

Direct and Indirect Effects of Androgens on Survival of Hematopoietic Progenitor Cells In Vitro

Androgens remain a common treatment for certain type of anemia, based upon its myelostimulating effects; however, it has not been established whether androgens affect apoptosis of hematopoietic progenitor cells (HPCs). We investigated the effects of the androgens, such as testosterone, 5 β -dihydrotestosterone (5-DHT), and oxymetholone, on apoptosis of normal hematopoietic progenitor cells in vitro. Androgens did not rescue normal bone marrow (BM) CD34+ cells and colony-forming cells (CFCs), other than mature erythroid CFCs, from apoptosis induced by serum- and growth factor deprivation. Oxymetholone did not affect growth factor-mediated survival of normal CD34+ cells or its inhibition by interferon-gamma (IFN- γ). In a standard methylcellulose clonogenic assay, low concentrations of oxymetholone and 5-DHT stimulated the clonal growth of colony-forming unit (CFU)-erythroid, but did not affect growth of CFU-granulocyte/macrophage or burst-forming unit-erythroid. Oxymetholone and 5-DHT stimulated the production of stem cell factor in normal bone marrow stromal cells (BMSCs) via transcriptional regulation. In agreement with this, oxymetholone-treated BMSCs better supported the survival of HPCs. These data indicate that survival-enhancing or growth-stimulatory effects of androgens on hematopoietic progenitor cells are minimal and mostly restricted to mature erythroid progenitors, and its myelostimulating effects could be attributed, at least in part, to the stimulation of production of hematopoietic growth factors in BMSCs.

Key Words : Androgens; Erythroid Progenitor Cells; Myeloid Progenitor Cells; Apoptosis

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INTRODUCTION

Androgens have a wide variety of biological effects, including several actions in hematopoiesis (1). The administration of testosterone was shown to increase hemoglobin levels in mice (2), and experiments with plethoric mice suggested that its major erythropoietic effect was exerted through the stimulation of erythropoietin (EPO) production by the kidney (3). This mechanism is unlikely to be involved in the beneficial effects of androgens in aplastic anemia patients, who produce abundant EPO; moreover, androgens have been shown to exert actions on hematopoietic cells independent of their effect on EPO secretion. In addition to increased hemoglobin levels, mice injected with testosterone derivatives had increased granulocyte and platelet numbers (4, 5). In vitro, androgens stimulated hematopoiesis in marrow suspension cultures (6-8) and enhanced both erythroid and myeloid colony formation in semisolid cultures (9, 10). It was demonstrated that testosterone was taken up by the nuclei of rat bone marrow cells and that it stimulated total RNA metabolism (11). Androgens have also been implicated in increasing the production of colony-stimulating factors by adherent cells from normal human blood

(12). In addition, androgens have diverse, generally suppressive effects on the immune system (13, 14), which might be involved in their therapeutic effects in aplastic anemia.

In most of the previous studies regarding the in vitro effects of androgens on hematopoietic cells, whole bone marrow cells were used, rather than purified hematopoietic progenitor cells such as CD34+ cells. Furthermore, the question of whether androgens affect the survival of hematopoietic progenitor cells has not been well studied. In addition, the effects of androgens on the production of hematopoietic growth factors by bone marrow stromal cells (BMSCs), which might affect the growth and survival of hematopoietic progenitors, are poorly understood. With these questions in mind, we investigated the effects of androgens, such as testosterone, 5 β -dihydrotestosterone (5-DHT), and oxymetholone, on the survival of purified normal hematopoietic progenitor cells and on the production of cytokines by normal BMSCs. Here, we show that direct survival-enhancing or growth-stimulatory effects of androgens on hematopoietic progenitor cells were minimal and mostly restricted to mature erythroid progenitors. Oxymetholone and 5-DHT stimulated the production of several cytokines in bone marrow stromal cells, and oxymetholone-

stimulated stromal cells better supported the survival of hematopoietic progenitor cells.

