Pergamon

0960-0760(95)00005-4

J. Steroid Biochem. Molec. Biol. Vol. 52, No. 5, pp. 459–467, 1995

0960-0760/95 $9.50 + 0.00

Androgens Induce Divergent Proliferative Responses in Human Breast Cancer Cell Lines

S. N. Birrell,* J. M. Bentel, T. E. Hickey, C. Ricciardelli, M. A. Weger, D. J. Horsfall and W. D. Tilley

Department of Surgery, School of Medicine, The Flinders University of South Australia, Bedford Park, 5042 SA, Australia

Although the majority of primary human breast cancers express the androgen receptor (AR), the role of androgens in breast cancer growth and progression is poorly understood. We have investigated the effects of the naturally occurring androgen, dihydrotestosterone (DHT), and a synthetic non-metabolizable androgen, mibolerone, on the proliferation of six human breast cancer cell lines. The anti-proliferative and proliferative effects of androgens were only observed in cell lines that expressed the AR. Two of the AR-positive cell lines, T47-D and ZR-75-1 were growth inhibited in the presence of either DHT or mibolerone, while the proliferation of MCF-7 and MDA-MB-453 cells was increased by both androgens. Co-incubation of cultures with 1 nM DHT and a 100-fold excess of the androgen receptor antagonist, hydroxyflutamide, resulted in reversal of both inhibitory and stimulatory effects of DHT on T47-D, MCF-7 and MDA-MB-453 cell proliferation, indicating that DHT action is mediated by the AR in these lines. Hydroxyflutamide only partially reversed the DHT-induced growth inhibition of ZR-75-1 cultures, which suggests that growth inhibition of these cells may be mediated by non-AR pathways of DHT (or DHT metabolite) action. Mibolerone action on breast cancer cell growth was similar to that of DHT, with the exception that growth stimulation of MCF-7 and MDA-MB-453 cells was only partially reversed in the presence of a 100-fold excess of hydroxyflutamide. Anandron, another androgen receptor antagonist, was able to reverse all inhibitory and stimulatory actions of the androgens. AR antisense oligonucleotides reduced the level of immunoreactive AR expression in MDA-MB-453 and ZR-75-1 cells by more than 60%, but only reversed the growth inhibitory action of mibolerone in ZR-75-1 cultures. The results suggest that androgen action in breast cancer cell lines may not be solely mediated by binding of androgen to the AR. For example, metabolites of DHT with oestrogenic activity, or androgen binding to receptors other than the AR, may explain the divergent responses to androgens observed in different breast cancer cell lines.

J. Steroid Biochem. Molec. Biol., Vol. 52, No. 5, pp. 459–467, 1995

INTRODUCTION

The proliferation of breast cancer is regulated by a complex interplay of steroid hormones including oestrogens [1], progestins [2] and androgens [3, 4]. Approximately 60–70% of primary breast tumours express oestrogen receptor (ER) and progesterone receptor (PR) which is usually indicative of a well differentiated breast tumour amenable to hormonal therapy [5, 6]. In addition, approx. 75% of primary breast cancers are immunoreactive for the androgen receptor (AR) [7]. In a recent study, specific binding of [^{3}H]methyltrienolone ([^{3}H]R1881) was detected in 85% of 852 primary breast cancers, while ER and PR were expressed in 71 and 61% of the tumours, respectively [8]. In contrast to ER and PR, the functional significance of AR expression in human breast cancer is poorly understood. The high frequency of AR expression observed in a study of Lea *et al.* [8] implies

*Correspondence to S. N. Birrell.
Received 22 Aug. 1994; accepted 20 Dec. 1994.

that androgens may influence the growth and functional activity of breast tumours. This may be of clinical significance, since epidemiological studies indicate that low urinary androsterone and serum free testosterone levels are strongly related to early onset of breast cancer, a much higher relapse rate after diagnosis and a worse response to endocrine therapy [9, 10a]. Recent data from our laboratory suggests that the duration of response to medroxyprogesterone acetate (MPA) in patients with metastatic breast cancer is related to the level of AR in primary breast tumours [10b]. Since MPA is a synthetic progestin with androgen agonist activity [11], the association between AR and response to MPA observed in our study suggests that MPA may act via the AR to inhibit breast cancer cell growth in advanced breast cancer.

Androgens such as fluoxymestrone [12] have been used in the treatment of advanced breast cancer in both pre- and post-menopausal women. A higher response rate and a longer time to disease progression are observed when androgens are combined with an anti-oestrogen compared to treatment with anti-oestrogen alone [12, 13]. In addition, studies using animal models have demonstrated that androgens can inhibit breast cancer cell growth. Both the growth of human breast cancer cell lines in nude mice [14] and dimethyl benzanthracine-(DMBA)-induced mammary tumours in rats [15] are inhibited by dihydrotestosterone (DHT) [16]. Since these breast cancer cell lines and the DMBA-induced tumours express AR, and growth inhibition is specifically reversed by the AR antagonist, hydroxyflutamide, it appears that androgens inhibit breast tumour growth via an AR-mediated mechanism.

There is limited data available regarding the effect of androgens on the growth of breast cancer cells *in vitro*. The most extensively studied breast cancer cell line is ZR-75-1 which expresses ER, PR and AR [17]. Androgens inhibit the proliferation of this cell line to 40% of untreated controls and growth inhibition is reversed by a saturating concentration of hydroxyflutamide [18]. The inhibitory effect of DHT is more prominent when cell proliferation has been stimulated by prior treatment with oestradiol [18]. In contrast, proliferation of EFM-19 [3], EVSA-T [19] or MCF-7 [3] breast cancer cell lines is stimulated by physiologically equivalent doses of testosterone and DHT to a maximum of 140% of untreated controls. The aim of the present study was to examine the effects of the more active metabolite of testosterone, DHT, and a synthetic non-metabolized androgen, mibolerone on the proliferation of six human breast cancer cell lines, and to investigate which of these effects are mediated by the androgen receptor.

