The Androgen Derivative 5α-Androstane-3β,17β-Diol Inhibits Prostate Cancer Cell Migration Through Activation of the Estrogen Receptor β Subtype

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Abstract

Prostate cancer growth depends, in its earlier stages, on androgens and is usually pharmacologically modulated with androgen blockade. However, androgen-ablation therapy may generate androgen-independent prostate cancer, often characterized by an increased invasiveness. We have found that the 5α-reduced testosterone derivative, dihydrotestosterone (the most potent natural androgen) inhibits cell migration with an androgen receptor–independent mechanism. We have shown that the dihydrotestosterone metabolite 5α-androstane-3β,17β-diol (3β-Adiol), a steroid which does not bind androgen receptors, but efficiently binds the estrogen receptor β (ERβ), exerts a potent inhibition of prostate cancer cell migration through the activation of the ERβ signaling. Very surprisingly, estradiol is not active, suggesting the existence of different pathways for ERβ activation in prostate cancer cells. Moreover, 3β-Adiol, through ERβ, induces the expression of E-cadherin, a protein known to be capable of blocking metastasis formation in breast and prostate cancer cells. The inhibitory effects of 3β-Adiol on prostate cancer cell migration is counteracted by short interfering RNA against E-cadherin. Altogether, the data showed that (*a*) circulating testosterone may act with estrogenic effects downstream in the catabolic process present in the prostate, and (*b*) that the estrogenic effect of testosterone derivatives (ERβ-dependent) results in the inhibition of cell migration, although it is apparently different from that linked to estradiol on the same receptor and may be protective against prostate cancer invasion and metastasis. These results also shed some light on clinical observations suggesting that alterations in genes coding for 3β-hydroxysteroid dehydrogenases (the enzymes responsible for 3β-Adiol formation) are strongly correlated with hereditary prostate cancer. (Cancer Res 2005; 65(12): 5445-53)

Introduction

Prostate cancer develops in response to an abnormal activation of the androgen receptor, which may induce a variety of misregulated and unbalanced events leading to tumor progression (1). In the early stages, prostate cancer growth is dependent on circulating androgens (2) and is pharmacologically modulated by androgen blockade. Most patients initially respond to androgen ablation therapies, but relapse with hormone-refractory prostate cancer (3), as a consequence of clonal selection of androgen-independent foci. Androgen-resistant tumors are generally characterized by an increased invasiveness. Therefore, it is important to find new approaches to control prostate cancer progression towards androgen-insensitivity.

In prostate cells, testosterone, is first irreversibly reduced by the 5α-reductase enzyme, to dihydrotestosterone and subsequently reversibly 3α- or 3β-hydroxylated by the 3α- and 3β-hydroxysteroid dehydrogenases. The 5α-reductase exerts a well-established activating physiological role in the control of the prostate development (4), because dihydrotestosterone activates androgen receptor–dependent transcription more efficiently than the precursor itself. Moreover, 5α-reductase not only provides a potent amplification of the androgenic signal (4–6), but it also prevents estrogen formation by subtracting testosterone from the action of aromatase (7, 8), thus blocking activation of the estrogen receptor subtypes (ERα and ERβ; refs. 9, 10). In prostate, ERβ is the prevailing subtype (11), and a growing body of evidence points to the protective role of this receptor in prostate cancer (12, 13). It has been shown that the transformation of the dihydrotestosterone to 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-Adiol), generates two metabolites unable to bind the androgen receptor, but possessing a very high affinity for the estrogen receptors (14–16). Therefore, the effects of testosterone may result from the balance between the androgenic and the estrogenic molecules originating from its catabolism. Recent data have been published postulating a direct estrogenic role of the 3β-hydroxylated derivatives of dihydrotestosterone in the prostate development and homeostasis (11–13, 17–23).

Several cell lines are available to study prostate cancer: DU145 cells express the highly efficient, androgen-inducible type 2 subtype of the 5α-reductase enzyme, the aromatase (24, 25) as well as the ERβ subtype (26). We have recently generated novel prostate cancer cells in which the androgenic control has been restored (27). These models, obtained by stable transfection of DU145 cells with human androgen receptor, has been named DU145-AR.

In the present study, we have analyzed the influence of dihydrotestosterone on the migratory capabilities of androgen receptor–positive and androgen receptor–negative cell lines, and found that in both cases, dihydrotestosterone greatly inhibits cell migration in an androgen receptor–independent way. The inhibition of DU145 cell motility has been associated with the formation of the testosterone/dihydrotestosterone metabolite 3β-Adiol acting through the activation of the ERβ receptor.

Materials and Methods

Reagents, hormones, and receptor agonist and antagonist ligands. All chemicals, estradiol, dihydrotestosterone, and 3β-Adiol were obtained from Sigma (St. Louis, MO); ICI 182,780 was kindly donated by AstraZeneca, London, United Kingdom. Trans-hydroxytamoxifen and tetrahydrocrysene (R,R-THC), an ERα agonist and ERβ antagonist compound were prepared in the laboratories of Drs. Benita and John Katzenellenbogen (University of Illinois, Urbana-Champaign, IL).

Cell culture of prostate cancer cell lines. The cell line DU145 was originally obtained from American Type Culture Collection (Rockville, MD) and was stably transfected in our laboratory using LipofectAMINE Plus (Life Technologies, Grand Island, NY) either with pcDNA3hAR expressing androgen receptor protein (to obtain DU145-AR) or with pcDNA3 (mock; ref. 27). Cells were routinely grown in RPMI 1640 medium (Biochrom KG, Berlin, Germany), supplemented with 5% charcoal stripped-FCS (Life Technologies), glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), and geneticin (25 mg/mL).

PC3-neo and PC3-AR, were kindly obtained from Dr. Baldi, University of Florence, Italy (28) and grown in the same condition described for DU145 cells.

Reverse transcription-PCR and Southern blot analysis. Transfected cells were harvested and homogenized in 4 mol/L guanidine thiocyanate containing 25 mmol/L sodium citrate, 0.5% lauryl sarcosine, and 0.1 mol/L 2-mercaptoethanol. RNA was precipitated in 70% ethanol. For androgen receptor expression, RT-PCR was done as previously described (27). ERαβ expression was analyzed using 2 μg of total RNA that were reverse transcribed using RT-PCR kit (Perkin-Elmer Corp., Wellesley, MA; ERαβ downstream primer 5'-CATCTCCAGCAGCAGGTCAT). The PCR reaction [35 cycles (95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute)] was done using the upstream specific primer (upstream ERα 5'-TGATGAAAGGTGG-GATACGA; upstream ERβ 5'-TTTGTGGAGCTCAGCCTGTT).