MATERIALS AND METHODS

Cells and cell lines

CD34⁺ cells were purified from bone marrow obtained from normal donors undergoing bone marrow harvest, after informed consent. Following Ficoll separation, CD34⁺ cells were isolated using the MACS system (Miltenyi Biotec, Auburn, CA, U.S.A.) according to the manufacturer's instructions. Only cell preparations with purity of 95% CD34⁺ or greater by flow cytometric analysis were used in the experiments. Primary human BMSCs were prepared by culturing CD34-negative cells (the cells that remained after purification of the CD34⁺ cells from the bone marrow mononuclear cells) in MEM- α (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.) supplemented with 12.5% fetal bovine serum (FBS), 12.5% horse serum (Gibco), 10⁻⁶ M hydrocortisone (Sigma, St. Louis, MO, U.S.A.), 5 \times 10⁻⁵ M 2-mercaptoethanol (Sigma), streptomycin, and penicillin (hereafter referred to as LTC medium). After confluence was reached, the adherent cells were harvested and maintained in LTC medium with weekly passage. After three passages, neither CD45⁺ cells nor PECAM-1⁺ cells were detected by flow cytometric analysis. The murine bone marrow stromal cell line MS-5 was grown in MEM- α supplemented with 10% FBS and was passaged weekly.

Hormones and cytokines

Testosterone, 5 β -dihydrotestosterone (5-DHT), and β 2-estradiol were purchased from Sigma. Oxymetholone was kindly provided by Hanseo Pharmaceutical Co. (Seoul, Korea). The hormones were formulated as 1 \times 10⁻² M stock solutions using ethanol as diluent and stored at -20°C. The following cyto-

Table 1. Primers used for the determination of cytokine and growth factor expression by RT-PCR

Cytokine or Factor	Sense	Antisense
IL-6	GTACCCCGAGGAG-AAGATTC	CAAACCTGCATA-GCCACTTTC
IL-7	TCA ATT ATT GGA CAG CAT GA	TCT TTA GTG CCC ATC AAA AT
SCF	CCA TTG ATG CCT TCA AGG AC	CTT CCA GTA TAA GGC TCC AA
TPO	GGA CTT TAG CTT GGG AGA AT	CAA CAA TCC AGA AGT CCT GT
Fik-2 ligand	GGG TCT TGA TAA CGG ATA CA	ATG TTG TCT TGG ATG AAA GG
GAPDH	TGA AGG TCG GAG TCA ACG GAT TTG GTC	CAT GTG GGC CAT GAG GTC CAC GAC

kines were used: interleukin-3 (IL-3), stem cell factor (SCF), *flk-2* ligand (FL), thrombopoietin (TPO), EPO, and stromal cell-derived factor-1 α (SDF-1 α) (all purchased from R&D Systems, Minneapolis, MN, U.S.A.).

Flow cytometry

A total of 5 \times 10⁴ to 1 \times 10⁵ cells were incubated at 4°C for 30 min with phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (HPAC-2; Becton-Dickinson, San Jose, CA, U.S.A.) and were analyzed using a Coulter Elite flow cytometer (Coulter Electronics Ltd., Hialeah, FL, U.S.A.). Isotope-identical antibodies served as controls. To detect apoptosis, cells were stained with FITC-conjugated annexin V (BD PharMingen, San Diego, CA, U.S.A.) alone or together with propidium iodide (PI; BD PharMingen) according to the manufacturer's instructions and analyzed by flow cytometry.

Clonogenic assay in methylcellulose

Cells (1 \times 10³ CD34⁺ cells or the equivalent) were plated in triplicate in 35-mm tissue culture dishes containing 1 mL of assay medium consisting of Iscove's modified Dulbecco medium (IMDM; Gibco), 1.2% methylcellulose (Stem Cells, Vancouver, Canada), 30% FBS, 50 μ M 2-mercaptoethanol, 2 mM L-glutamine (Gibco), and 0.5 mM hemin (Sigma), supplemented with 20 ng/mL SCF, 50 ng/mL IL-3, and 6 U/mL EPO. After 7-14 days of incubation at 37°C in 5% CO₂ in air, the numbers of colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-erythroid (CFU-E) were scored under the inverted microscope.

Preparation of conditioned media (CM)

Conditioned media were prepared from MS-5 cells and primary human BMSCs grown in the appropriate medium in T25 culture flasks. When confluence was reached, the cultures were thoroughly rinsed, and 3 mL of serum-free X-VIVO medium (BioWhittaker, Walkersville, MA, U.S.A.) was added to each flask, with or without the inclusion of androgens. After a 72-hr incubation at 37°C, the supernatants were harvested, filtered through 2-micron membranes, and stored at 4°C until analyzed.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of SDF-1 α and SCF in the conditioned media were measured using commercial ELISA kits (R&D Systems) according to the manufacturer's instructions. The optical density was measured using a spectrophotometer (Molecular Device Co., Sunnyvale, CA, U.S.A.). The standard curves and calculated levels of the cytokines were obtained using the program SoftmaxTM (Molecular Device Co.).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from primary human BMSCs using Trizol™ (Gibco) according to the manufacturer's instructions. After purification, 1 µg of RNA was reverse-transcribed using SuperScript reverse transcriptase (Gibco) and the universal primer oligo dT15 (Promega, Madison, WI, U.S.A.). One µL of the cDNA products was then added to 24 µL of PCR buffer (Gibco) supplemented with 2 mM MgCl₂, 0.2 µM specific primers (Table 1), and 1 U/reaction tube of Koma *Taq* polymerase (Koma International, Seoul, Korea). The PCR cycles consisted of 1 min at 94°C, 45 sec at 55-65°C, and 1 min at 72°C; 30 cycles were carried out using a GeneAmp PCR system (Perkin Elmer, Norwalk, CT, U.S.A.).