EXPERIMENTAL

Chemicals

All chemicals unless otherwise indicated were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). 7α,17α-dimethyl-19-nortestosterone (mibolerone) was kindly provided by Upjohn Pharmaceuticals. RU23908 [5,5-dimethyl-3-{4-nitro-3-(trifluoromethyl)phenyl}-2,4-imidazolinedione (anandron)] was kindly provided by Dr J. P. Raynaud of Roussel-Uclaf, Romainville, France. [^{3}H]methyltrienolone ([^{3}H]R1881) (126 Ci/mmol) was supplied by Amersham (Sydney, Australia).

Cell culture

Six human breast cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured in RPMI 1640 medium supplemented with 5% foetal bovine serum (FBS), 4 nM glutamine, 1 μg/ml insulin, 100 ng/ml streptomycin, 100 ng/ml penicillin (Commonwealth Serum Laboratories, Melbourne, Australia). Prior to confluence, cells were replated into 96-well tissue culture plates (Corning, Crown Scientific, Moorebank, Australia) in phenol red-free DMEM/F12 medium supplemented with 10% dextran-coated charcoal treated FBS (DCC-FBS) [20], 4 mM glutamine, 100 ng/ml streptomycin and 100 ng/ml penicillin (i.e. steroid-free medium).

Ligand-binding assays

Receptor content was measured in whole cells using a radioligand binding exchange assay [20]. Cells were grown as monolayers in 12-well tissue culture plates (Corning, Crown Scientific), washed with phenol red-free DMEM/F12 culture medium containing 1% w/v bovine serum albumin, then incubated with 0.2–5 nM [^{3}H]R1881 for 1.5 h in the presence or absence of a 200-fold excess of unlabelled R1881 to determine total and non-specific binding, respectively. Assays were performed in the presence of a 500-fold excess of triamcinolone acetonide to eliminate binding of [^{3}H]R1881 to PR or glucocorticoid receptor (GR). Following incubation, monolayers were washed in ice cold PBS/0.5% BSA (2 × 5 min) to remove unbound label. Specifically bound radioactivity was extracted with 1 ml ethanol (2 × 30 min, room temperature). Scatchard plots and linear regression analysis were used to calculate the concentration of hormone binding sites per cell, and the dissociation constant (K_d).

Androgen receptor immunohistochemistry

Immunohistochemical staining of the AR was performed as previously described [21, 22] using an affinity-purified rabbit anti-peptide antibody that recognizes an epitope in the NH_2-terminal 21 amino acids of the human AR (designated U402). The anti-serum was generously provided by Drs Carol M. Wilson, Michael J. McPhaul and Jean D. Wilson, Department of Internal Medicine, University of Texas Southwestern Medical Centre, Dallas, TX.

Quantitative analysis of AR immunocytochemistry

The immunostained sections were examined using an Olympus BH-2 microscope (× 200 magnification) coupled to a computer aided colour video image analysis (VIA) system (Video Pro 32®, Leading Edge Pty Ltd, Adelaide, South Australia, and Leica Australia Pty Ltd) as previously described [22]. AR positive nuclei were stained brown with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and AR negative nuclei were counterstained with weak Lillie Mayers haematoxylin. VIA measurements were made of the following features of AR staining for each cell line: (i) the mean area of AR staining per field, which is an estimate of the number of nuclei positively stained; and (ii) the integrated optical density (IOD) per field, which is proportional to the total amount of AR staining. The following parameters were derived from the primary measurements of AR staining: (i) the mean optical density, MOD (i.e. IOD/area of AR staining) which is a measure of the average concentration of AR in the positively stained nuclei, and (ii) the percentage of AR positive nuclei (i.e. [area DAB/area of DAB + area of haematoxylin staining] × 100).

VIA measurements were made for at least 20 fields per section, the exact number of fields being determined by the cumulative quotient principle [23] to achieve variance below 5% for the mean number of tumour nuclei stained.

Isolation of RNA

Cells were plated into 175 cm^2 culture flasks at 2–4 × 10^4 cells/cm^2 in steroid-free DME/F12 medium. After 3 days in culture the cells were recovered by trypsinization, centrifuged and stored as a pellet at −70°C. Total cellular RNA was isolated as previously described [24]. The cell pellet was thawed in denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M (β-mercaptoethanol) and homogenized using an Ultraturrax (IKA-Werk, Germany) homogenizer for 2 × 15 s bursts. RNA was extracted with water-saturated phenol and precipitated with isopropanol. The RNA pellet was washed twice in 75% ethanol to remove guanidinium salts, dissolved in water treated with 0.1% diethyl pyrocarbonate (DEPC) and the concentration of RNA determined by measuring the absorbance at 260 nm. This method typically yielded 5 μg of total RNA from 10^6 cells.

cRNA probe synthesis

pBSKM13 + plasmids containing segments of the human AR cDNA (713 bp *Hind*III-*Eco*R1 fragment) or β-actin cDNA (247 bp *Eco*R1–*Bam*H1 fragment) were linearized with an appropriate restriction endonuclease (*Hind*III for AR and *Bam*H1 for (β-actin), and *in vitro* transcription performed as described previously using T3 and T7 RNA polymerases, respectively [24, 25]. For synthesis of the hAR cRNA probe, the *in vitro* transcription mixture contained the linearized DNA template (250–500 ng), 0.5 mM unlabelled CTP, ATP and GTP, 5 μM unlabelled UTP and 2.5 μM [^{32}P]UTP (specific activity 800 Ci/mmol; Bresatec, Adelaide, SA, Australia). The β-actin cRNA probe was labelled with one-fifth the amount of [^{32}P]UTP while maintaining the same total UTP molar concentration used for synthesis of the human AR cRNA probe. After a 1 h incubation at 30°C, the DNA template was digested with RNase-free DNase (RQ1 DNase, Promega Corp., Sydney, Australia) and unincorporated nucleotides were removed by ammonium sulphate precipitation.

