0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(2):893–903

doi: 10.1210/jc.2004-0801

Membrane Androgen Receptor Activation Induces Apoptotic Regression of Human Prostate Cancer Cells *in Vitro* and *in Vivo*

Anastassia Hatzoglou,* Marilena Kampa,* Christina Kogia,* Ioannis Charalampopoulos, Panayiotis A. Theodoropoulos, Ploutarchos Anezinis, Constantina Dambaki, Evangelia A. Papakonstanti, Efstathios N. Stathopoulos, Christos Stournaras, Achille Gravanis, and Elias Castanas

Departments of Experimental Endocrinology (A.H., M.K., C.K., E.C.), Pharmacology (I.C., A.G.), Biochemistry (P.A.T., E.A.P., C.S.), Urology (P.A.), and Pathology (C.D., E.N.S.), University of Crete School of Medicine, Heraklion GR-71110, Greece

Nongenomic androgen actions imply mechanisms different from the classical intracellular androgen receptor (iAR) activation. We have recently reported the identification of a membrane androgen receptor (mAR) on LNCaP human prostate cancer cells, mediating testosterone signal transduction within minutes. In the present study we provide evidence that activation of mAR by nonpermeable, BSA-coupled testosterone results in 1) inhibition of LNCaP cell growth (with a 50% inhibitory concentration of 5.08 nM, similar to the affinity of testosterone for membrane sites); 2) induction in LNCaP cells of both apoptosis and the proapoptotic Fas protein; and 3) a significant decrease in migration, adhesion, and invasion of iAR-negative DU145 human prostate cancer cells. These actions persisted in the presence of antiandrogen flutamide or after decreasing the content of iAR in LNCaP cells by iAR antisense oligonucleotides. Testosterone-BSA was also effective in inducing apoptosis of DU145 human prostate cancer cells, negative for iAR, but expressing mAR sites. In LNCaP cell-inoculated nude mice, treatment with testosterone-BSA (4.8 mg/kg body weight) for 1 month resulted in a 60% reduction of tumor size compared with that in control animals receiving only BSA, an effect that was not affected by the antiandrogen flutamide. Our findings suggest that activators of mAR may represent a new class of antitumoral agents of prostate cancer. (*J Clin Endocrinol Metab* 90: 893–903, 2005)

THE BIOLOGICAL ACTIVITY of testosterone occurs predominantly through binding to the intracellular androgen receptor (iAR), a member of the nuclear receptor superfamily, functioning as a ligand-activated transcription factor (1). However, in recent years, a number of reports indicate additional, rapid androgen actions, including the rapid activation of kinase-signaling cascades, modification of the cytoskeleton, and modulation of intracellular calcium levels (2–7). These effects are considered to be nongenomic in nature, because they occur in cell types that lack a functional iAR or in the presence of inhibitors of transcription and translation, or are observed too rapidly to involve changes in gene transcription (8). We have recently reported the identification of an androgen-specific membrane receptor (mAR) in the LNCaP human prostate cancer cell line (9), which modifies, upon activation, actin cytoskeleton dynamics within minutes through a specific signaling cascade (10). This membrane receptor was also identified in human prostate tumors, been highly expressed on cancer cells (11).

First Published Online December 7, 2004

* A.H., M.K., and C.K. contributed equally to this work.

Abbreviations: CMO, Carboxymethyloxime; DHT, dihydrotestosterone; FasL, Fas ligand; FITC, fluorescein isothiocyanate; iAR, intracellular androgen receptor; mAR, membrane androgen receptor; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

In the present study we tested the effects of nonpermeable, BSA-coupled testosterone on proliferation and apoptosis of either iAR-negative (DU145 cells) or iAR antisense oligonucleotide-treated iAR-positive LNCaP human prostate cancer cells. Our findings suggest that activation of mAR may result in apoptotic regression of prostate cancer cells both *in vitro* and *in vivo*.

Materials and Methods

Cell cultures

LNCaP and DU145 cells (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 C in a humidified atmosphere of 5% CO_2 in air. They were subcultured once a week and incubated in serum-free medium for 24 h before experiments with the steroids. Culture media were purchased from Invitrogen Life Technologies, Inc. (Paisley, UK). All steroids [testosterone and dihydrotestosterone (DHT)] and antiandrogens (flutamide and cyproterone acetate; Sigma-Aldrich Corp., St. Louis, MO) were added, dissolved in PBS buffer. Testosterone 3-(*O*-carboxymethyl)oxime, testosterone 3-(*O*-carboxymethyl)oxime-BSA, testosterone-3-(*O*-carboxymethyl)oxime-BSA-fluorescein isothiocyanate [testosterone-3-(*O*-carboxymethyl)oxime-BSA-FITC; 10 mol testosterone/mol BSA], BSA-FITC, and testosterone-3-(*O*-carboxymethyl)oxime were obtained from Sigma-Aldrich Corp. In the case of testosterone-BSA, before each experiment, stock solutions of BSA conjugates were mixed with dextran (0.05 mg/ml) and charcoal (50 mg/ml) for 30 min, centrifuged at 3000 × *g* for 10 min, and passed through a 0.22-mm pore size filter to remove any potential contamination with free testosterone. We also assayed the medium for the presence of free testosterone with a specific RIA method. In all cases, free testosterone was lower than the detection limit of the assay. At different incubation periods, cell number was assayed by the tetrazolium salt assay (12).

Detection of mAR

Binding assays: membrane preparation. Cells cultured in 150-cm^2 flasks without serum were washed twice with PBS, removed by scraping, and centrifuged at 1500 rpm. Pelleted cells were homogenized by sonication in 50 mM Tris-HCl buffer, pH 7.4, containing freshly added protease inhibitors (10 μg/ml phenylmethylsulfonylfluoride and 1 μg/ml aprotinin). Unbroken cells were removed by centrifugation at 2,500 $\times$ *g* for 15 min. Membranes were obtained by centrifugation at 45,000 $\times$ *g* for 1 h and washed once with the same buffer. Protein concentration was measured by the method of Bradford (13).

Binding conditions. Saturation binding experiments were performed in a final volume of 0.1 ml, containing cell membranes at a final protein concentration of 2 mg/ml and at least six different concentrations of [^{3}H]testosterone (ranging from 2–50 nM; specific activity, 95 Ci/mmol; Amersham Biosciences, Little Chalfont, UK) without (total binding) or with (nonspecific binding) a 1000-fold molar excess of unlabeled androgen (DHT). For displacement binding experiments, cell membrane preparations at a final concentration of 2 mg/ml were incubated with 5 nM [^{3}H]testosterone in the absence or presence of various concentrations of an unlabeled steroid (DHT, estradiol, or progesterone), ranging from 10^{-12}–10^{-6} M. Nonspecific binding was estimated in the presence of 5 μM DHT. In both types of binding experiment, after an overnight incubation at 4 C, bound radioactivity was separated by filtration under reduced pressure through GF/B filters (Whatman, Clifton, NJ) previously soaked in 0.5% polyethylenimine in water and rinsed three times with ice-cold Tris-HCl buffer. Filters were mixed with 4 ml scintillation cocktail, and the bound radioactivity was counted in a scintillation counter (Tri-Carb, series 4000, Packard Instrument Co., Downers Grove, IL) with 60% efficiency for tritium.

Flow cytometry. Cells cultured in serum-free medium for 24 h were detached from the culture flask by scraping and suspended in PBS at a density of 10^6 cells/ml. They were incubated at room temperature with 10^{-7} M testosterone-BSA-FITC conjugate for various periods (1 min to 1 h). A 1,000-fold concentration of BSA-FITC was used to determine nonspecific binding. Cells were analyzed by flow cytometry using a FACSArray apparatus (BD Biosciences, Franklin Lakes, NJ) in a sample size of 10,000 cells gated on the basis of forward and side scatter. Testosterone-3-(O-carboxymethyl)oxime-BSA-FITC and BSA-FITC were obtained from Sigma-Aldrich Corp.

