0013-7227/89/1243-1576$02.00/0
Endocrinology

Vol. 124, No. 3
Printed in U.S.A.

ANDROGENS DIRECTLY STIMULATE PROLIFERATION OF BONE CELLS IN VITRO

Christian H. Kasperk, Jon E. Wergedal, John R. Farley, Thomas A. Linkhart, Russel T. Turner, David J. Baylink

Departments of Medicine and Biochemistry, Loma Linda University and Pettis VA Hospital
Loma Linda, Ca 92357

Abstract: This report describes the first observation of a direct mitogenic effect of androgens on isolated osteoblastic cells in serum-free culture. [^{3}H]thymidine incorporation into DNA and cell counts were used as measures of cell proliferation. The percentage of cells that stained for alkaline phosphatase was used as a measure of differentiation. Dihydrotestosterone (DHT) enhanced mouse osteoblastic cell proliferation in a dose dependent manner over a wide range of doses (10^{-8} to 10^{-11} molar), and was maximally active at 10^{-9} M. DHT also stimulated proliferation in human osteoblast cell cultures and in cultures of the human osteosarcoma cell line, TE89. Testosterone, fluoxymesterone (a synthetic androgenic steroid) and methenolone (an anabolic steroid) were also mitogenic in the mouse bone cell system. The mitogenic effect of DHT on bone cells was inhibited by antiandrogens (hydroxyflutamide and cyproterone acetate) which compete for binding to the androgen receptor. In addition to effects on cell proliferation, DHT increased the percentage of alkaline phosphatase (ALP) positive cells in all three bone cell systems tested, and this effect was inhibited by antiandrogens. We conclude that androgens can stimulate human and murine osteoblastic cell proliferation in vitro, and induce expression of the osteoblast-line differentiation marker ALP, presumably by an androgen receptor mediated mechanism.

Forty years ago Albright and Reifenstein (1) observed that testosterone has a positive effect in male senile osteoporosis. A number of other clinical observations indicate that androgens play a role in the maintenance of bone mass (2); that severe osteoporosis is found in male patients with hypogonadism (3); that decreased serum testosterone levels in elderly males are a risk factor for fractures (4) and that androgenic steroids increase bone mass (5,6). Thus, androgens along with other factors are important in the maintenance of skeletal volume. Treatment of osteoporotic patients with anabolic steroids significantly increased serum levels of skeletal alkaline phosphatase (ALP) (7). This latter effect suggests that androgens increase bone formation (8). The present study was undertaken to determine whether androgens have a direct effect on osteoblastic cells by examining their action on isolated osteoblast-line cells.

Materials and Methods

Materials: Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY), and calf serum was a product of Hyclone (Logan, Ut). Fast blue BB salt, naphthol- AS-MX phosphate, dimethylsulfoxide, dihydrotestosterone (DHT) and fluoxymesterone were received from Sigma Chemical Co. (St Louis, Mo). The compounds methenolone acetate and cyproterone acetate were generously provided by Schering AG, Berlin, West Germany; hydroxyflutamide was a gift from Dr.Neri, Schering Corporation, NJ. Human osteosarcoma cells (TE89) were obtained from American Tissue Culture Collection (ATCC # CRL 7738). These cells contain ALP and respond to parathyroid hormone with increased cAMP levels.

Cell culture: Primary mouse calvarial cell cultures were prepared from 1-2 day old neonatal mouse calvariae (Swiss-Webster strain) as described (9) and were plated (20000 cells per well) in serum-free DMEM in 48-well plates. All media contained Penicillin-G (10 U/ml) and Streptomycin (10 µg/ml). Androgens were added 24 hrs after plating. Human bone cells were isolated and cultured from femoral heads as described (9). Normal human bone cells of the 4th-6th passage and human osteosarcoma cells (TE89), maintained in DMEM containing 10 % calf serum, were passaged and plated (10000 per well) in 5 % calf serum in 48-well plates. After 24 hrs the medium was changed to serum-free DMEM for an additional 24 hrs before the androgenic compounds were added.