Northern blot analysis

The expression of SDF-1 mRNA in primary human BMSCs was detected by Northern hybridization. Briefly, 20 µg of total RNA was loaded in each lane, separated on 1.2% agarose gels containing 2.4% formaldehyde, and then transferred onto nylon membranes (Schleicher & Shuell, Keene, NH, U.S.A.) for 24 hr by the capillary transfer method. All probes were radiolabeled using a random priming protocol (Amersham Life Science, Arlington Heights, IL, U.S.A.). The membranes were hybridized with random primer-labeled SDF-1 probe for 24 hr, washed, and then subjected to autoradiography.

Western blot analysis

Western blotting was used to detect the expression of SDF-1 protein in primary human BMSCs. Cells were pelleted, washed in phosphate-buffered saline, and lysed by the addition of SDS sample buffer composed of 62.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125 mM DTT, and 0.03% (w/v) bromophenol blue. Total cell samples were sonicated, lysed, and denatured by boiling for 5 min. Equal amounts of protein from each sample were electrophoresed on 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham). The membranes were blocked for

1 hr with TBS containing 5% (w/v) milk and 0.1% Tween, and then incubated with the primary mouse monoclonal antibody (12G5; R&D Systems) overnight at 4°C. The blots were washed with TBST buffer, incubated with anti-mouse secondary antibody (Amersham) for 2 hr, and developed using West-Zol™ Plus (iNtRON Biotechnology, Seoul, Korea).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) of experiments conducted in triplicate or greater replicates. Data were analyzed using Student's t-test for paired or non-paired samples. A *p* value of less than 0.05 was considered significant.

RESULTS

Androgens stimulates clonal growth of CFU-E, but not BFU-E or CFU-GM

We examined the effect of the addition of 1×10^{-7} to 1×10^{-5} M oxymetholone or other hormones to normal BM CD34⁺ cells in a standard methylcellulose assay supplemented with the hematopoietic growth factors SCF, IL-3, and EPO. Oxymetholone did not influence the numbers of CFU-GM or BFU-E; however, there was a slight increase in the number of CFU-E in the presence of 1×10^{-7} or 1×10^{-6} M, but not 1×10^{-5} M, oxymetholone compared with controls grown without added hormones. The addition of testosterone, 5-DHT, or β-estradiol also induced an increased number of CFU-E, but did not affect the number of CFU-GM or BFU-E in this assay (Table 2).

Short-term treatment of CD34⁺ cells with androgens in the absence of HGFs enhances the survival of CFU-E, but not CFU-GM or BFU-E

Normal BM CD34⁺ cells were pre-incubated for 36 hr in the absence of serum and androgens before being plated in a

Table 2. Effects of androgens on the clonal growth of normal bone marrow CD34⁺ cells in methylcellulose clonogenic assays

Hormone	CFU-GM				BFU-E				CFU-E			
	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M
Oxymetholone	42.7±3.6	44.0±4.1	38.0±3.5	36.0±5.3	18.3±2.9	16.0±2.6	16.3±0.6	17.7±3.2	12.3±2.9	17.0±2.6*	18.0±1.6*	14.3±2.5
5-DHT		38.7±3.2	42.7±4.0	38.0±2.0		18.7±1.5	16.0±2.6	18.0±1.0		18.3±2.7*	22.0±2.6*	21.3±3.1*
Testosterone		38.7±2.7	35.7±2.0	37.3±3.1		14.3±1.2	14.7±0.6	14.2±1.3		13.7±4.0	14.7±2.8	13.7±2.5
Testradial		27.0±4.0*	22.7±1.5*	26.0±3.2*		16.7±3.2	14.3±1.2	16.7±2.1		14.7±2.1	14.3±1.2	10.0±0.5

Bone marrow CD34⁺ cells (1×10^3) from normal donors were plated in 1 mL methylcellulose medium supplemented with 30% FBS and SCF (20 ng/mL), IL-3 (50 ng/mL), and EPO (6 U/mL). CFU-E and BFU-E (and CFU-GM) were enumerated on day-7 and day-14 of incubation, respectively. Hormones were added at the indicated concentrations when the cultures were started. The presented data are the means±SD of the number of colonies from triplicate cultures. The experiments were repeated three times, with similar results. Representative results are shown. **p*<0.05 compared with untreated controls (containing 0.1% ethanol).

