Role of the Androgen Receptor in Human Breast Cancer

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Although the androgen receptor (AR)[3] is often co-expressed with the estrogen receptor (ER) and progesterone receptor (PR) in human breast tumors, its role in breast cancer is poorly understood. Specific growth stimulatory and inhibitory actions of androgens have been described in human breast cancer cell lines. The mechanisms by which androgens exert these contrasting growth effects are unknown. A commonly utilized second line therapy for the treatment of advanced breast cancer is high dose medroxyprogesterone acetate (MPA). Although MPA, a synthetic progestin, was thought to act exclusively through the PR, the androgenic side-effects observed in women taking MPA suggest that its action may also be mediated in part by the AR. In support of this hypothesis, the level of AR measured by radioligand binding in primary breast tumors was correlated with the duration of response to MPA treatment following failure of tamoxifen therapy. Recent data suggest that the presence of structurally altered AR in breast cancers may account for unresponsiveness to MPA in some of these cases. Further studies are warranted to determine the role of AR mediated pathways in regulating breast tumor growth. In particular, identification of androgen-regulated genes may lead to new possibilities for the hormonal treatment of breast cancer.

KEY WORDS: Androgens; androgen receptor; medroxyprogesterone acetate; receptor variants.

INTRODUCTION

Numerous studies indicate that 70–80% of primary breast tumors express ER and 50–70% express the PR (1–3). The AR is present in 70–90% of primary breast tumors (4–6). Moreover, the AR is frequently expressed in breast cancer metastases (75%) and is the sole sex steroid receptor expressed in approximately 25% of metastatic deposits (4). More recent immunohistochemical studies using antibodies specific for the AR have confirmed the earlier biochemical findings (7,8). These immunohistochemical studies also demonstrated that ER, PR, and AR are frequently co-expressed in breast tumors, again confirming earlier biochemical findings.

Approximately three quarters of ER and PR positive tumors are responsive to endocrine therapies (reviewed in Ref. 9). Although androgens have not been used as a primary hormonal treatment for breast cancer since the 1960s due to their masculinising side-effects (e.g., hirsutism, acne), androgens such as fluoxy-mestrone have a therapeutic efficacy comparable to current hormonal therapies such as tamoxifen (10).

The synthetic progestin, medroxyprogesterone acetate (MPA), is commonly used in the treatment of breast cancer as a second line hormonal therapy following failure of treatment with tamoxifen. The androgenic side-effects of MPA suggest that its action may in part be mediated by the AR. Response to MPA therapy is difficult to predict and the relative contribution of the PR and the AR to its action in human breast cancer is poorly understood. Synthetic progestins bind with a high affinity to PR, glucocorticoid receptor (GR)

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[3] Abbreviations: androgen receptor (AR); estrogen receptor (ER); progesterone receptor (PR); medroxyprogesterone acetate (MPA); gluocorticoid receptor (GR); 5α-dihydrotestosterone (DHT); 17β-estradiol (E_2).

and AR (reviewed in Ref. 11). In one clinical study the progestin response was significantly associated with high levels of AR, was weakly associated with high levels of GR, and was unrelated to the expression of ER and PR (12). In that study, 7 of 17 responders lacked detectable levels of PR. In another clinical study, high doses of MPA significantly reduced the level of both AR and PR in metastatic tumors (13). Unfortunately, methodological limitations with these studies, for example competition between MPA and the radioligand used in the biochemical assays, make it difficult to interpret the relationship between receptor levels and hormone responsiveness.

In this article, we examine the evidence supporting the hypothesis that the AR is an important mediator of the growth regulatory effects of androgens and synthetic progestins on human breast cancer cells. While no previous study has directly addressed the hypothesis that the actions of synthetic progestins on human breast cancer cells may in part be mediated by the AR, several research groups have provided data to support this hypothesis (14–16).

