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Regulation of c-*fos* Expression and TGF-β Production by Gonadal and Adrenal Androgens in Normal Human Osteoblastic Cells

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Although the role of estrogens in bone formation is becoming clarified, the function of androgens in this process remains to be defined. Consequently, we have explored the mechanism of action for both gonadal and adrenal androgens in normal human osteoblastic (hOB) cells, which are responsible for the synthesis and mineralization of bone. Changes in the steady-state mRNA levels for two nuclear proto-oncogenes (c-*fos* and c-*jun*) and one cytokine (TGF-β1) were quantified in response to short (30 min) and long (24–48 h) treatments of these cells with physiologic concentrations of steroids. In addition, the levels of TGF-β protein in the hOB cell conditioned-media were measured using a bioassay. The results indicated that neither 10 nM dihydrotestosterone, 10–20 nM testosterone, nor 10–100 nM androstenedione had a significant effect on the steady-state levels of c-*fos*, c-*jun*, or TGF-β1 mRNAs. Interestingly, 10–1000 nM dehydroepiandrosterone (DHEA) and 1–10 μM DHEA-sulfate rapidly reduced the steady-state level of c-*fos* mRNA by 60–80% in a dose-dependent manner within 30 min. In contrast, neither of these adrenal steroids had a significant effect on the message levels for c-*jun* or TGF-β1. Surprisingly, although TGF-β1 mRNA levels remained unchanged, the total amount of TGF-β activity in the hOB cell conditioned-media increased 2–5-fold in response to 24–48 h treatments of the cells with gonadal or adrenal androgens. This increase in TGF-β activity by DHEA-sulfate was both time- and dose-dependent, and was not blocked by cotreatment with the specific aromatase inhibitor 4-hydroxyandrostenedione (1 μM). Immunoprecipitations of the hOB cell conditioned-media with isoform-specific TGF-β neutralizing-antibodies indicated that TGF-β2 was predominantly produced by the cells in response to DHEA-sulfate treatment. These results demonstrate that differences exist between the actions of estrogens and androgens on normal human osteoblasts with regard to the regulation of c-*fos* expression and TGF-β production. Moreover, these data suggest that DHEA and DHEA-sulfate may play a distinct role in the regulation of human osteoblast function via the rapid repression of c-*fos* message levels and the slower increase in TGF-β2 protein levels.

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INTRODUCTION

Osteoblasts are the cells that synthesize and mineralize the bone matrix [1, 2]. Receptors for estrogens and androgens are present in a variety of primary-culture and osteosarcoma-derived osteoblastic cells from both human and rodent tissues [3–10], including normal human osteoblastic (hOB) cells [11–13]. These proteins are members of the nuclear receptor superfamily, and are hormone-activated transcription factors [3, 14]. In human osteoblastic cells, 17β-estradiol (E_2) has been observed to increase the expression of type I procollagen, TGF-β1, progesterone receptors, and c-*fos* [4, 11, 12, 15, 16]. Similarly, testosterone (T) has been shown to enhance the

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production of type I procollagen, TGF-β1, c-*fos*, and c-*jun* messages in human osteosarcoma (HOS TE85 and SAOS-2) cells [6, 17]. Dihydrotestosterone (DHT) has been reported to induce the expression of alkaline phosphatase and TGF-β2 (but not TGF-β1) in hOB and human osteosarcoma (TE89 and SAOS-2) cells [5, 18].

Besides responding to sex hormones, bone forming cells also synthesize these steroids. Normal hOB cells from both sexes are able to convert the adrenal androgen, androstenedione (ASD), to T, DHT, estrone (E_1), and E_2 [19, 20]. In addition, several human osteosarcoma cell lines metabolize ASD and E_1-sulfate to E_1 and E_2 [21, 22]. These results imply that sex steroids have intracrine, autocrine, and paracrine activities, as well as endocrine functions, in bone. Although ASD is metabolized to more potent sex hormones by human osteoblasts, it is not known if this adrenal steroid or the two most predominant adrenal androgens, dehydroepiandrosterone (DHEA) and DHEA-sulfate [23], can exhibit biological activities on cultured osteoblastic cells.

Because relatively little is known about the molecular actions of androgens on hOB cells, we chose to examine the effects of approximate physiologic concentrations of both gonadal (T and its more potent metabolite DHT) and adrenal (ASD, DHEA, and DHEA-sulfate) androgens on the expression of c-*fos*, c-*jun*, and TGF-β1 in these cells. The isoforms of TGF-β are important cytokines for bone remodeling, because these polypeptides regulate the activities of both osteoblasts and osteoclasts, which are the multinucleated giant cells that resorb bone [1, 24, 25]. Moreover, the AP-1 transcription factor, which is composed of Fos/Jun or Jun/Jun dimers, is known to modulate TGF-β transcription [25]. In rat calvarial-derived osteoblastic cells, the down-regulation of AP-1 and TGF-β expression precedes the advent of osteoblast maturation and mineralization [26]. Furthermore, transgenic mice which over-express the c-*fos* gene exhibit increased bone formation [27], while mice bearing a null mutation in the c-*fos* gene showed symptoms of severe osteopetrosis [28].

The current results with hOB cells indicated that DHT, T, and ASD did not affect the steady-state levels of c-*fos*, c-*jun*, or TGF-β1 mRNAs as determined by Northern hybridizations. On the other hand, DHEA and DHEA-sulfate rapidly reduced the amount of c-*fos* mRNA by 60–80%. In contrast, neither of these adrenal steroids had an effect on the message levels for c-*jun* or TGF-β1. However, the total amount of TGF-β activity in the hOB cell conditioned-media increased 2–5-fold in response to gonadal and adrenal androgen treatment as determined by a bioassay. Finally, immunoprecipitations of the hOB cell conditioned-media with TGF-β antibodies indicated that TGF-β2 was primarily released by the cells in response to DHEA-sulfate treatment.