For Southern analysis, 10 μL of amplified products were electrophoresed on 2% agarose gel. The DNA was denatured with 0.5 N NaOH, 1 mol/L NaCl for 30 minutes at room temperature, and then neutralized in 1.5 mol/L Tris-HCl (pH 7.4), 3 mol/L NaCl for 30 minutes at room temperature and transferred to a nylon membrane (Hybond N, Amersham, Buckinghamshire, United Kingdom). The membrane was washed with 2× SSC and hybridized with a specific ERαβ internal oligoprobe 5'-ATCTTTGACATGCTCCTGGC at 50°C for 24 hours. The membrane was then washed with 3× SSC [10× Denhardt's, 5% SDS, 25 mmol/L NaH$_2$PO$_4$ (pH 7.5)] for 30 minutes at 50°C and exposed to X-ray films.

Western blot analysis. Extracts of transfected cells were prepared by lysing in SDS sample buffer and sonicated at 4°C. Total proteins were determined with bicinchoninic acid assay (Pierce, Rockford, IL), 50 μg of protein were resolved on 7.5% SDS-PAGE for E-cadherin. Proteins blotted to a nitrocellulose filter paper were analyzed using the anti-E-cadherin clone 4A2C7 (Zymed, San Francisco, CA; 1:200) and a secondary antibody conjugated with peroxidase (1:5,000). Immunoreactive bands were visualized using the enhanced chemiluminescence detection kit reagents (Amersham). Protein normalization was done using an anti-Actin antibody I-19 (Santa Cruz, Santa Cruz, CA, 1:1,000); bands were visualized as described above.

Chemotaxis tests using Boyden chamber. The assay was done using a 48-well Boyden's microchemotaxis chamber according to the manufacturer's instructions (Neuroprobe, Cabin John, MD). Briefly, the cells were grown in complete medium until subconfluence, then harvested, resuspended (150,000 cells/50 μL serum-free/phenol red–free RPMI) and placed in the open-bottom wells of the upper compartment. Each pair of wells were separated by a polyvinylpyrrolidone-free polycarbonate porous membrane (8 μm pores) precoated with gelatin (0.2 mg/mL in PBS); for PC3 derived cells, the membrane was precoated with laminin for 5 hours [mouse laminin, 20 μg/mL in PBS (Chemicon, Temecula, CA)]. For chemotaxis experiments, the chemoattractants (phenol red–free RPMI + charcoal stripped-FCS 10%) were placed into the wells of the lower chamber compartment. The Boyden's chamber was then placed in cell culture incubator: DU145 derived cells were allowed to migrate for 5 hours at 37°C; PC3 derived cells O/N at 37°C. After incubation, the cells were fixed and stained (Diff-Quick stain kit, Dade Behring AG, Dudingen, Switzerland) and

mounted onto glass slides. Quantitative analysis was done using an oil immersion 100× objective on a light microscope. Six random objective fields of stained cells were counted for each well, and the mean number of migrating cells per square millimeter was calculated. The number of migrated cells, obtained from 5 independent wells for each group, was compared by ANOVA and Dunnett's tests. Each experiment has been repeated thrice.

Short interfering RNA (siRNA) against E-cadherin (Santa Cruz, #SC35242; at a final concentration of 12 nmol/L as specified by the siRNA protocol from Santa Cruz) was transfected using LipofectAMINE Plus; transfection efficiency, estimated by cotransfection with pEGFP-N1 (0.5 μg/well, Clontech, BD Biosciences, Mountain View, CA), was ~60%. In these experiments, Boyden assay was done on DU145-pcDNA3 cells after 24 hours of treatments with the test steroids to achieve the maximal activity of the E-cadherin siRNA.

Analysis of dihydrotestosterone metabolism in DU145 cells. DU145-pcDNA3 or DU145-AR have been incubated in standard conditions with 100 nmol/L of [^{14}C]labeled dihydrotestosterone (s.a. 2.2 GBq/mmol, NEN, Dupont, Boston, MA) for 48 hours at 37°C in a CO$_2$ incubator, then the whole medium and the cells were collected and frozen at −20°C. The metabolites formed were extracted twice with diethylether, nonradioactive dihydrotestosterone, 3α-diol, and 3β-Adiol (40 μg/100 mL) were added to each sample to aid visualization of the steroids on the TLC plates. Extracted samples were separated on a thin layer silica gel plate (Merck 60F$_{254}$, DC) by eluting thrice with dichloromethane-diethylether (4:1 v/v). The plate was then exposed to X-ray film to evaluate the radioactive spots. The retention factor of the three unlabeled standard compounds spots for dihydrotestosterone, 3α- and 3β-Adiols were identified with iodine vapors and the spots corresponding to dihydrotestosterone and diols were scraped off and the radioactivity counted in a Packard 300C liquid scintillation spectrometer as previously described (8, 29, 30).

The major (99%) dihydrotestosterone metabolites formed were 3α-diol and 3β-diol; a densitometric analysis of the spots associated with labeled metabolites derived from [^{14}C]dihydrotestosterone, obtained using ImageJ, showing the perfect overlapping of radioactive spots with unlabeled diols. 3α- and 3β-Adiol retention factors were similar and did not allowed the discrimination between the two metabolites.

Transcriptional activity. DU145-pcDNA3 cells were transfected using the Lipofectin-transferrin method as described (31). Cells, plated in 24-well plates, were transfected with the ERE-containing reporter construct (1 μg 2ERE-TK-CAT), 0.4 μg pCMVβ [β-galactosidase internal control plasmid (Clontech)], 1 μg pBK-CMV-SRC-1 (32) and carrier DNA. At 8 hours posttransfection, cells were treated with estradiol, estrogen receptor antagonists (trans-hydroxytamoxifen and the ERβ selective R,R-THC); transcriptional activity was assayed as previously described (31).

3β-Adiol-mediated transcriptional activity in DU-145 cells was evaluated cotransfecting 1 μg p(ERE)2× TATA-luc reporter construct (33), together with 10 ng of pCMV-ERβ (kindly provided by Dr. P.J. Kushner, Department of Medicine, University of California, San Francisco, CA) expression vector, and 200 ng of pCMV-β-gal (Promega, Madison, WI) as control of transfection efficiency. Transient transfection was done as above and treatments started 24 hours after transfection. Luciferase activity was measured 48 hours after transfection using the luciferase assay system (Promega); ERβ transcriptional activity assay, done to determine the dose response of dihydrotestosterone and 3β-Adiol, was conducted in the conditions described for Boyden test (48 hours of treatment started 24 hours after transfection). Light intensity was measured with a luminometer (Lumat LB 9501/16, Berthold) >10 seconds and expressed as relative light units per 1 mg of protein extract. β-Galactosidase activity was determined with a colorimetric assay. The final values are expressed as relative light units/LacZ activity and are mean values from three replicas of three distinct experiments.