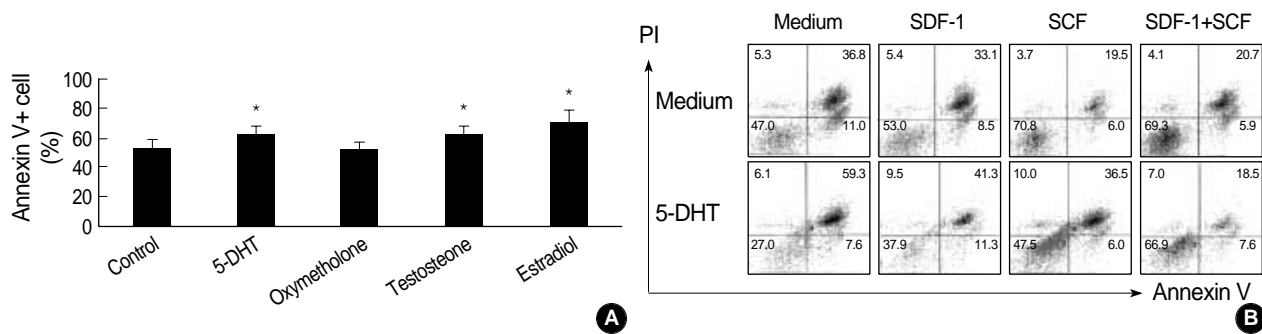


Fig. 1. Effects of androgens on apoptosis of normal bone marrow CD34+ cells induced by growth factor deprivation. BM CD34+ cells were incubated in IMDM for 36 hr in the presence or absence of the indicated hormones (10^{-6} M), stained with annexin V and propidium iodide, and analyzed on a flow cytometer. (A) Mean percentages and SD of annexin V-positive cells from five independent experiments are shown. * $p < 0.05$ compared with untreated control (IMDM containing 0.1% ethanol) (analyzed by t-test for paired samples). (B) 5-DHT (10^{-5} M) increased the percentage of annexin-positive cells. The addition of either SDF-1 (100 ng/mL) or SCF (100 ng/mL) in combination with 5-DHT partially relieved the increase in apoptosis induced by 5-DHT, and the addition of both SDF-1 and SCF completely reversed it. A representative result is shown.

Table 3. Effects of short-term androgen treatment on the clonal growth of normal bone marrow CD34+ cells

Hormone	CFU-GM				BFU-E				CFU-E			
	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M
Oxymetholone	32.6 ± 4.8	31.0 ± 4.5	28.3 ± 3.0	26.7 ± 1.5	13.0 ± 1.6	16.0 ± 1.7	15.7 ± 4.0	14.0 ± 2.6	15.7 ± 1.2	20.7 ± 1.5*	19.0 ± 3.0*	18.7 ± 2.9
5-DHT		30.3 ± 5.5	25.3 ± 2.1*	24.3 ± 2.0*		15.0 ± 2.6	14.3 ± 0.6	18.0 ± 2.6		22.7 ± 1.5*	19.0 ± 1.6*	17.0 ± 0.0
Testosterone		26.0 ± 5.5*	25.3 ± 4.5*	24.0 ± 2.5*		18.0 ± 2.3	14.3 ± 2.3	15.7 ± 2.1		23.3 ± 4.5*	20.0 ± 2.6*	21.3 ± 4.2*
Bestradol		28.3 ± 4.5*	26.3 ± 3.7*	25.7 ± 2.5*		15.7 ± 1.2	13.0 ± 2.6	16.3 ± 2.1		20.0 ± 2.6*	19.3 ± 2.6*	18.3 ± 1.5*

Bone marrow CD34+ cells from normal donors were incubated in serum-free medium in the presence or absence of hormones. After 36 hr, the cells were washed, and the number of cells equivalent to 1×10^3 input cells were plated in a methylcellulose clonogenic assay. CFU-E and BFU-E (and CFU-GM) were enumerated on day-7 and day-14 of incubation, respectively. The presented data are the means ± SD of the number of colonies from triplicate cultures. The experiments were repeated three times, with similar results. Representative results are shown. * $p < 0.05$ compared with untreated controls (containing 0.1% ethanol).

