

Breast cancer: from estrogen to androgen receptor

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Abstract

To investigate the link existing between androgens and human breast cancer, the hormonal milieu present in pre- and post-menopausal women has been translated in an in vitro model utilizing a hormone dependent breast cancer cell line MCF-7 exposed to DHEA, DHEAS, androstenediol, T, DHT with or w/o E₂. DHEAS and androstenediol stimulate the growth of MCF-7 cell line but reduce cell proliferation induced by E₂ (1 nM). T and DHT (1–100 nM) instead inhibit MCF-7 cell proliferation independently on E₂ presence. When we focused our study on the most powerful androgen, DHT alone (100 nM) consistently inhibits MCF-7 cell proliferation by 50% of the basal growth rate and counteracts E₂ proliferative action by 68%. These data correlate well with cell cycle analysis showing an enhanced number of cells in G₀/G₁ phase after 6 days of DHT treatment. Upon prolonged DHT exposure, Western blotting analysis shows a markedly increased AR content, while immunohistochemistry indicates that it was mostly translocated into the nucleus. So we assumed that the enhanced activation of the AR might inhibit MCF-7 cells proliferation. This assumption is corroborated by the fact that the inhibitory effects induced by DHT on MCF-7 cell proliferation are abrogated in the presence of hydroxyflutamide. Therefore to better investigate the role of AR in inhibiting E₂ action at genomic level, MCF-7 cells were transiently cotransfected with the reporter plasmid XETL carrying firefly luciferase sequence under the control of an estrogen responsive element and the full length AR or with an AR carrying a mutation (Cis 574→Arg 574) which abolishes its binding to DNA. The over-expression of the AR markedly decreases E₂ signalling which furthermore appears inhibited by simultaneous exposure to DHT but reversed by addition of hydroxyflutamide. The inhibitory effect was no longer noticeable when MCF-7 cells were cotransfected with XETL and the mutant AR. Taken together these data demonstrate that gonadal androgens antagonize MCF-7 proliferation induced by E₂. This seems to be related to the inhibitory effects of the over-expressed AR on E₂ genomic action. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Breast cancer; estrogen; Androgen

1. Introduction

In the last decade the molecular mechanism related to the hormone dependency of breast tumors has been extensively investigated. The prognostic impact of ERs and PRs in breast cancer is well established since they do predict a good response rate of breast cancer to hormone treatment (Thorpe, 1988; Fockens et al., 1989). However, the clinical significance and functional role of the androgen receptor expression are less well defined. Studies attempting to establish a link between

adrenal androgens circulating levels and breast cancer started at the beginning of the 1950s (Allen et al., 1957).

However data on urinary steroid metabolites were very controversial. This could probably have been explained by distortions due to the non-specific effect of illness or operative stress (Zumoff et al., 1982). A problem of this type has been ruled out in the perspective studies based on hormone assessment of urine collected from healthy subjects.

Three such perspective studies, each involving 5000 healthy women on the island of Guernsey, were carried out by Bulbrook and his colleagues between 1981 and 1986 (Bulbrook et al., 1986).

Results from women who developed the disease in the first 9 years of the study, mainly premenopausal, evidenced that they had significantly lower levels of

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urinary androgens metabolites than age-matched controls (Bulbrook and Thomas, 1989).

However, as the follow up continued over 25 years, these results became less clear-cut. Women with androgen metabolites at the lower end of the normal range were generally diagnosed with their disease at a late premenopausal period. Those patients with higher levels developed breast cancer at older ages. With the advent of the radioimmunoassay techniques, serum concentrations of adrenal androgens were able to be determined in breast cancer subjects and controls.

These studies all converge towards the finding that in contrast to the marked decline of the adrenal androgen levels with age in blood of normal women, the concentration of adrenal androgens (like dehydroepiandrosterone and dehydroepiandrosterone sulfate) were age invariant in the breast cancer patients. The premenopausal patients had subnormal while the postmenopausal patients had supernormal levels of each steroid hormone (Zumoff et al., 1981; Gordon et al., 1990; Dorgan et al., 1997; Helzloner et al., 1992).

