Regulation of Interleukin-6, Osteoclastogenesis, and Bone Mass by Androgens

The Role of the Androgen Receptor

Teresita Bellido,* Robert L. Jilka,* Brendan F. Boyce,§ Giuseppe Girasole,‡ Hal Broxmeyer,‡ Stacie A. Dalrymple,‖ Richard Murray,‖ and Stavros C. Manolagas*

**Division of Endocrinology and Metabolism, the Center for Osteoporosis and Metabolic Bone Diseases, University of Arkansas for Medical Sciences, and the McClellan VA Medical Center GRECC, Little Rock, Arkansas 72205; ‡Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202; §University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284; and ‖DNAX Research Institute, Palo Alto, California 94304*

Abstract

Interleukin-6 is an essential mediator of the bone loss caused by loss of estrogens. Because loss of androgens also causes bone loss, we have examined whether the IL-6 gene is regulated by androgens, and whether IL-6 plays a role in the bone loss caused by androgen deficiency. Both testosterone and dihydrotestosterone inhibited IL-6 production by murine bone marrow-derived stromal cells. In addition, testosterone, dihydrotestosterone, and adrenal androgens inhibited the expression of a chloramphenicol acetyl transferase reporter plasmid driven by the human IL-6 promoter in HeLa cells cotransfected with an androgen receptor expression plasmid; however, these steroids were ineffective when the cells were cotransfected with an estrogen receptor expression plasmid. In accordance with the in vitro findings, orchidectomy in mice caused an increase in the replication of osteoclast progenitors in the bone marrow which could be prevented by androgen replacement or administration of an IL-6 neutralizing antibody. Moreover, bone histomorphometric analysis of trabecular bone revealed that, in contrast to IL-6 sufficient mice which exhibited increased osteoclast numbers and bone loss following orchidectomy, IL-6 deficient mice (generated by targeted gene disruption) did not. This evidence demonstrates that male sex steroids, acting through the androgen-specific receptor, inhibit the expression of the IL-6 gene; and that IL-6 mediates the upregulation of osteoclastogenesis and therefore the bone loss caused by androgen deficiency, as it does in estrogen deficiency. (*J. Clin. Invest.* 1995. 95:2886–2895.) Key words: osteoclast development • testosterone • osteoporosis • adrenal androgens • interleukin-6 knockout mouse

Introduction

A series of recent observations by us and others have elucidated a link between estrogens, IL-6, and the bone loss associated with estrogen deficiency (1). Thus, 17β-estradiol inhibits the production of IL-6 by cells of the stromal/osteoblastic lineage from rodents and humans (2). This effect is apparently mediated at the level of gene transcription, as evidenced by the fact that 17β-estradiol inhibits the stimulated activity of the human IL-6 gene promoter by an estrogen receptor-dependent mechanism (3). Upon loss of ovarian function, IL-6 production is increased in ex vivo cultures of marrow cells from ovariectomized mice, compared with cultures of marrow cells from sham-operated animals (4). Similarly, IL-6 secretion is increased in human bone marrow after menopause or after discontinuation of estrogen replacement therapy (5). Furthermore, loss of ovarian function in mice causes an upregulation of osteoclast precursor formation in the marrow as well as an increase in the number of osteoclasts in trabecular bone. These changes are apparently mediated by IL-6 as evidenced by the ability of an IL-6 neutralizing antibody to prevent them (6). Consistent with this evidence, IL-6–deficient mice do not lose bone upon ovariectomy (7).

Inherited or acquired disorders of testicular function are associated with bone loss. Indeed, congenital male hypogonadism is associated with osteoporosis (8–10); and bone mass in these individuals can be increased by administration of testosterone. Similarly, castration in men causes increased bone resorption and bone loss (11, 12). In addition, low levels of testosterone correlate with decreased bone mineral density and increased risk of fracture in elderly men (13–15). As in humans, orchidectomy in the rat causes bone loss that is characterized by increased endosteal bone resorption, as well as loss of trabecular bone and increased osteoclast numbers. These changes can be prevented by administration of either testosterone or dihydrotestosterone (16).

Based on the evidence linking IL-6, osteoclastogenesis and bone homeostasis with estrogen loss (2–7), and the observation that testosterone also inhibits IL-6 production by bone marrow stromal cells (2), we have hypothesized that the bone sparing effect of androgens is due to their ability to inhibit IL-6 production; and that IL-6 is the mediator of the bone loss that follows loss of testicular function. To test this hypothesis, we have performed in vitro studies to examine the mechanism of the regulation of the IL-6 gene by androgens and the role of the androgen receptor in this effect. In addition, we have investigated whether orchidectomy in mice causes an IL-6–mediated upregulation of osteoclastogenesis and bone loss in the male, as occurs upon ovariectomy in the female.

Methods

Chemicals and reagents. Recombinant human IL-1β, s.a. 1.8×10^7 U/mg, was donated by the Biological Resources Branch of the Biological

Dr. Bellido and Dr. Jilka should both be considered as first authors of this manuscript.

Address correspondence Stavros C. Manolagas, Division of Endocrinology and Metabolism, University of Arkansas for Medical Sciences, 4301 West Markham, Mail Slot 587, Little Rock, AR 72205. Phone: 501-686-5130; FAX: 501-686-8148.

Received for publication 14 March 1994 and in revised form 10 March 1995.

The Journal of Clinical Investigation, Inc.
Volume 95, June 1995, 2886–2895

Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, (Frederick, MD). Recombinant murine TNFα, s.a. 4 × 10^7 U/mg, and recombinant murine IL-6, s.a. 1.66 × 10^8 U/mg, were purchased from Genzyme Corp. (Boston, MA). 17β-estradiol, dexamethasone, diethylstilbestrol (DES), triamcinolone (TRM), testosterone, dihydrotestosterone, androstenediol, androstenedione, dehydroepiandrosterone sulfate, and phorbol 12-myristate 13-acetate (TPA)[1] were purchased from Sigma Chemical Co. (St. Louis, MO). The androgen receptor ligand methyltrienolone (R1881), [17α-methyl-^{3}H] methyltrienolone (^{3}H-R1881, 86.9 Ci/mmol) and promegestone (R5020) were purchased from New England Nuclear (Boston, MA). D-[dichloroacetyl-1-^{14}C]Chloramphenicol (50–60 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Plasmid pCH110 which encodes β-galactosidase was purchased from Pharmacia (Piscataway, NJ). pBluescript II was purchased from Stratagene (La Jolla, CA). pUCAT-1 was generously provided by Dr. Dan Spandau (Indiana University). The human estrogen receptor expression plasmid was provided by Dr. P. Chambon (INSERM Unit 184, Strasbourg, France). The human androgen receptor expression plasmid was provided by Dr. J. W. Pike (Ligand Pharmaceuticals, San Diego, CA).

Cells and culture conditions. The +/+ LDA.11 stromal cell line was established from hematopoietically inactive long-term murine marrow cultures as previously reported (2). Cells were maintained in phenol red-free McCoy's 5A medium (Sigma Chemical Co.) supplemented with 10% FBS (Hyclone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml), at 37°C in 5% CO_2 in air. At confluence, the media were removed and replaced with fresh media containing 2% FBS, and cultures were continued for another 24 h before performing the receptor binding studies. HeLa cells used for the transfection experiments were obtained from American Type Culture Collection (Rockville, MD) and maintained in DME supplemented with 5% FBS.

Quantification of IL-6 secretion by +/+ LDA.11 cells. +/+ LDA.11 cells were seeded in 2 cm^2 wells at 10^5/well and cultured in 1.0 ml of phenol red-free McCoy's 5A medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. After 24 h, the medium was replaced with fresh medium containing 2% FBS without or with various concentrations of testosterone or dihydrotestosterone. 20 h later, recombinant human IL-1β (500 U/ml) and recombinant human TNFα (50 U/ml) were added and the cultures maintained for an additional 24 h. Then, the media was collected and stored at −70°C. The cell layer was rinsed with PBS and cells were harvested by trypsinization and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). IL-6 levels in culture supernatants were determined using the B9 cell bioassay as we have previously described (2). Briefly, B9 cells (5 × 10^3/well of a 96-well plate) were cultured in a series of dilutions of the supernatants in a final volume of 200 μl of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 5 × 10^{-5} M 2-mercaptoethanol and 10% FBS. After 42 h, 0.5 μCi of [^{3}H]thymidine (Amersham, Arlington Hts., IL) was added. 6 h later, the cells were harvested and incorporated radioactivity was counted. IL-6 was quantified using a standard curve set up with known amounts of recombinant murine IL-6. The quantity of IL-6 measured in the culture supernatant of each well was corrected for the number of cells present in each well and is expressed as ng/ml per 10^5 cells. Culture supernatants were diluted at least 400-fold before bioassay. Neither testosterone nor dihydrotestosterone influenced IL-6-stimulated B9 cell proliferation when tested at concentrations corresponding to those present in the diluted culture supernatants (< 2.5 × 10^{-10} M).

