Androgen receptor localisation and turnover in human prostate epithelium treated with the antiandrogen, Casodex

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ABSTRACT

In vitro models of normal and malignant human prostate are currently limited to a few well established cell lines that, with a single exception (LNCaP), fail to express the androgen receptor (AR) – a common characteristic of prostatic epithelium grown in culture. To investigate the molecular mechanism of action of the non-steroidal antiandrogen Casodex (bicalutamide) against wild-type AR, we have established a transient AR expression model in non-tumorigenic prostate cells of both epithelial and mesenchymal origin. In this

model, both dihydrotestosterone and Casodex can effectively transport the AR protein into the nucleus of prostate cells. Whereas the natural ligand, dihydrotestosterone, stabilises the receptor, the AR is rapidly degraded at a nuclear location when the transfected cells are treated with Casodex. In contrast, whereas the mutant AR in the LNCaP line is also degraded on Casodex treatment over the same time period, its intracellular targeting is defective.

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INTRODUCTION

Metastatic carcinoma of the prostate is a common and potentially life threatening disease that can be effectively treated by administration of anti-androgen drugs. These drugs, of either steroidal or non-steroidal structure (Fig. 1), bind to the androgen receptor (AR) protein in competition with androgens, and block the ability of the AR to transactivate androgen-responsive genes (Teutsch *et al.* 1994, Kuil & Mulder 1994, Kuil *et al.* 1995). Such therapy has been shown to result in the remission of androgen-dependent tumours in 80% of patients for an average duration of 3 years (Montie 1996, Prostate Cancer Trialists' Collaborative Group 1996). As androgen-regulated gene expression is essential for maintenance of normal prostatic function, blocking of the AR by either surgical or chemical castration results in loss of prostatic tissue, probably by an apoptotic mechanism (Isaacs *et al.* 1994, Westin *et al.* 1993, 1995). However, clones of tumour cells that are resistant to the hormone withdrawal treatment, and may possess a greater malignant potential than the original tumour eventually arise within the patients,

probably as a result of mutations in the AR gene or in related genes from the response pathway (Culigi *et al.* 1993a,b, Suzuki *et al.* 1993, Gaddipati *et al.* 1994, Elo *et al.* 1995, Taplin *et al.* 1995, Visakorpi *et al.* 1995, Yeh & Chang 1996, Koivisto *et al.* 1998).

The AR is a ligand-activated transcription factor of molecular mass 105 kDa composed, like most steroid receptors, of a hormone-binding C-terminal domain, a DNA-binding domain and a transactivation domain (reviewed by Sultan *et al.* (1993)). The AR is known to exist as a monomer in the cytoplasm of responsive cells, sequestered in a complex with heat-shock proteins (HSPs). On administration and binding of androgens, the AR changes conformation, and the HSPs are released (Veldscholte *et al.* 1992b). Phosphorylation (Kuiper & Brinkmann 1995), dimerisation (Wong *et al.* 1993) and AR translocation into the nucleus can then occur, followed by binding to the hormone response element upstream of AR-regulated genes, resulting in transactivation.

The study of AR interactions at the molecular level in normal prostate has been restricted by the downregulation of AR expression in almost all

ANDROGEN/ESTROGEN RECEPTOR LIGAND	RECEPTOR BINDING	NUCLEAR TRANSLOCATION	RECEPTOR DEGRADATION
1. 17 β -estradiol	YES	YES	NO
2. ICI 164,384	YES	NO	YES
3. Dihydrotestosterone	YES	YES	NO
4. Casodex (bicalutamide)	YES	YES (Not with mutant AR)	YES
5. Hydroxyflutamide	YES	YES	NO

FIGURE 1. Structures of steroidal and non-steroidal antiandrogen drugs.

in vitro cultures of prostate cells within a few weeks of establishment. This downregulation appears to be at the transcriptional level (Hayward *et al.* 1995 and our unpublished observations), and only transfection of the cells with an AR cDNA can restore wild-type AR protein to a measurable level (Yuan *et al.* 1993). Accordingly, most studies of antiandrogen drug action have been carried out in the LNCaP prostate cancer cell line, which does continue to express AR protein. This cell line was developed from a metastatic deposit in the lymph nodes of a patient after both chemotherapy and radiotherapy, and has a mutant AR with a Thr→Ala change at amino acid 877 (Veldscholte *et al.* 1990). The growth of cells expressing the mutant AR₈₇₇ is stimulated by androgen treatment, but the cells also respond positively to oestrogen, progesterone and even some antiandrogens (Veldscholte *et al.* 1990, Schuurmans *et al.* 1991). However, the antiandrogen bicalutamide (Casodex; AstraZeneca,