On the basis of these findings, androgens in breast cancer progression appear to be protective in premenopausal women and stimulating in postmenopausal ones. Thus, in order to investigate the link between androgens and human breast cancer, we have simulated the hormonal environment in pre/postmenopausal women in an *in vitro* model utilizing an hormone-dependent breast cancer cell line, MCF-7, exposed to dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstendiol, testosterone, dihydrotestosterone with or without estradiol.

2. Materials and methods

2.1. Cell lines and culture conditions

MCF-7 cells were kindly supplied by Dr B. Van der Burg (Utrecht, The Netherlands). Cell cultures were routinely maintained in DMEM supplemented with 10% FCS in a 5% CO₂ humidified atmosphere.

2.2. Cell proliferation assay

Cell proliferation assay was performed as previously described (Van der Burg et al., 1988). Briefly, cells were seeded on six well plates (10⁵ cells/well), grown for 2 days in complete medium, starved without serum for 24 h and then exposed to various concentration of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstendiol (Δ_5), testosterone (T), dihydrotestosterone (DHT), hydroxyflutamide (OH-Fl), estradiol (E₂) in phenol-free DMEM containing 5% charcoal-treated FCS (5% CT-FCS-DMEM) to reduce the endogenous steroid concentration (for details, see

the figure legends). The medium was renewed every 3 days. At the indicated time points, cells were harvested by trypsin and counted using Burker's chamber.

2.3. Gel electrophoresis and Western blot analysis of androgen receptor expression

Cells were plated in 10 cm Petri dishes at a density of 1 \times 10⁶ and cultured in complete medium, starved without serum for 24 h and then exposed to steroids in 5% CT-FCS DMEM. Cells were pelleted and resuspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerine, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride). The extracts were centrifuged, aliquots of the supernatant were used for protein determination and the remainder was diluted 1:1 in Laemli sample buffer (Biorad Laboratories, Richmond, CA) containing 5% mercaptoethanol.

After boiling, the samples were separated by SDS-PAGE. Thirty micrograms of total protein were loaded in each lane. Electrophoresis was carried out in 4–15% polyacrylamide mini-gels (Biorad Laboratories, Richmond, CA) at 100 V and 250 mA for 1.5 h. High molecular mass markers (Rainbow Markers Amersham Pharmacia biotech) were applied to one of the lanes. Proteins were electroblotted onto Hybond TM nitrocellulose membrane (Amersham Pharmacia biotech). The membranes were blocked in 5% non fat powder milk (Carnation CO) and incubated for 1 h with the AR (441) mouse monoclonal IgG₁ (Santa Cruz Biotechnology), against the N-terminal 21 aminoacids of the human AR.

After washing with Tris-buffered saline, the membranes were incubated in peroxidase-linked secondary antibody (goat anti-mouse IgG) for 1 h, washed again, and developed using a chemiluminescence method (ECL, Amersham Pharmacia biotech).

2.4. Cell cycle analysis

Cell cycle kinetics were studied by flow cytometry (Sciorati et al., 1997). MCF-7 cells were seeded in 10 cm Petri dishes in 7.5% FCS. To obtain cells synchronization in the G₀ phase, cells were starved for 48 h in serum free medium. Cells were then incubated in presence or absence of 1 nM T or 1 nM DHT in 5% CT-FCS DMEM. At chosen intervals cells were washed with PBS, trypsinized, pelleted, resuspended in 5% CT-FCS DMEM and washed twice with PBS. Cells were then resuspended in 1 ml of PBS, fixed by adding 5 ml of 70% cold ethanol, incubated at 4 °C for 15 min and pelleted. Harvested cells were resuspended in PBS containing 75 M propidium iodide at a density of 1 \times 10⁶ cells/ml and treated for 30 min at 4 °C in the dark with 2.5 U/ml RNase A. Finally the cells were filtered to remove aggregates and analysed for DNA content by