PROLIFERATIVE EFFECTS OF ANDROGENS ON HUMAN BREAST CANCER CELLS

The *in vitro* proliferation of human breast cancer cell lines may be stimulated or inhibited by androgens. Physiological and pharmacological concentrations of androgens stimulated the growth of MCF-7 and EFM-19 cells (17), whereas growth was inhibited with physiological concentrations in ZR-75-1 (18) and MFM-223 cells (19), and growth was inhibited only by pharmacological concentrations of androgens in T-47D cells (20). These data raise the question of how androgens can have both stimulatory and inhibitory effects on breast cancer cell proliferation.

The effects of androgens on the ZR-75-1 breast cancer cell line have been examined extensively. This cell line expresses ER, PR, and AR and is estrogen responsive (21). After 12-days incubation in the presence of 0.1nM estradiol (E_2) in phenol red-free medium, the cell number was increased almost 3-fold above control (18). The addition of 1nM dihydrotestosterone (DHT) caused a 78% attenuation of E_2 induced cell proliferation. This effect of DHT could be completely reversed with a saturating concentration of the AR antagonist, hydroxyflutamide. DHT, in the absence of E_2, causes a 25% inhibition of ZR-75-1

cell proliferation, again reversed by co-incubation with hydroxyflutamide (18). Implantation of ZR-75-1 cells into ovariectomized athymic mice has provided a model to examine *in vivo* effects of androgens on breast cancer growth. Again, in the presence and absence of exogenous E_2, DHT inhibited tumor growth and this effect of DHT was reversed by simultaneous administration of hydroxyflutamide (22). Thus this cell line behaves similarly both *in vivo* and *in vitro* and is uniformly growth inhibited by androgens.

Our studies have confirmed this inhibitory effect of androgens on the proliferation of ZR-75-1 cells using the synthetic non-metabolized androgen, mibolerone (23). (See Fig. 1A.) In contrast, our studies also demonstrated that mibolerone stimulates the proliferation of the AR positive but ER and PR negative breast cancer cell line, MDA-MB-453 (Figure 1A) (23). Cotreatment of these two cell lines with mibolerone and specific anti-sense oligonucleotides to the AR, demonstrated that the inhibitory effect, but not necessarily the stimulatory effect of androgens on the proliferation of these breast cancer cell lines, was mediated by the AR (23). (See Fig. 1A.) Video image analysis (Fig. 1B) of AR immunostaining (Fig. 2) in both cell lines demonstrated that AR protein levels were significantly decreased by co-incubation with AR anti-sense oligonucleotides. While these observations are consistent with AR expression being a necessary requirement for androgenic effects on breast cancer cell proliferation (Table I); the absolute levels of AR (as well as ER and PR; Table I) in the cell lines were not associated with either a particular stimulatory or inhibitory proliferative response. It is likely therefore that additional cellular factors or the structure of the AR determine whether breast cancer cell proliferation is stimulated or inhibited in the presence of androgens.