RNase protection assay

RNase protection assays were performed as described previously [24, 25]. Samples of total RNA, and the AR (6 × 10^5 cpm) and β-actin (1 × 10^4 cpm) cRNA probes were resuspended in 25 μl of hybridization buffer (0.4 M NaCl, 40 mM PIPES, 4 mM EDTA and 60% deionized formamide (Clontech, Palo Alto, CA)), denatured at 80°C for 2 min and then hybridized for 16 h at 45°C. Following hybridization, the samples were digested with a combination of RNase A (20 μg/ml) and RNase T1 (2 μg/ml) for 40 min at 30°C. Digestions were terminated by addition of 2 μl proteinase K (25 mg/ml) and 10% SDS. Samples were then extracted with phenol:chloroform (1:1 v/v), ethanol precipitated twice, and resuspended in loading buffer containing 90% formamide, 0.01% bromophenol blue and 0.01% xylene cyanol. The AR and β-actin protected fragments were separated by electrophoresis on a denaturing 5% polyacrylamide gel containing 8 M urea and the protected bands were visualized using a Phosphor Imager (Molecular Dynamics Pty Ltd, Victoria, Australia). The amount of radioactivity present in the protected fragments was determined using Image Quant software (Molecular Dynamics Pty Ltd).

Cell proliferation

The effect of steroid hormones on breast cancer cell number was determined using a modification of a methylene blue whole cell binding assay as previously described [26, 27]. Stock cells were plated (2.0–4.0 × 10^3 cells/well) in 96 well plates in steroid-free DME/F12 medium. Cells were allowed to adhere to the culture surface prior to the addition of DHT (0.1–10 nM) or mibolerone (0.1–10 nM) on day 3. In some experiments, the breast cancer cell lines were also cultured in the presence of the androgen receptor antagonists, hydroxyflutamide, anandron or antisense AR oligonucleotides (see below). Control cell cultures contained vehicle (0.01% ethanol) alone.

At designated times, the cell monolayers were fixed with 10% (v/v) formaldehyde in normal saline for 30 min prior to staining with 1% (w/v) methylene blue in 0.01 M borate buffer (pH 8.5). The excess dye was

removed by 4 successive washes in 0.01 M borate buffer (pH 8.5) and bound dye was eluted in 1:1 (v/v) ethanol and 0.1 M HCl solution. The absorbance of the eluate was measured at 650 nm in a plate-reading spectrophotometer (Bio-Rad model 450, Bio-Rad, Australia) and cell number was determined from a standard curve generated for each cell line.

AR antisense oligonucleotides

The AR antisense oligonucleotides were designed to include the initiation methionine codon of the AR gene. Random sense and specific antisense phosphorothioate AR oligonucleotides (5′-CTGCACTTCCATCCT-3′) designed according to the sequence described by Tilley *et al.* [28] were synthesized using phosphoramidite chemistry on a model 280B Applied Biosystems oligonucleotide synthesizer. The oligonucleotides were purified according to the method described by Sizeland and Burgess [29].

Statistical analysis

Data are expressed as mean ± SEM. One-way analysis of variance was used to determine statistical significance between control and treatment groups. Cell number at specific timepoints was used to compare dose response data and statistical significance was accepted at the $P < 0.05$ level.

RESULTS

AR expression in breast cancer cell lines

The six human breast cancer cell lines used in this study varied widely in their level of AR expression (Table 1). Two of the cell lines examined, MDA-MB-231 and BT-20, demonstrated no detectable levels of specific [^{3}H]R1881 binding. The remaining cell lines specifically bound [^{3}H]R1881, with calculated AR levels ranging from 12,200 to 69,540 sites per cell for the MCF-7 and MDA-MB-453 cell lines, respectively. Although radioligand binding studies indicated that the MDA-MB-231 cell line was AR-negative, video image analysis of AR immunocytochemistry indicated that 5% of MDA-MB-231 cells were weakly stained for AR. Immunocytochemical analysis of the percentage of cells stained positively for AR varied from 5% in the MDA-MB-231 cell line to 88.1% in the MDA-MB-453 cell line (Table 1). In the cell lines with demonstrable AR immunostaining, the MOD (i.e. cellular concentration of receptor) varied more than 20-fold, from 1.8 arbitrary units for MDA-MB-231 to 42.0 arbitrary units for MDA-MB-453. The high level of AR expression in MDA-MB-453 cells was similar to that observed in the prostate cancer cell line, LNCaP (Table 1). Heterogeneity of AR staining within individual breast cancer cell lines was reflected by the variance of the MOD, which was lowest in the MDA-MB-231 and MDA-MB-453 cell lines (MOD variance = 5.5 and 5.4, respectively) and

Table 1. Measurement of androgen receptor levels in breast and prostate cancer (LNCaP) cell lines by radioligand binding and colour video image analysis of immunostained cells

Cell line	BT-20	MDA-MB-231	MCF-7	T47-D	ZR-75-1	MDA-MB-453	LNCaP
Androgen receptor level*	0	0	12,200 ± 1321	19,450 ± 2134	45,030 ± 2216	69,540 ± 2634	78,455 ± 2111
Mean optical density (MOD)†	0	1.8 ± 0.5	17.5 ± 2.1	22.1 ± 2.8	35.6 ± 1.7	42.0 ± 1.6	45.7 ± 1.1
Variance of MOD		5.5	15.2	18.1	20.3	5.4	37.9
Percentage of AR positive nuclei†	0	5.0 ± 1.0	24.0 ± 7.1	29.5 ± 6.4	75.7 ± 7.5	88.1 ± 2.5	94.0 ± 6.5

*Specific [^{3}H]R1881 binding sites per cell; values are the mean (± SEM) for 3 independent determinations using a whole cell binding assay.
†Values are mean (± SEM) determined by video image analysis for 20 random fields of cells immunostainced with AR antibody, U402, as described in the text.

greatest in the ZR-75-1 cell line (MOD variance = 20.3) (Table 1).