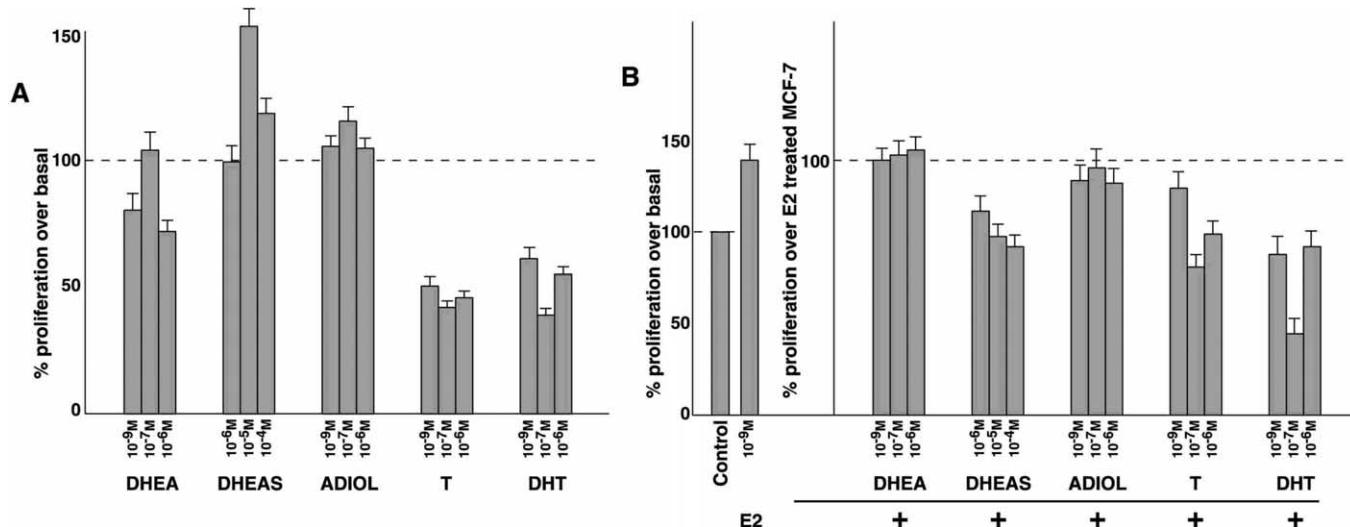


Fig. 1. Dose dependent effect of androgens on MCF-7 cell proliferation in presence (Panel B) or absence (Panel A) of E₂. Cells were seeded in six well plates in complete medium, starved in serum free medium for 24 h and then exposed for 6 days to different concentrations of hormones. These data represent a mean \pm S.E.M. of three separate experiments, each in duplicate.

quantitating the red fluorescence in a FACSCAN apparatus (Becton & Dickson). The percentage of cells in G₀/G₁, S or G₂/M phases of cell cycle was determined by analysis of the results by use of the CELLFIT computer program (Becton & Dickson).

2.5. Plasmids

The full length androgen receptor expression plasmid and CMV P881 plasmid containing the cloned human AR complementary DNA carrying a mutation in the DNA Binding Domain (Cys 574 \rightarrow Arg 574) (Zoppi et al., 1992) were kindly supplied by Dr M.J. McPhaul, Southwestern Medical Center, University of Texas at Dallas (USA).

Firefly luciferase reporter plasmids used were XETL (Bunone et al., 1996). The Renilla luciferase expression vector pRL-CMV (Promega) was used as a transfection standard.

2.6. Transfection of MCF-7 cells

MCF-7 cells were seeded at a density of 5×10^4 /well in 24 well plates; after 24 h cells were transfected in serum-free conditions with Fugene 6 Transfection Reagent (Roche) according to the manufacturer's protocol. After 6 h medium was renewed, ligands were added (for detail see the figure legend) in 5% CT-FCS DMEM and cells were incubated for additional 24 h. One-tube assays for firefly and Renilla luciferases were performed using the Dual luciferase reporter assay system (Promega) as specified by the manufacturer.

2.7. Immunocytochemical staining

MCF-7 cells were cultured for 48 h on chamber slides in DMEM containing 7.5% FCS. After 24 h of serum starvation cells were exposed to 1 nM DHT in 5% CT-FCS DMEM for an additional 24 h. Cells were fixed for 30 min in freshly prepared para-formaldehyde (2%). Cells were then incubated for further 30 min with 10% normal goat serum to block the non-specific binding sites.