EFFECTS OF ANDROGENS ON ER, PR AND AR LEVELS AND BREAST CANCER CELL PROLIFERATION

The data summarized in the previous section indicate that androgens have either inhibitory or stimulatory effects on the growth of human breast cancer cells *in vitro*. In addition, androgens either up- or downregulate AR mRNA expression in breast cancer cell lines. The cloning of the human AR (24–26) and subsequent production of specific antibodies against the receptor facilitated measurement of AR mRNA levels and more sensitive detection of AR protein expression
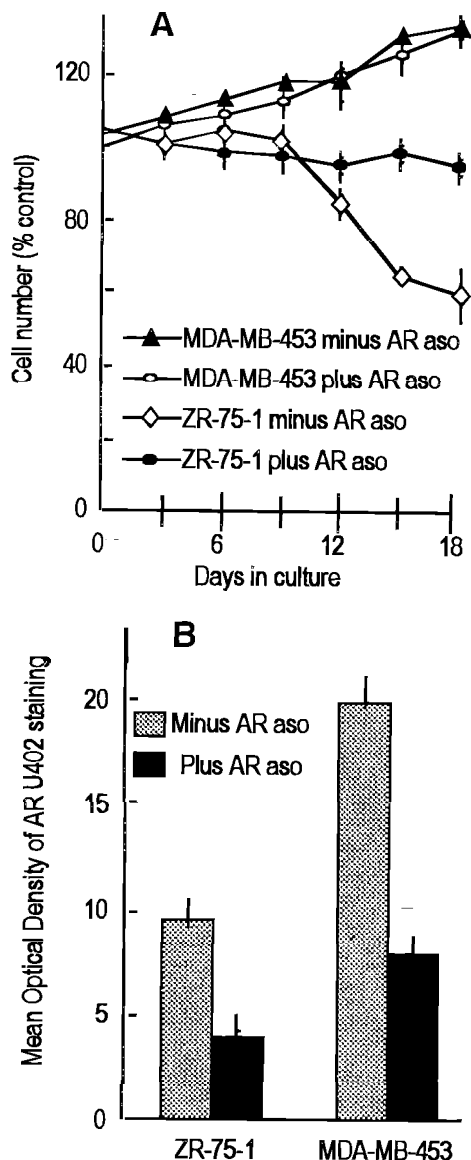
Fig. 1. (A) Proliferation of MDA-MB-453 and ZR-75-1 breast cancer cells in the presence of 1nM mibolerone with or without 10mM AR antisense oligonucleotides (aso). Proliferation was assessed using a methylene blue whole cell uptake assay in 96 well plates (23). Values are expressed as percentage of untreated controls and are the mean of two independent experiments comprising 12 observations per time point. *SEM* ≤ 2.2 for all data points. (B) Androgen receptor expression in ZR-75-1 and MDA-MB-453 breast cancer cells cultured in the presence of 10μM random sense (control) or 10μM AR aso. Cells were cultured in 96 well plates as for proliferation assays. Oligonucleotides were replenished daily for 3 days prior to cells being trypsinised, cytospun and stained with the anti-amino-terminal AR antibody, U402 (23). Mean optical density (MOD; proportional to the cellular concentration of AR) of positive staining was determined using video image analysis (23). Results represent mean ± *SEM*.

in breast cancer cell lines and tumors. The levels of both AR mRNA and AR binding in T47D and MDA-MB-453 breast cancer cells were reduced following treatment with DHT or the synthetic progestin, Organon 2058 (27). Hackenberg *et al.* (28) examined the effects of DHT on AR mRNA and protein levels in the ER negative human breast cancer cell line, MFM-223. This cell line contains high levels of AR and is growth-inhibited by physiological concentrations of DHT. AR protein levels measured by immunocytochemistry and ligand binding were reduced to 57% of control levels by 10 nM DHT after 24h, and at the corresponding time the AR mRNA level was also reduced (28).

In another study, prolonged exposure (i.e., 14 days) of ZR-75-1 cells to physiological concentrations of DHT inhibited both basal and estrogen-induced proliferation (29). Treatment with sub-nanomolar concentrations of DHT markedly reduced the ER and PR content, as measured by radioligand binding and anti-ER monoclonal antibodies. These effects of DHT on breast cancer cell proliferation and receptor levels were reversed by the androgen receptor antagonist, hydroxyflutamide, indicating that they were mediated via an interaction with the AR (29).

In vivo studies using the hormone-dependent DMBA rat mammary tumor model have shown that treatment with testosterone results in tumor regression and a concomitant reduction in ER levels (30). One possible mechanism for this decrease in ER levels in the tumours is that androgen directly regulates ER expression. An alternative explanation is that pharmacological doses of testosterone may be aromatized to E_2 resulting in autologous down-regulation of ER. Thirdly, androgens bind with a low affinity to ER, which may result in an apparent reduction in ER levels following treatment with high doses of testosterone propionate due to interference with ligand binding in biochemical assays for ER.