Confocal laser microscopy. Cells were allowed to grow on poly-L-lysine-coated glass coverslips for at least 48 h before culture medium was replaced with serum-free medium. After a 24-h period, cells were washed twice with PBS and incubated with testosterone-BSA-FITC for 30 min in the presence or absence of DHT. As a negative control, BSA-FITC was used. Cells were then washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 30 min. Coverslips were mounted onto slides using a 1:1 (vol/vol) mixture of glycerol and Vestashield (Vector Laboratories, Inc., Burlingame, CA). Specimens were analyzed using a confocal laser scanning microscope (TCS-NT, Leica, Heidelberg, Germany).

Measurement of apoptosis

Apoptotic proteins were assayed after SDS-PAGE and immunoblotting using specific antibodies, as previously described (14). Briefly, at the end of each experiment, cells were washed twice with PBS, removed by scraping, and centrifuged at 1,500 rpm. Cell lysis was completed at 4 C by vigorously shaking the pellet for 30 min reconstituted in a lysis buffer composed of 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Nonidet P-40, and freshly added protein inhibitors (10 μg/ml phenylmethylsulfonylfluoride and 1 μg/ml aprotinin). Solid cellular debris was removed by centrifugation at 12,000 $\times$ *g* for 15 min. The cytoplasmic fractions were collected and stored at −80 C. Protein concentration was measured with the Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA). Samples of cytoplasmic protein fractions, containing 20 μg protein, were solubilized with SDS-PAGE sample buffer and electrophoresed through a 12% sodium dodecyl sulfate gel. The resulting protein bands were transferred to nitrocellulose membranes using an electroblot apparatus (LKB, Bromma, Sweden). Standard Western blotting procedures were employed. Band intensities were quantified by PC-based Image Analysis (Image Analysis, Inc., Ontario, Canada). The following antibodies were used: as primary: antihuman Bcl-2 monoclonal antibody (clone 124, DakoCytomation, Glostrup, Denmark; 1:200), rabbit polyclonal antisera against Bax, Bak, Bcl-$x_{s/l}$, and Bad (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:100), and anti-Fas (1:2,500) and anti-Fas ligand (anti-FasL; 1:1,000, Transduction Laboratories, Lexington, KY); and as secondary: goat peroxidase-conjugated antimouse IgG (Chemicon International, Inc., Temecula, CA; 1:10,000) or antirabbit IgG (Immunotech, Marseille, France; 1:4,000). For purposes of normalization, the blots were also stained with a monoclonal antiactin antibody in a dilution of 1:400 (Amersham Biosciences).

For the detection of cell cycle, cells were incubated with the selective agents, stained with propidium iodide, and analyzed in a FACSArray apparatus (BD Biosciences), as described previously (15). Confocal laser microscopy was performed using a confocal laser scanning microscope (Leica). The percentage of mitotic cells was calculated after counting a specimen of more than 1000 cells, stained with 4′,6-diamido-2-phenylindole hydrochloride.

Apoptosis quantitation was assayed by the use of the following methods

1) The APOPercentage apoptosis assay (Biocolor Ltd., Belfast, Ireland) to quantify apoptosis. This assay uses a dye that stains red the apoptotic cells undergoing the membrane flip-flop event when phosphatidylserine is translocated to the outer leaflet. Detection of apoptosis can be readily observed under inverted microscopy. For apoptosis quantitation, the amount of dye within the labeled cells can subsequently be released into solution, and the concentration is measured at a wavelength of 550 nm, using a color filter microplate colorimeter (MicroElisa reader, Dynatech Laboratories, Chantilly, VA) (13).

2) With flow cytometry of annexin V-propidium iodide-stained cells. Cells were transferred to a staining tube and washed with 4 ml PBS containing 1% BSA at 4 C. After medium removal (200 rpm, 10 min, 4 C), 100 μl 2 μg/ml annexin V-FITC were added in a staining buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$, pH 7.4) and incubated for 10 min in the dark. Then 1 μg/tube propidium iodide was added, and cells were analyzed within 20 min by flow cytometry using a FACSArray apparatus (BD Biosciences) and with CellQuest (BD Biosciences) and ModFit LT (Verify Software, Topsham, MN) software.

Antisense oligonucleotides

Oligonucleotide treatment of cells was performed according to the protocol of Eder *et al.* (16), using the following phosphorothioate oligonucleotides: 5′-CTGCTGCTGCTGCTG-3′ (antisense), 5′-ATCGTGGTGTTGATC-3′ (missense), and 5′-TTGCAGCTGATGCTA-3′ (mismatched), against the CAG repeat region (nucleotides 703–750) of the AR. All nucleotides were synthesized by MWG Biotec (Ebersberg, Germany). The same FITC-labeled antisense oligodeoxynucleotide was also synthesized to control transfection efficiency. Transient transfection of cells was performed by the addition of 1687 μM of the corresponding oligonucleotide and incubation of cells for 24 h. Thereafter, cells were washed and coincubated with the corresponding agents. Efficacy of transfection was assayed by the corresponding FITC-labeled oligonucleotide, and detection of iAR was performed by ligand binding assay, as described previously (17).

Cell migration and adhesion

Cell migration was assayed by the *in vitro* wound-healing assay, as described previously (18). Briefly, confluent monolayers of cells were scratched with a pipette tip to create a cell-free area. Testosterone-BSA (10^{-8}, 10^{-7}, and 10^{-6} M) was added, and wound closure was documented by photography of the same region at different times. The cell-free gap was measured and reported. Adhesion of cells on collagen-treated plates was performed as previously described (19, 20). Briefly, subconfluent cultures (5×10^4 cells) preincubated for 2 h with testosterone-BSA were seeded on collagen I ELISA plates (Sigma-Aldrich) for various periods of time. After washing, adherent cells were stained with crystal violet in methanol and lysed with1% sodium dodecyl sulfate.

Absorbance, linearly proportional to the number of attached cells, was measured at 600 nm. Quantitation of migration and invasion of DU145 cells was performed in Transwell cell culture chambers provided with 8-μm pores (Costar, Cambridge, MA). Cells were pretreated with 10^{-7} M testosterone-BSA for 2 h, then they were added to the upper compartment of a Transwell cell culture chamber provided with a polycarbonate filter with 8-μm pores. Twenty-four hours later, filters were stained with crystal violet. Attached cells were lysed with 30% acetic acid, and absorbance (proportional to the number of cells) was counted (21). In all experiments a fixed concentration of 10^{-7} M testosterone-BSA was used, which was found to produce submaximal inhibition of cell growth.

Animal experiments

Male BALB/c$^{-/-}$ nude mice (10 wk old) with intact gonads were purchased from Harlan (San Pietro al Natisone, Italy). They were housed in a sterile environment and fed *ad libitum*. One week after acclimatizing, animals were injected in the back with 5×10^6 LNCaP cells diluted in Matrigel (Sigma-Aldrich Corp.) in a total volume of 0.1 ml. After 4 wk, macroscopic tumors had developed. Thereafter, animals were treated with ip injections (three times per week for 4 wk) of testosterone-BSA (4.8 mg/kg body weight, corresponding to a calculated circulating concentration of 5×10^{-7}), diluted in PBS three times per week in a total volume of 0.5 ml, as shown in Fig. 4. Tumors were measured with a Vernier (Metrax, Rotweil, Germany) every week, and animals were killed after 4 wk of treatment. Tumors were excised, measured, and weighed, and pathology was analyzed. They were stained with hematoxylin-eosin and observed by the same pathologist. The labeled streptavidin-biotin method with the SuperSensitive Biotin-Streptavidin Immunodetection System (QA200-OX, Biogenex, San Ramon, CA) according to the manufacturer's instructions was used to immunostain formalin-fixed and paraffin-embedded 3-μm-thick tissue sections for mitotic activity with the mouse antihuman monoclonal antibody MIB-1 (M7240, DakoCytomation; dilution 1:50). Fast Red was used as chromogen, and Mayer's hematoxylin was used for counterstaining. The indirect terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling (TUNEL) enzymatic labeling technique was used to detect apoptotic activity of the tumors, (*in situ* cell death detection kit with alkaline phosphatase/AP, Roche, Mannheim, Germany), according to the manufacturer's instructions.