Immunohistochemical staining was performed using an affinity purified rabbit anti-peptide antibody that recognizes an epitope in the NH₂-terminal 21 amino-acids of the human AR (designated U402) kindly provided by Dr M.J. McPhaul, UT-SWMed. Center at Dallas, TX. The cells were then incubated with the secondary antibody biotinylated goat-antirabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature followed by incubation with avidin–biotin–horseradish peroxidase complex (Strept ABC Complex-HRP, Vector Laboratories). The peroxidase reaction was developed using Stable DAB (Sigma Chemical, Italy) for 3 min.

3. Results

Among the androgens tested, androstendiol and DHEAS appear to be the strongest androgens in stimulating MCF-7 cells in absence of estradiol with a maximum response to 100 nM. In contrast both androgens show a tendency to down-regulate MCF-7 cell proliferation in the presence of E₂.

T and DHT inhibited MCF-7 cells proliferation independently on E₂ presence.

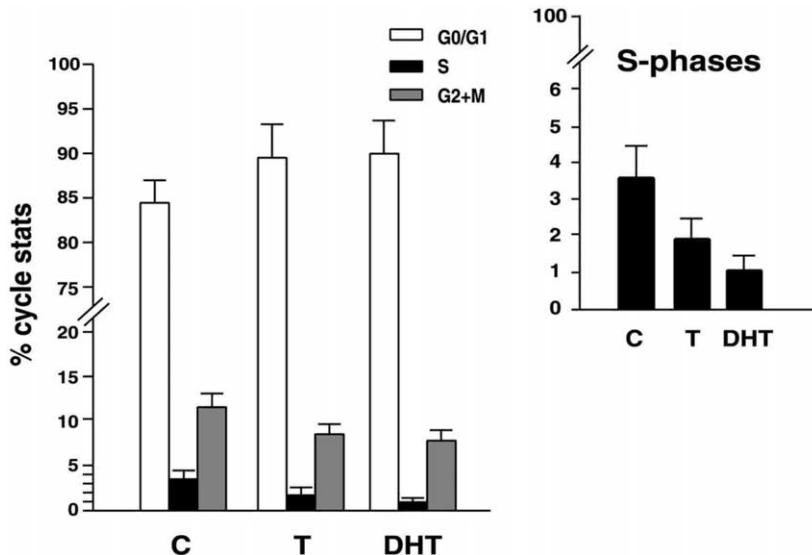


Fig. 2. Effect of T and DHT on the cell cycle of MCF-7 cells. Cells monolayers synchronized by starvation were incubated with or without (control) 1 nM T or DHT in DMEM supplemented with 5% charcoal-treated FCS and then analyzed for DNA content. These data represent a mean \pm S.E.M. of three separate experiments, each in duplicate.