MATERIALS AND METHODS

Cell culture

Normal hOB cells were cultured according to the procedure of Robey and Termine [29] with trabecular bone explants obtained from orthopedic procedures on patients who showed no signs of metabolic bone disease [11]. The cells were grown at 37°C (5% CO_2/95% humidified air, v/v) in T-75 flasks with the equivalent of phenol red-free/calcium-free DME/Ham's F-12 medium containing 10% fetal calf serum (v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were >95% homgeneous and displayed a mature (normal) osteoblastic phenotype [11, 29]. All experiments were performed with pooled male or female cells that were subcultured at the end of the first passage and grown to over 90% confluency (~3 million cells/flask). The experimental medium (phenol red-free DME/Ham's F-12) was devoid of fetal calf serum, but contained 0.25% (w/v) bovine serum albumin (BSA) (Pentex crystallized BSA; Miles, Kankakee, IL) and 1 mM $CaCl_2$ (BSA-medium).

Steroid treatments of the hOB cells were performed as follows. Flasks of cells were rinsed 1–2 times with 10–20 ml of warm (37°C) phosphate-buffered saline (PBS) (to remove endogenous steroids present in the serum) and subsequently placed in 10 ml of BSA-medium (which is devoid of steroids) for 24 h (TGF-β experiments) or 48 h (c-*fos* and c-*jun* experiments). On the day of steroid treatment, the cells were placed in 10 ml of fresh BSA-medium containing either vehicle (0.1% ethanol, v/v; evaporated to dryness) or steroid. For the c-*fos* and c-*jun* experiments, the cells were treated with vehicle or steroid at 37°C for 30 min, at which time the flasks were rinsed 1–2 times with 10–20 ml of warm PBS, and total RNA was isolated for Northern hybridization as described in the following section. For the TGF-β experiments, the cells were treated in a similar manner for 24–48 h, and then processed for Northern hybridization. In addition, the hOB cell conditioned-media was saved and stored at −70°C for the TGF-β bioassay as described below.

RNA quantitation

Total cellular RNA (30–60 μg/flask) was isolated from confluent flasks of male or female hOB cells using TRI reagent (a mixture of phenol and guanidine thiocyanate) according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH) [30], but with the following changes: the cells were scraped from the flasks after lysis, a chloroform extraction (1:1, v/v) was added, and both the isopropanol precipitation and ethanol wash were carried out overnight at −20°C. 10–20 μg of each RNA sample (quantified by A260/A280; >85% pure) were denatured by glyoxal/dimethyl sulfoxide [31], and resolved by size using electrophoresis (70 V/95 mA, 4–5 h; along with DNA

size standards) on a 1% (w/v) agarose gel [32]. The RNA was transferred overnight from the gel to a Hybond-N nylon membrane (0.45 μm; Amersham, Arlington Heights, IL) by capillary action in 3 M NaCl/0.3 M sodium citrate, pH 7.0 (20X SSC) as described for DNA [33]. The RNA was cross-linked to the membrane by UV exposure (120 mJ/cm^2 at 260 nm) using a Stratalinker 1800 (Stratagene, La Jolla, CA). The blot was then air dried and boiled for 15 min in 20 mM Tris–HCl, pH 8.0.

Plasmids containing the full-length cDNAs that were used in this study were obtained from the following sources: human c-*fos* cDNA was provided by Dr I. M. Verma (Salk Institute, San Diego, CA); human c-*jun* cDNA was obtained from Dr R. Tijan (University of California, Berkeley, CA); human TGF-β1 cDNA was a gift from Dr R. Derynck (Genentech, South San Francisco, CA); rat GAPDH cDNA was donated by Dr P. Fort (University De Montpellier, Montpellier, France); and human 18S RNA cDNA was purchased from the American Type Culture Collection. The plasmids were purified from 500 ml chloramphenicol-induced bacterial cultures using the Xprep Maxi Plasmid DNA Purification System (XGen, Cambridge, MA). After cutting the plasmids with the appropriate restriction enzyme(s), the cDNAs were isolated by electrophoresis and purified from the low-melt agarose gels using the SpinBind DNA Recovery System (FMC BioProducts, Rockland, ME). The cDNAs were labeled to >1 million cpm per ng with [^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) by random primer extension using a Ready-To-Go DNA Labeling Kit (Pharmacia, Piscataway, NJ); the [^{32}P]-cDNAs were then purified by gel-filtration (Pharmacia Nick columns).

Northern hybridization of the RNA blots was performed in a hybridization incubator (Model 400; Robbins Scientific, Sunnyvale, CA) at 67°C with 10 ml of QuikHyb hybridization solution (Stratagene) containing 250 μg/ml of sonicated salmon sperm DNA. The blots were pre-hybridized for 1–2 h, and then hybridized with a fresh solution containing 25 ng of [^{32}P]-cDNA (2–4 million cpm/ml) for 2–3 h. The [^{32}P]-blots were washed once for 5 min at 25°C with 50 ml of 2X SSC/0.1% (v/v) SDS, and then washed twice for 30 min at 60–65°C with 50 ml of 0.5X SSC/0.1% (v/v) SDS (c-*fos* and GAPDH) or with 0.1X SSC/0.1% (v/v) SDS (c-*jun*, TGF-β1, and 18S RNA). The washed blots were exposed to X-ray film (Kodak X-Omat AR5; Eastman Kodak, Rochester, NY) with intensifying screens at −70°C. The films were developed by a Kodak X-Omat M20 Film Processor, and the bands are quantified using a Shimadzu CS 9000 Dual Wavelength Flying Spot Scanning Laser Densitometer (Kyoto, Japan). Several films of varying exposure time were developed for each hybridization, and the optimal one was chosen for quantification. Prior to re-probing with a different cDNA, the blots were stripped of [^{32}P]-cDNA by boiling for 15 min in 20 mM Tris–HCl, pH 8.0, 0.1X SSC/0.1% (v/v) SDS.