For toxicity studies, BALB/c$^{-/-}$ mice were treated with 4.8, 48, and 96 mg/kg body weight, achieving calculated plasma concentrations of 5×10^{-7}, 5×10^{-6}, and 10^{-5}, every other day. Another group of animals, injected with vehicle, was included in the study and served as the control. After 1 month, animals were killed and weighed, and liver, prostate, and testes were isolated. Slices were stained with hematoxylin-eosin and examined by the same pathologist. All animal experiments were approved by the Faculty of Medicine animal committee.

Statistical analysis

Statistical analysis was performed by parametric methods (*t* test or ANOVA), using the Origin V5.0 (Microcal Software, Northampton, MA) and the Systat V10.0 (SPSS, Inc., Chicago, IL) microcomputer programs. In the figures, an asterisk indicates statistical significance at the 0.05 level at least.

Results

In vitro effects

Activation of mAR decreases the growth of LNCaP human prostate cancer cells. We incubated LNCaP cells with variable concentrations of testosterone-BSA and assayed cell number 72 h later, taking into account the doubling time of this cell line (~70 h). Testosterone-BSA induced a dose-dependent inhibition of cell growth (Fig. 1A), with a 50% inhibitory concentration (IC_{50}) of 5.08 ± 1.3 nM, comparable to the affinity of testosterone for membrane binding sites (9). Exposure of LNCaP cells to free testosterone (10^{-7}) induced a typical increase in cell number (Fig. 1C). Such long incubation times could not exclude the possibility of a dissociation of testosterone-BSA conjugate and a subsequent action of the free steroid molecule. However, serial detection of free testosterone in the culture medium with a specific RIA was negative, confirming the stability of the conjugate. Additionally, free testosterone bound to iAR would be expected to produce an induction of cell proliferation and not the inhibition of cell growth we observed. To exclude such an action, we performed a number of additional experiments. 1) We introduced into the cell medium a constant concentration of the antiandrogen flutamide (10^{-6}). This addition did not modify the antiproliferative effect of testosterone-BSA, suggesting the involvement of receptors different from the classical iAR (Fig. 1A). Incubation of cells with flutamide alone did not have any effect on cell proliferation (Fig. 1A) (2). After the initial 24-h incubation, cells were washed with fresh medium and deprived of testosterone-BSA, and the incubation was continued in this medium for an additional 48-h period. The decrease in cell proliferation persisted in this case also (Fig. 1B), but with a significantly lower IC_{50} (45.8 ± 13.2 nM; $P < 0.01$). These findings suggest that the antiproliferative effect of the conjugate persists at least 48 h after the elimination of testosterone-BSA (3). We transiently transfected LNCaP cells with iAR antisense oligonucleotides directed against the CAG repeat region (bases 703–750) of iAR. Transfection with this agent resulted in a 70% decrease in iAR expression, confirmed by radioligand binding (Fig. 1C). Missense or mismatched oligonucleotides did not modify the expression of iAR. The antisense iAR manipulation did not significantly affect the antiproliferative effect of testosterone-BSA (Fig. 1D), also suggesting the involvement of membrane androgen-binding sites different from classical iAR. These data indicate that in iAR-positive LNCaP cells, long-term incubation in the presence of testosterone-BSA induces cell growth inhibition, with an IC_{50} at the nanomolar level. This effect appears to be iAR independent, probably involving the activation of mAR.

Testosterone-BSA induces apoptosis of iAR-positive (LNCaP) and iAR-negative (DU145) human prostate cancer cells. To test the hypothesis that the decrease in cell number seen in the presence of testosterone-BSA might be due to apoptotic regression of prostate cancer cells, we quantitated apoptosis with the ApoPercentage assay in LNCaP cells (Fig. 2, A and B). Apoptosis was significantly higher (by 55 ± 0.09% of control; n = 8; $P < 0.01$) only after long (24- and 48-h) incubation periods. The number of cells in mitosis after testosterone-BSA incubation was not different from that of control cells, suggesting that the antiproliferative effect of the agent is mainly due to its proapoptotic properties. Flow cytometry analysis of propidium iodide-stained cellular DNA confirmed this observation (data not shown). Quantitation of the apoptotic proteins (Fig. 2C) revealed a strong increase in the expression of Fas protein. Indeed, testosterone-BSA preincubation for 24 h, followed by 48 h in the absence of the agent, resulted in a 6-fold increase in Fas protein (6.59 ± 0.57 of control; n = 6; $P < 0.001$), whereas FasL was not significantly affected by the treatment. Additionally, testosterone-BSA provoked a slight, but significant, increase in anti-apoptotic