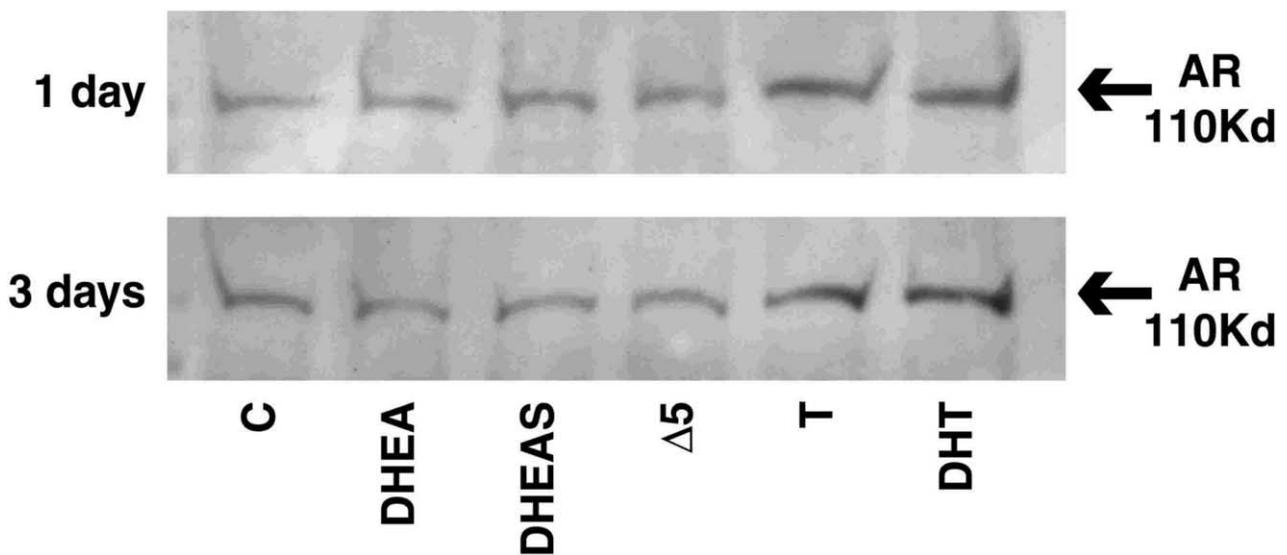


Fig. 3. Effect of androgens on the AR expression in MCF-7 cells. Cells were incubated for 1 or 3 days in presence or absence (control) of androgens. Proteins in cell lysates were separated by SDS PAGE (4–15% polyacrylamide mini gels), blotted and processed for immunodetection of AR as described in Section 2. These data are representative of three separate experiments, each in triplicate.

The most powerful androgen tested DHT (100 nM) consistently inhibits MCF-7 cell proliferation by 50% of the growth basal rate and counteracts E₂ proliferative action by 68% (Fig. 1).

All these data correlate well with cell cycle analysis showing an enhanced number of cells in G₀/G₁ phase after 6 days of DHT treatment (Fig. 2).

Upon prolonged DHT exposure Western blotting analysis shows a marked increase of AR content (Fig. 3) while immunohistochemistry addresses a translocation of AR into the nucleus (Fig. 4). So we assumed that

an enhanced activity of AR induced by DHT might inhibit MCF-7 cell proliferation.

This assumption was corroborated by the fact that the DHT effects on cell proliferation were abrogated by hydroxyflutamide, a selective antagonist of AR, either in absence or in presence of E₂ (Fig. 5).

To make this assumption less speculative and to better understand the role of AR in inhibiting E₂ action at genomic level, we cotransfected MCF-7 cell with firefly luciferase reporter plasmid containing an ERE sequence upstream of a TK promoter together with a