Androgen binding studies. Androgen binding studies were performed using cytosol preparations of +/+LDA11 cells as previously described (17), using [^{3}H]R1881 as the ligand. Briefly, 150–200 × 10^6 cells were harvested by trypsinization and washed twice in PBS, resuspended at 4°C in a buffer consisting of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 0.5 mg/ml pepstatin, 1 mg/ml aprotinin, 0.3 M KCl, and 1 mM sodium molybdate. The cells were sonicated on ice (Sonifier cell disrupter, Heat Systems-Ultrasonics, Inc., Plainview, NY), and centrifuged for 1 h at 105,000 *g* in a Beckman L8-55 ultracentrifuge (Beckman Instruments, Palo Alto, CA). Aliquots of the cytosolic fractions (100 μl) were incubated at 4°C for 3 h (in triplicate) with 0.05–1.0 nM [^{3}H]R1881. Parallel aliquots were incubated in the presence of 100-fold molar excess of nonradioactive R1881. At the end of the incubation, bound and free steroids were separated by a 15-min incubation with dextran-coated charcoal suspension on ice (1.25% Norit A and 0.125% Dextran T-70 in 0.1% gelatin buffer containing 0.1 M NaH_2PO_4, 0.15 M NaCl, and 0.015 M sodium azide), and subsequent centrifugation at 2,500 *g* for 20 min. Radioactivity was determined in supernatants using a liquid scintillation counter. Specific binding was determined by subtracting the mean cpm in the presence of unlabeled R1881 (or other unlabeled steroid) from the mean cpm of the corresponding tubes containing only ^{3}H-R1881. The protein concentration of cytosol preparations was determined using a Bio-Rad protein assay kit (Hercules, CA). For Scatchard analysis, the best fit line was determined by linear regression and a 3 test influence diagnostic (Cook's distance, leverage coefficient, and DFFITS) was performed to detect outliers that could have a disproportionately strong influence on the calculation of the regression line (18).

Construction of the reporter plasmid. The human IL-6 promoter was amplified by polymerase chain reaction using human genomic DNA as the template, and primers and conditions described elsewhere (3). The 1.2-kb long promoter was subsequently subcloned into the reporter plasmid pUCAT-1 and its identity with the known sequence of the human IL-6 promoter was confirmed by restriction mapping and sequencing. For the work presented in this paper, a reporter plasmid containing the 225-pb proximal portion of the promoter (IL-6-CAT) was prepared by digesting the 1.2 kb IL-6 promoter-CAT plasmid with BamHI and NheI, filling in with Klenow fragment of DNA polymerase to create blunt ends, and religation.

Transfections. HeLa cells were cultured in DME containing 5% FBS in 10-cm tissue culture dishes. Cells were transfected when they reached 50–60% confluence. 3 h before transfection, cells were washed with PBS and phenol red-free DME medium without serum and containing 0.5% BSA was added. Transfections were performed using the calcium phosphate precipitation method followed by glycerol shock (19). Cells were transfected with 10 μg of the reporter plasmid IL-6-CAT, 5 μg of the androgen receptor or estrogen receptor expression plasmid and 10 μg of pCH110 to provide a measure of transfection efficiency. After transfection, cells were maintained in serum-free media in the absence or presence of the steroids for 16 h, and were then stimulated with TPA (160 nM). 24 h later, cells were harvested and whole-cell extracts were prepared by three freeze/thaw cycles in TEN buffer (0.04 M Tris-HCl, pH 7.4; 1.0 mM EDTA; 0.15 M NaCl).

Assay of reporter gene activity. Chloramphenicol acetyl transferase (CAT) activity in cell extracts was determined after heat treatment at 60°C for 10 min to inactivate cellular deacetylation activity (20). The reaction mixture contained 0.1 μCi ^{14}C-chloramphenicol, 1 μM acetyl CoA and 50–100 μg of cellular protein extract in a total volume of 100 μl; incubation was carried out for 4 h at 37°C. Subsequently, the acetylated [^{14}C]chloramphenicol was separated from the unacetylated by thin layer chromatography in 95% chloroform–5% methanol. Radioactivity was quantified using a beta scanner (AMBIS Systems, Inc., San Diego, CA). β-galactosidase activity of the cell extracts was determined colorimetrically from the rate of hydrolysis of *o*-nitrophenylgalactoside. β-galactosidase activity was not affected by activation of the cells with TPA nor by treatment with the steroids used in this study. Values obtained for CAT activity (percent acetylation) were normalized for β-galactosidase activity. Activities described in the legends of the figures are expressed in terms of the % acetylation per unit of β-galactosidase activity.

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CFU-GM, colony forming units-granulocytes/macrophages; CT, calcitonin; IL-1ra, interleukin-1 receptor antagonist; R1881, methyltrienolone; TPA, phorbol 12-myristate 13-acetate; TRAPase, tartrate-resistant acid phosphatase.

Animals. Male C57B1/6 or Swiss Webster mice, weighing 22–30 g and ~ 60 d old, were obtained from Taconic Farms (Germantown, NY). IL-6–deficient mice were generated using standard homologous recombination techniques in which 95% of exon IV of the IL-6 gene was replaced with the neomycin resistance gene and inserted in E14.1 embryonic stem cells (from strain 12901a) as previously described (21). After establishing germline transmission, animals heterozygous for the IL-6 mutation were intercrossed to yield mice that were homozygous for either the wild type IL-6 gene (designated as "+/+") or the mutated IL-6 gene (designated as "−/−"). Subsequently, a colony of +/+ mice and a colony of −/− mice were maintained by random breeding of the respective homozygous +/+ or −/− mice. Three month old male progeny (four generations after establishing each colony) of either +/+ or −/− homozygous parents were used in these experiments. The IL-6 genotype (+/+ or −/−) was confirmed by Southern blotting using DNA prepared from the distal portion of the tail (Qiagen, Chatsworth, CA). After digestion with BamH1 and electrophoresis, blots were probed with a 1.1-kb EcoRI fragment corresponding to exon V of the IL-6 gene. DNA from wild type mice exhibited a 6-kb band, whereas DNA from IL-6 deficient mice exhibited a 3.7-kb band due to the presence of a BamHI restriction site in the DNA construct inserted into the IL-6 locus (21).

Male mice were sham-operated or orchidectomized. In the case of experiments using C57B1/6 or Swiss-Webster mice, some animals were implanted subcutaneously, immediately after orchidectomy, with pellets containing testosterone (Innovative Research of America, Toledo, OH), which was released from the pellet over a 3-wk period. In the case of experiments lasting 4 wk between orchidectomy and sacrifice of the animals, testosterone pellets were again implanted at 3 wk after the operation. Approximately 24 h after the operation, some orchidectomized animals were injected (i.p.) with 1 mg of a neutralizing rat monoclonal antibody to murine IL-6 (20F3) (22) provided by DNAX Research Institute for Cellular and Molecular Biology (Palo Alto, CA). The IL-6 neutralizing antibody was administered at 7-d intervals thereafter.

Animals were anesthetized and then killed by heart puncture, at either 4 wk (see Fig. 5), or two weeks (see Figs. 6 and 7) after the operation. Serum was collected for determination of total testosterone by radioimmunoassay (Diagnostics Products Corp., Los Angeles, CA) to confirm loss of androgens. The level of testosterone in serum of sham-operated mice was 30–600 ng/ml, whereas it was undetectable (< 10 ng/ml) in serum from orchidectomized mice. Tibiae were removed for histomorphometric analysis and femurs were removed for harvest of bone marrow cells. To isolate marrow cells, the ends of the bones were cut and the cells were obtained from the marrow by flushing with MEM (GIBCO BRL) containing 10% FCS (Sigma Chemical Co.), using a syringe fitted with 25-gauge needle. After the cells were rinsed, the nucleated cell count was determined using a Coulter Counter.

Bone histomorphometry. Tibiae were fixed in 10% neutral buffered formalin, decalcified in 14% EDTA, and embedded in paraffin. Serial sections were cut and stained with Orange G, phloxine B, hematoxylin, and eosin. Three representative sections from each specimen were used for histomorphometric analysis of osteoclast numbers and the percentage of the area of the distal metaphysis occupied by secondary spongiosa bone, as determined with a Bioquant image analysis system (R and M Biometrics, Nashville, TN) and a light microscope with a digitizing tablet and drawing tube attachment. The identity of the specimen was unknown to the examiner.

Clonal growth assays. Marrow cells from each animal were cultured separately to establish three replicate cultures (23). For determination of CFU-GM, cultures of marrow cells were established by plating 10^5 cells in 35-mm tissue culture dishes containing 1 ml of 0.3% agar in McCoys 5A medium with essential and nonessential amino acids, 10% FCS, and 10% pokeweed mitogen-stimulated mouse spleen cell conditioned medium. Cultures were incubated at 37°C in lowered O_2 (5%) and scored after 5 d for colonies (> 50 cells per group). The fraction of CFU-GM in the S-phase of the cell cycle was determined by incubating bone marrow cells (1×10^6/ml) with 50 μCi/ml of [^{3}H]thymidine for 20 min at 37°C before plating for CFU assay. The reduction in the number of colonies after exposure of cells to [^{3}H]thymidine compared to cells not exposed to [^{3}H]thymidine provides an estimate of the number of cells in S-phase (24). The number of CFU-GM per femur was calculated using the marrow cell yield from the animal.