Steady-state AR mRNA levels were also determined for each of the cell lines using an RNase protection assay. A low level of AR mRNA was detected in the MDA-MB-231 cell line (Fig. 1). The level of AR mRNA in the other cell lines was related to the level of AR protein detected by both radioligand binding and immunocytochemistry (Fig. 1, Table 1).

Effects of androgens on breast cancer cell proliferation

Standard curves generated for each of the breast cancer cell lines using the methylene blue assay produced linear optical densities between 0 and 8×10^4 cells per well (results not shown). Culture in 96 well plates in medium containing 10% DCC–FBS resulted in cell growth with a doubling time for all cell lines of 3–4 days between days 3 and 12 of culture.

Effect of DHT. Three distinct proliferative responses to DHT were observed: (i) stimulation of proliferation of the MDA-MB-453 and MCF-7 cell lines, with maximum growth equal to 122 and 115% of controls, respectively; (ii) no effect on the proliferation of MDA-MB-231 or BT-20 cells; and (iii) inhibition of the proliferation of the T47-D and ZR-75-1 cell lines to 88 and 71% of controls, respectively [Fig. 2(A)]. DHT effects on proliferation were not observed until 6 days following addition of the steroid to the culture medium, and the magnitude of the stimulatory and inhibitory effects on breast cancer cell proliferation was similar with all concentrations of DHT used (i.e. 0.1, 1.0 and 10.0 nM ($P > 0.05$)) [Fig. 2(B)]. With the exception of the ZR-75-1 cell line, co-incubation of cells with a 100-fold excess of the AR antagonist, hydroxyflutamide, completely reversed the effects of DHT on breast cancer cell proliferation [Fig. 2(C)]. All stimulatory and inhibitory proliferative responses were reversed by a 100-fold excess of the androgen antagonist,

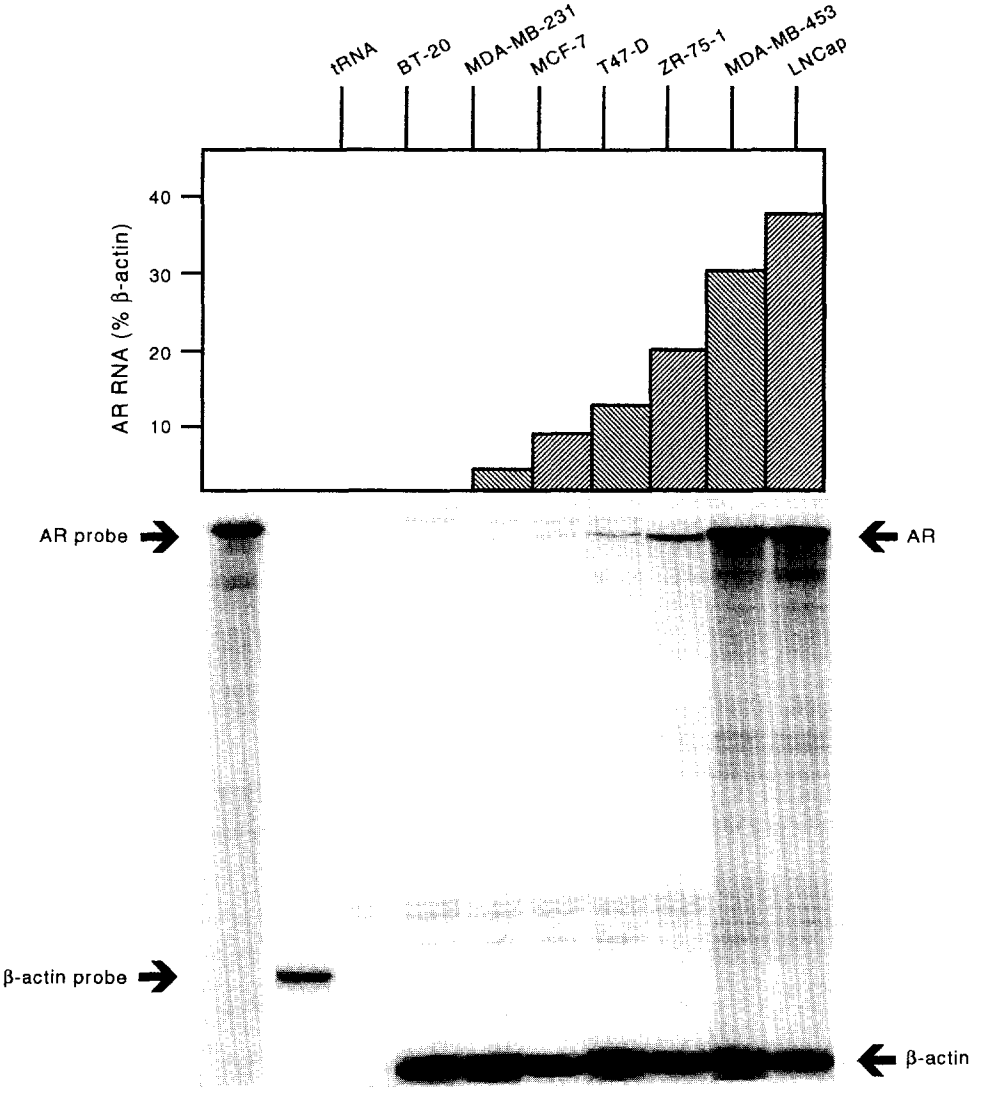

Fig. 1. RNAse protection assay. Androgen receptor mRNA expression in breast cancer cell lines determined using RNase protection assay. Levels of AR mRNA were calculated as a percentage of the β-actin internal control.