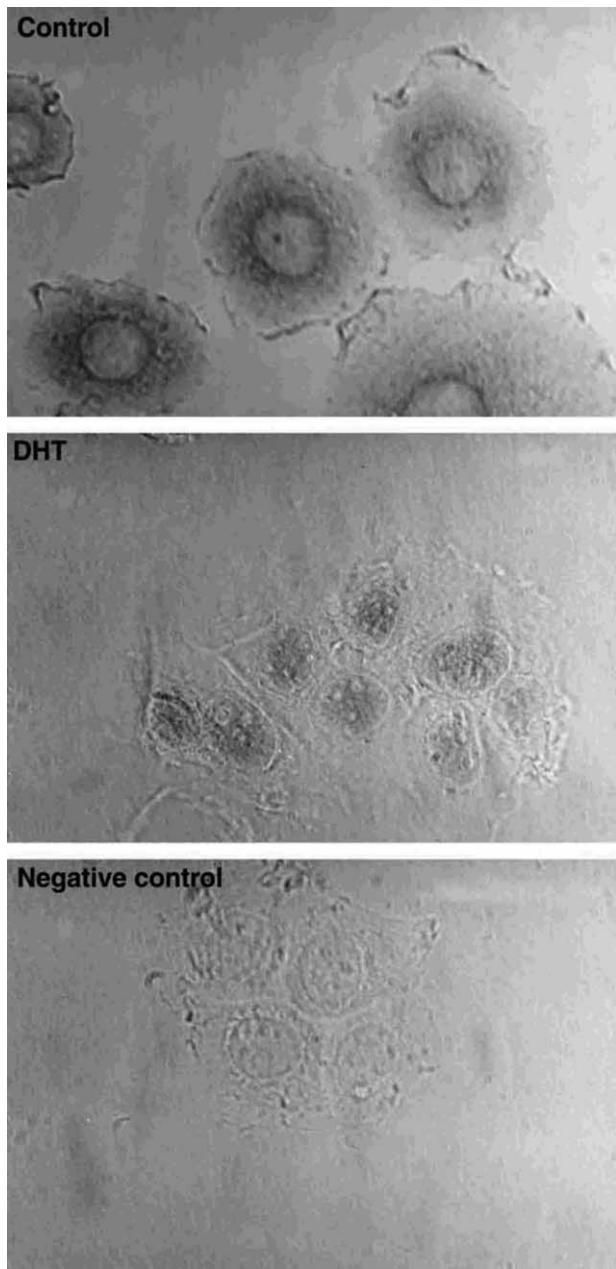


Fig. 4. Immunohistochemical staining of AR. MCF-7 cells were incubated for 24 h in DMEM supplemented with 5% charcoal-treated FCS in presence or absence (control) of 1 nM DHT. Cultured MCF-7 cells were processed for AR immunostaining using an affinity purified rabbit anti-peptide antibody against an epitope in the NH₂-terminal 21 aminoacid of the human AR. No immunoreactivity was detected when MCF-7 cells were incubated without the primary antibody (negative control). These data are representative of three separate experiments.

plasmid codifying the full length AR inducing an over-expression of AR.

In another set of experiments we cotransfected the cells with the mutant AR containing arginin instead of cisteine in the first zinc finger at position 574. This mutation found by Tilley in a patient with androgen resistance makes the AR unable to bind the DNA (Tilley et al., 1989).

The over-expression of AR markedly decreased E₂ (100 nM) signalling which appears furthermore inhibited by simultaneous exposure to 100 nM of DHT, but reversed by addition of hydroxyflutamide.

These inhibitory effects are no longer noticeable when MCF-7 cells were cotransfected with the mutant AR since the normal E₂ signal was restored (Fig. 6).

4. Discussion

Recently we have demonstrated by immunolocalization the existence of aromatase in MCF-7 cells utilizing a rabbit antibody against human placental aromatase. We have proved its biological activity measuring its enzymatic activity (Maggiolini et al., 2001).

Because all the androgens tested in the present study are aromatizable except for DHT, they potentially may activate ERs through their conversion into estrogens. It has been reported, however, that androgens 'per se' may activate ERs directly in the absence of E₂ (Maggiolini et al., 1999). In the same circumstances, the androgens T and DHT with higher binding affinity for AR, were able to do so only at a supraphysiological concentration.

Previous authors have shown how DHEA had inhibitory effects on the estrogen-induced growth of MCF-7 human breast cancer cells and how these effects were reversed by pure antiandrogens flutamide and hydroxyflutamide, postulating that AR activation plays a pivotal role in the inhibitory effect of DHEA on the E₂-induced MCF-7 growth (Bocuzzi et al., 1993).

In the present study we have evidenced that the two most potent androgens are able to inhibit MCF-7 cells proliferation either in presence or absence of E₂. This effect was abrogated by hydroxyflutamide suggesting a specific role of the AR in inhibiting MCF-7 cells proliferation.

A substantial increase of immunoreactive AR was detected following stimulation with DHT compared to parallel cultures that were not treated with hormones, while in another set of experiments with the same hormonal treatment immunohistochemistry revealed that it was exclusively localized around the nucleus.

These findings are in line with previous data which demonstrate that prolonged exposure of cultured human breast cancer cells to DHT markedly enhances ARs content which appear mostly translocated into the nucleus as a result of their functional activation (Marugo et al., 1992). Other authors reported that in MCF-7 cells over expressing AR, T, DHT and its non metabolizable analogue methyltrienolone (R1881) inhibited MCF-7 cells in a dose-dependent manner; DHT and R1881 were equally potent whereas T was 10-fold less potent.

