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Testosterone and 5α-dihydrotestosterone inhibit in vitro growth of human breast cancer cell lines

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ABSTRACT

Androgens are of biological and clinical importance for the growth and development of breast cancer in women, and the androgen receptor (AR) has been shown to be a predictor of tumor differentiation. In the present study, we investigated the relationship between AR status and testosterone and 5α-dihydrotestosterone (DHT)-dependent proliferation of the human breast carcinoma cell lines MCF-7, T47-D, MDA-MB 435S and BT-20. AR status was studied by means of immunocytochemistry and Western blot analysis. All four cell lines stained positively for AR. Western blot analysis revealed a strong expression of AR in MCF-7, in contrast to BT-20 cells. According to proliferation kinetics, we observed a significant (p ≤ 0.05) dose-dependent inhibition of cell growth by testosterone and DHT treatment in all four cell lines. In the estrogen receptor (ER)-negative cell lines BT-20 and MDA-MB 435S, testosterone was a more potent inhibitor of cell proliferation than DHT (p ≤ 0.05), in contrast to the ER-positive cells lines MCF-7 and T47-D, in which a stronger inhibition of proliferation was achieved by DHT. A partial transformation of testosterone to estrogen in ER-positive cells might be an explanation for this effect. Our data favor a possible role of androgens in growth regulation of breast cancer. Clinical studies are needed to analyze the importance of AR as a possible predictor in response to endocrine therapy of breast cancer.

INTRODUCTION

Breast cancer is the most common malignant tumor in women, with a lifetime risk ranging from 1/10 to 1/7. Various risk factors are responsible for breast cancer development, the most common being reproductive risk factors linked to the duration and strength of estrogen exposure (e.g. early menarche, late menopause). Endogenous estrogens are known to be related to individual breast cancer risk both premenopausally and postmenopausally. Furthermore, free endogenous testosterone (serum) seems to be another important risk factor for postmenopausal breast cancer. Additionally, alterations in the endogenous androgen metabolism might play a role in the development of breast cancer. Androgens are known to modulate the expression of genes such as those encoding...
human aromatase (CYP19)\textsuperscript{5}, leukocyte-specific actin-binding protein (L-plastin)\textsuperscript{6}, human glandular kallikrein (hK2)\textsuperscript{7} and prostate-specific antigen (PSA)\textsuperscript{8}, which are believed to be involved in the growth and development of breast cancer. Furthermore, the down-regulation of mammary cell proliferation and estrogen receptor (ER) expression seems to be induced by androgens, suggesting that estrogen--androgen hormone replacement therapy might reduce the risk of breast cancer associated with estrogen replacement\textsuperscript{9}. Androgens such as fluoximesterone\textsuperscript{10} have already been used in the adjuvant therapy of breast cancer in both pre- and postmenopausal women, showing an efficacy comparable to that with other types of endocrine treatment. Some 20–50% of women with metastatic disease responded to therapy with androgens (fluoximesterone). A higher response rate and a prolonged time to progression of the disease were observed in a combined androgen--antiestrogen therapy, compared with treatment with antiestrogen alone\textsuperscript{10}.

The absence of fundamental studies that have analyzed the potent inhibitory effect of androgens on breast cancer cell lines, and the side-effects, are possible explanations for the limited use of androgens in the clinical praxis of endocrine therapy, restricted classically to antiestrogens and aromatase inhibitors\textsuperscript{11}. Therefore, we have investigated the relationship between androgen receptor (AR) status and androgen-dependent proliferation in four human breast carcinoma cell lines, MCF-7, T47-D, MDA-MB 435S and BT-20, in vitro.

**MATERIALS AND METHODS**

**Cell lines, reagents and antibodies**

All cell lines were purchased from the American Type Culture Collection (ATCC).