Macclesfield) was demonstrated to behave as an AR antagonist even towards the 877 mutant in LNCaP (Veldscholte *et al.* 1992a, 1994). Unlike other AR therapeutic agents, Casodex was shown to inhibit the growth of LNCaP cells, and did not activate the AR₈₇₇ mutant in a HeLa cell co-transfection experiment with an androgen-responsive reporter gene. Casodex also failed to induce dissociation of HSPs from AR₈₇₇ in LNCaP cells (Veldscholte *et al.* 1994) – a prerequisite for successful nuclear translocation of the activated AR. Limited proteolytic digestion of AR (produced by *in vitro* translation) showed different cleavage patterns in the presence of androgens and various antiandrogens, providing evidence of ligand-induced conformational changes, which were maintained with the AR₈₇₇ mutant only in the presence of Casodex (Kuil & Mulder 1994, 1995, Kallio *et al.* 1994). Different androgenic ligands were also shown, in a rat model, to affect receptor stability and turnover (Zhou *et al.* 1995).

No studies have yet used a wild-type AR protein (the natural target for antiandrogens *in vivo*) in human prostate epithelial or fibroblast-derived models to examine the effects of antiandrogens. We have developed a model in which enhanced transient expression of a wild-type AR can be achieved in SV40-immortalised epithelial and fibroblast cultures derived from non-tumorigenic human prostates (Cussenot *et al.* 1991, Berthon *et al.* 1995) in order to study regulation of wild-type AR at the molecular level after exposure to androgens and the antiandrogen Casodex. We believe that this model more accurately reflects the *in vivo* situation compared with the LNCaP model. Treatment of the cells in the transient AR model with both androgens and antiandrogens results in correct nuclear targeting of the AR (not observed in LNCaP) and an accelerated turnover of AR protein at discrete locations within the cell nucleus. We propose that this model demonstrates both the correct conformational changes in the AR (Kuil & Mulder 1994, Kallio *et al.* 1994) and downstream responses to antiandrogens.

MATERIALS AND METHODS

Expression vectors

The human androgen receptor cDNA was kindly provided by Prof. Liao (University of Chicago). It was transferred into the intermediate vector pT7Blue (Novagen, Madison, WI, USA) via PCR amplification of the 5' region of the AR incorporating a Not1 site in front of the ATG and modifying the ATG to include the Kozak sequence (Kozak 1989). The full AR cDNA was reconstructed using an internal Nar1 site and BamH1 at the 3' end of the cDNA from the original clone. This was then ligated into pCEP4 vector (Invitrogen, Groningen, The Netherlands) utilising the Not1 and BamH1 sites. The cDNA was then completely sequenced to ensure wild-type authenticity. As a transfection control we used pCEP4.CAT (Invitrogen) plasmid, which contains the bacterial chloramphenicol acetyl transferase (CAT) gene under the control of the cytomegalovirus promoter.

Cell culture

LNCaP.FGC cells were obtained from the American Tissue Culture Collection (catalogue number CRL1740). The immortalised prostatic epithelial cells PNT1A have previously been described by Cussenot *et al.* (1991). The prostatic fibroblast cell line pf1SV1 was generated and characterised recently by Berthon *et al.* (1995). All cell lines were maintained in R10 (RPMI with 10%

decomplemented fetal bovine serum (FBS), 2 mM glutamine and 10 mM Hepes (Life Technologies)) at 37 °C, 5% CO₂ with media changes twice weekly. Charcoal-stripped medium (R10dcc) was obtained by twice-repeated incubations of FBS with 5% activated charcoal (Sigma) for 30 min at 50 °C followed by 0.2 µm filtration.

Transfection of prostate cells

To carry out the transient assay, transfection conditions for the various prostate cell lines were optimised with a number of commercial liposome-based products. LNCaP cells were best transfected with Lipofectin (Ruokonen *et al.* 1996), but PNT1A- and pf1SV1-immortalised normal prostate cells demonstrated transfection efficiencies that varied over a tenfold range but were optimal with LipofectAmine (Life Technologies, Paisley, UK; catalogue numbers 18292-011 and 18324-012, used according to the manufacturer's instructions). Approximately 25% of each cell type expressed the recombinant AR or CAT gene constructs, as assessed by staining with monoclonal antibodies against the AR (Novocastra, Newcastle upon Tyne, UK; see below) or CAT (Boehringer, Roche Diagnostics, Lewes, UK) proteins.