The studies described earlier indicate that androgens can regulate breast cancer cell growth via an interaction with the AR. However, the effects of androgens on breast cancer cell proliferation may be either stimulatory or inhibitory, suggesting that cell specific differences (e.g., the level of expression of co-factors and co-repressors) or structural alterations in the receptor, as discussed later, may modulate receptor function. Furthermore, the effects of androgens on breast cancer cell growth may be modulated by interactions with ER and PR and other growth factor pathways.
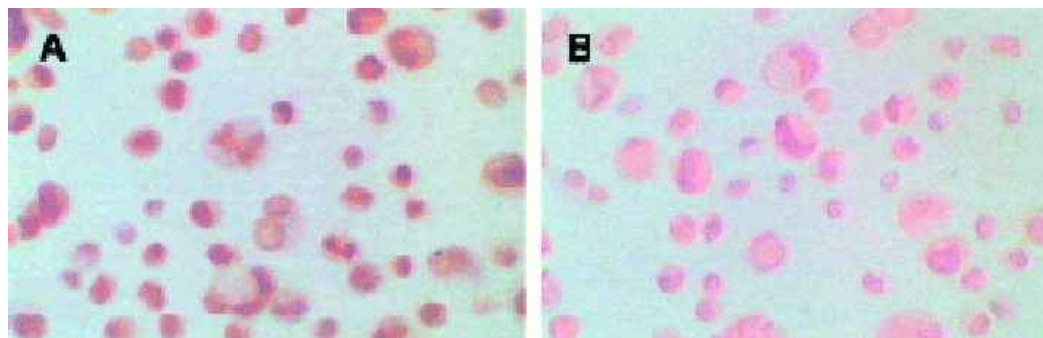
Fig. 2. AR immunostaining of MDA-MB-453 breast cancer cells after 3 days of treatment with 10μM AR random sense (control) (A) or 10μM AR antisense oligonucleotides (B). (×200). Cells were cultured in 96 well plates as for proliferation assays. Oligonucleotides were replenished daily for 3 days prior to cells being trypsinised, cytospun and stained with the anti amino-terminal AR antibody, U402 (23).
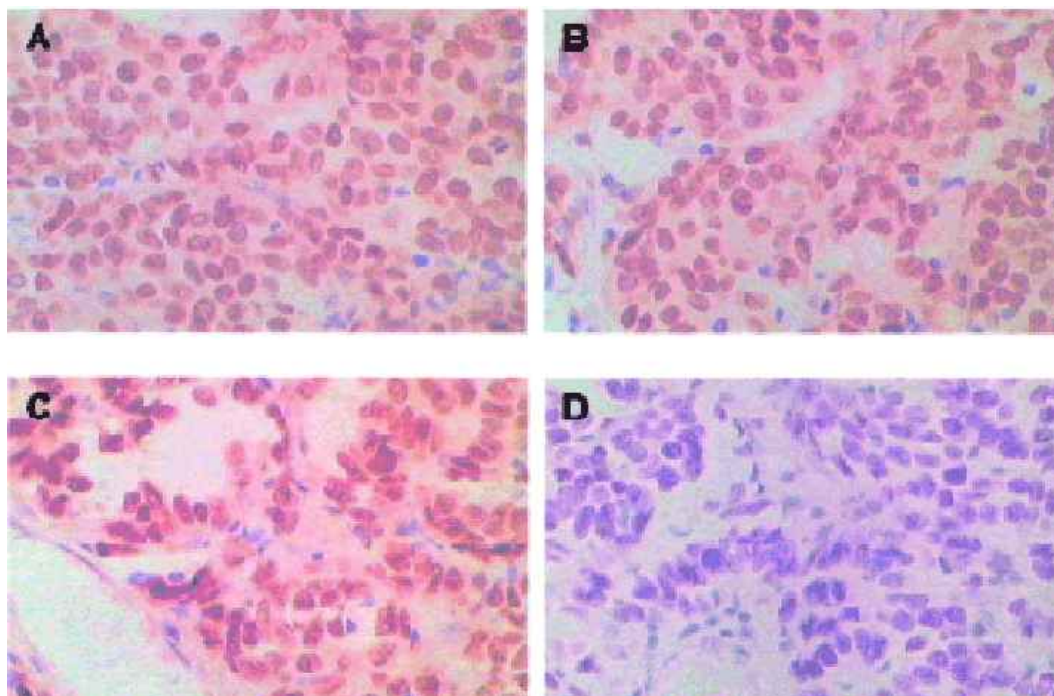