TGF-β bioassay

The TGF-β bioassay, which measures the inhibition of CCL-64 (mink lung epithelial) cell DNA synthesis by this peptide, was performed as described by Danielpour *et al.* [15, 34]. Briefly, CCL-64 cells were seeded into 48-well tissue culture plates at a concentration of 5000–10,000 cells per well, and allowed to proliferate for 2 days at 37°C (5% CO_2/95% humidified air, v/v) in modified McCoy's 5A medium containing 10% fetal calf serum (v/v), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Two ml aliquots of the hOB cell conditioned-media (obtained as described above) were dialyzed in 1 × 15 cm segments of membrane tubing (12–14 kDa cutoff; Spectrapor, Los Angeles, CA) vs 2 l of pH 1.5 modified McCoy's 5A medium (without phenol red and $NaHCO_3$) for 20–24 h at 4°C. This procedure activates latent TGF-β [15, 25]; over 95% of the peptide secreted by the hOB cells in response to E_2 treatment was latent [15]. The segments of tubing were next rinsed in water and dialyzed again vs 2 l of pH 7.4 modified McCoy's 5A medium for another 20–24 h at 4°C. The dialyzed conditioned-media were then diluted 1:1 (v/v) with modified McCoy's 5A medium contaning 20% fetal calf serum (v/v) and 2% penicillin/streptomycin (v/v), and filter-sterilized with 0.2 μm syringe filters (Microgen, Laguna Hills, CA).

The CCL-64 cells were treated overnight (in triplicate) with 400 μl samples of the sterile-dialyzed conditioned-media. In a similar manner, cells were also treated with various concentrations (0–160 pM) of purified-activated TGF-β1 (R and D Systems, Minneapolis, MN) in order to generate a standard dose–response curve. The cells were next pulsed for 4–6 h with 0.5 μCi of [^{3}H]thymidine (2 Ci/mmol; New England Nuclear), then fixed and washed 4 times with 1 ml of ice cold 10% (w/v) trichloroacetic acid, and finally dissolved in 200 μl of 2% (w/v) NaOH. The NaOH extracts were dissolved in liquid scintillation fluid (Ultima Gold; Packard, Meriden, CT), and radioactivity was measured by liquid scintillation counting with an average efficiency of ~60% for ^{3}H using a Beckman Model LS-5801 Liquid Scintillation Spectrometer (Irvine, CA). The bioassay results (expressed in pmol of TGF-β/l, molecular weight = 25,000) were multiplied by 2 in order to correct for the 1:1 dilution of the conditioned-media prior to assay.

In order to determine which isoform of TGF-β was being produced by the hOB cells in response to the steroid treatments, 0.5 ml aliquots of the dialyzed (i.e. activated) conditioned-media were mixed with 10 μg/ml of neutralizing-antibodies specific for either TGF-β1 or TGF-β2 (AB-101-NA and AB-112-NA; R and D Systems), and immunoprecipitated overnight at 4°C (while mixing) with 60 μg/ml of recombinant

protein A/G (Calbiochem, San Diego, CA) [15]. The precipitates were pelleted by centrifugation at 16,000 *g* in a microcentrifuge (at 4°C). The resulting supernatants were diluted 1:1 (v/v) with modified McCoy's 5A medium containing 20% fetal calf serum (v/v) and 2% penicillin/streptomycin (v/v), filter-sterilized, and duplicate 400 μl samples were then assayed for TGF-β bioactivity as described above.

RESULTS

Previous work from our laboratories has shown that E_2 maximally induces the expression of c-*fos* mRNA in hOB cells after 30 min of treatment [16]. However, it is not known if androgens also enhance c-*fos* expression in these cells. Consequently, we examined the effects of approximate physiologic concentrations of both gonadal (T and DHT) and adrenal (ASD, DHEA, and DHEA-sulfate) androgens on the steady-state mRNA levels for the immediate early c-*fos* and c-*jun* nuclear proto-oncogenes [14], as well as the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hOB cells.

As indicated in Fig. 1, treatment of male or female hOB cells for 30 min with 10 nM each of T, DHT, R1881 (a potent synthetic androgen) or ASD did not have a significant effect on the steady-state levels of c-*fos* for GAPDH mRNAs ($\pm$10–30%). Likewise, cotreatment of the cells with 10 nM DHT and 1 μM hydroxyflutamide (a synthetic antiandrogen) did not affect these message levels either. In contrast, treatment of the hOB cells with 100 nM DHEA or 10 μM DHEA-sulfate rapidly decreased the steady-state level of c-*fos* mRNA by 60–70% from control values within 30 min. Furthermore, the reduction of c-*fos* mRNA levels in response to these adrenal steroids was dose-dependent [Fig. 1 (B, lanes 7–11)]. The 50% effective concentrations of DHEA and DHEA-sulfate for c-*fos* repression were $\sim$80 nM and $\sim$5 μM, respectively; these concentrations approximate the normal physiologic range of the steroids [23]. On the other hand, DHEA and DHEA-sulfate treatment did not have a significant effect on the level of GAPDH mRNA in the hOB cells ($\pm$10%). DHEA also reduced the level of c-*fos* mRNA in proliferating hOB cells (i.e. cells maintained in 5% charcoal-stripped fetal calf serum instead of BSA-medium), but the degree of repression was not as great as that observed in Fig. 1 (data not shown). However, no regulation of c-*fos* message levels were observed by the adrenal steroids with serum-induced cells (i.e. cells cotreated with 5% charcoal-stripped fetal calf serum and steroids) (data not shown).