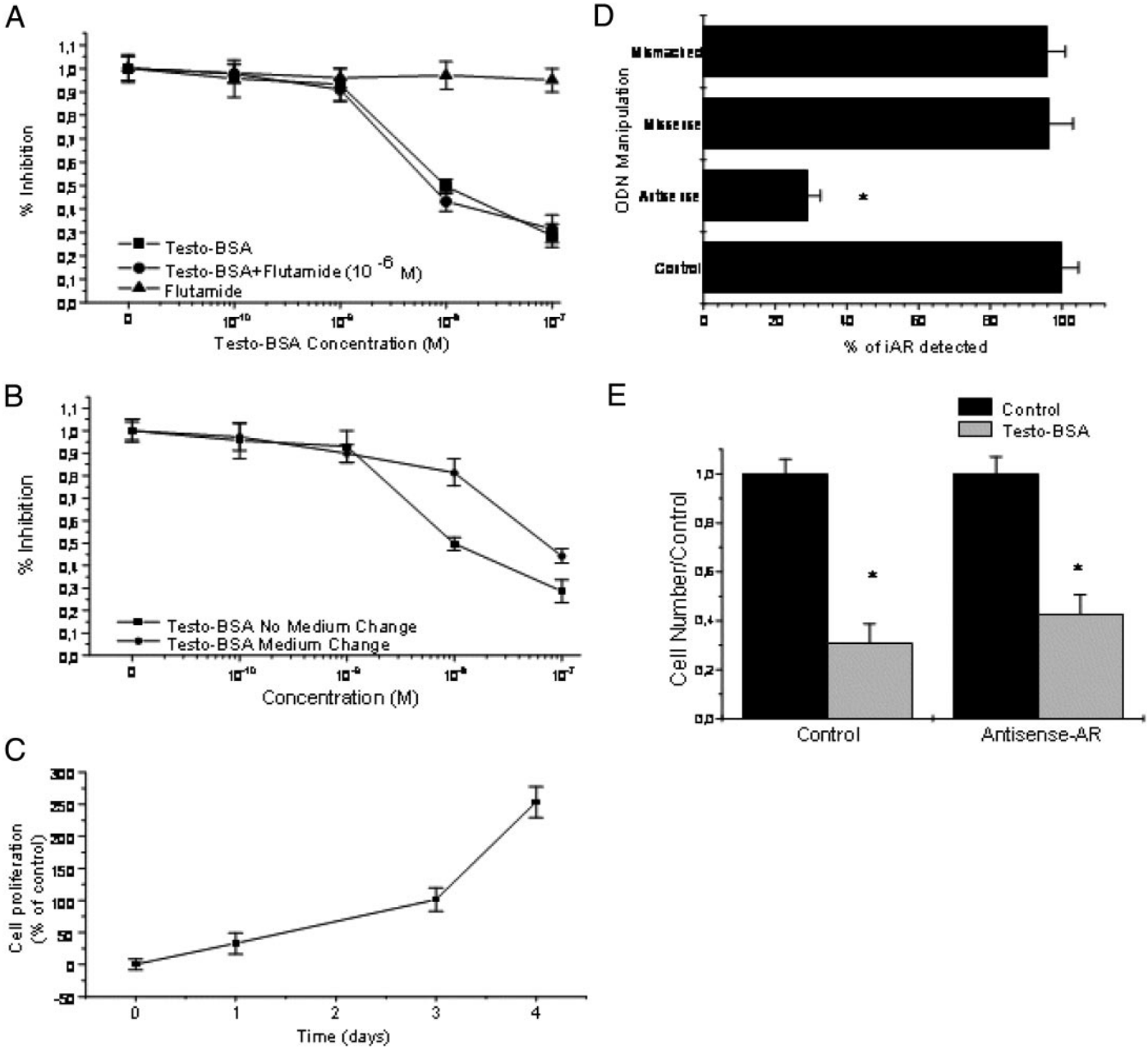

FIG. 1. Antiproliferative effect of testosterone-BSA on LNCaP human prostate cancer cells. A, Cells were incubated with the indicated concentrations of testosterone-BSA for 72 h in the absence or presence of 10^{-6} M flutamide. Results are presented as the ratio of the observed effect in the presence of the agent divided by the proliferation of control cells, incubated in the absence of testosterone-BSA (mean ± SEM of three different experiments performed in triplicate). The effect of the antiandrogen flutamide alone is also presented. Cell growth was measured with the MTT (3-[4,5-dimethlthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. B, LNCaP cells were incubated for 24 h in the presence of the indicated concentrations of testosterone-BSA. Thereafter, in one case (■) incubation was followed for another 48 h, whereas in the other (●) cells were washed with PBS, medium was replaced with fresh medium, deprived of testosterone-BSA, and incubation was continued for 48 h. At the end of the experiments, cell number was measured with the MTT assay. Results are presented as the ratio of the effect in the presence of the agent divided by the proliferation of control cells, incubated in the absence of testosterone-BSA. Shown are the mean ± SEM of four independent experiments performed in triplicate. C, Time effect of testosterone (10^{-7} M) on LNCaP cell growth. Cells were incubated with testosterone (10^{-7} M) for the indicated time periods. Cell number was assayed with the MTT method. Shown are the mean ± SEM of three independent experiments performed in triplicate. This figure presents relative growth, compared with that of control (nontreated) cells. D and E, Effect of transient transfection with an antisense oligonucleotide against CAG repeat region (nucleotides 703–750) of the iAR. Transfection *per se* induced a 20% decrease in cell growth. In that case, the effect of testosterone-BSA (10^{-7} M) was compared with that of cells transfected with a missense oligonucleotide. The effect of the agent was assayed after 3 d. D, Number of iAR, assayed with a radioligand binding assay. Shown are the mean ± SEM of three independent experiments performed in triplicate. *, $P < 0.01$. E, Effect of the transient transfection on testosterone-BSA-induced inhibition of cell growth. Shown are the mean ± SEM of three independent experiments performed in triplicate. *, $P < 0.01$ compared with the corresponding controls.

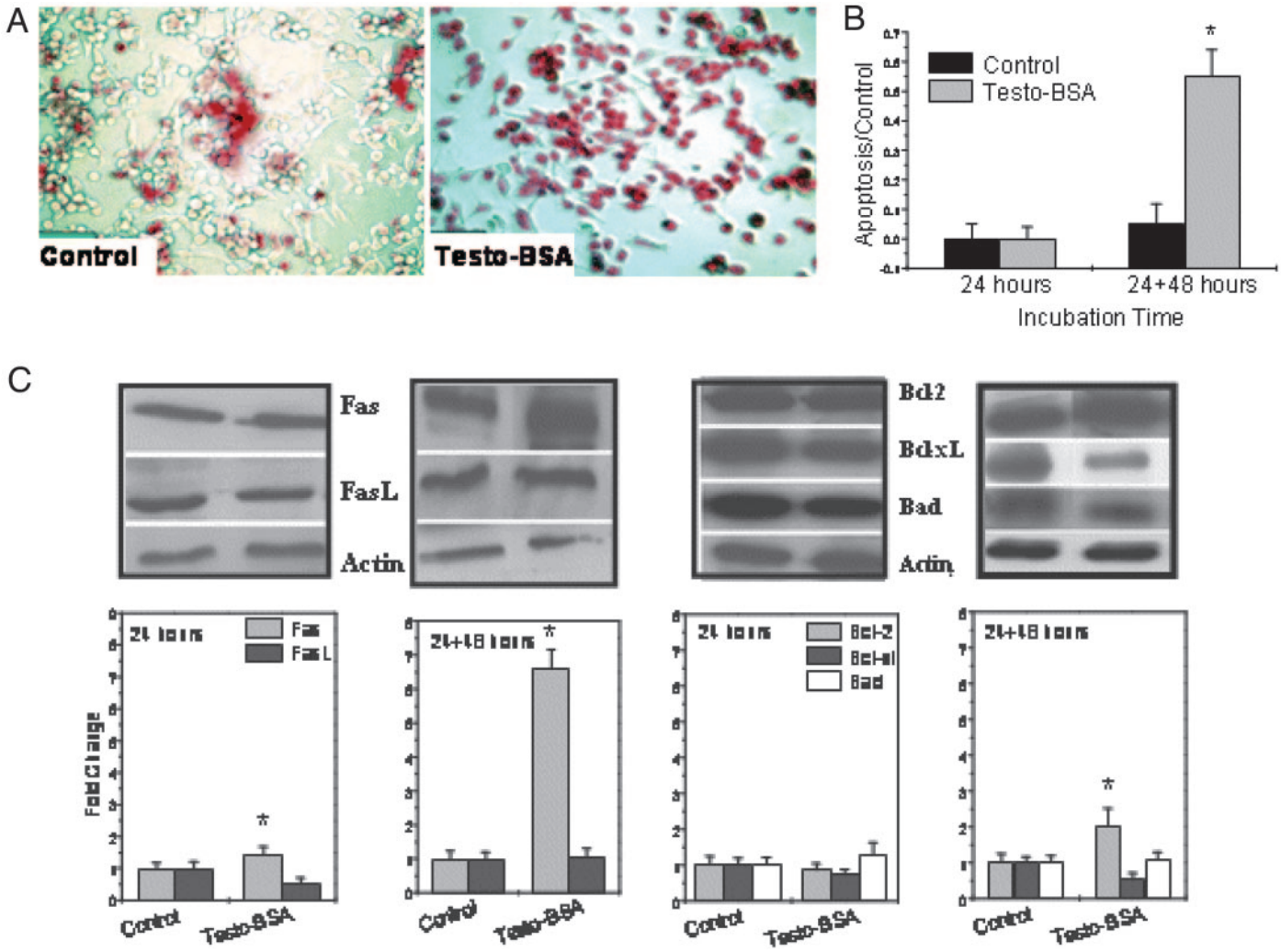

FIG. 2. Proapoptotic effect of testosterone-BSA on iAR-positive LNCaP human prostate cancer cells. A, Cells, stained by the ApoPercentage kit, according to the manufacturer's instructions. Apoptotic cells are stained in *red*. Control (BSA-treated) cells are shown at the *left*. Cells were treated with testosterone-BSA (10^{-7} M) for 24 h, followed by incubation in normal medium for an additional 48 h. B, Quantitation of results as the mean ± SEM of three independent experiments. C, Quantification of proapoptotic and antiapoptotic proteins. Shown are the mean ± SEM of three independent experiments and a representative blot.

Bcl-2 protein (2.03 ± 0.48 of control; n = 5; $P < 0.01$). This effect persisted when incubation was prolonged for 48 h more in the absence of the drug, probably representing an inherent rescue response of the cells to the apoptotic challenge.

To explore the antiproliferative effect of mAR, we used DU145 human prostate cancer cells. This cell line does not express iAR (22), but strongly expresses mAR, as shown by flow cytometry (Fig. 3A), confocal laser microscopy (Fig. 3B), and radioligand binding (K_d = 9.45 nM; 361 fmol/mg protein; Fig. 3C). Testosterone-BSA induced a dose-dependent decrease in DU145 cell proliferation (Fig. 3D), with an IC_{50} of 10.67 ± 2.22 nM. It is of note that in DU145 cells the same effect was seen with free, non-BSA-conjugated testosterone (Fig. 3D). These findings strongly support the hypothesis that the observed antiproliferative effect of testosterone-BSA is unrelated to the presence of intracellular AR.