Assay of osteoclast formation in bone marrow cultures. Marrow cells were cultured at 1.5×10^6 per 2-cm^2 well on 13-mm^2 round Thermanox tissue culture cover slips. Marrow cells (4–6 replicate cultures) from each animal were cultured separately and maintained for 9 d in the presence of 10^{-8} M $1,25(OH)_2D_3$, with replacement of half of the medium every 3 d. At the end of the experiment, cells were processed for autoradiographic detection of ^{125}I-calcitonin (CT) binding and tartrate-resistant acid phosphatase (TRAPase) as we have previously described (2, 6). In each experiment, the specificity of the ^{125}I-CT binding was established by the absence of autoradiographic grains on TRAPase-positive cells when incubation with ^{125}I-CT was carried out in the presence of 300 nM CT. The number of osteoclasts formed per femur was calculated using the marrow cell yield from the animal.

Statistics. Data were analyzed by Student's *t* test or by one- or two-way analysis of variance (ANOVA) after performing the Kolmogorov-Smirnov test to establish that the data were normally distributed, and the Spearman rank correlation test to establish homogeneity of variances. Dunnet's test or the Student-Neuman-Keuls test was used to estimate the level of significance of differences between means.

Results

The effect of testosterone and dihydrotestosterone, the non-aromatizable and bioactive metabolite of testosterone, on IL-6 production was compared in murine stromal cells. The +/+LDA11 cells used as a model in these experiments exhibit characteristics of adventitial reticular cells of the marrow stroma, and secrete IL-6 upon stimulation with IL-1 and tumor necrosis factor (TNF) (2). Pretreatment with either testosterone or dihydrotestosterone caused a dose dependent inhibition of IL-1 + TNF-stimulated IL-6 production by +/+LDA11 cells (Table I). The potency of the two steroids was similar.

To investigate whether the inhibitory effect of testosterone and dihydrotestosterone on IL-6 production was mediated by the classical androgen receptor, as opposed to the estrogen receptor that is expressed in these cells (25), we performed binding studies of the androgen analog ^{3}H-R1881 to cytosol preparations of +/+ LDA11 cells. Scatchard analysis of the binding of ^{3}H-R1881 demonstrated the presence of 334±46 high affinity (K_d = 1.15±0.10 × 10^{-9} M) binding sites per cell (Fig. 1 *A*). This binding was specific for the tritiated ligand as evidenced by the ability of unlabeled R1881 to displace ^{3}H-R1881 (Fig. 1 *B*). Consistent with androgen receptor specificity, the binding of ^{3}H-R1881 was not displaced by the progesterone analog R5020, or the synthetic glucocorticoids dexamethasone and triamcinolone, or the synthetic estrogen analog diethylstilbestrol. This binding profile is similar to that displayed by the androgen receptor in osteoblastic cells (26–28).

The role of the androgen receptor in the regulation of IL-6 production was firmly established in experiments whereby HeLa cells were transiently cotransfected with a plasmid containing the proximal 225-pb of the human IL-6 promoter driving the chloramphenicol acetyl transferase (CAT) reporter gene and an androgen receptor expression plasmid. As we have reported earlier (3), the activity of this promoter was stimulated by treatment of the cells with 160 nM phorbol 12-myristate 13-acetate (TPA) for 24 h (Fig. 2 *A*). Pretreatment of the transfected cells with testosterone (10^{-8} M) or dihydrotestosterone (10^{-8} M or 10^{-6} M) completely inhibited the TPA-stimu-

Table I. Effect of Androgens on Cytokine-induced IL-6 Production by +/+ LDA11 Murine Bone Marrow Stromal Cells

Treatment	IL-6
	(ng/ml per 10^5 cells)
IL-1 + TNF	4.27±1.43
IL-1 + TNF + testosterone (10^{-12} M)	3.87±0.33
IL-1 + TNF + testosterone (10^{-11} M)	2.90±0.42*
IL-1 + TNF + testosterone (10^{-10} M)	2.09±0.33*
IL-1 + TNF + testosterone (10^{-9} M)	1.12±0.49*
IL-1 + TNF + testosterone (10^{-8} M)	1.03±0.04*
IL-1 + TNF + testosterone (10^{-7} M)	1.01±0.48*
IL-1 + TNF + dihydrotestosterone (10^{-12} M)	4.05±0.19
IL-1 + TNF + dihydrotestosterone (10^{-11} M)	2.97±0.48*
IL-1 + TNF + dihydrotestosterone (10^{-10} M)	2.31±0.86*
IL-1 + TNF + dihydrotestosterone (10^{-9} M)	1.72±0.43*
IL-1 + TNF + dihydrotestosterone (10^{-8} M)	0.65±0.21*
IL-1 + TNF + dihydrotestosterone (10^{-7} M)	1.41±0.82*

+/+LDA11 cells were cultured for 20 h in the absence or the presence of different concentrations of either testosterone or dihydrotestosterone. Then IL-1 (500 U/ml) and TNF (500 U/ml) were added and cells were maintained for another 24 h in culture. Values indicate means (±SD) of triplicate cultures from one experiment. Data were analyzed by one-way ANOVA. * $P < 0.05$, versus cells not treated with steroids as determined by Dunnet's test. These results were highly reproducible; in replicate experiments, IL-6 production in the presence of 10^{-8} M testosterone or 10^{-8} M dihydrotestosterone was 39.8%±15.8% ($n = 2$) and 51.8%±12.3% ($n = 4$), respectively, of that seen in untreated cells ($P < 0.05$, Student's t test). IL-6 production by cells not stimulated with IL-1 and TNF was less than 0.01 ng/ml per 10^5 cells. Neither testosterone nor dihydrotestosterone had an affect on cell number.

lated transcription of the construct. Although in the experiment shown in Fig. 2 *A*, pretreatment of the cells with 10^{-8} M 17β-estradiol appeared to have a small inhibitory effect on the activity of the IL-6 promoter, in five replicate experiments, the mean effect of 17β-estradiol in HeLa cells cotransfected with the IL-6-CAT construct and the androgen receptor was not statistically different from cells not exposed to any steroids (Fig. 2 *B*). On the other hand, dihydrotestosterone exhibited a consistent inhibiting effect on the activity of the IL-6 promoter. The potency of dihydrotestosterone and testosterone in inhibiting the activity of the IL-6 promoter was similar in these transfection experiments, as it was in the inhibition of bioassayable IL-6 production by +/+LDA11 cells.

The dependency of the inhibiting effect of sex steroids on their respective receptors was determined by examining the effect of testosterone, dihydrotestosterone, and 17β-estradiol, on CAT expression in HeLa cells cotransfected with the IL-6-CAT promoter construct and an estrogen receptor expression plasmid instead of the androgen receptor plasmid. We found that, as opposed to the androgen receptor transfected cells, in HeLa cells transfected with the estrogen receptor plasmid and the IL-6-CAT construct, 17β-estradiol exerted a potent inhibiting effect on the IL-6 promoter activity; whereas testosterone and dihydrotestosterone, even at concentrations as high as 10^{-6} M, were ineffective (Fig. 3). Preincubation of the transfected cells with 17β-estradiol, testosterone or dihydrotestosterone had no effect on TPA-stimulated activity of the IL-6 promoter when cells were cotransfected with a control plasmid (pBluescript)