R1881 treatment was associated with an increased number of cells at G₀/G₁ phase of cell cycle postulating

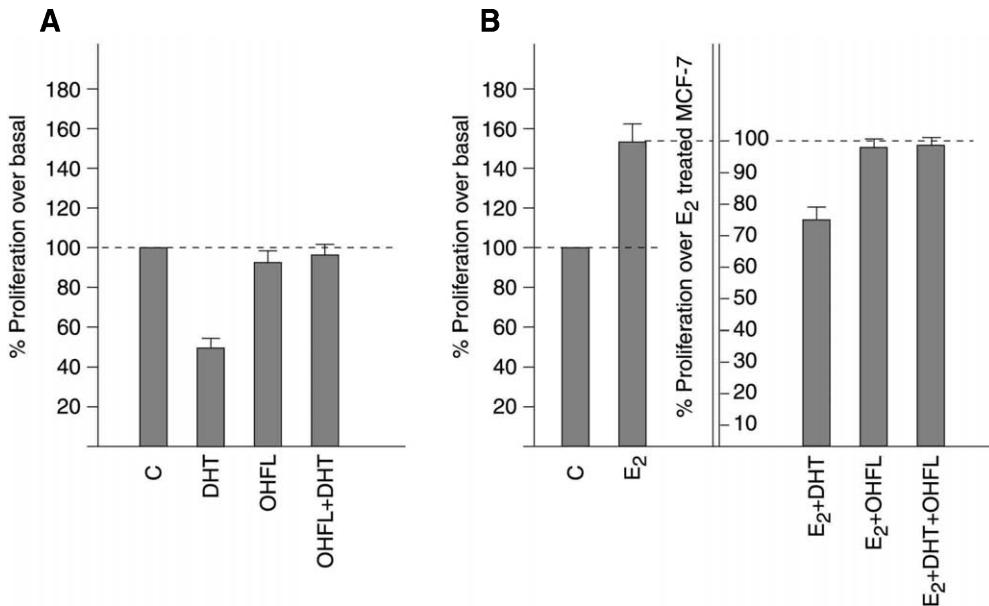


Fig. 5. Effect of hydroxyflutamide and DHT with or without E₂ on MCF-7 cell growth. Cells were seeded in 6-well plates in DMEM supplemented with 7.5% FCS, starved in serum-free medium for 24 h and then exposed for 6 days to 100 nM DHT, 1 μ M hydroxyflutamide with (Panel B) or without (Panel A) E₂. These data represent a mean \pm S.E.M. of three separate experiments, each in duplicate.

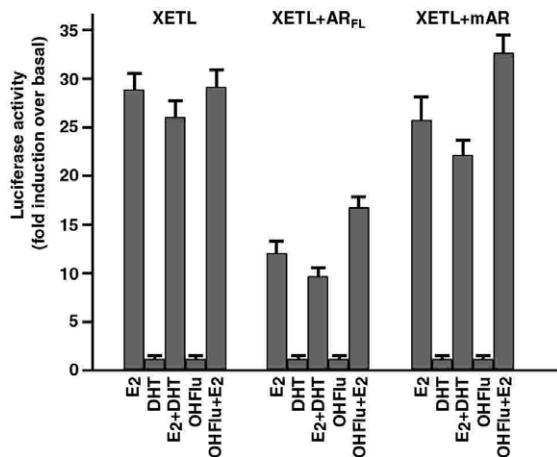


Fig. 6. Effect of transient overexpression of AR on an estrogen-responsive-element-luciferase reporter plasmid. MCF-7 cells were transiently cotransfected with the reporter plasmid XETL, carrying Firefly luciferase sequence under the control of an estrogen-responsive-element (ERE) and the full length androgen receptor expression plasmid (AR_{FL}) or CMV P881 plasmid (mAR) containing a mutation in the DNA binding domain of AR (Cis 574 \rightarrow Arg 574) which abolishes its binding to DNA. Cells were then treated for 24 h with ligands as indicated. These data represent a mean \pm S.E.M. of three separate experiments, each in triplicate.

the androgen specific induction of the synthesis of one or more gene products whose function is to prevent the entry of these target cells into the next cell cycle (Szelei et al., 1997).