In contrast to the c-*fos* results, Fig. 2 shows that a 30 min treatment of the hOB cells with gonadal or adrenal androgens did not have a significant effect on the steady-state levels of c-*jun* or GAPDH mRNAs. Thus, the rapid repression of c-*fos* mRNA levels observed with DHEA and DHEA-sulfate treatment appeared to be message specific.

Additional studies from our laboratories have demonstrated that E_2 optimally induces the expression of TGF-β1 mRNA and protein in hOB cells after 24 h of treatment [15], while DHT has been reported to repress TGF-β1 expression in these cells [18]. Therefore, we examined the effects of both gonadal and adrenal androgens on the steady-state level of TGF-β1 and GAPDH mRNAs in hOB cells. As depicted in Fig. 3, treatment of male or female hOB cells for 24 or 48 h with 10 nM DHT, 20 nM T, 10 nM ASD, 100 nM DHEA, or 10 μM DHEA-sulfate did not have a significant effect on the steady-state levels of TGF-β1 or GAPDH mRNAs ($\pm$10–20%). These results suggest that unlike the action of E_2 [15], androgens do not appear to induce the expression of TGF-β1 mRNA in these cells.

Since osteoblasts produce several different isoforms of TGF-β (mostly β1, β2, and some β3) [25], it was of interest to determine whether the levels of any specific isoform were regulated by the gonadal and adrenal androgens in hOB cells. Consequently, male or female hOB cells were treated for 24 or 48 h with 10 nM DHT, 20 nM T, 10 nM ASD, 100 nM DHEA, or 10 μM DHEA-sulfate and the resulting cell conditioned-media were assayed for total TGF-β activity. As indicated in Fig. 4, treatments of these cells with gonadal or adrenal androgens for 24 h failed to show significant effects on the level of TGF-β activity in the conditioned-media. In contrast, 48 h of steroid treatment increased the amount of total TGF-β activity 2–5-fold above control levels in the hOB cell conditioned-media. Interestingly, the increase in TGF-β activity observed with 10 nM DHT was only 2-fold, while 10 μM DHEA-sulfate increased the production of this cytokine over 4-fold. In fact, approximate physiologic levels of the adrenal androgens appeared to be more potent at increasing TGF-β production than approximate physiologic levels of DHT or T.

Because a physiologic concentration (10 μM) of DHEA-sulfate exhibited the greatest effect on the level of TGF-β activity in the hOB cell conditioned medium, we chose to pursue the action of this steroid on the human osteoblastic cells more thoroughly. As shown in Fig. 5, treatment of male or female hOB cells for 48 h with DHEA-sulfate increased the level of total TGF-β activity in the cell conditioned-media in a dose-dependent manner. While the increase in TGF-β activity observed with 1 μM DHEA-sulfate was only 2-fold, 5–10 μM of the adrenal steroid increased the production of this cytokine 3–5-fold above control levels. Moreover, the increase in TGF-β activity produced with 10 μM DHEA-sulfate was not blocked by the specific aromatase inhibitor, 4-hydroxyandrostenedione (1 μM); this concentration of inhibitor completely blocks aromatase activity in human breast cancer and osteosarcoma cells [21]. This result suggests