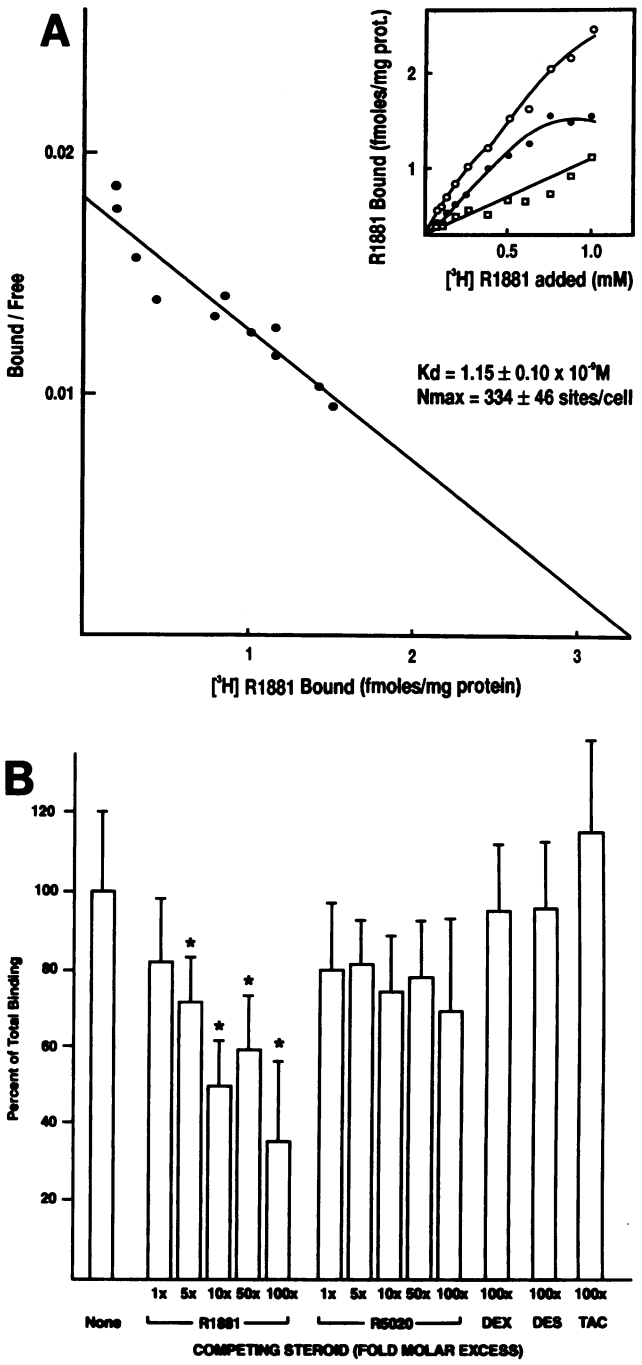

Figure 1. Androgen binding in +/+LDA11 murine marrow stromal cells. (*A*) (*Inset*) Saturation analysis of ^{3}H-R1881 binding. 100 μl of cytosol were incubated with increasing concentration of ^{3}H-R1881 alone (○) or in the presence of 100× unlabeled R1881 (□) for 3 h at 4°C, as described in Methods. The latter conditions were used to estimate nonspecific binding. Bound ^{3}H-R1881 was separated from free ^{3}H-R1881 with 1.0 ml of ice-cold dextran coated charcoal suspension. Specific binding (●) of ^{3}H-R1881, derived by subtracting nonspecific from total binding, was used to generate the Scatchard plot. Each point represents the mean of triplicate determinations. Similar results were obtained in two additional experiments. (*B*) Competition analysis of ^{3}H-R1881 binding. Aliquots of cytosol (100 μl) were incubated with 0.5 nM ^{3}H-R1881 in the absence (none) or in the presence of unlabeled steroids at the indicated molar excess. Bars represent the mean (±SD) of triplicate determinations. Data were analyzed by one-way ANOVA. * $P < 0.05$ vs. "none" as determined by Dunnet's test.

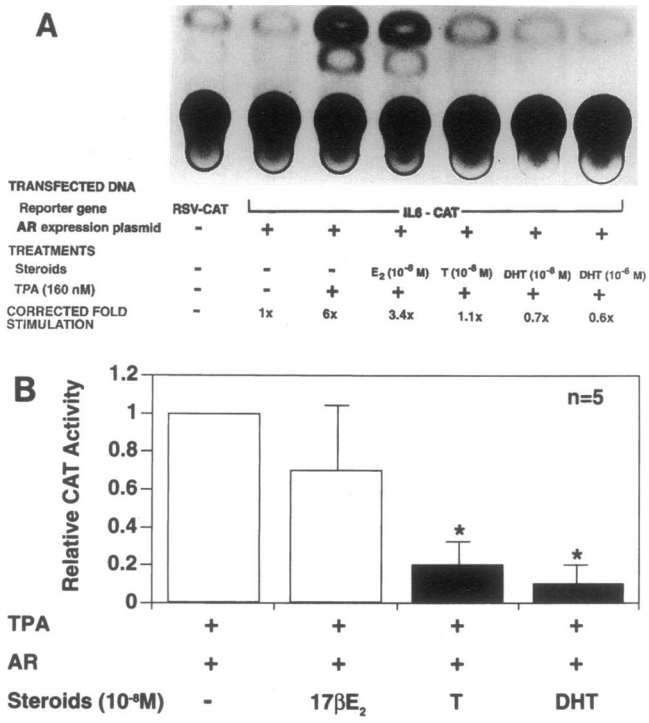

Figure 2. Inhibition of the expression of IL-6-CAT construct by androgens. (*A*) HeLa cells were cotransfected with the proximal 225-bp of the human IL-6 promoter driving the CAT gene (IL6-CAT) and an androgen receptor expression plasmid using the calcium phosphate precipitation method. After transfection, cells were cultured in phenol red-free DME (without serum) in the absence or presence of the indicated steroids. Sixteen hours later, cells were exposed to 160 nM TPA for 24 h, after which cell extracts were prepared and used for assay of CAT activity. An autoradiogram of a representative experiment is shown. For each extract, the relative CAT activity was calculated by determining the percent acetylation of chloramphenicol per unit of β-galactosidase activity, as described in Methods. Relative CAT activity in extracts of cells not treated with TPA was 8.55% acetylation of chloramphenicol per unit of β-galactosidase activity. This value was designated as 1× and was used to calculate the "corrected fold stimulation" of CAT activity in the TPA-treated cells. CAT activity in cells transfected with a plasmid containing the CAT gene driven by a Rous Sarcoma Virus (RSV) promoter is also shown. (*B*) Five replicate experiments similar to the one shown in *A* were performed. In each experiment, the relative CAT activity of extracts from cells treated with TPA in the absence of steroids was designated as 1.0. This value was then used to calculate the relative CAT activity in extracts from cells pretreated with 17β-estradiol, testosterone or dihydrotestosterone in each experiment. Bars represent the mean (±SD) relative CAT activity. Data were analyzed by one-way ANOVA. * $P < 0.05$ vs. cells not treated with steroids as determined by Dunnet's test.

instead of the estrogen receptor or androgen receptor expression plasmid (data not shown).

Adrenal androgens have high affinity for the androgen receptor (29). We have therefore also examined whether androstenediol, androstenedione or dehydroepiandrosterone sulfate had an effect on the transcriptional activity of the IL-6 promoter. Pretreatment of HeLa cells cotransfected with the IL-6 reporter plasmid and the androgen receptor expression plasmid with adrenal androgens caused inhibition of the activity of the IL-6 promoter (Fig. 4).

The relevance of the in vitro observations to the in vivo

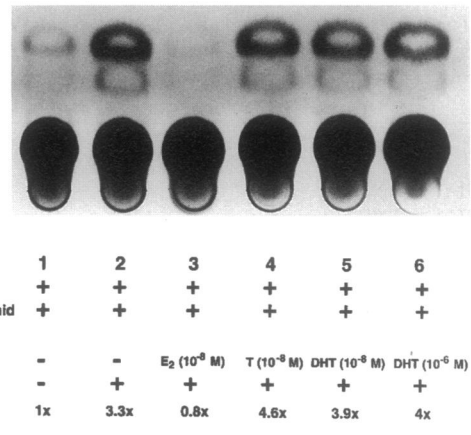

Figure 3. Lack of effect of androgens on ER-mediated suppression of IL-6-CAT activity. HeLa cells were cotransfected with the IL-6–CAT construct and an estrogen receptor expression plasmid. After transfection cells were incubated in the absence or in the presence of the indicated steroids, stimulated with TPA, and extracts prepared for CAT assay. An autoradiogram of a representative experiment is shown. The corrected fold stimulation of CAT activity was calculated as described in Fig. 2 *A*. Relative CAT activity in extracts of cells not treated with TPA was 24.57% acetylation of chloramphenicol per unit of β-galactosidase activity.

situation was assessed by performing orchidectomy in mice and examining its impact on the bone. Orchidectomy caused a decrease in the volume of trabecular bone in the secondary spongiosa of the proximal metaphysis of the tibia (Fig. 5 *A*); as well as an increase in the number of osteoclasts (Fig. 5 *B*). Orchidectomized animals implanted with slow-release pellets containing 0.5 or 2.5 mg of testosterone immediately after orchi-

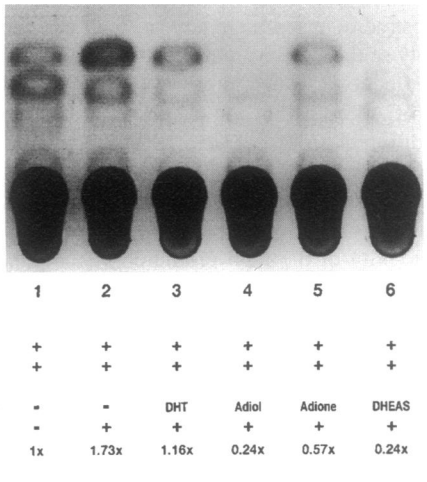

Figure 4. Inhibition of the expression of IL-6–CAT construct by adrenal androgens. HeLa cells were cotransfected with the IL-6–CAT construct and an androgen receptor expression plasmid. After transfection, cells were incubated as in Fig. 2 *A* in the absence or in the presence of the indicated androgens, stimulated with TPA, and extracts prepared for CAT assay. An autoradiogram of a representative experiment is shown. Corrected fold stimulation of CAT activity was calculated as described in Fig. 2 *A*. Relative CAT activity in extracts of cells not treated with TPA was 6.85% acetylation of chloramphenicol per unit of β-galactosidase activity.