Real-time PCR for E-cadherin in DU145-pcDNA3. DU145-pcDNA3 were plated in 6 well culture dishes with RPMI at 5% charcoal stripped-FCS. Cells were treated with dihydrotestosterone or 3β-Adiol 10^{-7} mmol/l; control cells were treated with vehicle (ethanol). Following 48 hours' treatment, cells were lysed and total RNA extracted using RNeasy Mini Kit with DNase 1 (Qiagen, Valencia, CA).

Forward and reverse primers used in real-time reverse transcription-PCR for E-cadherin (34) produced a product of 200 bases; E-cadherin upstream primer 5′-TGAAGGTGACAGAGCCTCTGGAT; E-cadherin downstream primer 5′-TGGGTGAATTCGGGCTTGTT. The primers are designed to bind at two neighboring exons to assure amplification of only cDNA products (34).

The primers were also tested in accordance with recommendations accompanying the ABI Prism 7700 sequence detection system on C-G base content and 5′ content and using the program Primer Express 1.5 (provided by Applied Biosystems, Foster City, CA).

SYBR Green Master Mix 2× (Applied Biosystems) containing SYBR Green enzyme, the internal fluorescent control ROX, and all components required for PCR, excluding cDNA and primers was used for real-time PCR analysis. Preliminary assays were done to optimize the primer concentration to avoid nonspecific binding according to manufacturer recommendations (two sets of primers recognizing E-cadherin and the 18s rRNA housekeeping gene, Applied Biosystems). The resulting amplification plot was used to select the optimal primer concentrations and insure similar reaction efficiencies for both primers. A set of fluorescence levels was selected at which samples amplifying E-cadherin and 18s were both in a point of exponential PCR amplification.

Treatments were done in triplicate, and then each sample read in triplicate and mean cycles corresponding with the selected fluorescence level for each triplicate sample assessed; E-cadherin values were normalized for 18s. Statistical analysis was done using ANOVA; single dose assays were analyzed by one-way ANOVA. To determine the levels of significance of the responses, the t values were compared using an unpaired Student's t test.

Results

The objective of this study was to analyze the effects of androgen derivatives on the adhesive properties of prostate cancer cells.

Effects of dihydrotestosterone on the migratory properties of androgen receptor–containing stable DU145 cell lines. We have confirmed that stably transfected DU145-AR cells (previously described in ref. 27), maintain androgen receptor expression using RT-PCR. Figure 1*A* shows the presence of an intense amplicon corresponding to that expected for androgen receptor in DU145-AR (lane 6) and in the control cells LNCaP, expressing endogenous androgen receptor (lane 3); no androgen receptor mRNA was detected in mock-transfected DU145-pcDNA3 (lane 5), confirming the absence of the androgen receptor mRNA, even in lower amounts, in these cells.

To evaluate the dihydrotestosterone effects on the adhesive and migratory properties of prostate cancer cells, we have used the Boyden assay and tested the effect of dihydrotestosterone on cell migration using both the androgen receptor–positive and the androgen receptor–negative DU145 cells. Figure 1*C* shows that dihydrotestosterone significantly reduced ($P < 0.01$) migration of DU145-AR at all doses tested; however, dihydrotestosterone also significantly inhibited ($P < 0.01$) cell migration of androgen receptor–negative mock-transfected DU145-pcDNA3 (Fig. 1*B*), suggesting that dihydrotestosterone must control cell migration via androgen receptor–independent mechanisms.

Conversion of dihydrotestosterone to 3-hydroxylated compounds and their effects on stably transfected DU145 cell lines. To ascertain whether the inhibition of cell migration induced by dihydrotestosterone might be linked to dihydrotestosterone metabolites, we have analyzed the metabolic conversion of radiolabeled [^{14}C]dihydrotestosterone in the two cell clones considered. The major dihydrotestosterone metabolites formed in our prostate cancer cells were the 3-hydroxyderivatives, 3α-diol and 3β-Adiol (Fig. 2*A*); a densitometric analysis is shown in Fig. 2*B*. The radioactivity overlapped the signals of the two unlabeled diols (data not shown). The retention factors in the two lanes corresponding to DU145-pcDNA3 and DU145-AR were in proximal regions, with similar intensity, but peaked differently, as expected by the similar chromatographic mobility of the two diols; therefore, the spots might exhibit a different ratio of the diols (presumably, 3α-diol prevailing in DU145-pcDNA3, and 3β-Adiol in DU145-AR). From radioactivity counts of the spots, we have estimated a conversion rate of dihydrotestosterone to the two 3-hydroxylated steroids, of ∼40%.

Both diols are unable to bind to androgen receptor, but 3β-Adiol binds the estrogen receptor proteins with a higher efficiency for the ERβ subtype (both diols bind ERβ, but the binding capacity of 3β-Adiol is much higher than that of 3α-diol; relative binding affinities, ERβ: RBA versus E2 = 3α-diol, 0.3; 3β-Adiol, 7-ERα: RBA versus E2 = 3α-diol, 0.07; 3β-Adiol, 3; ref. 15), it is possible that this 3-hydroxylated derivative is the effector of dihydrotestosterone in the control on cell migration. Therefore, we next focused on the enzyme forming the most active metabolite, which in prostate is the 3β-hydroxysteroid dehydrogenases type 1 (35); we analyzed
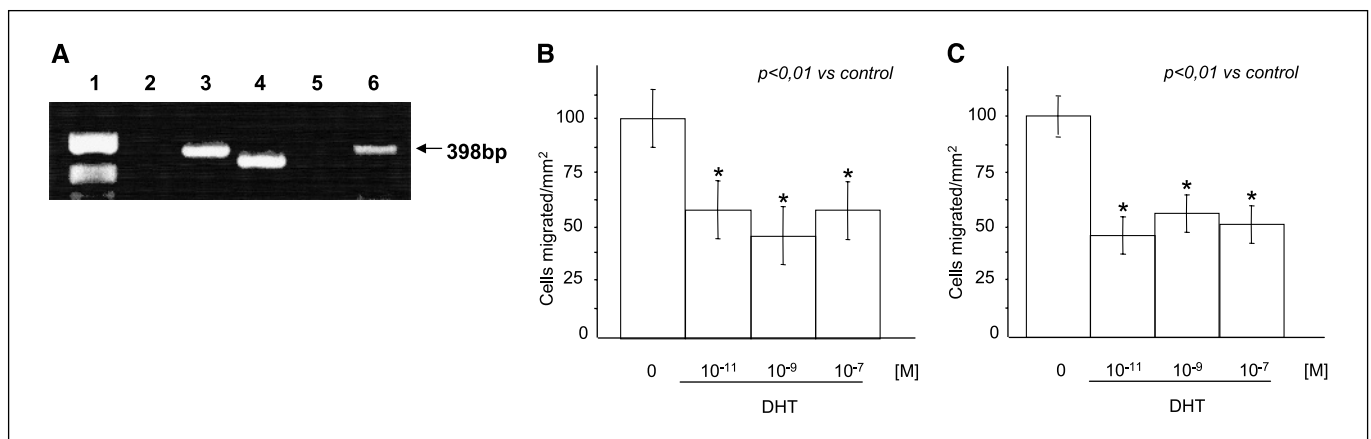
Figure 1. Characterization of stably transfected DU145 cells and migration test done using Boyden chamber on prostate cancer cell lines. *A,* RT-PCR analysis done on total RNA extracted from prostate cancer cell lines. Lane 1, DNA molecular weight markers; lane 2, water; lane 3, LnCaP; lane 4, pWA109RNA RT-PCR control RNA; lane 5, DU145-pcDNA3; lane 6, DU145-AR. *B,* DU145-pcDNA3 cells. *C,* DU145-AR cells. Cells were treated for 48 hours and Boyden assay was done using a 5-hour migration time in the absence of FCS in the upper chamber using medium and 10% FCS as chemotactic reagent in the lower chamber medium (see Materials and Methods for details).