Interestingly, in DU145 cells not expressing iAR, both free testosterone and testosterone-BSA induced apoptosis (Fig. 3E). Indeed, incubation of DU145 cells with 10^{-7} M free testosterone or testosterone-BSA for 48 h resulted in a time-dependent induction of apoptosis; the maximal effect was seen at 48 h (64.3 ± 1.16% and 90.7 ± 1.05% of control, respectively; n = 5; $P < 0.01$; Fig. 3E). Both testosterone and DHT (probably due to their faster metabolism) showed a lower apoptotic ratio with a longer incubation period (72 h) compared with testosterone-BSA. These findings also support the hypothesis that the proapoptotic effect of testosterone-BSA is iAR independent and probably involves mAR.

The proapoptotic effect of testosterone is shared by its carboxymethyloxime derivative. To explore whether the action of testosterone-BSA was due to its action on membrane androgen sites, or inversely, whether the chemical modification of the molecule (through its esterification with a carboxymethyloxime molecule), we incubated DU145 cells with testosterone and testosterone-carboxymethyloxime (testosterone-CMO). As shown in Fig. 4, both agents induced a time- and dose-

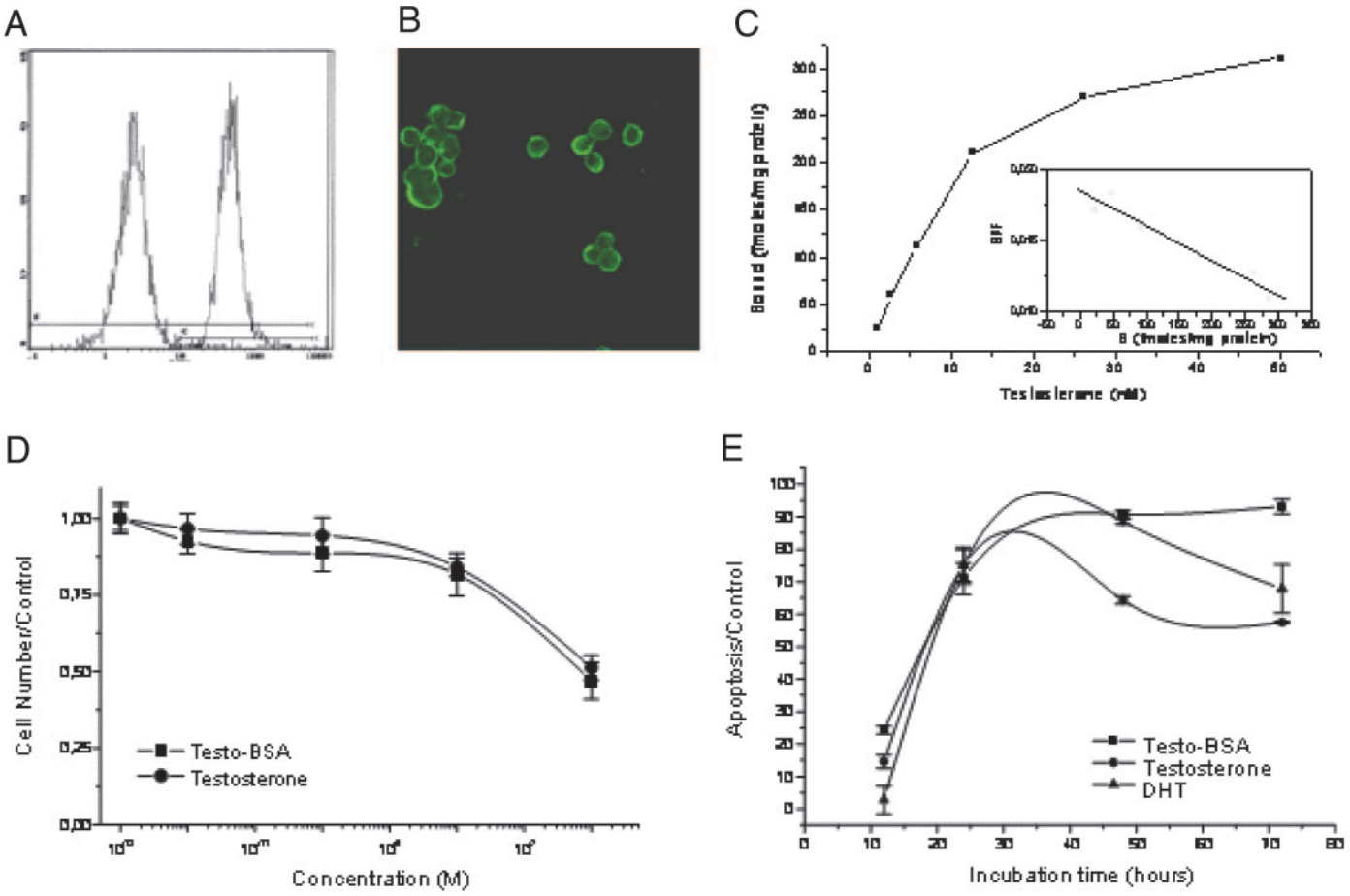

FIG. 3. Antiproliferative and proapoptotic effects of testosterone-BSA in iAR-negative DU145 human prostate cancer cells. A, Detection of membrane androgen binding in iAR-negative DU145 cells by flow cytometry. The figure depicts a typical flow cytometric analysis of specific testosterone-BSA-FITC conjugate (*right curve*) and corresponding BSA-FITC binding *(left curve*), performed as described previously (9). The difference between the two curves represents specific testosterone-BSA binding. B, Representative confocal section of DU145 cells, incubated for 10 min with testosterone-BSA-FITC. Note the peripheral membrane labeling of the cells. Under the same conditions, BSA-FITC did not show any labeling of cells. C, Typical saturation binding of [^{3}H]testosterone on membrane fractions of DU145 cells. *Inset*, Scatchard analysis of data. The saturation binding was repeated three times with similar results. D, Effect of incubation for 3 d with testosterone (●) and testosterone-BSA (■) on proliferation of DU145 cells. Shown are the mean ± SEM of three independent experiments. E, Effects of testosterone and testosterone-BSA on the apoptosis of DU145 cells. DU145 cells were incubated with 10^{-7} M testosterone (●), DHT (▲), or testosterone-BSA (■) for 48 h. Apoptosis was assayed with the ApopTag assay. The figure presents the apoptosis measured in the presence of the agents, divided by that in the corresponding control (cells incubated in the absence of the agents). BSA had no effect on apoptosis. Shown are the mean ± SEM of three different experiments performed in quadruplicate.

related apoptosis of cells. Patterns of apoptosis were similar with both agents: a 60% peak (58.2 ± 1.66 increase after testosterone; 57.8 ± 5.77 increase after testosterone-CMO; not significantly different) was observed after 12 h, and a gradual decline of action was observed thereafter. Nevertheless, even after 72-h incubation in the presence of 10^{-7} M testosterone or testosterone-CMO, a significant increase over the control value was apparent (31.1 ± 3.9 and 30.6 ± 3.9; $P < 0.01$ *vs.* control). These results indicate that addition of CMO to the molecule of testosterone does not modify its action on membrane androgen-binding sites. Binding experiments (not shown) revealed that testosterone and testosterone-CMO displace radiolabeled testosterone with the same affinity.