DNA synthesis assay: 24 hrs after plating cells, steroids were added to the cell cultures. Androgenic steroids alone or in combination with antiandrogens were dissolved in dimethylsulfoxide and diluted such that the final dimethylsulfoxide concentration in the culture medium did not exceed 0.005 % (v/v), unless otherwise noted. Controls contained the same amount of solvent. Replicate (n=6) culture wells were incubated with the androgenic compounds for a total of 24 hrs, and [^{3}H]thymidine (1.5 uCi/ml) was added to the cultures for the last 6 hrs of the incubation period. The incorporation of [^{3}H]thymidine into trichloroacetic acid precipitable material (DNA) was measured as an index of cell proliferation as described (10).

Cell counts: To determine the effect of androgenic steroids on cell number, osteoblast-line cells were incubated with androgens for 3 days (mouse, Fig.1b) or 4 days (human, Table 2 and 3, Fig.2 and 3), before determining cell number. Cultures were stained for ALP using naphthol-AS-MX phosphate and fast blue BB in tris buffer (pH 8.6) as described (9). Total cell number and the number of ALP$^+$ and ALP$^-$ cells were evaluated blind in two fields in each of 6 replicate culture wells using a microscope with an eyepiece reticule.

Statistical methods: Statistical significance was evaluated by the two-tailed Student's T-Test. Data are shown as % of vehicle control, mean ± SEM.

Results

DHT stimulated the incorporation of tritiated thymidine into mouse bone cell DNA in a dose dependent manner (Fig.1a). At DHT doses higher than 10-8 M the response decreased. To establish that the observed stimulation of [^{3}H]thymidine incorporation into bone cell DNA was accompanied by an actual increase in bone cell proliferation, changes in the total cell number were determined after three days of androgen treatment. We found

Received in Iowa City December 6, 1988

that DHT increased murine bone cell number in a dose dependent manner (Fig.1b).

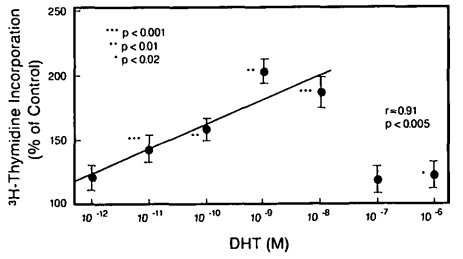

Fig.1a. Dose response of [^{3}H]thymidine incorporation into primary mouse calvarial cells to DHT, in serum-free DMEM. For DHT doses greater than 10^{-8} M, control wells received appropriate dimethylsulfoxide dilutions (0.5 % for 10^{-6} M DHT; 0.05 % for 10^{-7} M DHT). Doses greater than 10^{-8} M did not increase DNA synthesis (corresponding data points not included in the regression analysis).

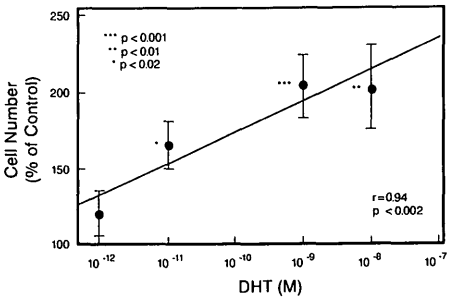

Fig.1b. Dose response of mouse calvarial cell number to DHT. Control cell number was 27±5.

As shown in Table 1, testosterone (the major circulating androgen), methenolone (an anabolic steroid), and fluoxymesterone (a synthetic androgen), also stimulated mouse bone cell DNA synthesis. All of the androgenic or anabolic steroids tested stimulated mouse osteoblast-line cell proliferation. Progesterone did not stimulate [3H]thymidine incorporation into DNA (1nM progesterone 121±11, n.s.) at the same doses used for the androgen experiments, indicating that not all steroids stimulate.

To determine whether androgens have a direct effect on bone cell differentiation, we stained mouse bone cells for ALP after 3 days of DHT treatment and counted the total number of cells and

Table 1. Effect of androgens (1nM) on [^{3}H]thymidine uptake into DNA of mouse bone cells.

	CPM	% control	p
Control	235±14	100±6	
DHT	479±23	204±9	< 0.001
Testosterone	429±47	182±20	< 0.01
Fluoxymesterone	423±44	180±18	< 0.01
Methenolone	633±55	269±23	< 0.001

the number of ALP$^+$ stained cells. The results of these studies indicate that the percentage of ALP$^+$ staining as well as the total number of murine bone cells was increased by DHT treatment (Table 2). Thus, DHT stimulated differentiation as measured by the percentage of ALP$^+$ cells.