Fig. 3. Photomicrographs (×200) of AR immunostaining of primary breast carcinomas derived from two patients treated with medroxyprogesterone acetate (MPA). Figure A and B shows intense nuclear staining for AR in a tumour of a patient that responded to MPA treatment. Staining is positive for the anti-carboxyl (i.e., R489; A) and anti-amino-terminal (i.e., U402; B) AR antibodies. Figures C and D illustrate AR immunostaining in a tumour which did not respond to treatment with MPA. Nuclear immunoreactivity is apparent in the tissue section stained with the anti-carboxyl AR antibody; (R489; C), but no immunostaining was evident with the anti-aminoterminal antibody (U402; D).

Table I. ER, PR and AR Expression in 6 Human Breast Cancer Cell Lines

Cell line	Proliferative response to DHT[b,c]	Receptor levels[a]		
		ER	PR	AR
MCF-7	Stimulated	++	+	+
T47-D	Inhibited	+	+++	+
ZR-75-1	Inhibited	+	+	++
MDA-MB-453	Stimulated	−	−	+++
BT-20	No response	−	−	−
MDA-MB-231	No response	−	−	−

[a] Determined by radioligand binding (34).
[b] Proliferative response was measured using a methylene blue colorimetric assay as previously described (23).
[c] Cell lines were exposed to 1nM DHT for 10 days.

EFFECT OF MPA ON HUMAN BREAST CANCER CELLS

In Vitro Effects of MPA in Human Breast Cancer

While MPA inhibits the proliferation of breast cancer cells *in vitro,* this effect is not correlated with PR levels (15,20,31,32). Poulin *et al.* (33) suggested that MPA effects on ZR-75-1 cells were largely by direct interaction with the AR. However, this conclusion was based on the use of saturating doses of glucocorticoids and the synthetic progestin, R5020, in the culture medium to block MPA interaction with steroid hormone receptors other than the AR.

To further clarify the interaction of MPA with the AR, we examined effects of MPA on the human breast cancer cell line, MDA-MB-453. This cell line expresses high levels of AR mRNA and protein (34). In contrast to ZR-75-1 cells, expression of ER or PR mRNA and protein could not be detected in MDA-MB-453 cells (34). Evidence that the AR is functional in these cells is provided by the observation that androgens induce the activity of endogenous androgen-responsive genes (e.g., fatty acid synthetase, gross cystic disease fluid protein 15 and the prolactin receptor) (34). This cell line therefore is an important model for studying androgenic effects in the absence of ER or PR.

In contrast to the stimulatory effect of androgens on the proliferation of MDA-MB-453 cells (37), 100nM MPA inhibits the proliferation of these cells by approximately 35% after 12 days in culture (unpublished data). In addition, binding studies with radiolabeled MPA in the presence or absence of various competitor steroids and receptor antagonists demon-strated specific binding of MPA to AR in MDA-MB-453 cells (35). Equimolar concentrations of MPA, DHT and the synthetic androgen, R1881, displaced approximately 50% of bound tritiated MPA indicative of high affinity binding of MPA to the AR. A 100-fold excess of the synthetic progestin, R5020, only partially displaced bound tritiated MPA, consistent with a lower relative binding affinity of this progestin for the AR (36). Furthermore, saturation analysis of tritiated MPA binding to MDA-MB-453 cells demonstrated high affinity binding ($Kd = 0.5nM$) to the AR (35), similar to the relative binding affinity reported for MPA binding to the PR (36).