Table 4. Effects of oxymetholone on cytokine-induced modulation of the survival of BM CD34+ cells

Treatment	Annexin V-negative CD34+ cells (%)			
	Day 1	Day 3	Day 5	Day 7
Untreated	61.5 ± 3.8	21.2 ± 3.2	12.8 ± 2.7	1.5 ± 0.6
Oxymetholone	60.9 ± 4.2	22.8 ± 3.9	13.9 ± 3.5	1.7 ± 0.9
TFS	70.4 ± 5.3	50.5 ± 4.3	32.5 ± 3.6	18.7 ± 3.3
TFS+ oxymetholone	71.2 ± 5.9	49.8 ± 4.7	30.6 ± 2.8	17.9 ± 2.8
TFS+IFN- γ	65.7 ± 5.1	27.8 ± 3.9	8.2 ± 2.4	3.2 ± 0.7
TFS+IFN- γ + oxymetholone	66.3 ± 4.6	26.5 ± 4.4	9.1 ± 2.9	3.9 ± 0.6

Bone marrow CD34+ cells from normal donors were incubated in IMDM for the indicated periods in the absence or presence of oxymetholone (1×10^{-5} M), alone or in combination with a mixture of cytokines (5 ng/mL TPO+5 ng/mL FL+5 ng/mL SCF; TFS) or IFN- γ (100 ng/mL), stained with annexin V and anti-CD34 antibody, and subjected to flow cytometric analysis. The presented data are the means ± SD of annexin V-negative/CD34-positive cells from three independent experiments.

standard methylcellulose clonogenic assay. The addition of 1×10^{-7} to 1×10^{-5} M oxymetholone during the pre-incubation period did not affect the formation of CFU-GM or BFU-

Table 5. Effects of androgens on the production of SDF-1 in MS-5 cells

Hormone	SDF-1 (ng/mL)			
	Control	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M
Oxymetholone	64.3 ± 6.4	63.9 ± 4.1	65.9 ± 3.5	61.7 ± 5.3
5-DHT		55.3 ± 1.8*	54.3 ± 6.9*	31.4 ± 9.8*
Testosterone		NT	70.5 ± 2.0	69.3 ± 3.1
β -estradiol		NT	68.1 ± 3.5	70.9 ± 5.2

Murine bone marrow stromal cells MS-5 were incubated in serum-free medium X-VIVO, with or without androgens at the indicated concentrations. After a 72-hr incubation, the concentrations of SDF-1 α in the culture supernatants were measured using an ELISA. The presented data are the means ± SD of three independent experiments. * $p < 0.05$ compared with the untreated control (containing 0.1% ethanol) (analyzed by t-test for paired samples).

E; however, 1×10^{-6} M oxymetholone increased the number of CFU-E compared with controls pre-incubated without added hormones. Similarly, pre-incubation with 5-DHT or testosterone induced an increase in the number of CFU-E. Unexpectedly, the addition of 5-DHT, testosterone, or β -estradiol caused a slight decrease in the number of CFU-GM (Table 3).

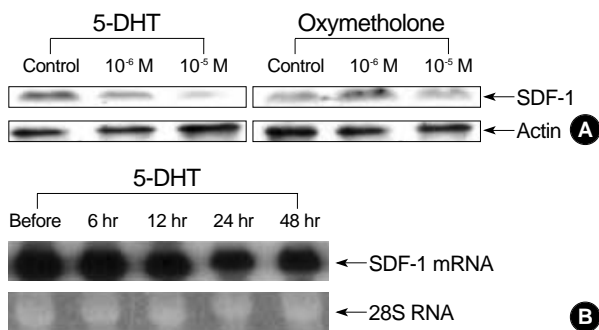


Fig. 2. 5-DHT, but not oxymetholone, down-regulates the production of SDF-1 in bone marrow stromal cells. (A) Primary human bone marrow stromal cells (BMSCs) were incubated in serum-free medium X-VIVO with or without (control) the indicated androgens. After a 72-hr incubation, the expression of SDF-1 was analyzed in total cell lysates by Western blotting. (B) 5-DHT down-regulates the expression of SDF-1 mRNA in BMSCs. Primary human BMSCs were incubated in serum-free medium X-VIVO in the presence of 5-DHT (10⁻⁵ M). At the indicated time points, the level of SDF-1 mRNA in the cells was analyzed by Northern hybridization. A blot of 28S ribosomal RNA is shown to control for RNA loading.