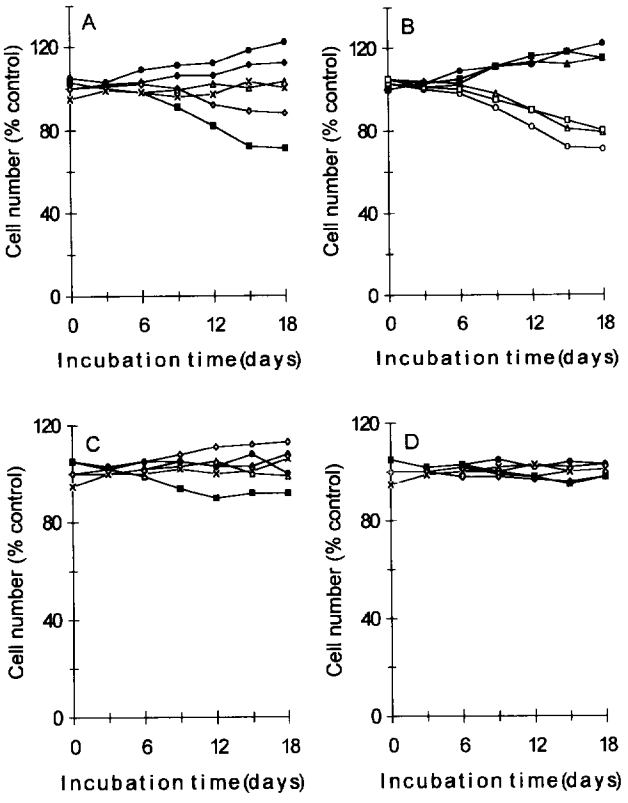

Fig. 2. (A) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) breast cancer cell lines in the presence of 1 nM DHT, expressed as % of untreated controls. Results are the mean of 2 independent experiments showing 12 observations per time point. SEM ≤ 3.5. (B) Growth of MDA-MB-453 and ZR-75-1 with the addition of 0.1 nM (▲ and △), 1 nM (● and ○) and 10 nM (■ and □) DHT respectively, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.5. (C) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) with the addition of 1 nM DHT and 100 nM hydroxyflutamide, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.6. (D) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) in the presence of 1 nM DHT and 100 nM anandron, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.2.

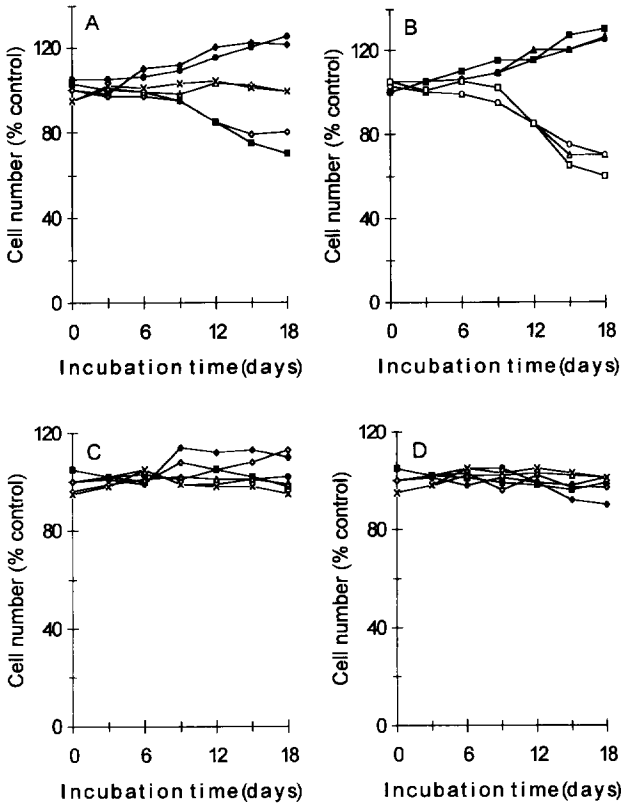

Fig. 3. (A) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) with the addition of 1 nM mibolerone, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.1. (B) Growth of MDA-MB-453 and ZR-75-1 respectively, with the addition of 0.1 nM (▲ and △), 1 nM (● and ○) and 10 nM (■ and □) mibolerone expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.8. (C) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) with the addition of 1 nM mibolerone and 100 nM hydroxyflutamide, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.4. (D) Growth of MDA-MB-453 (●), MCF-7 (◆), MDA-MB-231 (△), BT-20 (×), T-47D (◇) and ZR-75-1 (■) with the addition of 1 nM mibolerone and 100 nM anandron, expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.9.

anandron [Fig. 2(D)]. Neither hydroxyflutamide nor anandron alone had a significant effect on cell proliferation (data not shown).

Effect of mibolerone. Qualitatively similar effects to those of DHT were observed when breast cancer cell lines were incubated with 0.1, 1.0 and 10.0 nM mibolerone (Fig. 3). Mibolerone stimulated the proliferation of the MDA-MB-453 and MCF-7 cell lines to 130 and 121% of controls, respectively [Fig. 3(A)]. No effects on proliferation of MDA-MB-231 or BT-20 cells were observed, and the T47-D and ZR-75-1 cell lines were growth inhibited to a maximum of 80 and 70% of controls, respectively [Fig. 3(A)]. No significant differences in proliferation were evident between the three concentrations of mibolerone tested ($P > 0.05$) [Fig. 3(B)]. While co-incubation of T47-D and ZR-75-1 cells with 1 nM mibolerone and a 100-fold excess (i.e. 100 nM) hydroxyflutamide resulted in reversal of the growth inhibitory effects of this concentration of mibolerone, the growth stimulatory effects of 1 nM mibolerone on MDA-MB-453 and MCF-7 cell cultures were only partially reversed in the presence of 100 nM hydroxyflutamide [Fig. 3(C)]. In contrast, 100 nM anandron completely abolished mibolerone effects on the growth of all breast cancer cell lines [Fig. 3(D)].