The regulation of bone cell proliferation may vary among different species (11). We determined therefore whether androgens have similar effects in human bone cells. Consistent with our findings in the mouse bone cell system, androgens (a) elicited direct mitogenic responses in human bone cell cultures (Table 3) and (b) increased the ALP$^+$ cell number more than ALP$^-$ cell number in both normal and osteosarcoma cells (Fig.2). This indicates that the direct effects of androgens on osteoblast-line cell proliferation and differentiation are not specific for murine bone cells and can also be seen with human bone cells in vitro.

Table 2. Effect of 3 day treatment with DHT (1nM) on mouse osteoblast-line cell number and percentage of ALP$^+$ cells.

	Cells/mm^2	% ALP$^+$	p
Control	60±9	5±1.6	<0.001
DHT	103±10	12±1.7	<0.01

Table 3. Effects of 4 day treatment with androgenic steroids (1nM) on the number of normal human bone cells.

	Cells/mm^2	% control	p
Control	83±13	100±16	
DHT	130±15	151±17	<0.01
Methenolone	150±19	176±22	<0.01

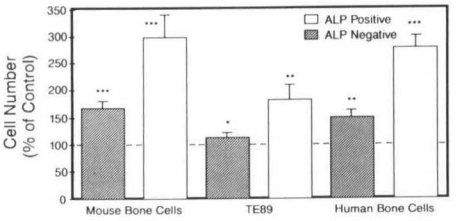

Fig.2. Effect of DHT on ALP positive (ALP$^+$) and ALP negative (ALP$^-$) cells in normal mouse, normal human osteoblast-line and human osteosarcoma (TE89) monolayer cell culture. (*** = p<0.001;** = p <0.01; * = p<0.1). Control values in cells per mm^2 for mouse bone cells were: 90±5; TE89 cells: 75±7; human bone cells: 83±14.

These data indicate that androgens can have direct effects on cells of the osteoblast-lineage in vitro. We reasoned that these bone cells contain androgen receptors. To test this hypothesis indirectly, antiandrogens (which compete with androgens for the androgen receptor [12]) were added to the human bone cell cultures, either alone or in combination with an androgen. We used hydroxyflutamide, a non-steroidal antiandrogen, and cyproterone acetate, a steroidal antiandrogen. Hydroxyflutamide inhibited the mitogenic effects of DHT and methenolone (Fig.3a), prevented the stimulatory effects of DHT and methenolone to increase the number of ALP$^+$ human bone cells (Fig.3b) and blocked the mitogenic effects of androgens on mouse bone cells (data not shown). Cyproterone acetate inhibited the mitogenic effects of methonolone on mouse bone cells (thymidine incorporation as % of control ± SE: 1nM methenolone 269±23, 1 nM methenolone + 1 nM cyproterone 182±11, p < 0.01)

Discussion

These studies represent the first in vitro demonstration that androgens can directly stimulate

bone cells to proliferate and differentiate (as measured by ALP activity). These observations suggest that the increase in skeletal mass in androgen treated subjects could be due to a direct effect of androgens on bone formation. In some tissues testosterone can be converted to estrogens which are thought to play a role in controlling bone mass (13). Our observation that fluoxymesterone, a synthetic androgen, and DHT, both of which can not be metabolized to estrogens, increased mouse bone cell proliferation argues against the possibility that testosterone was converted to estrogens by the bone cells and exerted its effect through the estrogen receptors which are present in human bone cells (14,15).

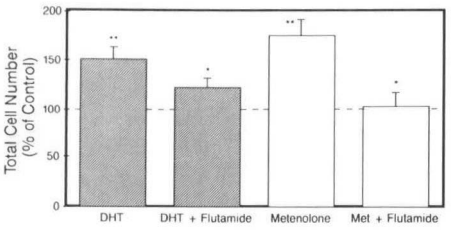

Fig.3a. Effect of the antiandrogen hydroxyflutamide (FL)(10 nM) on the stimulation of human bone cell number by DHT (1 nM) and methenolone (Met)(1nM). (** = p<0.01;* = n.s.). Total cell number in the control was 86±13 cells per mm².