Testosterone-BSA decreases iAR-negative DU145 human prostate cancer cell migration, adhesion, and invasiveness. The adhesion and motility of tumor cells through the extracellular matrix are considered important steps in the invasive process. We thus examined whether testosterone-BSA could affect these processes. We used DU145 cancer cells, which possess mAR and are devoid of iAR. Preincubation of cells with 10^{-7} M testosterone-BSA significantly inhibited (24 ± 1.2; n = 8; $P < 0.001$) within 4 h the adhesion of cells to type I collagen (Fig. 5A). Furthermore, we used the *in vitro* wound-healing assay to assess the effect of testosterone-BSA on cell migration. As depicted in Fig. 5B, testosterone-BSA induced an inhibition of wound healing at 20 h by 60.4 ± 7.3% of the control value (n = 18; $P < 0.01$). This effect was dose dependent (Fig. 5B, *inset*). Quantitation of the effect of testosterone-BSA (10^{-7} M) on migration of DU145 cells using the Transwell migration assay (Fig. 5C) revealed a 70 ± 13.1% inhibition of migration through the filter 24 h after testosterone-BSA application (n = 8; $P < 0.001$). Finally, the same concentration of testosterone-BSA (10^{-7} M) inhibited by 42 ± 15.6% (n = 10; $P < 0.05$) the invasion of DU145 cells in our Matrigel Transwell assay (Fig. 5D).

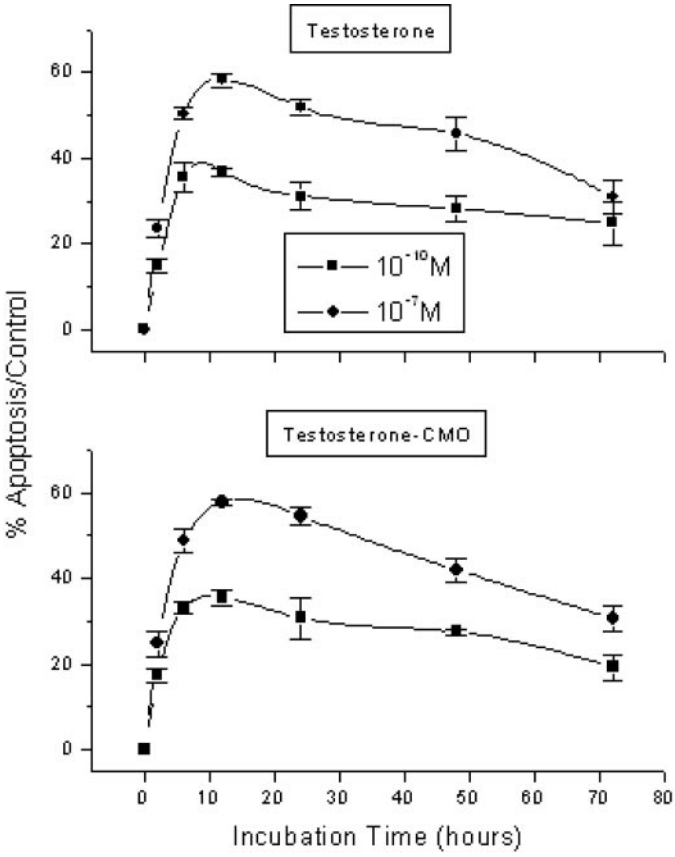

FIG. 4. Proapoptotic and binding affinity of testosterone-CMO on DU145 prostate cancer cell apoptosis. DU145 prostate cancer cells were incubated for the indicated time periods with 10^{-10} M (■) and 10^{-7} M (●) testosterone (*top*) and testosterone-CMO (*bottom*, ●). Apoptosis, normalized for that of control cells (without the addition of hormone), is presented as the mean ± SEM of two different experiments performed in triplicate.

In vivo effects

Testosterone-BSA administration decreased tumor mass in LNCaP cell-inoculated male nude mice. We tested the antitumoral, proapoptotic actions of testosterone-BSA *in vivo*. For this purpose we inoculated BALB/$c^{-/-}$ male nude mice with LNCaP cells in Matrigel and, after the development of macroscopic tumors, 1 month later treated them with ip injections (three times per week for 4 wk) of testosterone-BSA (4.8 mg/kg body weight, corresponding to a circulating concentration of $\sim 5 \times 10^{-7}$). Our results shown in Fig. 6A suggest that testosterone-BSA produced a linear, time-related reduction of tumor size, which became significant after 4 wk. Cyproterone acetate, a pure antiandrogen, coadministered with testosterone-BSA (52.11 mg/kg body weight, 10^{-6} plasma concentration) did not significantly alter the testosterone-BSA-induced decrease in tumor size, suggesting that no major dissociation of the conjugate and free hormone release occurred. The volume distribution of excised tumors at the end of the experiment (4 wk) is presented in Fig. 6B. The intermittent administration of testosterone-BSA induced a significant ($P < 0.01$) decrease in tumor volume (55.8 ± 3.9 compared with 87.2 ± 7.2 in control animals receiving only BSA). The histological analysis of tumors showed that mitoses (Fig. 7C) predominated at the periphery of all tumors. Apoptotic cells were present in significant numbers (Fig. 7B), predominantly at the periphery of tumors of animals treated with testosterone-BSA, whereas they were significantly less in the animals treated with BSA alone (control). Cyproterone acetate administration did not alter this pattern.

We also tested the toxicity of testosterone-BSA in nude mice. A series of BALB/$c^{-/-}$ mice was treated with increasing concentrations of testosterone-BSA (4.8, 48, and 96 mg/kg body weight, achieving plasma concentrations of 5×10^{-7}, 5×10^{-6}, and 10^{-5}) repeatedly (three times per week) for 4 wk . One month later, animals were killed and weighed, and liver, prostate gland, and testes were dissected and examined for toxic (liver) or stimulatory effects (testes and prostate). All animals supported the treatment remarkably well. Body weight was not altered by the treatment at any dose used, and neither organ presented any histological modification. These findings suggest that testosterone-BSA treatment *in vivo* is not toxic for the experimental animals, and that dissociation of free testosterone from its BSA conjugate, if present, is minimal, because no sign of androgen-inducted stimulation was found in target organs (prostate and testes).

Discussion

In recent years a number of studies introduced the concept of nongenomic steroid hormone actions to explain observations related to rapid steroid effects. The classical model of steroid action involves binding to specific intracellular steroid-binding proteins (receptors), translocation to the nucleus, DNA binding, and activation of specific genes (23). This sequence requires hours to be completed. According to the Mannheim criteria (6), a nongenomic effect (proposed as membrane-initiated steroid signaling) might occur within minutes, being present in cells not expressing classical steroid receptors and being insensitive to inhibitors of transcription and translation. Furthermore, activation of these receptors might be triggered by nonpermeable (*e.g.* BSA-coupled) steroids, and it is, in most cases, insensitive to steroid antagonists. Nongenomic steroid actions have been reported for most prominent steroids (for recent reviews, see Refs. 8 and 24). Although the nature of these membrane steroid sites was elusive until recently, the identification of a membrane progesterone receptor (25–28) and the isolation of a membrane glucocorticoid-binding protein with homologies with κ-opioid receptors (29) show that these proteins might belong to the seven-transmembrane G protein-coupled receptors.