Figure 2. Dihydrotestosterone metabolism in prostate cancer cell lines and role of the dihydrotestosterone derivative 3β-Adiol on cell migration. *A,* radioenzymatic assay was done to evaluate the pattern of dihydrotestosterone metabolites in stably transfected DU145 cells. Living prostate cancer cells were incubated with [^{14}C]labeled dihydrotestosterone in the same growth conditions used for the migration tests. Labeled metaboli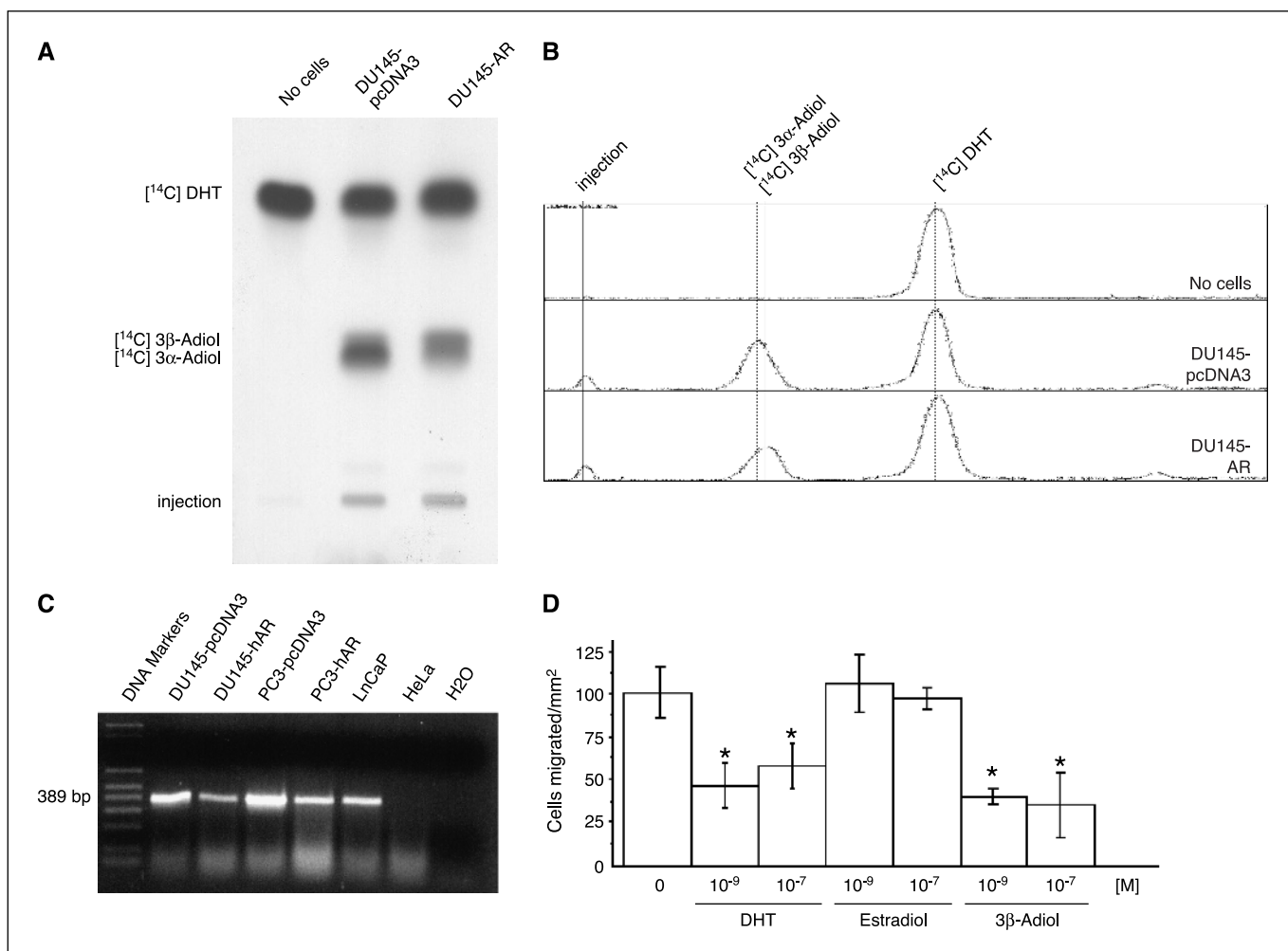tes formed after incubation were extracted with diethylether and resolved on TLC. *B,* radioactive spots were identified using X-ray and by densitometric analysis using ImageJ; retention factors indicated in (*B*) were determined using standard unlabeled steroids visualized by iodine vapors. *C,* evaluation of the 3β-hydroxysteroid dehydrogenases type 1 isozyme expression in prostate cancer cells using RT-PCR analysis. The analysis was done on total RNA extracted from androgen receptor–positive or androgen receptor–negative prostate cancer cell lines, and from HeLa cell-negative controls. *D,* effects of dihydrotestosterone, estradiol, and 3β-Adiol on migratory properties of DU145-pcDNA3 cells evaluated using Boyden chambers; statistical analysis was done using one-way ANOVA (*, $P < 0.01$).

the mRNA expression of this enzyme in mock and androgen receptor–positive DU145 cells. We also included (*a*) a new fully characterized prostate cancer cell line, the PC3 stably transfected with the androgen receptor (kindly donated by Prof. Baldi, University of Florence; ref. 28) as a comparative control, (*b*) LNCaP cells as positive control, and (*c*) the HeLa cells as negative control. Figure 2*C*, shows that 3β-hydroxysteroid dehydrogenases type 1 is expressed in all prostate cancer cells considered (apparently at higher levels in the androgen receptor–negative clones), suggesting that 3β-Adiol formation in DU145 clones is linked to this enzyme.

