterone, in the absence of estrogen action, may not be sufficient for optimal skeletal development. Since the hFOB/AR cells have high numbers of AR but virtually no ERs, they similarly permit assessment of direct in vitro androgen effects on bone cell function in the absence of estrogen effects. The lack of estrogen responsiveness in these cells may account, in part, for the differences between our results and previous studies using other model systems [Bodine et al., 1995; Kasperk et al., 1997b].

In addition to direct effects on osteoblasts, steroid hormones may modulate the proliferation, recruitment, and differentiation of osteoblasts by regulating the local production of cytokines and growth factors. A pivotal role of TGF-β as a potential mediator of androgen action on adult human osteoblasts has been previously suggested [Bodine et al., 1995; Kasperk et al., 1997b]. In this study, hFOB/AR-6 cells displayed high constitutive steady-state levels of TGF-β mRNA which decreased after treatment with 5α-DHT, whereas in other cells studied (hFOB, hOB), there was no consistent effect on TGF-β mRNA expression following treatment with 5α-DHT. Our finding that 5α-DHT-induced growth inhibition could be reversed by exogenous administration of rhTGF-β_1 suggests that 5α-DHT inhibits the proliferation of hFOB/AR-6 cells by reducing TGF-β production. This hypothesis is also supported by the finding that TIEG mRNA expression was similarly inhibited in hFOB/AR-6 cells by 5α-DHT, but not in other cells studied. Since TIEG repre-

TABLE II. Effects of Androgens and Anti-androgens on Osteocalcin Secretion*

	Percent of Vehicle Controls
Vehicle	100 ± 7.4
5α-DHT	107 ± 8.1
Hydroxyflutamide	98 ± 7.1
5α-DHT and hydroxyflutamide	110 ± 5.0
Testosterone	113 ± 4.8
DHEA	127 ± 14

*Human FOB/AR-6 cells were treated (as described in the legend to Fig. 6), and osteocalcin secretion was measured in the conditioned medium. Mean ± SEM of three replicate measurements normalized to vehicle controls.

sents a downstream gene of TGF-β action in osteoblasts, and osteoblasts overexpressing TIEG mimic the effects of TGF-β [Hefferan al., 1997], our data are consistent with an androgen-induced inhibition of the TGF-β-TIEG signaling pathway in hFOB/AR-6 cells.

In summary, we have characterized the effects of gonadal and adrenal androgens in a novel androgen-responsive fetal osteoblastic cell line. In these cells, which express a mature osteoblast phenotype, androgens inhibit the proliferation rate and the expression of differentiated osteoblastic functions, including ALP activity and PICP synthesis. All these effects can be abolished or attenuated by treatment with the selective AR antagonist OHF, indicating direct mediation by the AR. These studies thus suggest that the potential "anabolic" effect of androgens on bone may not be mediated at the level of the mature osteoblast. More studies, using similarly well-defined cell populations at different stages of osteoblast differentiation are needed to further assess androgen effects on bone.

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