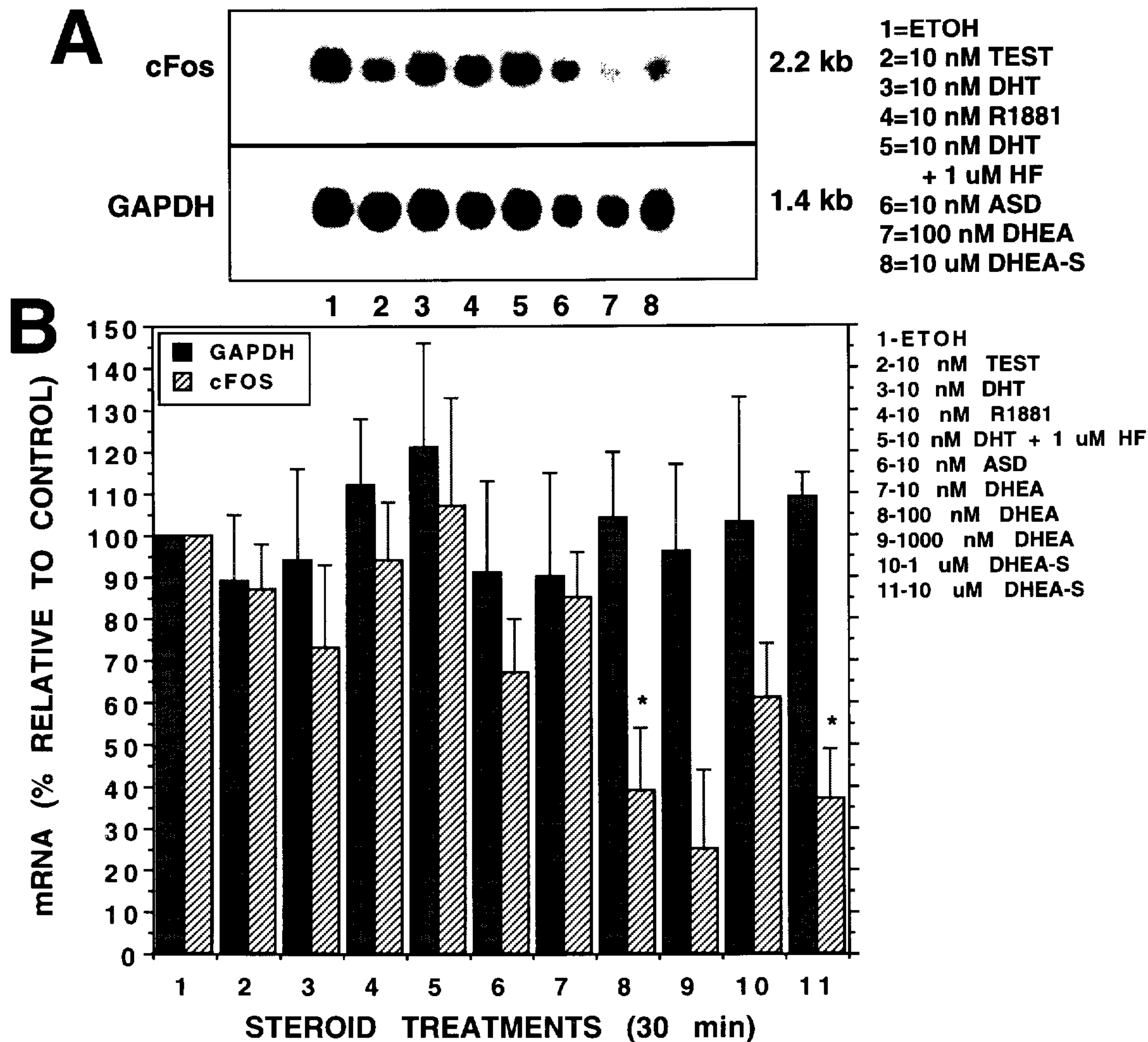

Fig. 1. Rapid repression of c-*fos* steady-state mRNA levels by DHEA and DHEA-sulfate in hOB cells. (A) Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 48 h. The cells were then treated for 30 min with vehicle or steroids. After treatment, total RNA was isolated from the cells and electrophoresed (along with DNA size standards) on agarose gels. Northern hybridization was next performed with a [^{32}P]dCTP-labeled cDNA probe for human c-*fos*. After autoradiography, the blots were stripped and subsequently reprobed with [^{32}P]-labeled cDNAs for rat GAPDH (a constitutively expressed control message) and human 18S RNA (to control for variations in gel lane loading) as described in the Materials and Methods. (B) The autoradiographs were quantified by scanning laser densitometry, and variations in gel lane loading (10–30%) were corrected for by standardizing the data to the 18S RNA results (not shown). The results are presented as the mean ± SD of 2–5 experiments; $^*P < 0.05$ (Behren's–Fisher t-test) compared to the ethanol control. kb, kilobase pairs; GAPDH, glyceraldehyde phosphate dehydrogenase; ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; HF, hydroxyflutamide; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate.

that the increase in TGF-β production by DHEA-sulfate was apparently not due to metabolism of the steroid to E_1 and E_2 by the hOB cells.

Finally, we wanted to determine which isoform of TGF-β was being produced by the hOB cells in response to DHEA-sulfate treatment. As indicated above, previous work from our laboratories demonstrated that E_2 induces TGF-β1 expression by hOB cells, while Kasperk *et al.* [18] showed that DHT increases the expression of TGF-β2 by these cells. Therefore, male or female hOB cells were treated for 48 h with 10 μM DHEA-sulfate, and the cell conditioned-media were immunoprecipitated with neutralizing-antibodies specific for either TGF-β1 or TGF-β2. The resulting supernatants were then assayed for total TGF-β activity. As depicted in Fig. 6, immunoprecipitation of the hOB cell conditioned-media with the TGF-β1-specific antibody neutralized only 20% of the total TGF-β activity. However, immunoprecipitation of the conditioned-media with the TGF-β2 specific antibody removed approximately 90% of the cytokine activity. These results indicate