We then evaluated the effects of 3β-Adiol on the migratory properties of DU145-pcDNA3 cells; because the 3β-hydroxysteroid dehydrogenases type 1 is a reversible enzyme, we have omitted DU145-AR in which the retroconversion of 3β-Adiol to dihydrotestosterone may generate androgenic responses. Figure 2*D* confirms the inhibitory effect of dihydrotestosterone on cell migration and shows that estradiol has no effect on cell migration. More

importantly, the data shows that 3β-Adiol significantly reduces the migratory capacity of DU145-pcDNA3 cells; we have excluded, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay that the inhibition of migration induced by 3β-Adiol might be linked to altered cell proliferation (data not shown).

Characterization of DU145-AR cells estrogen-responsiveness. To prove that 3β-Adiol inhibits cell migration through an estrogen signaling pathway, we first analyzed whether our DU145 clones express the ERβ subtype, as already seen in different population of DU145 lacking androgen receptor protein (26). Figure 3*A* shows a Southern blot analysis done on RT-PCR products obtained using primers specific against the two estrogen receptor subtypes; the ERβ primers readily amplified ERβ mRNA (band size, 536 bp) both in mock-transfected DU145-pcDNA3 cells, in DU145-AR and in control MCF-7 cells (a subclone of breast cancer–derived cells known to express both estrogen receptor subtypes; ref. 36). On the contrary, ERα selective primers generated an amplicon of 889 bp clearly detectable in control MCF-7 cells, but not present in both
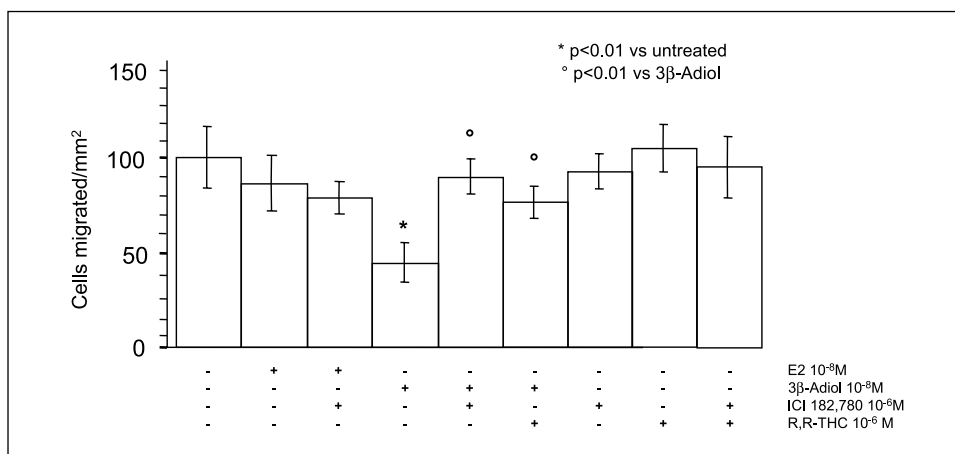
DU145 clones (ERα was not detectable even after androgen treatment, data not shown). These data show that ERα is not expressed in our DU145 clones; thus, a possible estrogenic effect in these cells should be ascribed to the ERβ subtype.

We then analyzed the function of endogenous ERβ in DU145-pcDNA3, by characterizing the estradiol effects on transcription using the reporter plasmid 2ERE-TK-CAT. It seems that 10 nmol/L of estradiol induced a 2-fold increase (Fig. 3B) in the transcriptional activation of the 2ERE-TK-CAT (normalized for β-galactosidase); the cotransfection of the 2ERE-reporter construct with a plasmid overexpressing SRC-1 (a coactivator of the p160 family of nuclear receptor coactivators; ref. 32), potentiated up to 3.5-fold the transcriptional competence of endogenous ERβ activated with the same estradiol concentration. The transcriptional effect induced by estradiol could be fully counteracted by trans-hydroxytamoxifen, a well-known ERβ antagonist (9), and with tetrahydrocrysene (R,R-THC), an ERβ-selective antagonist (37). Similar results were obtained using another ERE-responsive artificial promoter, the 2ERE-pS2-luc (data not shown).

ERβ transcriptional activation mediated by the testosterone derivatives used in the Boyden test was measured with dose-response curves (Fig. 3C). It seems that both dihydrotestosterone and 3β-Adiol activate ERβ-mediated transcriptional activity, although dihydrotestosterone is less active than 3β-Adiol by about 0.5 log difference; this difference could be explained by the fact that dihydrotestosterone cannot directly bind ERβ and must be metabolized prior to 3β-Adiol to activate ERβ. Moreover, dihydrotestosterone may also be converted to 3α-diol, which possesses a much lower affinity for the ERβ. Therefore, the estrogenic action of testosterone metabolites is influenced not only by ERβ cellular levels, but also by the 3α and/or 3β-hydroxysteroid dehydrogenase levels that may undergo different methods of regulation in a given prostate cancer cell.

To evaluate whether the effects of 3β-Adiol we described are due to transcription activated by the 3β-Adiol/ERβ complex, we analyzed the transcriptional competence of ERβ activated by 3β-Adiol and by estradiol in DU145-pcDNA3. Figure 3D shows that both estradiol and 3β-Adiol efficiently activated the transcription

Figure 3. Characterization of the ERβ expression in DU145 cells lacking (DU145-pcDNA3) or expressing (DU145-AR) the androgen receptor. A, Southern analysis done on the amplified products obtained in the RT-PCR assay using selective amplification primers recognizing ERα or ERβ mRNAs and generating amplicons of different sizes (ERα = 889 bp; ERβ = 536 bp; a single downstream primer, directed against a sequence present in both subtypes, was used; two selective upstream primers were designed in nonconserved regions of the two receptors). The analysis was done on total RNA extracted from androgen receptor–positive or androgen receptor–negative DU145 cell lines in comparison to ERα- and ERβ-positive control MCF-7 breast cancer cell lines. The analysis was done using a single [^{32}P]labeled oligoprobe capable of recognizing both the ERα and the ERβ amplicons. B, transcriptional activity of the endogenous ERβ protein in DU145-pcDNA3. The ERβ transcriptional competence was determined using the 2ERE-TK-CAT reporter plasmid (cotransfected with pCMVβ). The results were normalized for β-galactosidase activity; TOT, trans-hydroxytamoxifen; R,R-THC, tetrahydrocrysene, are ERβ-selective antagonists; SRC-1 was overexpressed by cotransfection with pBK-CMV-SRC-1. C, effects of different doses of dihydrotestosterone and 3β-Adiol on the ERβ-mediated transcriptional activity measured in DU145-pcDNA3 cells; transcriptional activity in conditions identical to those adopted for the Boyden test. D, effects of estradiol (E2) and 3β-Adiol on the transcriptional activity of the ERβ present in DU145-pcDNA3 cells. Transcriptional activity was measured using p(ERE)2× TATA-luc reporter construct and evaluated using the luciferase assay normalized with β-galactosidase (see Materials and Methods for details).