The inhibition of MCF-7 cell proliferation could be due to an inhibited cell cycle progression through activation of the kinase inhibitor protein p21^(WAF1/CIP1), an Ar target gene (Lu et al., 1999), which is under the

transcriptional control of p53 (El-Deiry et al., 1993). This suggests that the exposure of MCF-7 cells to a powerful androgen like DHT may sustain p53-dependent cell cycle arrest or apoptosis. On the other hand it has been raised how p21^(WAF1/CIP1) expression also in MCF-7 cells is enhanced upon androgen receptor activation and reversed by hydroxyflutamide (Yeh et al., 2000).

A direct growth inhibitory effect of androgens has been demonstrated in another estrogen-responsive human breast cancer cell line (ZR-75-1) where it has been demonstrated that androgens down-regulate the expression and RNA levels of the antiapoptotic Bcl-2 protein (Lapointe et al., 1999).

Together these data further support the view that the antiproliferative effects of DHT are due to the activation of AR.

The effect of DHT in breast cancer cell lines has been reported to be divergent (Birrel et al., 1995). Possible explanations for the observed divergent effects of androgens on breast cancer cells proliferation may include clonal variation in the cell lines also related to AR content, differences in media conditions, different plating densities and cellular metabolism of the steroids additives.

In the present study in order to eliminate some of these variables, MCF-7 cells were seeded at the same density on six-well plates (10^5 cells/well), grown for 2 days in complete medium, starved for 48 h in serum free medium to obtain cells synchronization in the G₀ phase, and then exposed to various concentrations of the different compounds in DMEM containing 5% charcoal-treated FCS.

In MCF-7 transiently over expressing AR, we observed that the E₂ signalling was drastically reduced, while it was restored in presence of AR carrying a mutation in the DNA binding domain. This addresses a role of AR in inhibiting E₂ action at genomic level.

It is well-documented that transactivation of target genes by steroid receptors is dependent on the context of both the target promoter and the transfected cells (Berry et al., 1990; Nagpal et al., 1992).

To regulate transcription steroids have to convey their activating or repressing signals to the basal transcription machinery. During recent years increasing body of evidence has emerged indicating that steroid receptors can down-regulate the expression of certain genes by interfering with the function of other transcription factors (McKenna et al., 1999). In addition to interact directly with the components of this apparatus, steroid receptors associate with various co-activators and repressors that in turn may function as bridging factors to the basal transcription machinery.

Katzenellenbogen and O'Malley proposed a tripartite system (ligand–receptor–coactivator) to explain the molecular interactions of steroid receptors that may define the potency and biological character of steroid hormones (Katzenellenbogen et al., 1996). In this hypothesis, the ligand–receptor interaction alone may not be able to control the response at the transcriptional level and the interaction between ligand–receptor complexes while cofactors may be essential for steroid hormones function and selectivity.

The current model to account for cell specific regulation of estrogen target gene expression suggest that target cells express different levels of co-activators (e.g. SRC-1, RIP-140, p300 and CBP) and co-repressors (Misiti et al., 1998) as well as different concentration of ER α , ER β and ligand, allowing fine-tuning of target gene transcription in response to estrogens (Klinge, 2000).

Furthermore, transcriptional interferences/squelching has been observed between the activator factors of the various steroid receptors.

For instance it emerges by recent studies that the over expressed AR may interfere with eterologous transcription factors by direct protein–protein contact or competition for common co-regulators (Arnisalo et al., 1998).

On the basis of these findings it remains to be investigated if the antagonist effect of AR on E₂ signal, as it emerges in the present study, is dependent on the interaction between ER and the over expressed AR and if this interaction is direct or mediated by common co-regulators.

This broadens our next area of investigation, which attempts to ascertain the mechanism by which AR may interfere at genomic level with E₂ signal and may

antagonize the E₂-induced proliferative effects on hormone dependent breast cancer cell lines.

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