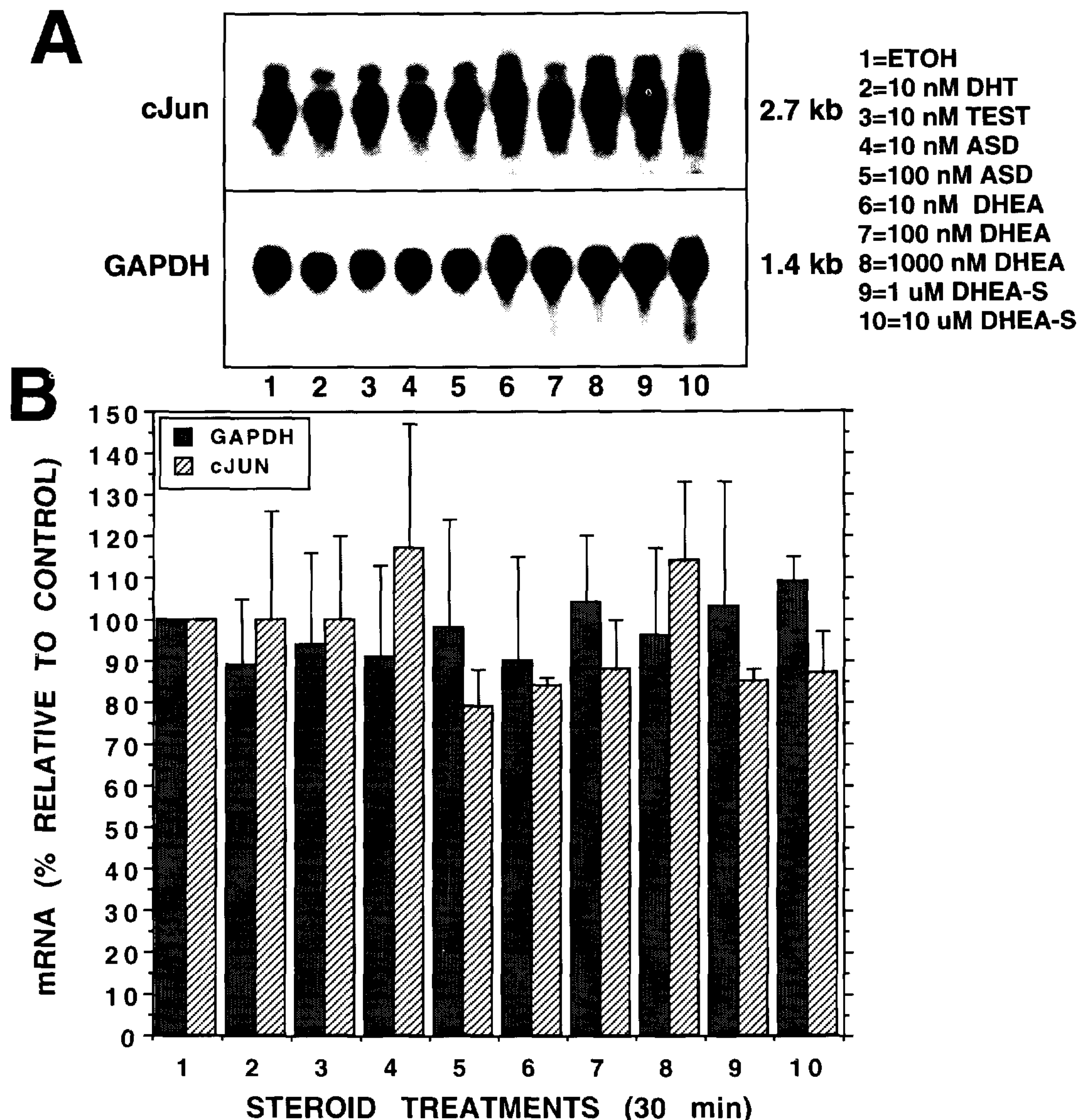

Fig. 2. No effect of gonadal and adrenal androgens on the steady-state level of c-*jun* mRNA in hOB cells. (A) Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 48 h. The cells were then treated for 30 min with vehicle or steroids. After treatment, total RNA was isolated from the cells and electrophoresed on agarose gels. Northern hybridization was next performed with a [^{32}P]dCTP-labeled cDNA probe for human c-*jun*; in some experiments, blots that were initially probed for c-*fos* mRNA (Fig. 1) were stripped and reprobed for c-*jun* mRNA. After autoradiography, the blots were stripped and reprobed again with [^{32}P]-labeled cDNAs for rat GAPDH and human 18S RNA as described in the materials and methods. (B) The autoradiographs were quantified by scanning laser densitometry, and variations in gel lane loading (10–30%) were corrected for by standardizing the data to the 18S RNA results (not shown). The results are presented as the mean $\pm$ SD of 2–5 experiment. kb, kilobase pairs; GAPDH, glyceraldehyde phosphate dehydrogenase; ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate.

that hOB cells primarily produce TGF-β2 in response to treatment with a physiologic concentration of DHEA-sulfate. It is important to note that the experiments presented in Figs 4–6 were performed with pools of hOB cells isolated from different patients. Consequently, the baseline levels of TGF-β expression by the cells also varied, although the degree of increase observed with DHEA-sulfate treatment remained constant.

DISCUSSION

Although gonadal androgens appear to enhance bone formation [35, 36], the precise molecular mechanisms by which these steroids regulate human osteoblastic activity are not well defined. Furthermore, no studies describing the actions of adrenal androgens on cultured osteoblastic cells have been reported.