Figure 4. Effects of estradiol (E2) and 3β-Adiol on the migratory properties of DU145-pcDNA3 cell lines. The pure estrogen receptor antagonist ICI 182,780 and the ERβ-selective antagonist R, R-THC counteract the estrogenic effects of the two ligands.

of the luciferase reporter gene, ICI 182,780 (ICI in figure, a pure antagonist), fully counteracted the luciferase expression induced by both steroids confirming that 3β-Adiol has an estrogenic activity; as expected, ICI 182,780 alone was not able to activate transcription. It is intriguing to underline that only 3β-Adiol reduced migration of DU145 cells.

The inhibitory effects of 3β-Adiol on migratory properties of DU145 cells are mediated by ERβ. To determine whether the ERβ protein is the molecular mediator of the 3β-Adiol effects on DU145 cell migration, we used the Boyden assay to test the effects of 3β-Adiol in the presence of the two estrogen receptor antagonists, ICI 182,780 and R,R-THC. Interestingly, the results in Fig. 4 show that 3β-Adiol reduced the control values by up to 50% ($P < 0.01$) of the cell migration of DU145-pcDNA3, an effect significantly counteracted either by the ERβ antagonist ICI 182,780 or by the ERβ-selective antagonist R,R-THC ($P < 0.01$) proving that ERβ is involved in this process. On the other hand, estradiol alone, estradiol plus ICI 182,780, or ICI 182,780 alone or in combination with R,R-THC did not modify cell migration.

The estrogenic effect of 3β-Adiol on cell migration is not specific for DU145 cells. In fact, we have also applied the Boyden chamber test to different prostate cancer cell lines: PC3-neo cells, an androgen receptor–negative PC3 cell line and PC3-AR, stably transfected androgen receptor–expressing cells (28). We have observed that both dihydrotestosterone and 3β-Adiol inhibited cell migration of PC3-neo (Fig. 5A) and of PC3-AR (Fig. 5B); the inhibitory effect overlapped those effects measured in DU145-derived clones, confirming again that androgen receptor activation is not involved in this process.

E-cadherin is a potential molecular mediator of the estrogenic action of 3β-Adiol. It is known that E-cadherin is one of the major factors capable of counteracting metastasis of breast and prostate cancer cells (38–40). Thus, we have analyzed whether 3β-Adiol may restore high levels of E-cadherin, normally expressed at very low levels in DU145-pcDNA3 cells. We have initially evaluated, using real-time PCR analysis, E-cadherin mRNA levels in DU145-pcDNA3 treated with different steroids (Fig. 6A). It seems clear that dihydrotestosterone (column 2) and 3β-Adiol (column 3) are both able to increase E-cadherin mRNA in prostate cancer cells; 3β-Adiol was extremely efficient because it induced a 6-fold increase of E-cadherin mRNA over control. This effect was counteracted by the estrogen receptor antagonist ICI 182,780 (column 6), indicating that this effect is due to ERβ. On the other

hand, the antiandrogen Casodex was unable to counteract the effect of dihydrotestosterone (androgen receptor is not present in these cells, column 5), once again supporting the notion that dihydrotestosterone also acts through the estrogenic molecules 3β-Adiol. Also estradiol induced (2-fold, column 4), although at lower levels, E-cadherin mRNA expression.

The E-cadherin protein levels have been analyzed using Western analysis (Fig. 6B). The protein is expressed at low levels in control cells (lanes 1 and 2), as well as in cells treated with 10 nmol/L of estradiol for 48 hours (lanes 3 and 4); on the other
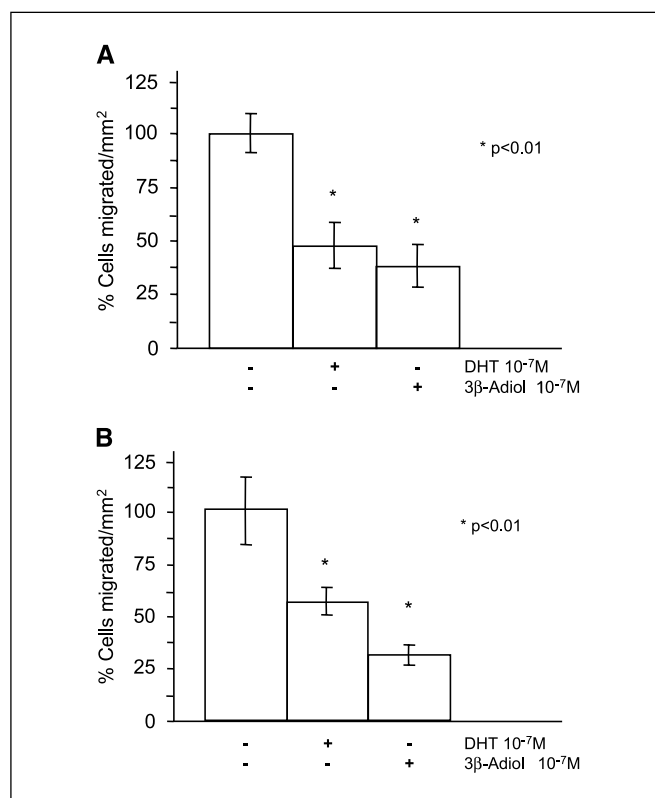