Effects of AR antisense oligonucleotides on androgen action

Incubation of both ZR-75-1 and MBA-MD-453 cell lines with 10 mM AR antisense oligonucleotides resulted in a 62 and 61.3% reduction of AR expression, respectively, as measured by video image analysis of immunostained cells (Fig. 4). Addition of 10 mM AR antisense oligonucleotides completely reversed the growth inhibitory effects of 1 nM mibolerone on ZR-75-1 cells (Fig. 5). In contrast, enhanced proliferation of MDA-MB-453 cells cultured in the presence of 1 nM mibolerone was not reversed by the addition of 10 mM AR antisense oligonucleotides to the medium (Fig. 5). Culture of either cell line with 10 mM AR antisense oligonucleotides or 10 mM random sense oligonucleotides alone had no effect on cell proliferation (data not shown).

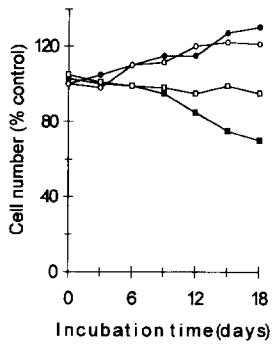

Fig. 5. Growth of MDA-MB-453 and ZR-75-1 in presence of 1 nM mibolerone (● and ■) and 1 nM mibolerone with 10 μM antisense AR oligonucleotides (○ and □), expressed as % of untreated controls. The mean of two independent experiments showing 12 observations per time point are shown. SEM ≤ 2.2.

DISCUSSION

We have examined the effects of an endogenous androgen, DHT and a synthetic non-metabolizable androgen, mibolerone, on the proliferation of six human breast cancer cell lines. The effects of DHT and mibolerone on breast cancer cell proliferation were qualitatively similar. Both androgens stimulated the proliferation of MDA-MB-453 and MCF-7 cells, and inhibited the proliferation of ZR-75-1 and T47-D breast cancer cells. These divergent proliferative responses to androgens were not related to the level of AR expression in individual breast cancer cell lines. Nevertheless, AR expression appeared to be an essential requirement for the modulation of breast cancer cell growth by androgens, since the proliferation of the cell lines MDA-MB-231 and BT-20, which expressed very low or undetectable levels of AR, was not affected by androgens. Possible explanations for the observed divergent effects of androgens on breast cancer cell proliferation may include clonal variation in the cell lines, differences in media conditions, different plating densities, and cellular metabolism of the steroid additives. In the present studies, with the exception of clonal variation of cell lines used by different investigators, we have been able to eliminate these variables by the use of a microplate assay to facilitate simultaneous examination of the effects of DHT and the non-metabolizable androgen, mibolerone, on multiple breast cancer cell lines cultured under identical conditions.

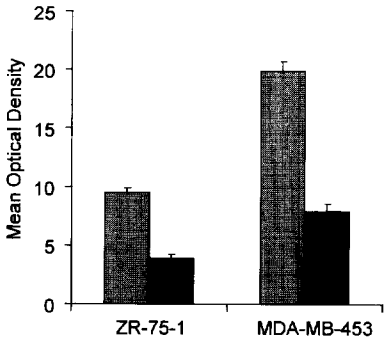

Fig. 4. Androgen receptor expression in ZR-75-1 and MDA-MB-453 cells cultured in the presence of 10 μM random sense (▩) or 10 μM AR antisense (■) oligonucleotides. Cells were cultured in 96 well plates as for proliferation assays. Oligonucleotides were replenished daily for 3 days and cells were trypsinized, cytospun and stained with U402 AR antibody as described in the text. Mean optical density (MOD) of staining was determined using VIA. Results represent mean ± SEM of 2 separate experiments.

Metabolism of androgens by *in vitro* cultured cells has been reported previously. Although DHT does not undergo aromatization, it has been shown that oestrogen-treated MCF-7 cells are able to glucuronidate DHT, resulting in the formation of various metabolites [30]. The biological activity of these metabolites is poorly understood. Recent data suggest that one of the metabolites of DHT, 5α-androstane-3β,17β-diol, increased proliferation of the MCF-7 cell line via interaction with the ER [31]. In addition, the proliferation of the breast cancer cell line MFM-223, which is inhibited by androgens, was also inhibited by physiological concentrations of this DHT metabolite [31]. Thus the growth stimulatory and inhibitory actions of DHT on the breast cancer cell lines may be mediated directly by DHT interaction with the AR. Alternatively, androgenic action may be mediated via interaction of DHT metabolites with AR, and in the MCF-7, ZR-75-1 and T47-D cell lines, via interaction of DHT metabolites with ER, which is expressed in these cells. In the present study we have used the non-metabolized androgen, mibolerone, to demonstrate that stimulation of MCF-7 and MDA-MB-453 cell growth, and inhibition of growth of ZR-75-1 and T47-D cell growth are due to the action of the parent compound.

Hydroxyflutamide completely blocked the inhibitory effect of mibolerone on the proliferation of the ZR-75-1

and T47-D cell lines, and the inhibitory action of DHT on T47-D cells. However, hydroxyflutamide only partially reversed the inhibitory effect of DHT on the growth of ZR-75-1 cells, indicating that DHT action may in part be mediated by pathways that are independent of the AR. The incomplete reversal of DHT effects by hydroxyflutamide was similar to data of Poulin *et al.* [18], at equivalent DHT and hydroxyflutamide concentrations. In contrast to hydroxyflutamide, all stimulatory and inhibitory effects of DHT and mibolerone on breast cancer cell proliferation were completely reversed by co-incubation with the AR antagonist, anandron. Differences in the binding sites and binding affinities of the two AR antagonists to the AR and other cellular proteins (e.g. steroid hormone-binding proteins and other steroid receptors) may account for the observed variation in the androgen antagonist activity of these compounds.