Furthermore, testosterone, which has been previously shown to bind with a lower affinity than DHT to the androgen receptor (16), and DHT were almost equipotent in stimulating cell proliferation, raising the possibility that testosterone may be converted into DHT in bone cells prior to stimulating cell proliferation. In all three osteoblastic cell systems examined, the relative increase in the number of ALP⁺ cells was greater than that of ALP⁻ cells. There are several

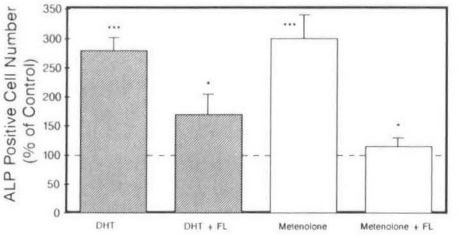

Fig.3b. Effect of hydroxyflutamide (10nM) on the number of ALP⁺ human bone cells by DHT (1nM) and Met (1nM) (*** = p<0.001; * = n.s.). Number of ALP⁺ cells in the control was 10±2 cells per mm².

mechanisms by which this could have occurred, including the possibility that ALP⁺ cells might be more sensitive than ALP⁻ cells to mitogenic stimulation by androgens or that androgens may have converted ALP⁻ cells to ALP⁺ cells. Although the mechanism for the increase in ALP⁺ cells is not understood, it is clear that androgens increased the number of differentiated bone cells.

Since our data indicate that androgens can have direct effects on cells of the osteoblast-lineage in vitro, we reasoned that these bone cells contain androgen receptors. Androgen receptor specific antiandrogens inhibited the mitogenic effects of DHT and methenolone (Fig.3a), prevented the stimulatory effects of DHT and methenolone to increase the number of ALP⁺ human bone cells (Fig.3b), and blocked the mitogenic effects of androgens on mouse bone cells (data not shown). These findings suggest that human (and mouse) bone cells have androgen receptors, although this possibility must be confirmed by direct measurement of androgen receptors.

Acknowledgements

We are grateful for the expert technical assistance of Mrs. Dana Long. One author (C.H.K.) is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft, Bonn, West Germany; the work was supported by a grant from the country of Schleswig-Holstein, West Germany and by medical research grants from the Veterans Administration.

References

1. Albright F, Reifenstein C 1948 The parathyroid glands and metabolic bone disease, Williams and Wilkens Company, Baltimore
2. Longcope C, Baker RS, Hui SL, Johnston CC 1984 Androgen and estrogen dynamics in women with vertebral crush fractures. Maturitas 6:309
3. Finkelstein JS, Klibanski A, Neer RM, Greenspan SL, Rosenthal DI, Crowley WF 1987 Osteoporosis in men with idiopathic hypogonadotropic hypogonadism. Ann Int Med 106:354
4. Swartz CM, Young MA 1988 Male hypogonadism and bone fracture. N Engl J Med 318:996
5. Keele DK, Vose GP 1969 A study of bone density. Amer J Dis Child 118:759
6. Dequeker J, Geusens P 1985 Anabolic steroids and osteoporosis. Acta Endocrinol Supple 271:45
7. Farley JR, Chesnut CH, Baylink DJ 1981 Improved method for quantitative determination in serum of alkaline phosphatase of skeletal origin. Clin Chemistry 27:2002
8. Lauffenburger T, Olah AJ, Dambacher MA, Guncaga J, Lentner C, Haas HG 1977 Bone remodeling and calcium metabolism a correlated histomorphometric, calcium kinetic, and biochemical study in patients with osteoporosis and Paget's disease. Metabolism 26:589
9. Wergedal JE, Baylink DJ 1984 Characterisation of cells isolated and cultured from human bone. Proc Soc Experiment Biol Med 176:60
10. Linkhart TA, Jennings JC, Mohan S, Wakley GK, Baylink DJ 1986 Characterisation of mitogenic activities from bovine bone matrix. Bone 7:479
11. Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD 1987 Osteoblasts synthesize and respond to TGF-beta in vitro. J Cell Biol 105:157
12. Tymoczko JL, Liao S 1976 Androgen receptors and the molecular basis for the action of antiandrogens in the ventral prostate. J Reprod Fert 24:147
13. Wilson JD 1987 Disorders of androgen action. Clin Res 35:1
14. Komm BS, Terpening CM, Benz OJ, Graeme KA, Gallegos A, Korc M, Greene GL, O'Malley BW, Haussler R 1988 Estrogen binding, receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science 241:81
15. Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL 1988 Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84
16. Wilson JD, Gloyna RE 1970 The intranuclear metabolism of testosterone in the accessory organs of reproduction. Rec Prog Hormone Res 26:309