In Vivo Effects of MPA in Human Breast Cancer

The selection of adjuvant or palliative therapy in breast cancer would be improved if a feature of the primary tumor were predictive of response to MPA. Towards this goal, 136 postmenopausal women who received secondary MPA treatment following failure of adjuvant tamoxifen therapy for lymph node positive breast cancer between 1984 and 1987, were studied (37). ER, PR, and AR levels in the primary tumor, size and degree of axillary node involvement were determined at the time of diagnosis. Median follow-up was 81 months, at which time 89 women had developed metastatic disease. Subsequently, 83 of these women received MPA. The objective response rate (i.e. complete response, partial response, or stable disease) and progression-free interval were assessed in response to secondary MPA therapy.

Forty eight of the 83 patients responded positively to MPA therapy of 500mg/day (37). The median progression free interval for these patients was 24 months. The response rate was significantly associated with the presence of AR ($p < 0.001$) but not with other primary tumor characteristics (e.g., ER or PR levels) or with duration of tamoxifen therapy. The progression free interval of responders to MPA was proportional to the concentration of AR in the primary tumor, that is a longer progression free interval with higher AR content (37).

Various studies have shown that ER levels are reduced in breast cancer patients who relapse while being treated with tamoxifen (38–41), indicative of a transition from an ER positive to negative phenotype following tamoxifen treatment. Furthermore, recurrence during adjuvant tamoxifen therapy has been associated with an increase in the frequency of an ER

and PR negative phenotype in breast tumors (41). This would fit with *in vitro* (42) and *in vivo* (9) data suggesting that chronic exposure to tamoxifen results in down-regulation of PR, as well as ER. In conjunction with the data on AR, these data are consistent with the hypothesis that adjuvant tamoxifen therapy results in a receptor phenotype in which the efficacy of second-line MPA therapy is partly dependent upon the presence of AR mediated growth regulatory pathways.

STRUCTURAL VARIANTS OF THE AR IN HUMAN BREAST CANCER

While we have demonstrated the potential importance of the AR in modulating and predicting MPA response in human breast cancer cells, it remains unclear why the expression of AR *in vivo* is not absolutely predictive of hormone response. To further evaluate the role of the AR in mediating the action of MPA *in vivo,* we utilized an immunocytochemical technique developed in our laboratory to detect potentially abnormal AR in prostate cancer biopsies (43). Serial sections of archival paraffin-embedded blocks of breast tumor biopsies from the same MPA treatment cohort described earlier were stained with polyclonal antibodies directed against either the amino- (i.e., U402) or carboxyl- (i.e., R489) termini of the AR. Although a correlation was found between AR levels measured by radioligand binding and response to MPA, the immunohistochemical studies demonstrated significant discordance between MPA responders and non-responders. Almost 50% (24/54) of non-responders lacked uniform immunostaining with the two antibodies (Fig. 3), compared with only 12.5% (4/32) in the responder cohort (unpublished data).

The immunohistochemical studies also demonstrated that 47% (16/34) of tumors in the subgroup of non-responders to MPA therapy contained AR by radioligand binding and immunostaining with the anti carboxyl-terminal AR antibody, R489, but not with the anti amino-terminal AR antibody, U402 (35). This finding is suggestive of the presence of an amino-terminal truncated AR variant such as the AR-A isoform described by Wilson and McPhaul (44). The pattern of AR immunostaining observed could result from a deletion in the amino-terminal region of the AR gene. However, this possibility is unlikely because such deletions have rarely been reported in the AR gene in the inherited syndrome of androgen insensitivity or prostate cancer (reviewed in Ref. 45). While mutations in the AR coding region may explain the heterogeneous staining pattern, the markedly discordant staining observed with the two antibodies to the AR termini is more consistent with a change in the ratio of the A and B isoforms of the receptor (where B is the full length AR). The amino-terminal truncated form (i.e., AR-A) lacking the first 187 amino acids of the receptor was originally identified in genital skin fibroblasts where the ratio of A:B forms is approximately 1:10 (44). These AR isoforms resemble the A and B forms of the human PR, also encoded by a single gene and differing by the absence or presence of an amino-terminal segment (46). At this time it is not known whether alterations in the ratio of the two AR isoforms could result in altered androgen responsiveness and, in particular, reduced responsiveness to treatment with MPA.