Dulbecco's modification of Eagle's medium (DMEM/F12) and fetal bovine serum (FBS) were purchased from CCPro (Neustadt, Germany). Antibiotic antymycotic solution (AAS), gentamycin, non-essential amino acids (NEAA), glutamax, trypsin–ethylenediaminetetra-acetic acid (EDTA), phosphate buffered saline (PBS) and Tris buffer were from Sigma Chemical Co. (Deisenhofen, Germany). All electrophoresis reagents were from Serva (Boehringer Ingelheim, Germany). Transfer membranes and Hyperfilm\textsuperscript{®} ECL were from Amersham Life Science (Braunschweig, Germany).

The primary antibody, directed against the androgen receptor, was a polyclonal immunoglobulin G (IgG) rabbit antibody from Santa Cruz Biotechnology (Heidelberg, Germany). The secondary antibody was an anti-rabbit IgG, fluorescein-conjugated antibody from Amersham Life Science.

Testosterone and 5α-dihydrotestosterone (DHT) were purchased from Sigma Chemical Co.

**Immunocytochemistry**

T47-D, BT-20 and MDA-MB 435S cells were routinely cultured in the exponential growing phase, transferred to cover slides and allowed to grow for another 24 h. After rinsing in PBS and fixation with 1% methanol in acetone, non-specific binding sites were blocked with 10% FBS in PBS for 20 min. After rinsing in PBS, cells were incubated with the primary antibody (1 μg/ml) for 1 h at 4°C. After three rinses in PBS, cells were incubated with the secondary antibody in a dark humid box at 24°C for 1 h. After another three rinses in PBS, cells were dried and fixed with paraformaldehyde solution on slides.

Negative controls were incubated only with the anti-rabbit IgG, fluorescein-conjugated antibody. Cells were visualized and photographed under a fluorescence microscope (Leica, Wetzlar, Germany).

**Western blot**

MCF-7, T47-D, BT-20 and MDA-MB 435S cells were washed twice with cold PBS, and scraped after incubation with 4–(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) lysis buffer. Cells were washed again with cold PBS and dispersed. The homogenate was centrifuged at 13 000 rpm for 10 min at 4°C, and proteinase and phosphatase inhibitors were added to the HEPES lysis buffer in which the pellet was resuspended. After cell incubation with Triton® for 15 min, cells were centrifuged at 13 000 rpm for 15 min, followed by protein determination\textsuperscript{12} utilizing the BioRad® kit (BioRad, Hercules, USA). Aliquots of the cell extracts were run on sodium dodecyl sulfate (SDS) 10% polyacrylamide gel. Then, the proteins were electrophoretically transferred in
Towbin® buffer (20% methanol) from the gel to a nitrocellulose membrane at 200 mA for 45 min. The membranes were incubated in blocking buffer (5% bovine serum albumin (BSA) in PBS, 0.02% sodium acid) for 2 h, followed by 60 min incubation with 1 : 5000 dilution of the AR antibody. After extensive washing, the membranes were incubated for 60 min with a 1 : 5000 dilution of the secondary antibody. After a second extensive washing, the antigen–antibody complex was visualized using ECL (Amersham), according to the manufacturer’s instructions.

**RESULTS**

**Expression of the androgen receptor**

To identify the androgen receptor status of the breast cancer cell lines MCF-7, T47-D, BT-20 and MDA-MB 435S, both Western blot and immunocytochemistry were performed. Detection of the AR by means of Western blot is shown in Figure 1. The AR was expressed in all cell lines but to varying degrees. It can be seen in Figure 1 that the AR was strongly expressed by MCF-7, in contrast to BT-20 cells. The AR could also be detected by immunocytochemistry (Figure 2). All cell lines stained positively for AR.

**Cell proliferation assay**

In cell proliferation experiments we used the MTT method, based on the splitting of the tetrazolium ring by dehydrogenases. This enzymatic reaction takes place only in active mitochondria. MCF-7, T47-D, BT-20 and MDA-MB 435S cells were routinely grown at a density of 1–5 × 10⁵/ml in DMEM/F12 supplemented with 1% NEAA, 2 mmol/l glutamax and 10% hormone-stripped FBS. Prior to measurement of proliferation kinetics, cells were switched to serum-free medium and cultivated for 72 h. Cells were seeded in 96-well plates at a density of 2 × 10³/well and incubated under conditions of 37°C, 5% CO₂, with testosterone and DHT at various concentrations of 10⁻⁷, 10⁻⁸ and 10⁻⁹ mol/l. Medium containing testosterone and DHT was changed every third day. Measurement was performed on days 3, 6, 9 and 12 according to the MTT method as described elsewhere. Untreated cells served as negative controls. Significance level was calculated using the Wilcoxon test. If the standard deviation was higher than 10%, experiments were repeated.