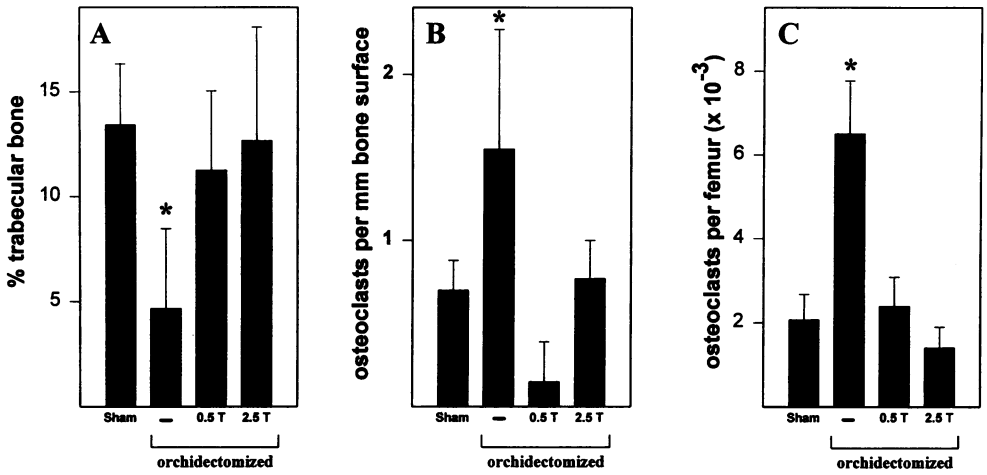

Figure 5. Effect of orchidectomy and testosterone replacement on trabecular bone and osteoclastogenesis. Male C57B1/6 mice were sham operated or orchidectomized. Orchidectomized animals were either left untreated or were implanted with slow release pellets containing 0.5 mg of testosterone (0.5 T) or 2.5 mg of testosterone (2.5 T). 4 wk after the operation, mice (four per group) were killed and the tibiae and femur were removed. The tibiae were placed into 10% neutral-buffered formalin and processed for measurement of the percentage of the area of the distal metaphysis occupied by trabecular bone of the secondary spongiosa (*A*) and the number of osteoclasts in this trabecular bone (*B*), as described in Methods. To assess the ability of the marrow to form osteoclasts (*C*), replicate ($n = 4-6$) cultures of marrow cells obtained from the femurs of these animals were established from each animal and maintained for 9 d in the presence of 10 nM $1,25(OH)_2D_3$. Osteoclast formation was quantified by enumerating cells which bind ^{125}I-CT and stain for TRAPase as described in Methods. The number of osteoclasts per femur formed in ex-vivo cultures of marrow was calculated by using the marrow nucleated cell yield. Bars represent the mean (±SD) % trabecular bone in the secondary spongiosa of the proximal tibia (*A*); the mean (±SD) number of osteoclasts per mm of secondary spongiosa bone surface in the proximal tibia (*B*); or mean (±SEM) number osteoclasts per femur formed in ex vivo cultures of marrow cells (*C*). Data were analyzed by one-way ANOVA. * $P < 0.05$ vs. sham operated mice, orchidectomized mice treated with 0.5 mg or 2.5 mg testosterone, as determined by the Student-Neuman-Keuls test. Similar data was obtained in an additional experiment using Swiss-Webster mice.

dectomy did not exhibit bone loss or increased numbers of osteoclasts. Because osteoclasts originate from hematopoietic precursors of the bone marrow, we also examined the effect of orchidectomy on osteoclast formation in ex-vivo cultures of marrow cells obtained from the femur of the same animals. Osteoclasts in the bone marrow cell cultures were identified by TRAPase staining and ^{125}I-CT binding, a combination of features unique to osteoclasts (30). Orchidectomy caused an increase in osteoclast formation in ex-vivo cultures of marrow cells, which was prevented by administration of testosterone (Fig. 5 *C*).

In a different experiment, ex vivo cultures of marrow cells were established from sham-operated or orchidectomized animals; and the number of CFU-GM, the presumed osteoclast progenitor, was determined. Orchidectomy had a relatively small effect on the absolute number of CFU-GM in the marrow (Fig. 6), but greatly increased CFU-GM replication, as assessed by determining the number of CFU-GM in S-phase. Administration of either testosterone (0.5 mg in slow release pellets) or weekly injections of 1 mg of an IL-6 neutralizing antibody, prevented the increase in CFU-GM numbers; and both of these agents completely suppressed CFU-GM cycling in orchidectomized mice to levels below that of sham-operated mice.

The role of IL-6 in orchidectomy-induced bone loss and upregulation of osteoclast formation was also examined by comparing the effects of orchidectomy in IL-6 sufficient and IL-6 deficient mice generated by targeted gene disruption (21). Unlike the experiments shown in Fig. 5 where we examined orchidectomized animals at 4 wk after the operation, in these studies, orchidectomized animals were studied at 2 wk after the operation. The reason for this change in the experimental protocol was to optimize our ability to detect changes in osteoclast numbers in bone sections. The amount of trabecular bone was not significantly different between sham-operated IL-6 replete vs. IL-6 deficient mice (Fig. 7 *A*). Similarly, there was no difference in the number of osteoclasts per mm bone surface between sham-operated IL-6 sufficient and sham-operated IL-6 deficient mice (Fig. 7 *B*). However, orchidectomy caused loss of trabecular bone in the secondary spongiosa of the tibia of the IL-6 replete mice, but it had no such effect in the IL-6 deficient mice (Fig. 7 *A*). Furthermore, orchidectomy caused an increase in the number of osteoclasts identified in sections of tibia from the IL-6 sufficient mice. This change was absent in the bone of the orchidectomized IL-6 deficient mice (Fig. 7 *B*). Consistent with the histomorphometric data, osteoclast formation in bone marrow cultures from orchidectomized IL-6 sufficient mice was increased when compared with sham-operated controls. In contrast, orchidectomy had no effect on osteoclast formation in bone marrow cell cultures from the IL-6 deficient mice (Fig. 7 *C*). In these experiments, we have utilized +/+ and −/− mice that are fourth generation descendants of the original intercross (F1) 12901a × C57B1/6 founder animals. Therefore, there might be slight strain background variation between the two groups. Nonetheless, it is very unlikely that such small variation could account for their strikingly different bone response to orchidectomy.

Discussion

Because of the striking similarities between the adverse effects of estrogen loss and androgen loss on bone homeostasis, we have investigated here the possibility that similar molecular, biochemical and cellular mechanisms underlie the bone protective effects of both classes of sex steroids.

Our findings demonstrate that stromal cells of the marrow contain androgen receptors. This observation, together with the demonstration of receptors for estrogens in the same cells (25), indicates that the marrow stroma has the necessary prerequisites

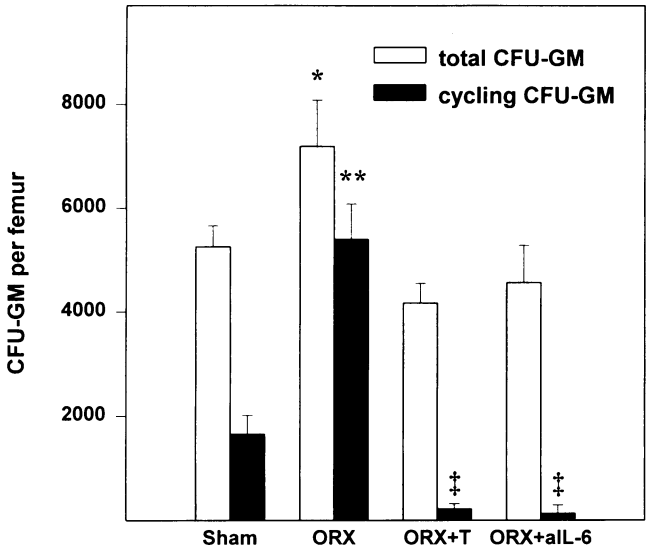

Figure 6. Effect of testosterone and IL-6 neutralizing antibody on CFU-GM in the bone marrow of orchidectomized mice. Swiss-Webster male mice were either sham-operated or orchidectomized. Orchidectomized animals were then left untreated (ORX) or were implanted with pellets containing 0.1 mg testosterone (ORX + T), or received weekly intraperitoneal injections of 1 mg of an IL-6 neutralizing antibody (ORX + aIL-6). 2 wk after surgery, animals were killed and bone marrow cells were obtained from the femur; the total number of CFU-GM and the number of cycling CFU-GM in the femur of each animal was determined as described in Methods. Open bars represent the mean (±SEM) number of CFU-GM per femur; closed bars represent the mean (±SEM) number of cycling CFU-GM per femur ($n = 4$ animals per group). Data were analyzed by one-way ANOVA * $P < 0.05$, vs. Sham, ORX + T, and ORX + aIL-6 total CFU-GM; *** $P < 0.01$ versus Sham, ORX + T, and ORX + aIL-6 cycling CFU-GM; ‡ $P < 0.01$ versus Sham cycling CFU-GM as determined by the Student-Neuman-Keuls test. Similar data was obtained in a second experiment.

to respond to either class of sex steroids. Our estimate of the concentration of the androgen receptor in the bone marrow-derived stromal cells is similar to the concentrations reported in osteoblastic cells (26–28). In addition, the affinity for the analog R1881 and the binding specificity of the androgen receptor in the bone marrow stromal cells is practically identical to the affinity and specificity of the androgen receptor in prostate and osteoblastic cells ($\sim 10^{-10}$ M) (26–29). A portion of the binding of the labeled androgen analog could not be displaced by the unlabeled analog in our experiments. This may be due to the presence of residual low affinity androgen binding proteins such as sex steroid binding globulins from the serum, or other low affinity intracellular binding proteins (28).