Further analysis of AR immunoreactivity and radioligand binding in the MPA treated cohort identified distinct differences between the responder and non-responder groups. Eleven of the tumors in the non-responder group stained positively with the amino-terminal antibody, U402, and did not have detectable radioligand binding for AR (unpublished data). A possible explanation for this observation is that those tumors that failed to respond to MPA treatment contain mutations in the hormone binding domain of the AR. In the inherited syndrome of androgen insensitivity, single base changes in the AR gene result in loss of receptor function (45). Preliminary analysis has identified similar inactivating mutations in the ligand binding domain of the AR gene in two out of the eleven tumors. In view of our finding that MPA binds to the AR with a high affinity, mutations in the AR gene could result in the loss of MPA binding and failure of MPA therapy.

A germline mutation in the ligand binding domain of the AR gene has been reported in a human prostate tumor (47). A consequence of this mutation was promiscuity of ligand binding resulting in stimulation of AR transcriptional activity by estradiol with comparable efficacy to DHT. Two daughters and one of the grand daughters of this patient were heterozygous for the mutation. As the development of the normal breast requires a coordinated and balanced stimulation by steroid hormones, the long-term consequences of this AR gene mutation for the development of the breast and breast tumorigenesis are unknown.

Recently, Dowsett's group (48) identified an exon 3 deleted splice variant of AR mRNA in three human breast cancer cell lines (MCF-7, BT-474 and MDA-

MB-231) as well as in some breast tumors using RT-PCR and direct DNA sequencing. The variant AR mRNA was co-expressed with wild-type mRNA. *In vitro* translation of the variant mRNA resulted in an AR protein approximately 5kDa smaller than the wild type protein; the second zinc finger within the DNA-binding domain was lacking. The AR variant is predicted to have reduced ability to activate gene transcription and could inhibit the activity of wild-type receptor by heterodimerisation. While the relatively high expression of this AR variant in some breast tumors suggests a role in regulating the growth of breast cancer, the corresponding truncated proteins have not been identified.

CONCLUSIONS

The studies described earlier provide evidence for the involvement of AR mediated pathways in the regulation of breast cancer growth. Furthermore, it appears that the AR may in part mediate the anti-proliferative effects of synthetic progestins such as MPA on human breast cancer cells. The presence of AR in primary breast tumors is also predictive of response to MPA therapy. The predictive power of AR, however, was not absolute, possible due in part to alterations in the structure of the AR. Alternatively, ligand-independent activation of the AR and modulation of the transcriptional activity of the receptor could explain the failure to demonstrate an absolute correlation between the presence of AR in the primary tumor and response to MPA treatment. There is considerable cross-talk between different growth factor pathways in androgen target tissues. For example, protein kinase A and the polypeptide growth factors, insulin-like growth factor I, epidermal growth factor and keratinocyte growth factor can stimulate AR-mediated reporter gene transcription and activate AR signaling pathways (49–50). The pure anti-androgen, casodex, is able to completely reverse AR transcriptional activity induced by these growth factors, suggesting that they may have a direct effect on the AR.

In order to develop better androgenic therapies for breast cancer, it is critical to understand the nature of gene pathways regulated by the AR in breast tissues. Identification of androgen-regulated genes that mediate growth-stimulating versus growth-inhibitory effects of androgens will improve our understanding of the role of androgens and the AR in breast cancer. Furthermore, characterization of gene pathways medi-ating the inhibitory effects of androgens and synthetic progestins on breast cancer cells may enable more precise prediction of the response to hormonal therapies, and define potential new targets for treating breast cancer. Such new treatments may be particularly important in metastatic disease where the AR is often the sole steroid receptor expressed.

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