Figure 5. Effects of dihydrotestosterone and 3β-Adiol on the migratory properties of PC3-derived cell lines lacking (PC3-neo) or expressing (PC3-AR) androgen receptor. A, Boyden test done on PC3-neo cells; B, Boyden test done on PC3-AR cells. *, $P < 0.01$ versus control untreated cells.
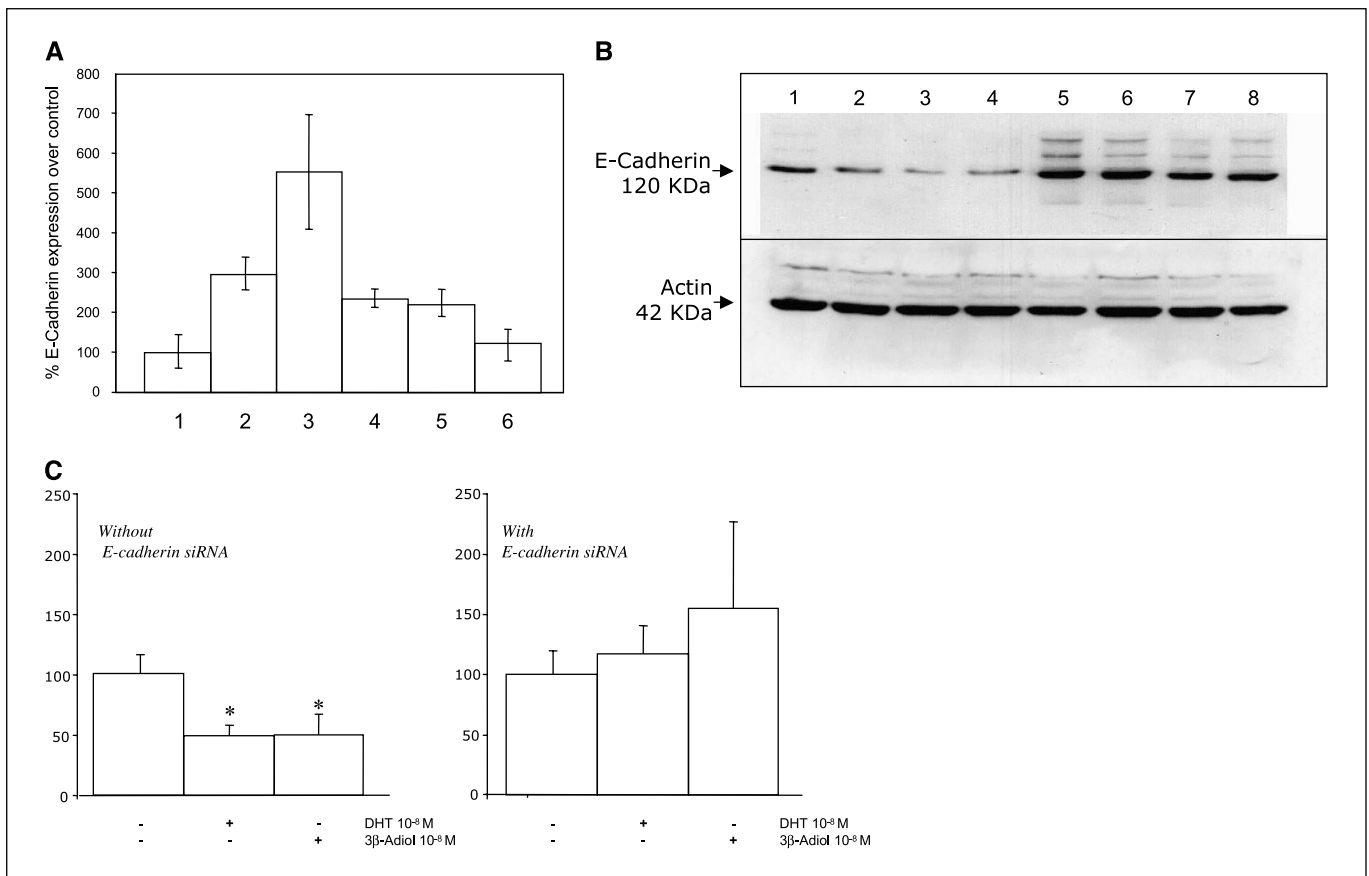
Figure 6. Effects of hormonal steroids on E-cadherin expression in DU145-pcDNA3. *A*, real-time reverse transcription-PCR analysis done using human E-cadherin amplification primers on total RNA extracted from DU145-pcDNA3 cells; column 1, control; column 2, dihydrotestosterone 10^{-8} mmol/L; column 3, 3β-Adiol 10^{-8} mmol/l; column 4, estradiol 10^{-8} mmol/L; column 5, dihydrotestosterone 10^{-8} mmol/L plus Casodex 10^{-7} mmol/L; column 6, 3β-Adiol 10^{-8} mmol/L plus ICI 182,780 10^{-7} mmol/L. *B*, Western blot assay done using an anti E-cadherin antibody (normalized using anti-actin antibody) on total protein extracted from DU145-pcDNA3 cells after 48 hours of treatment; lanes 1 and 2, untreated DU145-pcDNA3 as control; lanes 3 and 4, DU145-pcDNA3 treated with estradiol 10^{-8} mmol/L; lanes 5 and 6, DU145-pcDNA3 treated with 3β-Adiol 10^{-8} mmol/L; lanes 7 and 8, DU145-pcDNA3 treated with dihydrotestosterone 10^{-8} mmol/L. E-cadherin protein is detectable at 120 kDa and actin protein at 42 kDa. *C*, effect of E-cadherin siRNA on the migratory properties of DU145-pcDNA3 cells (*, $P < 0.01$ versus control untreated cells) treated with dihydrotestosterone or 3β-Adiol. The effects of dihydrotestosterone, and 3β-Adiol on migratory properties of DU145-pcDNA3 cells were evaluated after 24 hours of treatment (instead of 48 hours) in order to achieve the maximal levels of activity of the E-cadherin siRNA; the migratory properties of DU145-pcDNA3 cells in absence (left panel) or in presence of E-cadherin siRNA (right panel) was evaluated using Boyden chambers; statistical analysis was done using one-way ANOVA (*, $P < 0.01$).

hand, a robust increase in the intensity of the immunoreactive bands is easily detectable after treatment with 3β-Adiol (lanes 5 and 6) or dihydrotestosterone (lanes 7 and 8), suggesting that both steroids up-regulate the level of this protein in DU145 cells. It remains to be clarified what opposite effects estradiol plays at the transcriptional/translational levels, even though the degree of activation seems to be very limited when compared with that of 3β-Adiol.

To ascertain whether the inhibitory effects of 3β-Adiol on prostate cancer cell migration is directly linked to the over-expression of E-cadherin, we did the Boyden test on cells exposed to an E-cadherin siRNA (Santa Cruz, SC35242; transfection efficiency, determined with GFP, was estimated to be close to 60%); the Boyden test was done 24 hours after treatments (instead of 48 hours) to achieve the highest expression levels of siRNA. Figure 6*C* clearly shows that E-cadherin mRNA silencing completely abolished the antimigratory effects exerted by dihydrotestosterone and 3β-Adiol on DU145-pcDNA3 cells. Therefore, E-cadherin should be considered as one of the proteins mediating the protective effects of 3β-Adiol against prostate cancer invasion.

Discussion

In the present paper, we have analyzed the role of androgens and their physiologic derivatives on prostate cancer cell migration; surprisingly, using cells either lacking or expressing hAR (DU145- or PC3-derived clones), we have observed that dihydrotestosterone inhibits cell migration, acting through an androgen receptor–independent mechanism, at very low doses. 3β-Adiol, a dihydro-testosterone metabolite unable to bind the androgen receptor, even if at higher doses, also inhibits prostate cancer cell migration acting through ERβ activation.