Testosterone and 5-DHT at a high concentration, but not oxymetholone, enhance apoptosis of normal BM CD34⁺ cells induced by growth factor deprivation

Normal BM CD34⁺ cells were incubated for 36 hr in serum- and hematopoietic growth factor (HGF)-free IMDM, with and without androgens at a concentration of 10⁻⁵ M. After the cells were stained with propidium iodide and annexin V, the level of apoptosis was analyzed using flow cytometry. The percentage of annexin-positive apoptotic cells was not changed by the addition of oxymetholone (51.5 ± 5.1%) compared with controls (52.6 ± 5.6%). Similar results were obtained at lower concentrations of oxymetholone (10⁻⁹ to 10⁻⁶ M; data not shown). Again unexpectedly, both testosterone and 5-DHT at this concentration significantly increased the percentage of annexin-positive cells (62.2 ± 5.9%, *p* < 0.05; 61.7 ± 6.4%, *p* < 0.05, respectively) (Fig. 1A). The addition of either SDF1 or SCF in combination with 5-DHT partially relieved the increase in apoptosis induced by 5-DHT, and the addition of both SDF-1 and SCF completely reversed it (Fig. 1B).

Oxymetholone does not affect cytokine-mediated alteration of survival of normal BM CD34⁺ cells

To further define the role of androgens in the survival of hematopoietic progenitor cells, we examined the time course of apoptosis. Normal BM CD34⁺ cells were incubated for 7 days in IMDM in the absence or presence of a combination of low concentrations of growth factors (5 ng/mL TPO + 5 ng/mL FL + 5 ng/mL SCF, hereafter referred to as TFS). This combination of growth factors was expected to enhance the

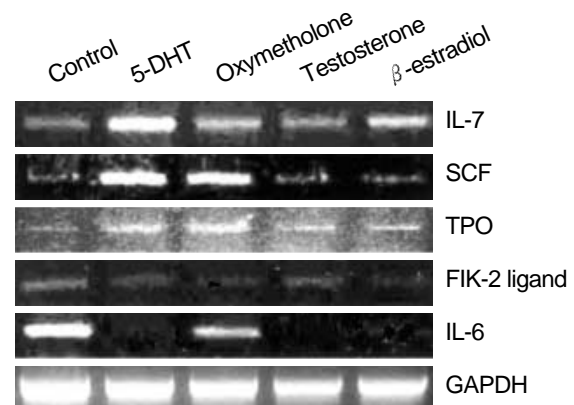


Fig. 3. Oxymetholone and 5-DHT modulate the levels of the mRNAs of various cytokines in bone marrow stromal cells (BMSCs). Primary human BMSCs were incubated in serum-free medium X-VIVO for 24 hr with or without (control) the indicated androgens (10⁻⁵ M) and then analyzed by RT-PCR.

survival, rather than the proliferation, of CD34⁺ cells. In the absence of all cytokines, annexin-positive cells markedly increased over time; the addition of oxymetholone did not delay the development of apoptosis. In the presence of TFS, the percentage of annexin-positive cells was much lower at every time point compared with that of cells incubated in the absence of TFS, regardless of CD34 positivity. Again, the addition of oxymetholone in combination with TFS did not result in any changes in the level of apoptosis (Table 4).

We next examined whether oxymetholone affects IFN- γ -mediated alterations in the clonal growth and survival of hematopoietic progenitor cells. In a standard methylcellulose clonogenic assay containing SCF, IL-3, and EPO, the addition of IFN- γ caused a significant decrease in the numbers of both CFU-GM and BFU-E; the inclusion of 10⁻⁹ to 10⁻⁵ M oxymetholone had no effect on this IFN- γ -induced inhibition of clonal growth (data not shown). Normal BM CD34⁺ cells were incubated for 7 days in IMDM in the absence or presence of TFS. The addition of IFN- γ in combination with TFS significantly decreased the percentage of annexin-negative CD34⁺ cells in a dose-dependent manner, while increasing the percentage of annexin-negative/CD34-negative cells and annexin-positive cells. Again, the inclusion of oxymetholone did not alter the effects of IFN- γ on the survival of CD34⁺ cells (Table 4).