Anti-sense oligonucleotides to the AR were used to determine whether both the inhibitory and stimulatory effects of androgens on the proliferation of the breast cancer cell lines were mediated by the AR. We have developed an immunohistochemical technique for measurement of cellular AR levels using computer-assisted video image analysis [32]. Using this method, we were able to demonstrate that co-incubation with 10 μM AR anti-sense oligonucleotides, but not sense oligonucleotides, resulted in a significant reduction of AR protein expression in both MDA-MB-453 and ZR-75-1 cells. The same concentration of AR antisense oligonucleotides completely reversed the growth inhibitory effect of 1 nM mibolerone on ZR-75-1 cells. In contrast, co-incubation with 10 μM AR antisense oligonucleotides did not alter the growth stimulatory effect of 1 nM mibolerone on MDA-MB-453 cells. These observations raise the possibility that the stimulatory effects of DHT and mibolerone on MDA-MB-453 cell growth are not mediated solely by the AR. It is also possible that the AR in MDA-MB-453 and MCF-7 cells is structurally altered such that hydroxyflutamide does not bind to AR in the normal manner required for antagonist activity. Although variant forms of the ER have been reported in breast cancer cell lines such as BT-20 [33], mutations in the AR gene in breast cancer cell lines have not been reported. The ER variant in breast cancer cell lines which has a deletion of exon 5, has also been demonstrated in breast tumour biopsies [34]. Overexpression of this variant form has been proposed to result in aberrant activation of wild type ER [34]. A point mutation in the ligand binding domain of the AR gene in the human prostate cancer cell line, LNCaP has been described [35]. This point mutation results in altered biological activity of pure antiandrogens, such as hydroxyflutamide, which act as AR agonists and enhance proliferation of LNCaP cells. While similar structural abnormalities of the AR gene in breast cancer cell lines have not been described, a single base change resulting in a critical amino acid substitution in the DNA binding domain of the AR gene has been found in males who develop breast cancer and have clinical evidence of androgen resistance [36].

In summary, our studies demonstrate that androgen action in breast cancer cell lines is cell type-specific and can result in either stimulation or inhibition of proliferation. Furthermore these effects on proliferation could not be attributed to differences in cell culture media conditions or plating densities. Androgens appear to regulate the proliferation of T47-D and ZR-75-1 cells via an interaction with the AR. However, in the case of MDA-MB-453 and MCF-7 breast cancer cells, both AR-mediated and AR-independent growth regulatory pathways may be involved in androgen-induced stimulation of proliferation. Activation of AR-independent pathways could result from the action of active metabolites of DHT that have oestrogenic-like actions [31]. Alternatively, androgen binding to receptors other than the AR may explain the divergent responses to androgens observed in different breast cancer cell lines. Thus, while it is clear that the expression of the AR is necessary for androgens to modulate the growth of breast cancer cells *in vitro*, additional cellular factors, and the structure of the AR itself, may determine whether cell proliferation will be stimulated or inhibited in the presence of androgens.

Acknowledgements—We would like to thank Dr Rosemary Hall for critical reading of this manuscript. Supported by The Anti-Cancer Foundation of the Universities of South Australia, The Flinders University of South Australia, The Royal Australasian College of Surgeons and the National Health and Medical Research Council of Australia.

REFERENCES

1. Beck J. S.: Oestrogen and the human breast. *Proc. Royal Soc. (Edin.)* **95B** (1989) 64–97.
2. Clarke C. L. and Sutherland R. L.: Progestin regulation of cellular proliferation. *Endocr. Rev.* **11** (1990) 266–301.
3. Hackenberg R., Hofmann J., Holzel F. and Schulz K. D.: Stimulatory effects of androgen and antiandrogen on the *in vitro* proliferation of human mammary carcinoma cells. *J. Cancer Res. Clin. Oncol.* **114** (1988) 593–601.
4. Labrie F., Simard J., deLaunoit Y., Poulin R., Theriault C., Dumont M., Dauvois S., Martel C. and Li S. M.: Androgens and breast cancer. *Cancer Detect. Prev.* **16** (1992) 31–38.
5. Horwitz K. B., McGuire W. L., Pearson O. H. and Segaloff A.: Predicting response to endocrine therapy in human breast cancer: a hypothesis. *Science* **189** (1975) 726–727.
6. McGuire W. L. and Clark G. M.: Prognostic factors and treatment decisions in axillary-node-negative breast cancer. *New Engl. J. Med.* **326** (1992) 1756–1761.
7. Kimura N., Mizokami A., Oonuma T., Sasano H. and Nagura H.: Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J. Histochem. Cytochem.* **41** (1993) 671–678.
8. Lea O. A., Kvinnsland S. and Thorsen T.: Improved measurement of androgen receptors in human breast cancer. *Cancer Res.* **49** (1989) 7162–7168.
9. Bulbrook R. D. and Thomas B. S.: Hormones are ambiguous risk factors for breast cancer. *Acta Oncol.* **28** (1989) 841–847.
10a. Thomas B. S., Bulbrook R. D., Hayward J. L. and Millis R. R.: Urinary androgen metabolites and recurrence rates in early breast cancer. *Eur. J. Cancer Clin. Oncol.* **18** (1982) 447–451.