The different dose responses of the two compounds suggest that dihydrotestosterone may also operate using additional androgen receptor–independent and complementary pathways (i.e., mem-brane effects, modulation of other types of receptors, etc.), which may synergize with the 3β-Adiol/ERβ pathway providing an exponentially based response. In fact, dihydrotestosterone might increase (or reduce) the efficiency of one (or more) factor(s) mediating the actions of 3β-Adiol (on: hydroxysteroid dehydro-genases enzymes, ERβ, E-cadherin, or other transcription factors,

activating other genes involved in the antimigratory complex cooperating with E-cadherin, etc.); at present, these putative pathways are unknown.

In any case, the effect of 3β-Adiol could be fully counteracted by classical ERβ-selective antagonist (i.e., R,R-THC; ref. 37) and ERβ is the subtype expressed in the cells used in our study. The 3β-Adiol/ERβ complex is transcriptionally active on classical ERE-reporter vectors at levels equivalent to those obtained using estradiol. Interestingly, a similar activation of the ERE-reporter vector can also be obtained using dihydrotestosterone. Surprisingly, the inhibition of prostate cancer cell migration seems to be highly specific for the 3β-Adiol/ERβ interaction, because prostate cancer cell migration is only partially modified by estradiol-activated ERβ, suggesting that the two steroids may induce different gene expression profiles (selective ERβ conformation?); this hypothesis is supported by the data presented here showing that 3β-Adiol and dihydrotestosterone seem to inhibit cell migration by inducing the overexpression of the E-cadherin protein (a single-pass transmembrane glycoprotein mediating Ca^{2+}-dependent cell-cell adhesion), whereas estradiol, which seems to be partially active at the transcriptional level, has no effect (or even an inhibitory effect) on the E-cadherin protein levels. Notably, the effect of 3β-Adiol is fully counteracted by estrogen receptor antagonists. E-cadherin is known to be highly protective against metastasis in several endocrine tumors (38–41), and germ line mutations in the E-cadherin gene (*CDH1*) strongly predispose to breast carcinoma (38, 42). Our results clearly showed that E-cadherin silencing using siRNA completely abolished the protective effects of 3β-Adiol (and dihydrotestosterone) against prostate cancer cell migration.

The discrepancy between 3β-Adiol and estradiol on E-cadherin expression may be explained by data showing that estradiol may control E-cadherin by activating several pathways. In fact, Fujita et al. (43) have recently produced intriguing data on the various mechanisms by which estradiol control expression of the E-cadherin tumor suppressor gene; estradiol-activated estrogen receptor that acts either via direct gene activation or through MTA3 an estrogen-dependent component of the Mi-2/NuRD transcriptional corepressor in breast epithelial cells; this dual control, at least in breast cancer, may also lead to E-cadherin down-regulation. It is possible that, in prostate cancer cells, the molecular mediator of the process is represented by 3β-Adiol, which enhances E-cadherin gene expression through ERβ activation; it is unknown, at present, whether one or both pathways are activated by 3β-Adiol in prostate cancer.

Altogether, our data show that androgens may control physiopathologic processes by acting through the estrogen receptor protein, in an aromatase-independent way. Therefore, cross-talk between androgenic and estrogenic signaling can simultaneously operate in target tissues and within target cells; in these structures, the two pathways can be easily discriminated by the specific expression of a given metabolic enzyme and/or a relative nuclear receptor. First, testosterone, converted into dihydrotestosterone, potentiates its androgenic signaling pathway, whereas converted to estradiol (via aromatase) starts an estrogenic signaling pathway. Second, whereas dihydrotestosterone cannot directly activate the estrogenic signaling pathway (because 5α-reduction is irreversible and dihydrotestosterone cannot be aromatized), it may interact with this pathway indirectly, via its conversion to 3β-Adiol. We have also provided data showing that the estrogenic signaling pathways activated by estradiol and 3β-Adiol may diverge at the gene transcription level, generating with 3β-Adiol, a protection against metastasis via E-cadherin expression.

This exciting novel hypothesis is strongly supported by the very recent clinical observation that genetic alteration of the *HSD3B1* and *HSD3B2* genes, encoding for 3β-hydroxysteroid dehydrogenases types 1 and 2, respectively, are closely associated with hereditary and sporadic prostate cancer susceptibility (linked to the *1p13* locus; ref. 44); the association of 3β-Adiol with prostate cancer was found when the joint effect of the two genes was considered; because, individuals with the variant genotypes at either *B1-N367T* or *B2-c7519g* have a significantly higher risk of developing hereditary type prostate cancer (44, 45). Our data provides a clear interpretation of these observations: a reduction of 3β-Adiol formations, consequent to these gene mutations, may be a risk factor for prostate cancer in these patients, because the 3β-Adiol "protective" effect will obviously disappear.

Together, the experimental and clinical observations seem to confirm that 3β-Adiol-activated ERβ is protective against prostate cancer progression, and that ERβ seems to be an active player against prostate cancer (ERβ "good" versus ERα "bad" as already proposed by the group of Gustaffson; refs. 10, 11, 13, 23). It is also possible that other factors may be involved *in vivo* after ERβ-3β-Adiol binding, including the interaction between the epithelial and stromal components, and the compartmentalization of the two estrogen receptors (ERα exclusively in the stroma and ERβ mostly in the epithelial compartment; ref. 46).

Finally, we believe that relevant clinical applications may arise from the observations here described. Few data have been already reported on the effects of selective estrogen receptor modulators on prostate cancer development, for example, in rat prostatic adenocarcinoma (androgen receptor–negative, but ERα- and ERβ-positive), which spontaneously generates metastasis, trioxifene (a common selective estrogen receptor modulator) significantly inhibits metastasis (47). It remains to be determined whether 3β-Adiol may have similar effects *in vivo*; however, these experimental findings support that 3β-Adiol like selective estrogen receptor modulators may have a potential antimetastatic efficacy for the treatment of androgen-independent prostate cancers (47). It must be underlined that androgen-depletion therapy should be reevaluated on the basis of the results presented in the present paper; in fact, the complete removal of androgens like dihydrotestosterone, obtained using the chemical castration based on the GnRH analogue treatments, will also remove the protection exerted by 3β-Adiol against cell migration. The action of 3β-Adiol will be maintained with the use of classical androgen receptor antagonists only; therefore, it will be interesting to compare the results obtained with these two classical therapeutic approaches for human disease, and to analyze whether androgen-insensitive prostate cancers may become more aggressive because the protection offered by 3β-Adiol is eliminated by the GnRH analogue-based therapy.

Acknowledgments

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