5-DHT, but not testosterone or oxymetholone, down-regulates SDF-1 production in bone marrow stromal cells

The possibility that androgens might be involved in the regulation of SDF-1 production in BMSCs was explored. Conditioned medium collected after a 72-hr incubation with untreated MS-5 cells contained a high concentration of SDF-1 α (64.3 ± 6.4 ng/mL). The addition of 5-DHT at the beginning of the culture period diminished the concentration of

Table 6. Effects of androgens on the production of SCF in primary human bone marrow stromal cells

Hormone	SCF (pg/mL)			
	Control	1 × 10 ⁷ M	1 × 10 ⁶ M	1 × 10 ⁵ M
Oxymetholone	153.6 ± 9.8	165.4 ± 7.5	188.2 ± 4.9*	220.5 ± 5.4*
5-DHT		167.2 ± 4.6	193.5 ± 7.2*	243.4 ± 6.3*
Testosterone		146.7 ± 10.1	162.1 ± 8.2	157.3 ± 5.4
β-estradiol		160.2 ± 8.2	158.8 ± 6.7	150.7 ± 3.9

Primary human bone marrow stromal cells were incubated in serum-free medium, with or without androgens at the indicated concentrations. After a 72-hr incubation, the concentrations of stem cell factor (SCF) in the culture supernatants were measured by ELISA. The presented data are the means ± SD of triplicate experiments. **p* < 0.05 compared with the untreated untreated control (containing 0.1% ethanol) (analyzed by t-test for paired samples).

SDF-1 in the conditioned medium in a dose-dependent manner, whereas the addition of oxymetholone, testosterone, or β-estradiol did not (Table 5). Based on these observations, Western blot analysis was performed, using primary human BMSC lysates. Treatment of the BMSCs with 5-DHT definitely decreased the amount of SDF-1 in a dose-dependent manner, indicating that this hormone down-regulates the production of SDF-1 in the cells (Fig. 2A). Northern blot analysis revealed that the expression of SDF-1 mRNA in the BMSCs was also down-regulated by 5-DHT (Fig. 2B).

Androgens modulate the production of HGFs in BMSCs

The possibility that androgens might influence the production of HGFs in BMSCs was also investigated. Primary human BMSCs were incubated in serum-free medium for 24 hr with or without androgens, and the expression levels of the mRNAs for various HGFs were analyzed by RT-PCR. Oxymetholone and 5-DHT induced increased expression of the mRNAs of IL-7, SCF, and TPO; decreased expression of IL-6 mRNA; and no change in the expression of FL mRNA (Fig. 3). We also used ELISA assays to examine the levels of SCF in the conditioned media collected after a 72-hr incubation with primary human BMSCs in the presence or absence of the hormones. The addition of 5-DHT and oxymetholone at the beginning of the culture period increased the concentration of SCF in the conditioned medium in a dose-dependent manner, whereas the addition of testosterone or β-estradiol did not (Table 6).

BMSCs treated with oxymetholone better support the survival of hematopoietic progenitor cells

Normal BM CD34⁺ cells were incubated for 36 hr in conditioned media harvested from primary human BMSCs incubated in the presence or absence of 10⁻⁵ M oxymetholone. There was a lower percentage of annexin-positive apoptotic cells in the culture incubated in conditioned medium from

Table 7. Effects of androgen-treated conditioned medium of primary bone marrow stromal cells on the survival of CD34⁺ cells

Medium	Annexin V-negative CD34 ⁺ cells (%)
X-VIVO	53.1 ± 5.1*
Non-treated CM	80.5 ± 4.6
Oxymetholone-treated CM	85.3 ± 2.7*
5-DHT-treated CM	84.5 ± 3.6
Testosterone-treated CM	79.8 ± 2.4
β-estradiol-treated CM	78.7 ± 3.3

Conditioned medium (CM) was prepared from primary human bone marrow stromal cells (BMSCs) in 25 culture flasks. When confluence was reached, the cultures were thoroughly rinsed, and 3 mL of serum-free X-VIVO medium was added to each flask, with or without the inclusion of hormones (10⁻⁵ M). After a 72-h incubation at 37°C, the supernatants were harvested. Normal BM CD34⁺ cells were incubated for 36 hr in CM, stained with annexin V and anti-CD34 antibody, and subjected to flow cytometric analysis. The presented data are the means ± SD of annexin V-negative/CD34-positive cells from three independent experiments. **p* < 0.05 compared with the non-treated CM (analyzed by t-test for paired samples).

oxymetholone-treated BMSCs than in the culture incubated in conditioned medium from untreated BMSCs (Table 7).