**Effect of testosterone and DHT on in vitro proliferation of breast cancer cell lines**

MCF-7, T47-D, BT-20 and MDA-MB 435S cells were incubated with testosterone and DHT at various concentrations. In all cell lines, testosterone or DHT administration led to a dose-dependent inhibition of cell proliferation (Figure 3).

In BT-20 cells, treatment with testosterone and DHT led to growth inhibition. Inhibition of proliferation was significantly higher (p ≤ 0.05) when treated with testosterone than with DHT at a concentration of 10⁻⁷ mol/l (Figure 4). In MCF-7 cells, inhibition of growth after DHT treatment was significantly higher (p ≤ 0.05) than inhibition with testosterone (Figure 4). In MDA-MB cells, a higher inhibition of proliferation was observed with testosterone. When T47-D cells were incubated with DHT, the inhibition of cell proliferation was higher, compared with testosterone treatment.

![Figure 1](image-url) Western blot analysis of androgen receptor in MCF-7, T47-D, BT-20 and MDA-MB 435S cells. After extraction of cytosol, electrophoresis and Western blot analysis were performed. AR was detected in all cell lines.
Figure 2  Immunocytochemical androgen receptor analysis of (a,b) MCF-7; (c,d) T47-D; (e,f) BT-20; and (g,h) MDA-MB 435S cells. After fixation on cover slides, cells were incubated with a polyclonal IgG-rabbit-antibody, directed against AR. (a, c, e, g). An anti-rabbit IgG fluorescein conjugated antibody was used as a secondary antibody. Cells incubated only with secondary antibody served as control (b, d, f, h). Cells were visualized and photographed under a Leica fluorescence microscope.
Figure 3  Dose dependence of proliferation of (a) MCF-7; (b) T47-D; (c) MDA-MB 435S; and (d) BT-20 after treatment with 10^{-7} mol/l (■), 10^{-8} mol/l (△), and 10^{-9} mol/l (▼) testosterone (left side) and 10^{-7} mol/l (■), 10^{-8} mol/l (△), and 10^{-9} mol/l (▼) dihydrotestosterone (right side). Untreated cells served as control (○). After cell incubation in serum free medium, MTT analysis was used for determination of cell proliferation on days 0, 3, 6, 9 and 12. Results are presented as mean ± SD
DISCUSSION

In the present study we investigated the effect of testosterone and DHT on the proliferation of four breast cancer cell lines. In particular, we found that MCF-7, T47-D, and MDA-MB 435S cells express the AR. This is in line with the results of Birrell and colleagues for AR expression in MCF-7 and T47-D cells. However, this is the first report of the presence of the AR in MDA-MB 435S cells.

According to the proliferation kinetics, an inhibition of proliferation was observed in all four cell lines when treated with testosterone or DHT in the concentrations 10^{-7}, 10^{-8} and 10^{-9} mol/l. Such an inhibition of cell growth was dose-dependent (Figure 3). Recent data demonstrate inhibitory effects of testosterone and DHT on the proliferation of MCF-7 cells, as seen in the present study. Our data as well as those of Birrell and colleagues show an inhibitory effect on proliferation of T47-D cells, but in MCF-7 cells the latter authors found a proliferative effect of the androgens. The incubation of BT-20 cells with various concentrations of androgens did not demonstrate any significant effect on proliferation in their study. It is noteworthy that in the study of Birrel and colleagues a different method other than MTT was used. In the present study, we found a density of 2000 cells/well at the start, and a period of 12 days was needed to achieve optimal proliferation conditions (exponential proliferation) and optimal effects of androgens on cell growth. After 12 days, steady state proliferation was observed.