We have previously reported that, like 17β-estradiol, testosterone inhibits IL-6 production by murine bone marrow derived stromal cells (2). However we did not know whether the inhibiting effect of testosterone on IL-6 production was mediated by the estrogen receptor or the androgen receptor; or whether the effect of testosterone was mediated by estrogenic metabolites of testosterone that were produced upon in vitro aromatization (31, 32). The demonstration of androgen receptors in stromal cells of the bone marrow, taken together with the finding that dihydrotestosterone can inhibit IL-6 production as effectively as testosterone, suggests that testosterone regulates IL-6 production directly, rather than through the conversion of testosterone to estrogenic metabolites.

The role of the androgen receptor in the androgen-induced inhibition of IL-6 was firmly established in these studies by the demonstration that the androgen receptor expression plasmid is required for eliciting the inhibiting effect of testosterone on the activity of the IL-6 promoter. In fact, the results of the experiments where HeLa cells were cotransfected with either the androgen receptor or the estrogen receptor expression plasmid indicate that whereas both androgens and estrogens can inhibit IL-6, their effects are strictly dependent on the expression of their respective receptor, and cannot be due to actions of androgens mediated by the estrogen receptor or vice versa.

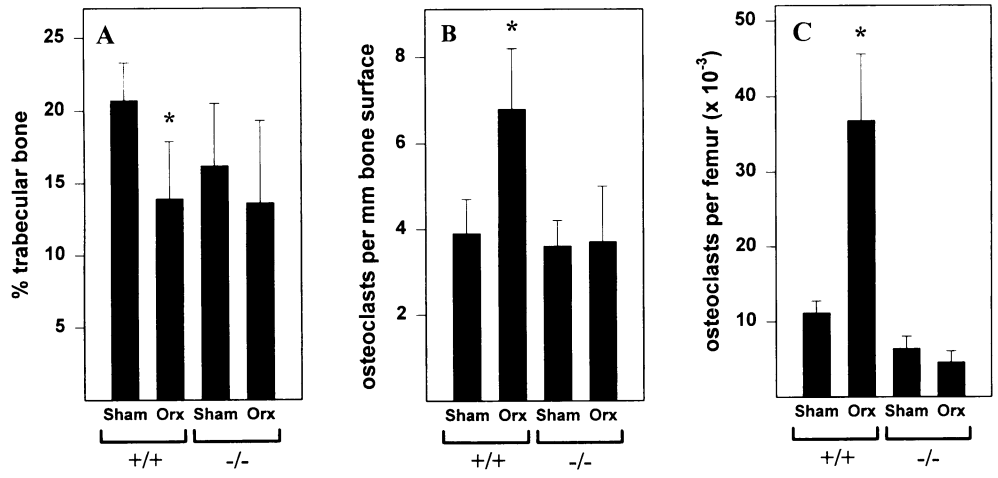

Figure 7. Effect of orchidectomy on trabecular bone and osteoclastogenesis in normal and IL-6 deficient mice. Normal mice (+/+) or IL-6 deficient mice (−/−) were either sham-operated or orchidectomized (ORX). 2 wk after surgery, animals (6 or 7 per group) were killed and the tibiae and femur removed. The percentage of the area of the distal metaphysis occupied by trabecular bone of the secondary spongiosa (*A*); the number of osteoclasts present in this trabecular bone (*B*); and the number of osteoclasts formed in ex-vivo cultures of marrow (*C*) was determined as described in Fig. 5. Bars represent the mean (±SD) % trabecular bone in the secondary spongiosa of the proximal tibia (*A*); the mean (±SD) number of osteoclasts per mm of secondary spongiosa bone surface in the proximal tibia (*B*); or mean (±SEM) number osteoclasts per femur formed in ex vivo cultures of marrow cells. Data were analyzed by two-way ANOVA. * $P < 0.05$ vs. sham operated +/+ mice. These results were reproduced in an additional experiment.

In our earlier studies, we had determined that the effect of estradiol on the transcriptional activity of the human IL-6 promoter is not mediated by direct interaction of the human estrogen receptor with the IL-6 promoter, but perhaps through an interference with events along the signal pathways initiated by the IL-6 stimulating agents (3). Identical conclusions were reached in studies reported by Ray et al. (33). We have not examined here whether the androgen receptor interacts directly with the IL-6 promoter. Nonetheless, the 225-bp segment of the IL-6 promoter used in the transient transfection experiments does not contain consensus DNA binding sites for the androgen receptor, nor does it contain glucocorticoid response elements which can bind the androgen receptor (34–37). In view of this, and the results of our earlier studies with estrogens, it is likely that the androgen receptor acts to inhibit the activity of the IL-6 promoter indirectly.

In accordance with the in vitro evidence for the inhibition of IL-6 gene expression by androgens and the role of this cytokine in osteoclast development (38–40), the results of the in vivo studies presented in this paper demonstrate that loss of androgens causes an IL-6–mediated increase in osteoclast formation and bone loss. In fact, the evidence that IL-6 knockout mice are protected from the loss of trabecular bone caused by orchidectomy argues in favor of the contention that IL-6–mediated upregulation of osteoclastogenesis in the bone marrow is the essential triggering event of this bone loss. The findings from the orchidectomized IL-6 deficient mice are practically identical to those of Poli et al. demonstrating that IL-6 knockout mice fail to upregulate CFU-GM and osteoclasts, and are protected from the loss of bone that follows ovariectomy (7). Hence, similar molecular and cellular mechanisms underlie the pathogenesis of bone loss following the loss of either female or male sex steroids.

Orchidectomy produced a much greater increase in the number of dividing CFU-GM, as compared to the total number of CFU-GM. Consistent with this evidence, administration of either testosterone or the IL-6 neutralizing antibody produced a greater decrease in the number dividing CFU-GM as compared to the total number of CFU-GM. These observations are in agreement with evidence that IL-6 promotes differentiation of hematopoietic cells by stimulating cell division of early progenitors (41–43); moreover, they strengthen the view that the cascade of events leading to bone loss, following loss of sex steroids, starts in the hematopoietic bone marrow.

An association between reduced levels of dehydroepiandrosterone sulfate and osteoporosis in humans (44–46) along with evidence that ovariectomy-induced bone loss in the rat is reduced by administration of dehydroepiandrosterone (47) has led to the idea that adrenal androgens may have bone protective properties. Our findings that dehydroepiandrosterone sulfate, androstenediol, and androstenedione suppress the activity of the human IL-6 promoter raise the possibility that the bone protective effects of this class of steroids as well might be due to their ability to suppress the production of IL-6.

Although the above evidence leads to the irrefutable conclusion that IL-6 is an essential mediator of the bone loss that occurs upon loss of either female or male sex steroids, other observations have led to the suggestion that IL-1 and TNF also play a role in the upregulation of osteoclastogenesis and the loss of bone that follows loss of ovarian function. Specifically, using a bioassay to quantify IL-1, Pacifici and coworkers have reported that spontaneous IL-1 production by cultured peripheral blood monocytes is elevated in patients with high-turnover osteoporosis (48). These findings, however, could not be confirmed by the results of studies whereby IL-1 levels were determined by an ELISA as opposed to the bioassay (49, 50). More recently, Kimble et al. (51) and Kitazawa et al. (52), have reported that IL-1 bioactivity is increased in the medium of cultures of bone marrow cells from ovariectomized rats and mice. Nonetheless, Miyaura et al. determined that IL-1 levels measured by an ELISA in bone marrow cell cultures from ovariectomized mice were not different from those in cultures from sham operated controls; however, when IL-1 was measured by a bioassay, its level was higher in the former (53). In direct conflict with the contention that IL-1 production increases after loss of estrogens, Hu et al. (54) and Flynn (55) have found that estrogens stimulate IL-1 production by rat peritoneal macrophages in vitro, and that ovariectomy decreases and estrogen replacement increases IL-1 production by these cells. Notably, Flynn observed that testosterone had no effect on IL-1. In line with these later studies, Keeting et al. reported that 17β-estradiol increases the levels of IL-1 mRNA in human osteoblastic cells (56). On the other hand, H. Fox (personal communication) has found that 17β-estradiol has no effect on IL-1 expression in the monocytic P388D1 and J774 cell lines, and in bone marrow-derived macrophages; whereas it does inhibit IL-6 production in all these cells. Taken together with the well recognized limitations of the IL-1 bioassay (57), including vulnerability to inhibitory molecules and the synergistic effects of more than one cytokine on the bioassay target cells, these observations dispute the contention that the IL-1 gene is upregulated following loss of estrogens (or androgens). Hence, the suggestion that IL-1 mediates the bone loss caused by loss of gonadal function lacks the prerequisite mechanistic foundation.