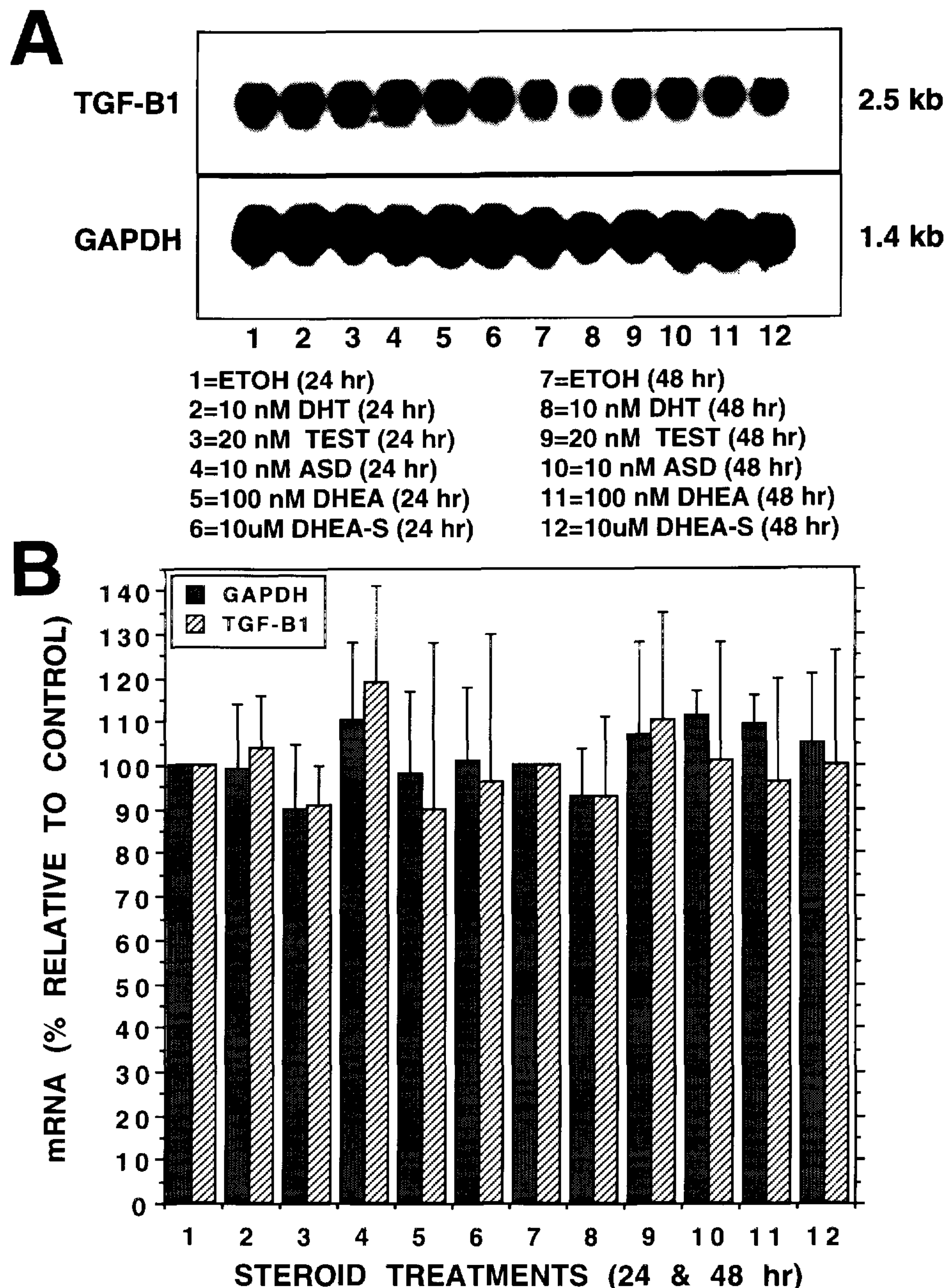

Fig. 3. No effect of gonadal and adrenal androgens on the steady-state level of TGF-β1 and mRNA in hOB cells. (A) Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 24 h. The cells were then treated for 24–48 h with vehicle or steroids. After treatment, total RNA was isolated from the cells and electrophoresed on agarose gels. Northern hybridization was next performed with a [^{32}P]dCTP-labeled cDNA probe for human TGF-β1. After autoradiography, the blots were stripped and reprobed with [^{32}P]-labeled cDNAs for rat GAPDH and human 18S RNA as described in the Materials and Methods. (B) The autoradiographs were quantified by scanning laser densitometry, and variations in gel lane loading (10–30%) were corrected for by standardizing the data to the 18S RNA results (not shown). The results are presented as the mean $\pm$ SD of 4–6 experiments. kb, kilobase pairs; GAPDH, glyceraldehyde phosphate dehydrogenase; ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate.

The results of the current study indicated that DHT, T, and ASD did not affect the steady-state levels of c-*fos*, c-*jun*, or TGF-β1 mRNAs in hOB cells. Since E_2 has been observed to induce the expression of TGF-β1 and c-*fos* in hOB cells [15, 16], these results suggest that differences exist between the actions of estrogens and androgens on normal human osteoblasts. Moreover, discrepancies may also occur between the actions of androgens on human osteosarcoma cells and hOB cells, since T has been reported to increase c-*fos*, c-*jun*, and TGF-β1 mRNA levels in osteosarcoma cells [6, 17].

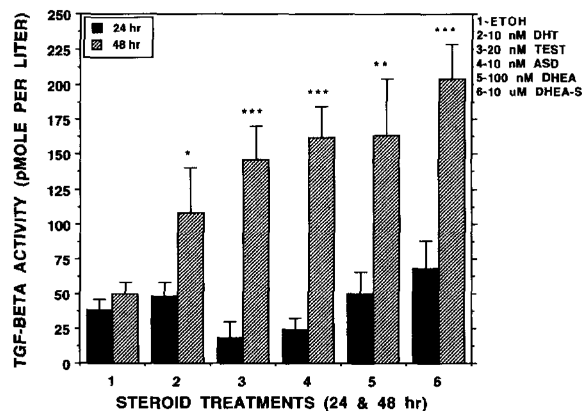

Fig. 4. Induction of total TGF-β activity by gonadal and adrenal androgens in hOB cells conditioned-media. Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 24 h. The cells were then treated for 24–48 h with vehicle or steroids. After treatment, the conditioned-media was saved and processed for the TGF-β bioassay as described in the Materials and Methods. The results are presented as the mean $\pm$ SEM of 3–4 experiments; $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.005$ (Behren's–Fisher t-test) compared to the 48 h ethanol control. Please note that these experiments were performed with confluent cultures of hOB cells ($\sim$3 million cells per T-75 flask); consequently, the cell numbers did not significantly change during the course of these experiments. ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate.

These results also demonstrated that DHEA and DHEA-sulfate rapidly and significantly reduced the steady-state amount of c-*fos* mRNA in hOB cells at approximate physiologic concentrations of the steroids. DHEA and DHEA-sulfate are the most abundant, but least understood, mammalian steroids. While the levels of DHEA in normal human plasma range from 10–50 nM, the concentration of its metabolite, DHEA-sulfate, varies from 2–10 μM [23]. Serum levels of DHEA-sulfate tend to be higher in men than in women, but these levels decline about 5-fold between the ages of 20 and 70 in both sexes [37]. DHEA and DHEA-sulfate are classified as weak androgens, and are 20 times less potent than T [23]. Interestingly, these steroids do not bind to the androgen receptor, and are androgens only by conversion to T and DHT [38]. Unlike other steroids, no convincing evidence is available for the existence of a DHEA receptor [39]. The best mechanistic data suggests that this steroid is an uncompetitive inhibitor of glucose-6-phosphate dehydrogenase with a K_i of 10–20 μM [40, 41]; however, this model cannot explain the repression of c-*fos* message levels by 100 nM DHEA. On the other hand, DHEA-sulfate, which is also a neurosteroid, has been reported to be an allosteric antagonist of the γ-aminobutyric acid-type A ($GABA_A$) receptor in the central nervous system ($K_d = 3\ \mu$M) [42]. The rapid reduction in c-*fos* mRNA levels by DHEA and DHEA-sulfate implies that a receptor-mediated event(s) is involved, but the precise mechanism for this repression is not known. The conversion of these steroids to ASD, T, DHT, and E_2 cannot explain the rapid repression of c-*fos* mRNA levels, since this effect occurred within 30 min and metabolism typically requires 12–24 h in order to reach steady-state conditions [19–22]; moreover, these metabolites either did not affect c-*fos* message levels or induced those levels [16].