10b. Birrell S. N., Roder D. M., Horsfall D. J., Bentel J. M. and Tilley W. D.: Medroxyprogesterone acetate therapy in advanced breast cancer: prognostic value of androgen receptor expression. *J. Clin. Oncol.* (1995) in press.
11. Labrie F., Poulin R., Simard J., Zhao H. F., Labrie C., Dauvois S., Dumont M., Hatton A. C., Poirier D. and Merand Y.: Interactions between oestrogens, androgens, progestins, and glucocorticoids in ZR-75-1 human breast cancer cells. *Ann. N.Y. Acad. Sci.* **595** (1990) 130–148.
12. Tormey D. C., Lippman M. E., Edwards B. K. and Cassidy J. G.: Evaluation of tamoxifen doses with and without fluoxymesterone in advanced breast cancer. *Ann. Inter. Med.* **98** (1983) 139–144.
13. Ingle J. N., Twito D. I., Schaid D. J., Cullinan S. A., Krook J. E., Mailliard J. A., Tschetter L. K., Long H. J., Gerstner J. G., Windschitl H. E., Levitt R. and Pfeifle D. M.: Combination hormonal therapy with tamoxifen plus fluoxymesterone versus tamoxifen alone in postmenopausal women with metastatic breast cancer—an updated analysis. *Cancer* **67** (1991) 886–891.
14. Engel L. W. and Young N. A.: Human breast carcinoma cells in continuous culture: a review. *Cancer Res.* **38** (1978) 4327–4339.
15. Asselin J., Melancon R., Moachon G. and Belanger A.: Characteristics of binding to oestrogen, androgen, progestin, and glucocorticoid receptors in 7,12,-dimethylbenz(a)anthracene-induced mammary tumours and their hormonal control. *Cancer Res.* **40** (1980) 1612–1622.
16. Dauvois S., Li S. M., Martel C. and Labrie F.: Inhibitory effect of androgens on DMBA-induced mammary carcinoma in the rat. *Breast Cancer Res. Treat.* **14** (1989) 299–306.
17. Horwitz K. B., Zava D. T., Thilagar A. K., Jensen E. M. and McGuire W. L.: Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Res.* **38** (1978) 2434–2437.
18. Poulin R., Baker D. and Labrie F.: Androgens inhibit basal and oestrogen-induced cell proliferation in the ZR-75-1 human breast cancer cell line. *Breast Cancer Res. Treat* 12 (1988) 213–225.
19. Marugo M., Bernasconi D., Miglietta L., Fazzuoli L., Ravera F., Cassulo S. and Giordano G.: Effects of dihydrotestosterone and hydroxyflutamide on androgen receptors in cultured human breast cancer cells (EVSA-T). *J. Steroid Biochem. Molec. Biol.* **42** (1992) 547–554.
20. Ricciardelli C., Horsfall D. J., Skinner J. M., Henderson D. W., Marshall V. R. and Tilley W. D.: Development and characterization of primary cultures of smooth muscle cells from the fibromuscular stroma of the guinea pig prostate. *In Vitro Cell Dev. Biol.* **25** (1989) 1016–1024.
21. Husmann D. A., Wilson C. M., McPhaul M. J., Tilley W. D. and Wilson J. D.: Antipeptide antibodies to two distinct regions of the androgen receptor localise the receptor protein to the nuclei of target cells in the rat and human prostate. *Endocrinology* **126** (1990) 2359–2368.
22. Tilley W. D., Lim-Tio S. S., Horsfall D. H., Aspinall J. O., Marshall V. R. and Skinner J. M.: Detection of discrete androgen receptor epitopes in prostate cancer by immunostaining: measurement by colour video image analysis. *Cancer Res.* **54** (1994) 4096–4102.
23. Aherne W. A. and Dunnill M. S.: Preparation of tissues-sampling. In *Morphometry*. Edward Arnold, London (1982) pp. 19–32.
24. Ricciardelli C., Horsfall D. J., Sykes P. J., Marshall V. R. and Tilley W. D.: Effects of 17β-oestradiol and 5α-dihydrotestosterone on guinea pig smooth muscle cell proliferation and steroid receptor expression *in vitro*. *J. Endocr.* **140** (1994) 373–383.
25. Tilley W. D., Wilson C. M., Marcelli M. and McPhaul M. J.: Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res.* **50** (1990) 5382–5386.
26. Oliver M. H., Harrison N. K., Bishop J. E., Cole P. J. and Laurent G. J.: A rapid and convenient assay for counting cells in microwell plates: application for assessment of growth factors. *J. Cell Sci.* **92** (1989) 513–518.
27. Hall R. E., Birrell S. N., Tilley W. D. and Sutherland R. L.: MDA-MB-453, an androgen-responsive human breast carcinoma cell line with high level androgen receptor expression. *Eur. J. Cancer* **30A** (1994) 484–490.
28. Tilley W. D., Marcelli M., Wilson J. D. and McPhaul M. J.: Characterization and expression of a cDNA encoding the human androgen receptor. *Proc. Natn Acad. Sci. U.S.A.* **86** (1989) 327–331.
29. Sizeland A. and Burgess A.: Anti-sense transforming growth factor alpha oligonucleotide inhibit autocrine stimulation of proliferation of a colon carcinoma cell line. *Molec. Cell Biol.* **11** (1991) 1235–1239.
30. Roy R., Dauvois S., Labrie F. and Belanger A.: Oestrogen-stimulated glucuronidation of dihydrotestosterone in MCF-7 human breast cancer cells. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 579–582.
31. Hackenberg R., Turgetto I., Filmer A. and Schulz K. D.: Oestrogen and androgen stimulation and inhibition of proliferation by androst-5-ene-3-beta, 17-beta diol in human mammary cancer cells. *J. Steroid Biochem. Molec. Biol.* **46** (1993) 597–603.
32. Tilley W. D., Bentel J. M., Aspinall J. O., Hall R. E. and Horsfall D. J.: Evidence for a novel mechanism of androgen resistance in the human prostate cancer cell line, PC-3. *Steroids* **60** (1995) 180–186.
33. Castles C. G., Fuqua S. A., Klotz D. M. and Hill S. M.: Expression of a constitutively active oestrogen receptor variant in the oestrogen receptor-negative BT-20 human breast cancer cell line. *Cancer Res.* **53** (1993) 5934–5939.
34. Zhang Q. X., Borg A. and Fuqua S. A.: An exon 5 deletion variant of the oestrogen receptor frequently coexpressed with wild-type oestrogen receptor in human breast cancer. *Cancer Res.* **53** (1993) 5882–5884.
35. Veldscholte J. C. R., Kuiper G. G., Jenster G., Berrevoets C., Claassen E., vanRooij H. C., Trapman J., Brinkmann A. O. and Mulder E.: A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding charcteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173** (1990) 534–540.
36. Wooster R., Mangion J., Eeles R., Smith S., Dowsett M., Averill D., Barrett L. P., Easton D. F., Ponder B. A. and Stratton M. R.: A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome. *Nat. Genet.* **2** (1992) 132–134.