The unsubstantiated evidence for the upregulation of IL-1 after loss of estrogens notwithstanding, Kimble et al. (51) reported that administration of IL-1 receptor antagonist (IL-1ra) immediately following ovariectomy had only a small effect compared to 17β-estradiol in preventing the ovariectomy-induced bone loss in the rat, as measured by densitometry. However, when IL-1ra was administered 4 wk after ovariectomy, it was as effective as 17β-estradiol in preventing subsequent bone loss. On the other hand, Kalu et al. found that IL-1ra administration initiated at 10 days following ovariectomy in the rat did not prevent the bone loss manifested at one month following the operation, when bone was analyzed by quantitative histomorphometry (58). Unlike their findings in the rat, Kitazawa et al. more recently reported that administration of the IL-1ra or even a TNF binding protein to ovariectomized mice immediately following the operation decreased osteoclast formation in vitro and the urinary excretion of pyridinoline cross-links (a marker of bone resorption), as did administration of 17β-estradiol (52).

Work from our laboratory may help to reconcile some of these apparent discrepancies. Indeed, we have found that blockade of the activity of IL-1, TNF, IL-11, GM-CSF, or TGFβ in vitro abrogates osteoclast formation in murine marrow cultures irrespective of the estrogen status of the donor animal, suggesting strongly that each of these cytokines is essential for osteoclast development (59, 60). Therefore, IL-1 and TNF antagonists may well inhibit the bone loss caused by the IL-6–mediated increased osteoclastogenesis, without needing to invoke upregulation of IL-1 or TNF production. In any event, the evidence that IL-6 knockout mice are protected from the

increased osteoclastogenesis and bone loss that follows the loss of either estrogens or androgens, suggests strongly that upregulation of IL-6 is the triggering event for these phenomena and that the putative increases in IL-1 or TNF after ovariectomy are, at best, consequences of the removal of the inhibitory effects of sex steroids on IL-6.

In summary, our findings and those of others strongly suggest that the cellular and molecular mechanisms which lead to the bone loss caused by androgen deficiency in the male are similar, if not identical, to the mechanisms which underlie the bone loss caused by estrogen deficiency in the female and that IL-6 is an essential pathogenetic factor in both of these conditions. Whether IL-6 is the sole pathogenetic factor will remain uncertain until definitive evidence for or against direct regulation of other cytokines by sex steroids in bone marrow cells is obtained.

Acknowledgments

The authors thank Dr. Giovanni Passeri, Beryl Story, Jena Derrick, Scott Cooper, and Dr. Hanna Mocharla for their help in various aspects of this work; and Niki Barker for the preparation of the manuscript.

This work was supported by National Institutes of Health grants AR-41313 and AR-43003 and by the Department of Veterans Affairs.

References

1. Manolagas, S. C., and R. L. Jilka. 1995. Bone marrow, cytokines and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N. Engl. J. Med.* 332:305–311.

2. Girasole, G., R. L. Jilka, G. Passeri, S. Boswell, G. Boder, D. C. Williams, and S. C. Manolagas. 1992. 17β-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in-vitro: a potential mechanism for the antiosteoporotic effect of estrogens. *J. Clin. Invest.* 89:883–891.

3. Pottratz, S., T. Bellido, H. Mocharla, D. Crabb, and S. C. Manolagas. 1994. 17β-estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. *J. Clin. Invest.* 93:944–950.

4. Passeri, G., G. Girasole, R. L. Jilka, and S. C. Manolagas. 1993. Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. *Endocrinology.* 133:822–828.

5. Bismar, H., I. Diel, R. Ziegler, and J. Pfeilschifter. 1994. Increased cytokine secretion by human bone marrow cells after menopause or discontinuation of estrogen replacement. *J. Bone Miner. Res.* 9(Suppl 1):S158. (Abstr.)

6. Jilka, R. L., G. Hangoc, G. Girasole, G. Passeri, D. C. Williams, J. S. Abrams, B. Boyce, H. Broxmeyer, and S. C. Manolagas. 1992. Increased osteoclast development after estrogen loss: mediation by interleukin-6. *Science (Wash. DC).* 257:88–91.

7. Poli, V., R. Balena, E. Fattori, A. Markatos, A. Yamamoto, H. Tanaka, G. Ciliberto, G. A. Rodan, and F. Costantini. 1994. Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1189–1196.

8. Greenspan, S. L., R. M. Neer, E. C. Ridgway, and A. Klibanski. 1986. Osteoporosis in men with hyperprolactinemic hypogonadism. *Ann. Intern. Med.* 104:777–782.

9. Finkelstein, J. S., A. Klibanski, R. M. Neer, S. H. Doppelt, D. I. Rosenthal, G. V. Segre, and W. F. Crowley, Jr. 1989. Increases in bone density during treatment of men with idiopathic hypogonadotropic hypogonadism. *J. Clin. Endocrinol. Metab.* 69:776–783.

10. Seeman, E., L. J. Melton, W. M. O'Fallon, and B. L. Riggs. 1983. Risk factors for spinal osteoporosis in men. *Am. J. Med.* 75:977–983.

11. Stepan, J. J., M. Lachman, J. Zverina, V. Pacovsky, and D. J. Baylink. 1989. Castrated men exhibit bone loss: effect of calcitonin treatment on biochemical indices of bone remodeling. *J. Clin. Endocrinol. Metab.* 69:523–527.

12. Goldray, D., Y. Weisman, N. Jaccard, C. Merdler, J. Chen, and H. Matzkin. 1993. Decreased bone density in elderly men treated with the gonadotropin-releasing hormone agonist decapeptyl (D-Trp^6-GnRH). *J. Clin. Endocrinol. Metab.* 76:288–290.

13. Murphy, S., K. Khaw, A. Cassidy, and J. E. Compston. 1993. Sex hormones and bone mineral density in elderly men. *Bone Miner.* 20:133–140.

14. Jackson, J. A., M. W. Riggs, and A. M. Spiekerman. 1992. Testosterone deficiency as a risk factor for hip fractures in men: a case-control study. *Am. J. Med. Sci.* 304:4–8.

15. Stanley, H. L., B. P. Schmitt, R. M. Poses, and W. P. Deiss. 1991. Does hypogonadism contribute to the occurrence of a minimal trauma hip fracture in elderly men? *J. Am. Geriatr. Soc.* 39:766–771.

16. Wakley, G. K., H. D. Schutte, Jr., K. S. Hannon, and R. T. Turner. 1991. Androgen treatment prevents loss of cancellous bone in the orchidectomized rat. *J. Bone Miner. Res.* 6:325–330.

17. Grino, P. B., J. E. Griffin, and J. D. Wilson. 1987. Transformation of the androgen receptor to the deoxyribonucleic acid-binding state: studies in homogenates and intact cells. *Endocrinology.* 120:1914–1920.

18. Glantz, S., and B. K. Slinder. 1990. Primer of Applied Regression and Analysis of Variance. W. Day and M. Navrozov, editors. McGraw-Hill, Inc., New York. 110–141.

19. Graham, F. L., and A. J. van der Eb. 1973. Transformation of rat cells by DNA of human adenovirus 5. *Virology.* 54:536–539.

20. Crabb, D. W., C. D. Minth, and J. E. Dixon. 1989. Assaying the reporter gene chloramphenicol acetyl transferase. *Methods Enzymol.* 168:690–701.

21. Dalrymple, S. A., L. A. Lucian, R. Slattery, T. McNeil, D. M. Aud, S. Fuchino, F. Lee, and R. Murray. 1995. IL-6 deficient mice are highly susceptible to *L. monocytogenes* infection: correlation with inefficient neutrophilia. *Infect. Immun.* In press.

22. Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J.-C. Zou, and J. S. Abrams. 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. *J. Immunol.* 145:4185–4191.

23. Maze, R., B. Sherry, B. S. Kwon, A. Cerami, and H. E. Broxmeyer. 1992. Myelosuppressive effects in vivo of purified recombinant murine macrophage inflammatory protein-1 alpha. *J. Immunol.* 149:1004–1009.

24. Broxmeyer, H. E., D. E. Williams, G. Hangoc, S. Cooper, P. Gentile, R. N. Shen, P. Ralph, S. Gillis, and D. Bicknell. 1987. The opposing actions in vivo on murine myelopoiesis of purified preparations of lactoferrin and their colony inhibiting factors. *Blood Cells.* 13:31–48.

25. Bellido, T., G. Girasole, G. Passeri, X. Yu, H. Mocharla, R. L. Jilka, A. Notides, and S. C. Manolagas. 1993. Demonstration of estrogen and vitamin D receptors in bone marrow derived stromal cells: upregulation of the estrogen receptor by $1,25(OH)_2D_3$. *Endocrinology.* 133:553–562.