Recently, we identified in the LNCaP human prostate cancer cell line, an androgen-specific membrane receptor (9) that modifies, upon activation, actin cytoskeleton dynamics within minutes through a specific signaling cascade (10). This membrane receptor was also identified in human prostate tumors, showing a higher expression on cancer cells (11). Different mechanisms of action of membrane steroid receptors have been reported. They include kinase regulation, cyclic nucleotide modulation, and intracellular calcium changes (2–5, 8, 24, 30–33) within minutes. In rare conditions where the effects of the nongenomic actions have been de-

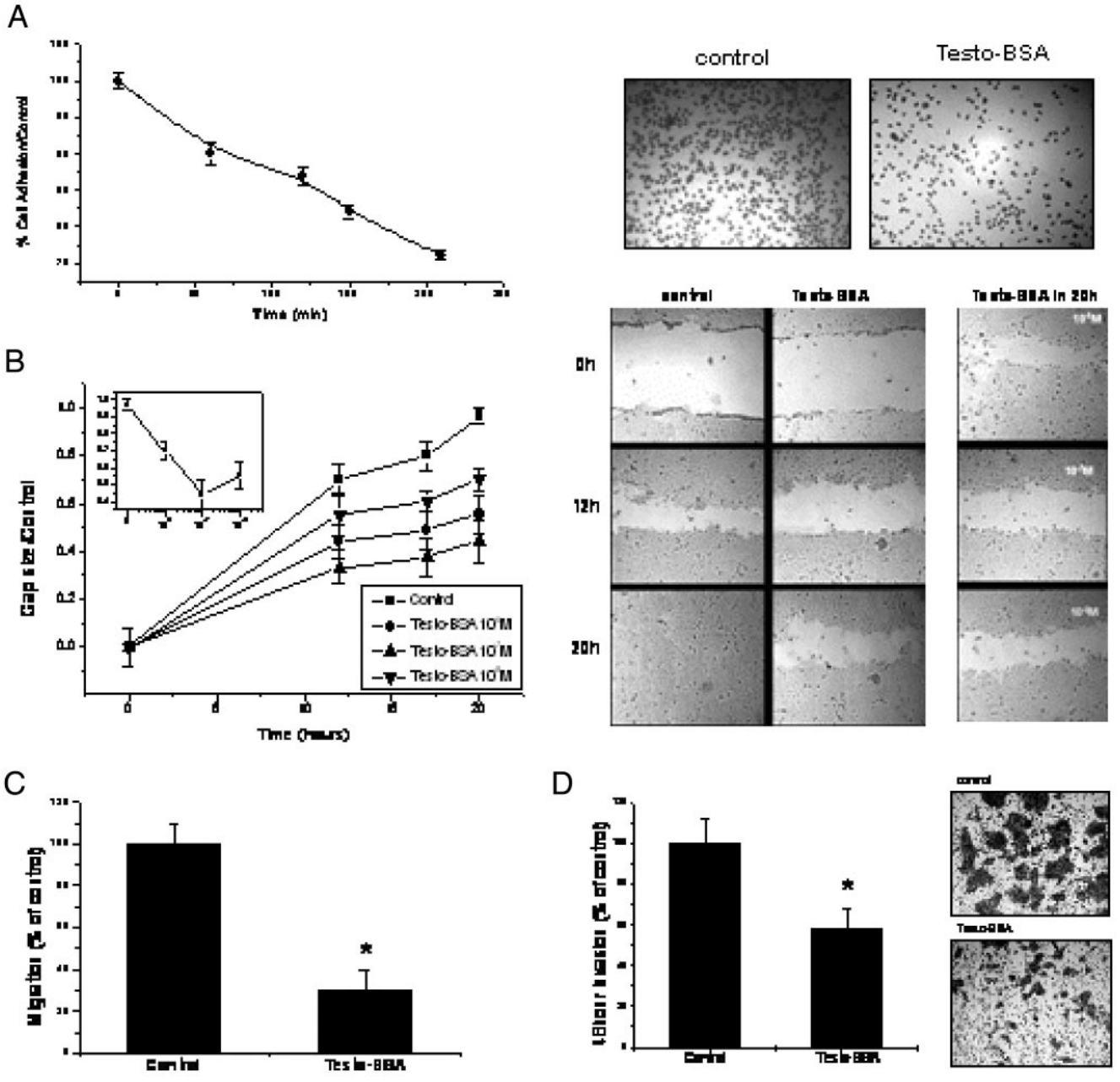

FIG. 5. Effect of testosterone-BSA on DU145 human prostate cancer cell migration and adhesion. A, Effect of testosterone-BSA (10^{-7} M) preincubation on the adhesion of DU145 cells on collagen I. Subconfluent cultures (5×10^4 cells), preincubated for 2 h with testosterone-BSA, were seeded on collagen I ELISA plates for various time periods. After washing, adherent cells were stained with crystal violet in methanol and lysed with 1% sodium dodecyl sulfate. Absorbance, linearly proportional to the number of attached cells, was measured at 600 nm. The *left curve* presents the time effect of testosterone-BSA compared with the corresponding control (mean ± SEM of eight independent experiments performed in triplicate). *Right* microphotographs depict typical cases of stained cells after incubation for 2 h. B, Effect of testosterone-BSA on migration of DU145 cells. Cells were cultured for 24 h, then the confluent monolayer was scratched with a pipette tip to create a cell-free area. Testosterone-BSA (10^{-8}, 10^{-7}, and 10^{-6} M) was added, and wound closure was documented by microphotography of the same region in different periods of time. Curves show the diameter of scratch in control cells and after the addition of various concentrations of testosterone-BSA. The *inset* presents the dose dependence of the phenomenon 20 h later (mean ± SEM of three different experiments with five repetitions per experiment). Pictures show the pattern of the initial scratch and the time-and dose-dependent healing. C, Quantitation of DU145 cell migration. Cells were pretreated with 10^{-7} M testosterone-BSA for 2 h, then they were added to the upper compartment of a Transwell cell culture chamber, provided with a polycarbonate filter with 8-μm pores. Twenty-four hours later, filters were stained with crystal violet. Attached cells were lysed with 30% acetic acid, and absorbance (proportional to the number of cells) was counted. Shown are the mean ± SEM of eight independent experiments. *, $P < 0.001$. D, Inhibition of DU145 cell invasion by testosterone-BSA. Cells were cultured in the presence of 10^{-7} M testosterone-BSA on the Matrigel (10 μg/well)-coated upper compartment of Transwell culture chambers, provided with an 8-μm pore size polycarbonate filter. Forty-eight hours later, Matrigel was removed by scraping, and invaded cells, attached to the lower surface of the filter, were stained with crystal violet and either photographed (as shown in the *right* photographs) or lysed with 30% acetic acid and counted (*left* graph). Shown are the mean ± SEM of 10 different assays. *, $P < 0.05$. In all cases, control cells were incubated in the presence of 10^{-7} M BSA.

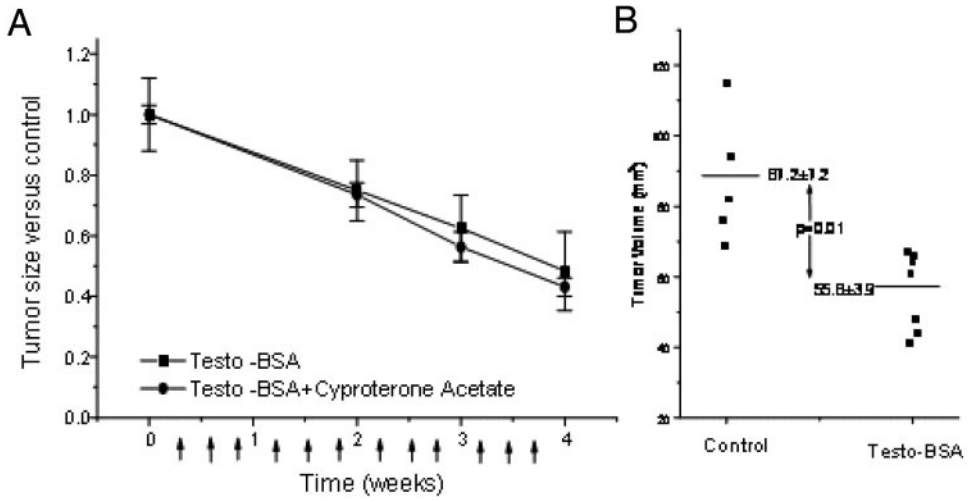

FIG. 6. *In vivo* effects of testosterone-BSA in LNCaP cell-inoculated nude mice. A, Effect of testosterone-BSA administration on LNCaP-induced tumor size in male nude mice. Each group consisted of seven mice. One month after inoculation, animals developed macroscopic tumors Thereafter, testosterone-BSA (4.8 mg/kg body weight, achieving a calculated plasma concentration of $\sim 10^{-7}$ M) was administered three times per week (*arrows*) for 4 wk . At the indicated time intervals, tumor size was measured with a Vernier. ■, Tumor size at a given interval, compared with the control (BSA-treated) animals. ●, Another series of animals treated with the combination of testosterone-BSA with 52.1 mg/kg body weight cyproterone acetate (achieving a calculated plasma concentration of $\sim 10^{-7}$ M). The mean tumor volume is presented. In the control group, two animals died at the end of the first month. B, Distribution of the tumor volume at the end of the experiment. Animals were killed, tumors were excised, and their exact volume was measured with a Vernier. Their volume (sphere) was calculated and depicted. *Horizontal bars* present the mean of each group.

tected after long incubation times, discrepancies have been found, indicating the possible participation of classical intracellular steroid receptors also (reviewed in Ref. 8).