Although the amount of TGF-β1 mRNA did not change in response to treatments of the hOB cells with gonadal or adrenal androgens, all of these steroids significantly increased the level of TGF-β activity in the conditioned-media. The observation that DHT and T increased TGF-β production confirms that hOB cells contain functional androgen receptors [5, 13, 18]. Furthermore, the majority of the TGF-β activity present in the hOB cell conditioned-media in response to DHEA-sulfate treatment was immunoprecipitated with a TGF-β2-specific antibody. One interpretation of these results is that DHEA-sulfate and the other adrenal androgens were metabolized to T and DHT by the hOB cells, and that these steroids were responsible for the increase in TGF-β2 production via the androgen receptor. If this were the case, then these data would confirm and extend previous studies which indicated that hOB cells metabolize ASD to T and DHT [19], and that DHT increases TGF-β2 production by these cells [18]. However, it is also

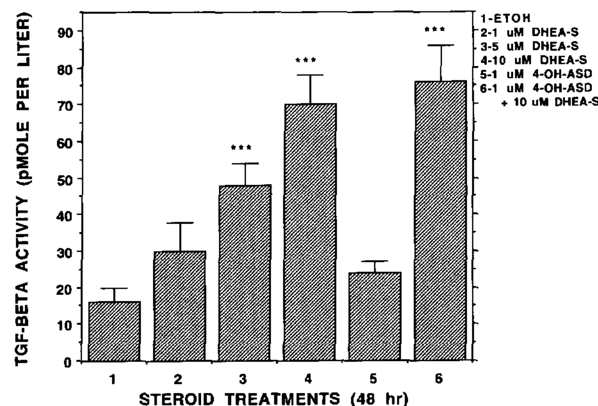

Fig. 5. Induction of total TGF-β activity by DHEA-sulfate in hOB cell conditioned-medium. Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 24 h. The cells were then treated for 48 h with vehicle or steroids. After treatment, the conditioned-media was saved and processed for the TGF-β bioassay as described in the Materials and Methods. The results are presented as the mean $\pm$ SEM of 5–8 experiments; $^{***}P < 0.005$ (Behren's–Fisher t-test) compared to the ethanol control. Please note that these experiments were performed with confluent cultures of hOB cells ($\sim$3 million cells per T-75 flask); consequently, the cell numbers did not significantly change during the course of these experiments. ETOH, ethanol; DHEA-S, DHEA-sulfate; 4-OH-ASD, 4-hydroxyandrostenedione.

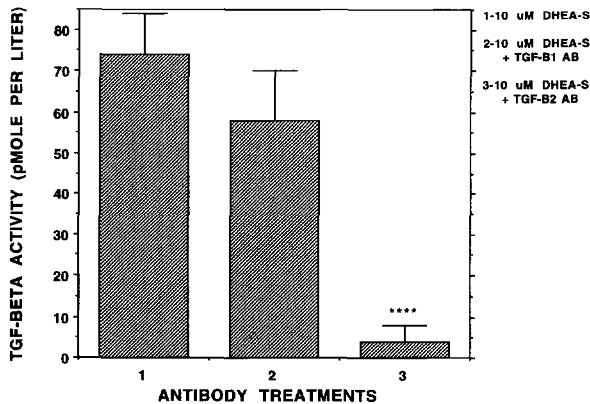

Fig. 6. Induction of TGF-β2 expression by DHEA-sulfate in hOB cell conditioned-medium. Confluent flasks containing pools of male or female hOB cells were rinsed with PBS and placed in BSA-medium for 24 h. The cells were then treated for 48 h with 10 μM DHEA-sulfate. After treatment, the conditioned-media was saved, immunoprecipitated with neutralizing-antibodies specific for TGF-β1 or TGF-β2, and then processed for the TGF-β bioassay as described in the Materials and Methods. The results are presented as the mean $\pm$ SEM of 4–6 experiments; ****$P < 0.001$ (Behren's–Fisher t-test) compared to the no antibody treatment control; $P < 0.01$ compared to the TGF-β1 antibody treatment. Please note that these experiments were performed with confluent cultures of hOB cells ($\sim$3 million cells per T-75 flask); consequently, the cell numbers did not significantly change during the course of these experiments. DHEA-S, DHEA-sulfate; AB, antibody.

conceivable that DHEA-sulfate might have directly stimulated TGF-β2 production by the hOB cells (i.e. without being metabolized). In either case, the current studies indicate that physiologic concentrations of ASD, DHEA, and DHEA-sulfate can have a significant biological effect on human osteoblastic cells. It is important to add that we do not yet know the precise mechanism(s) by which gonadal and adrenal androgens increase TGF-β production by hOB cells (i.e. enhanced expression and/or secretion).

Finally, these data lend support to the concept that adrenal androgens are not only important metabolic sources of sex steroids for bone in postmenopausal women and aging men [19, 21, 43–47], but that these steroids may have more direct actions on this tissue as well [43, 45, 48]. Several clinical investigations have found correlations between decreased serum DHEA and DHEA-sulfate concentrations, and an increased severity of osteoporotic symptoms in both women and men [43–47]. Furthermore, an experimental study with female rats concluded that DHEA has a physiologic role (beyond metabolism to E_2) in maintaining bone balance [48]. Consequently, the current results concerning the rapid repression of c-*fos* mRNA levels and the slower increase in TGF-β2 protein levels by DHEA and DHEA-sulfate in normal human osteoblastic cells may help to provide some mechanisms for the effects of these adrenal steroids on bone.

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