26. Colvard, D. S., E. F. Eriksen, P. E. Keeting, E. M. Wilson, D. B. Lubahn, F. S. French, B. L. Riggs, and T. C. Spelsberg. 1989. Identification of androgen receptors in normal human osteoblast-like cells. *Proc. Natl. Acad. Sci. USA* 86:854–857.

27. Orwoll, E. S., L. Stribrska, E. E. Ramsey, and E. J. Keenan. 1991. Androgen receptors in osteoblast-like cell lines. *Calcif. Tissue Int.* 49:183–187.

28. Benz, D. J., M. R. Haussler, M. A. Thomas, B. Speelman, and B. S. Komm. 1991. High-affinity androgen binding and androgenic regulation of α_1(I)-procollagen and transforming growth factor-β steady state messenger ribonucleic acid levels in human osteoblast-like osteosarcoma cells. *Endocrinology.* 128:2723–2730.

29. Wilson, E. M., and F. S. French. 1976. Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis, and prostate. *J. Biol. Chem.* 251:5620–5629.

30. Hattersley, G., and T. J. Chambers. 1989. Calcitonin receptors as markers for osteoclastic differentiation: correlation between generation of bone-resorptive cells and cells that express calcitonin receptors in mouse bone marrow cultures. *Endocrinology.* 125:1606–1612.

31. Purohit, A., A. M. Flanagan, and M. J. Reed. 1992. Estrogen synthesis by osteoblast cell lines. *Endocrinology.* 131:2027–2029.

32. Tanaka, S., M. Haji, Y. Nishi, T. Yanase, R. Takayanagi, and H. Nawata. 1993. Aromatase activity in human osteoblast-like osteosarcoma cell. *Calcif. Tissue Int.* 52:107–109.

33. Ray, A., K. E. Prefontaine, and P. Ray. 1994. Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J. Biol. Chem.* 269:12940–12946.

34. Tanabe, O., S. Akira, T. Kamiya, G. G. Wong, T. Hirano, and T. Kishimoto. 1988. Genomic structure of the murine IL-6 gene. High degree conservation of potential regulatory sequences between mouse and human. *J. Biol. Chem.* 141:3875–3881.

35. Roche, P. J., S. A. Hoare, and M. G. Parker. 1992. A consensus DNA-binding site for the androgen receptor. *Mol. Endocrinol.* 6:2229–2235.

36. Claessens, F., L. Celis, B. Peeters, W. Heyns, G. Verhoeven, and W. Rombauts. 1989. Functional characterization of an androgen response element in the first intron of the C3(1) gene of prostatic binding protein. *Biochem. Biophys. Res. Commun.* 164:833–840.

37. De Vos, P., F. Claessens, B. Peeters, W. Rombauts, W. Heyns, and G. Verhoeven. 1993. Interaction of androgen and glucocorticoid receptor DNA-binding domains with their response elements. *Mol. Cell. Endocrinol.* 90:R11–R16.

38. Ishimi, Y., C. Miyaura, C. H. Jin, T. Akatsu, E. Abe, Y. Nakamura, A. Yamaguchi, S. Yoshiki, T. Matsuda, T. Hirano, T. Kishimoto, and T. Suda. 1990. IL-6 is produced by osteoblasts and induces bone resorption. *J. Immunol.* 145:3297–3303.

39. Black, K., I. R. Garrett, and G. R. Mundy. 1991. Chinese hamster ovarian cells transfected with the murine interleukin-6 gene cause hypercalcemia as well

as cachexia, leukocytosis and thrombocytosis in tumor-bearing nude mice. *Endocrinology.* 128:2657–2659.

40. Lowik, C. W. G. M., G. van der Pluijm, H. Bloys, K. Hoekman, O. L. M. Bijvoet, L. A. Aarden, and S. E. Papapoulos. 1989. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem. Biophys. Res. Comm.* 162:1546–1552.

41. Liechty, K. W., and R. D. Christensen. 1990. In vivo effect of interleukin-6 on cycling status of hematopoietic progenitors from adults and neonates. *Pediatr. Res.* 28:323–326.

42. Pojda, Z., and A. Tsuboi. 1990. In vivo effects of human recombinant interleukin 6 on hemopoietic stem and progenitor cells and circulating blood cells in normal mice. *Exp. Hematol.* 18:1034–1037.

43. Emerson, S. G. 1991. The stem cell model of hematopoiesis. *In* Hematology. Basic Principles and Practice. R. Hoffman, E. J. Benz, S. J. Shattil, B. Furie, and H. J. Cohen, editors. Churchill Livingstone, New York. 72–81.

44. Spector, T. D., P. W. Thompson, L. A. Perry, H. H. McGarrigle, and A. C. Edwards. 1991. The relationship between sex steroids and bone mineral content in women soon after the menopause. *Clin. Endocrinol. (Oxf).* 34:37–41.

45. Nordin, B. E., A. Robertson, R. F. Seamark, A. Bridges, J. C. Philcox, A. G. Need, M. Horowitz, H. A. Morris, and S. Deam. 1985. The relation between calcium absorption, serum dehydroepiandrosterone, and vertebral mineral density in postmenopausal women. *J. Clin. Endocrinol. Metab.* 60:651–657.

46. Sambrook, P., J. Birmingham, D. Champion, P. Kelly, S. Kempler, J. Freund, and J. Eisman. 1992. Postmenopausal bone loss in rheumatoid arthritis: effect of estrogens and androgens. *J. Rheumatol.* 19:357–361.

47. Turner, R. T., E. T. Lifrak, M. Beckner, G. K. Wakley, K. S. Hannon, and L. N. Parker. 1990. Dehydroepiandrosterone reduces cancellous bone osteopenia in ovariectomzied rats. *Am. J. Physiol. Endocrinol. Metab.* 258:E673–E677.

48. Pacifici, R., L. Rifas, R. McCracken, I. Vered, C. McMurtry, L. V. Avioli, and W. A. Peck. 1989. Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. *Proc. Natl. Acad. Sci. USA* 86:2398–2402.

49. Hustmyer, F. G., E. Walker, X.-P. Yu, G. Girasole, Y. Sakagami, M. Peacock, and S. C. Manolagas. 1993. Cytokine production and surface antigen expression by peripheral blood mononuclear cells in postmenopausal osteoporosis. *J. Bone Miner. Res.* 8:51–59.

50. Zarrabeitia, M. T., J. A. Riancho, J. A. Amado, J. Napal, and J. Gonzalez-Macias. 1991. Cytokine production by peripheral blood cells in postmenopausal osteoporosis. *Bone Miner.* 14:161–167.

51. Kimble, R. B., J. L. Vannice, D. C. Bloedow, R. C. Thompson, W. Hopfer, V. T. Kung, C. Brownfield, and R. Pacifici. 1994. Interleukin-1 receptor antagonist decreases bone loss and bone resorption in ovariectomized rats. *J. Clin. Invest.* 93:1959–1967.

52. Kitasawa, R., R. B. Kimble, J. L. Vannice, V. T. Kung, and R. Pacifici. 1994. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. *J. Clin. Invest.* 94:2397–2406.

53. Miyaura, C., I. Owan, T. Masuzawa, K. Kusano, T. Tamura, Y. Ohsugi, and T. Suda. 1993. IL-1α is major endogenous bone-resorbing factor in estrogen deficiency. *J. Bone Miner. Res.* 8(S1):S130. (Abstr.)

54. Hu, S. K., Y. L. Mitcho, and N. C. Rath. 1988. Effect of estradiol on interleukin 1 synthesis by macrophages. *Int. J. Immunopharmacol.* 10:247–252.

55. Flynn, A. 1986. Expression of Ia and the production of interluekin 1 by peritoneal exudate macrophages activated *in vivo* by steroids. *Life Sciences.* 38:2455–2460.

56. Keeting, P. E., L. A. Pivirotto, and D. S. Cissel. 1994. Sex hormone treatment increases steady-state interleukin-1β mRNA levels in human osteoblastic HOBIT cells. *J. Bone Miner. Res.* 9(S1):S167 (Abstr.)

57. Dinarello, C. A. 1991. Interleukin-1 and interleukin-1 antagonism. *Blood.* 77:1627–1652.

58. Kalu, D. N., E. Salerno, C. C. Liu, F. Ferarro, B. N. Arjmandi, and M. A. Salih. 1993. Ovariectomy-induced bone loss and the hematopoietic system. *Bone Miner.* 23:145–161.

59. Girasole, G., G. Passeri, R. L. Jilka, and S. C. Manolagas. 1994. Interleukin-11: a new cytokine critical for osteoclast development. *J. Clin. Invest.* 93:1516–1524.

60. Jilka, R. L., G. Girasole, G. Passeri, M. Munshi, N. Howe, and S. C. Manolagas. 1994. Estrogen deficiency induces sensitivity of the osteoclastogenic process to IL-6. *J. Bone Miner. Res.* 9(S1):S143. (Abstr.)