Prostate cancer seems a tissue of choice for the detection of nongenomic actions of androgen. Indeed, in this tissue a preferential expression of mAR has been detected (11) compared with noncancerous cells, and a new mechanism of action has been identified, involving actin cytoskeleton modification, through a specific signaling cascade (9, 10). In the present work we explored the involvement of testosterone membrane receptor activation in the control of human prostate cell proliferation and growth. Our results suggest that activation of membrane sites by a BSA-conjugated androgen results in a strong and dose-dependent regression of prostate cancer cells. This effect persists for more than 48 h, even after withdrawal of BSA-conjugated androgen from the culture medium. In contrast to previously described, long-term effects (34), this action was not antagonized by most effective androgen antagonists, indicating that probably membrane testosterone receptors are the mediator of this action. The

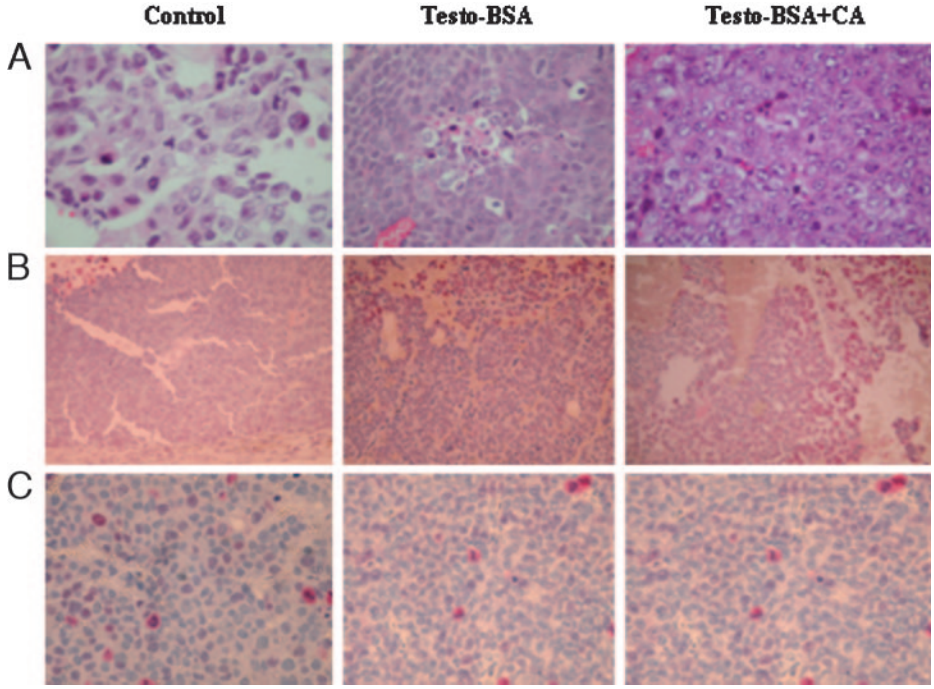

FIG. 7. Histological staining of tumor slides from LNCaP cell-inoculated male nude mice treated with testosterone-BSA. Typical histological preparations from each category (A, hematoxylin-eosin staining; B, TUNEL assay; C, MIB-1 staining). In TUNEL staining, apoptotic cells are stained deep purple. Note that apoptosis was more prominent in peripheral parts of the tumors. In MIB-1 staining, dividing cells are stained *deep purple*. No differences were observed between control and treated animals in the density or distribution of mitotic cells.

involvement of this membrane site in the apoptotic effects of testosterone-BSA was confirmed by the following observations. Testosterone-BSA was effective in an iAR-negative and mAR-positive cell line (DU145) or after transfection of iAR-positive LNCaP cells with an iAR antisense oligonucleotide, which produced a dramatic decrease in the content of intracellular AR. Finally, in iAR-negative DU145 cells, testosterone, testosterone-CMO, and testosterone-BSA effectively induced the same antiproliferative and proapoptotic effects.

Apoptosis is triggered in LNCaP cells both *in vitro* and *in vivo* after testosterone-BSA treatment. Testosterone-BSA caused a strong elevation of the apoptotic Fas receptor, suggesting its participation in the proapoptotic effects of this agent. It is interesting that testosterone-BSA did not significantly alter the FasL protein. It is thus possible that activation of mAR may cause an increase in the sensitivity of prostate cancer cells to the locally produced apoptotic signal, FasL. Indeed, human prostate cancer cells, including the cell lines LNCaP and DU145, express the FasL gene and produce its end product (35–37). Furthermore, we showed that testosterone-BSA, in parallel with its apoptosis-inducing action, participated in the regulation of the antiapoptotic component of the Bcl-2 family, Bcl-2 protein, causing its significant elevation. It is of note that other apoptosis-inducing substances, such as rapamycin, up-regulate antiapoptotic Bcl-2 proteins in human medullary thyroid carcinoma cell lines (38). A similar phenomenon has been described in pheochromocytoma PC12 cells, where prevention of apoptosis is accompanied by decreased antiapoptotic Bcl-2 proteins (39). Thus, it is possible that a temporal antiapoptotic, Bcl-2-related mechanism is activated to counteract the stress signals generated by the apoptosis-inducing factors to rescue cells from programmed death. Migration, adhesion, and invasion of DU145 prostate cancer cells were decreased significantly in testosterone-BSA-treated cells. Increased mobility and migration of malignant cells through the stroma are initial processes in the invasiveness of tumor cells, facilitating their spreading in distant sites (see Ref. 40 for a review). Furthermore, increased attachment of cells to basement membranes permit them to establish distant foci, generating metastases in other organs (41). Our findings suggest that activation of mAR may inhibit these processes.

The efficacy of testosterone-BSA in inhibiting human prostate cancer cell growth was tested *in vivo* in male nude mice inoculated with LNCaP cells. One month of treatment with minimal doses of the agent (those producing 10^{-7} M in blood) caused a 60% reduction of tumor size compared with that in control animals. The curve describing the change in tumor size was negatively linear and did not present a maximum at the time tested. It is thus, possible that longer treatment periods would produce a more significant reduction of tumor size. Testosterone-BSA treatment resulted in apoptosis of tumor cells and a reduction of cell proliferation. The agent was nontoxic for the animals, even at concentrations 100 times higher than the effective ones. In this respect, testosterone-BSA could represent a new antitumoral agent for prostate cancer, alone or in association with specific androgen antagonists actually in use, to rule out the possibility of acting as a growth-promoting factor, after possible dissociation of the conjugate and release of free hormone.

Prostate cancer generally starts as an androgen-dependent tumor and evolves to androgen insensitivity. To have a realistic chance to tackle the poor outcome of the advanced disease, identification of alternative therapeutic approaches is necessary. Our data provide the first evidence of an antitumoral effect of androgens in prostate cancer, acting through the activation of mAR both *in vitro* and *in vivo*. The preferential expression of these sites in prostate cancer (11) and the efficacy *in vitro* and *in vivo* of testosterone-BSA combined with its lack of toxicity point to a putative use of membrane testosterone activators as a new class of antitumoral agents in the treatment of prostate cancer.

Acknowledgments

Received April 29, 2004. Accepted November 23, 2004.

Address all correspondence and requests for reprints to: Dr. Elias Castanas, Laboratory of Experimental Endocrinology, University of Crete School of Medicine, P.O. Box 2208, Heraklion GR-71003, Greece. E-mail: castanas@med.uoc.gr.

This work was supported by grants from the General Secretariat of Research and Technology and the University of Crete.

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