During the time of incubation, testosterone and DHT levels changed. Analysis of testosterone and DHT metabolism in MCF-7, T47-D, MDA-MB 435S and BT-20 cells by means of radioassays performed by our group revealed that these androgens are partly transformed into various metabolites, resulting in a multifaceted effect on the in vitro
proliferation of the four cell lines (data not shown). Therefore, to ensure adequate hormone concentration, the medium was changed every third day, and the total duration of the experiments was limited to 12 days, whereas in other studies the duration of experiments ranged from 3 to 18 days\textsuperscript{19}. The aim of the present study was to observe accumulative effects of testosterone and DHT and their metabolites on the proliferation of the above four cell lines in vitro. Proliferation effects in vivo are more complex, and must be investigated in further studies.

There is evidence that growth regulation of breast cancer cell lines by androgens may be mediated through a specific interaction with the AR\textsuperscript{18}. Since all four cell lines were AR-positive and showed an inhibitory effect on cell growth, our data support this hypothesis.

Proliferative responses were also related to the ER status of individual breast cancer cell lines. In the ER-negative cell lines BT-20 and MDA-MB 435S, there was a higher inhibition of proliferation after treatment with testosterone. Interpretation of the role of androgens in breast cancer is not easy, because of the known dual affinity of testosterone for both the AR and the ER in female target tissues\textsuperscript{19}. It should also be considered that binding of androgens to the ER results in the induction of several characteristic estrogenic responses in human breast cancer cells\textsuperscript{20}, which could be a possible explanation for the present findings. Moreover, it is well known that complex metabolic pathways lead to metabolism of testosterone and DHT by various enzymes (e.g. aromatase) within breast cancer cells\textsuperscript{21}. Therefore, another mechanism might be a possible shift in the steady state towards the aromatase catalyzed reaction testosterone $\rightarrow$ estrogen, instead of the transformation testosterone $\rightarrow$ DHT. The newly formed estrogen will react with the specific ER forming an estrogen–ER complex, which stimulates cell growth\textsuperscript{22}.

DHT has not been reported to undergo aromatization. Therefore, inhibitory actions of DHT on breast cancer cell lines may be mediated directly by DHT interacting with the AR.

Thus, it seems likely that testosterone exhibits differential effects on the proliferation of breast cancer cells, reflecting the proportions of ER and AR expressed by each cell line. This model may explain the therapeutic advantage of the combined use of an antiestrogen plus an androgen over antiestrogen treatment alone.

The positive correlation of elevated serum testosterone levels and the development of postmenopausal breast cancer\textsuperscript{3}, and the possible induction of breast cancer by androgens, is not in disagreement with our findings of growth inhibition of breast cancer cells by testosterone and DHT. Different mechanisms, and possible explanations for the inhibitory effect of androgen action in breast cancer cells, are currently being investigated. Expression of the prolactin receptor can be induced by androgens\textsuperscript{23}, and inhibition of the binding of prolactin to its receptor inhibits breast cancer proliferation\textsuperscript{24}. Androgens suppress ER expression\textsuperscript{25} and, consequently, the estrogen-enhanced growth of breast cancer cell lines. Moreover, androgens can induce breast cancer cell apoptosis, like antiestrogens and progesterins\textsuperscript{26}. Furthermore, down-regulation of protooncogene bcl-2, showing antiapoptotic effects, has been found to be mediated by an androgen–androgen receptor–mediated mechanism\textsuperscript{27}.

In summary, we have shown that the breast cancer cell lines MCF-7, MDA-MB 435S, BT-20 and T47-D express the androgen receptor, and testosterone and DHT inhibit cell proliferation in these cell lines. Our results underline the importance of androgens in breast cancer development. In further studies, the predictive value of the determination of androgen receptor status, as well as markers of androgen metabolism (e.g. 5α-reductase, steroid sulfate), will be investigated in breast cancer tissue.

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