DISCUSSION

In the present study, we showed that androgens, including oxymetholone, have a modest growth- and survival-enhancing effect on CFU-E, but not on CFU-GM or BFU-E. These effects, which were restricted to mature erythroid progenitors, might be the basis for the observed androgen dependency among responders to androgen therapy for aplastic anemia (15). From the combined findings, it is clear that androgens do not exert their myelostimulatory effects at the level of hematopoietic stem cells or immature progenitor cells. It was quite unexpected that testosterone and 5-DHT at a high concentration enhanced to some extent the apoptosis of normal BM CD34⁺ cells induced by serum deprivation and growth factor deprivation, rather than decreased it. These results differ from observations in previous studies that found testosterone and its derivatives enhanced the survival of hematopoietic progenitor cells (8). This disparity could be attributable to the type of cells analyzed in the experiments. In the previous studies, bone marrow cells were used without purification and thus were a mixed population of hematopoietic progenitor cells and various accessory cells present in the BM. It is possible that the observations in the previous studies were the result of the modulation of survival by accessory cells, such as BMSCs, rather than the direct action of the hormones on the hematopoietic progenitor cells. To our knowledge, this is the first time that enhancement of apoptosis of normal BM CD34⁺ cells by testosterone and 5-DHT has been reported. This finding is less surprising than it initially might appear, because androgens have been shown to induce apoptosis and

growth inhibition of non-lymphocytic leukemic cells (16, 17).

IFN- γ is well known as a hematopoiesis-inhibitory cytokine (18-20) and has been implicated in the pathogenesis of bone marrow failure (21, 22). In the present study, oxymetholone did not rescue colony-forming cells from IFN- γ induced inhibition of clonal growth of BM CD34+ cells in methylcellulose cultures. Furthermore, oxymetholone did not mitigate IFN- γ -induced suppression of growth factor-mediated survival of CD34+ cells. These results suggest that direct action of oxymetholone on hematopoietic progenitor cells is minimal, raising the possibility that the myelostimulatory effects of oxymetholone are attributable to other mechanisms.

Stromal cell-derived factor-1 (SDF-1), which is constitutively produced in BMSCs, plays a central role in bone marrow homing of hematopoietic stem cells by signaling via its receptor, CXCR4 (23). Recently, it has also been demonstrated that SDF-1 is involved in the positive regulation of hematopoiesis. SDF-1 enhances both the survival and proliferation of hematopoietic progenitors, especially in synergy with other hematopoietic growth factors (24-26), while inhibiting the cycling of very primitive hematopoietic cells, both in vitro and in vivo (27). Therefore, it is possible that the down-regulation of SDF-1 production in BMSCs would lead to the suppression of hematopoietic cell survival and growth. In the present study, we showed that 5-DHT, but not testosterone or oxymetholone, down-regulated SDF-1 production in BMSCs and that 5DHT-induced increases in apoptosis were nearly completely relieved by the addition of SCF and SDF-1. This paradoxical interaction between androgens and SDF-1 might be part of a regulatory mechanism for in vivo homeostasis of hematopoiesis. Androgens are known to suppress B lymphocyte development (28), and it has been demonstrated that this suppression is mediated through the up-regulation of the production of tumor growth factor-beta in BMSCs (29). In this context, it is possible that the down-regulation of SDF-1 production in BMSCs by 5-DHT contributes to the suppression of B lymphocyte development, because SDF-1 also plays a role in the development of B lymphocytes (30).

Androgen receptor is ubiquitously expressed in the bone marrow cells of both males and females, with the exception of lymphoid and erythroid cells (31). Testosterone derivatives down-regulated IL-6 production in BMSCs in vitro (32), which was consistent with the observation that orchietomy increased bone marrow IL-6 levels in mice (33). However, little was known concerning whether testosterone or its derivatives are involved in regulating the production of hematopoietic growth factors. In the present study, we clearly showed that androgens modulate the mRNA expression of several hematopoietic growth factors in BMSCs. Oxymetholone up-regulated the production of SCF via transcriptional regulation, and conditioned medium derived from oxymetholone-treated BMSCs better supported the survival of BM CD34+ cells. These results could provide an explanation for the observed oxymetholone-mediated enhancement of granulopoiesis and

thrombopoiesis, as well as erythropoiesis, in vivo.

In summary, androgens including oxymetholone directly stimulated the clonal growth and survival of mature erythroid progenitor cells, but not that of immature erythroid progenitors or granulocytic progenitors. Oxymetholone increased the capacity of BMSCs to support hematopoietic progenitor cells, most likely by up-regulating the production of a couple of hematopoietic growth factors, including SCF.

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