Cells were grown to 80% confluency in six well plates or on poly-L-lysine-treated slides for immunofluorescence, washed twice in phosphate-buffered saline (PBS) and incubated for 6 h at 37 °C with 1 ml liposome-DNA solution (10 µl of either Lipofectin or LipofectAmine and 2 µg Qiagen-purified plasmid DNA in Opti-MEM (Life Technologies)). For RNA preparation, 10⁶ cells were transfected using Lipofectin (Life Technologies). After transfection, cells were again washed twice in PBS and allowed to recover overnight in R10. Medium was replaced the next morning with fresh R10 or R10dcc for androgen-negative controls. Treatment of the cells was carried out in R10 supplemented with 10⁻⁶ M dihydrotestosterone (DHT), hydroxyflutamide or Casodex (solubilised in ethanol). Cells were harvested for RNA and protein or fixed for immunofluorescence up to 72 h after transfection, although the cultures were stable up to 5 days after transfection.

Androgen-binding assays in transfected cells

To verify that the manipulated AR gene had wild-type activity, an equilibrium androgen-binding assay was carried out on cell extracts of pCEP4.AR and pCEP4.CAT-transfected cells at 24–48 h after transfection. The binding assay was carried out with whole cell extracts essentially as described by Zhou *et al.* (1995), except that the synthetic

androgen mibolerone (^3H -labelled and unlabelled competitor both obtained from New England Nuclear, Hounslow, UK, product numbers NET-919 and NLP-024 respectively) were used. Scatchard plots were produced for each binding assay.

Cell viability measurements with DAPI

To measure cell viability, the ability of inviable cells in a living tissue culture to incorporate 4',6-diamidino-2-phenylindole (DAPI) was exploited.

The stock DAPI was prepared at 0.5 mg/ml in PBS. This was further diluted 1 : 2000 in normal growth medium and added to viable cell cultures for 15 min. Excess DAPI was washed off and the cells were viewed under phase and ultraviolet illumination with a Nikon Eclipse inverted microscope (20 × phase objective) using an appropriate filter. Images of the living cells were captured using a Neotech frame grabber/VHS video camera linked to a Macintosh computer. After image capture, the cultures were washed in PBS and incubated with Annexin V-Alexa568 (Boeringer, catalogue number 1985485, used according to the manufacturer's procedure) to identify apoptotic cells (Vermes *et al.* 1995) and the fluorescent images again captured.

Western blotting

Cells were washed twice in ice cold PBS and then lysed on ice in 1 × SDS sample buffer (50 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 µg/ml phenylmethylsulphonyl fluoride, 1 µg/ml pepstatin A, 1 µg/ml E64). The lysate was immediately heated to 96 °C for 20 min followed by sonication on ice and centrifugation at 10 000 *g* for 10 min at room temperature.

The lysate was then transferred to a fresh Eppendorf tube and heated to 96 °C for a further 5 min before loading on a 7.5% SDS polyacrylamide

gel and submission to electrophoresis according to Laemmli (1970). After electrophoresis, the gels were soaked for 1 h in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol, final pH 8.3). Proteins were transferred by electroblotting on to Hybond ECL (Amersham International, Bucks, UK). Membranes were washed for 20 min in PBS and blocked for 1 h in PBS, 5% Marvel at room temperature. Before addition of the primary antibody, membranes were rinsed and then washed for 1 × 10 min and 2 × 5 min in PBS. AR monoclonal antibody 2F12 (Novocastra) was diluted 1/60 in blocking solution and incubated on the membrane overnight at room temperature. Membranes were washed as above and the secondary antimouse HRP-linked antibody (Amersham) added at a dilution of 1/1000 in blocking solution for 2 h at room temperature. After rinsing with PBS, blots were washed for a period of 1 h with several changes of PBS. Protein expression was visualised using the enhanced chemiluminescence (ECL detection kit; Amersham). Loading controls were CK8 (clone M20; Sigma, Poole, Dorset) for epithelial lines and vimentin (V-4630, Sigma) for pf1SV1.

Northern blotting

Cells were washed twice with sterile PBS and total RNA prepared using TriZol according to the

FIGURE 2. Effects of Casodex on AR expression in prostate cells. (a) AR protein degradation after Casodex treatment. Cells were transfected with either pCEP4.AR (first two lanes in each panel) or pCEP4.CAT (final two lanes in each panel) as described in Materials and Methods, and allowed to recover overnight in R10 medium. The medium was replaced with R10, followed by a further change after 48–72 h when the R10 was supplemented with either DHT (10^{-6} M) or Casodex (10^{-6} M) for 12 h. Protein extracts were prepared and the AR content of the cells was measured by western blot (top panel). Expression of cytokeratin 8 for LNCaP and PNT1A, or vimentin for pf1SV1 (bottom panels) was subsequently detected by stripping and reprobing the same blots. Molecular mass markers are shown to the left of each panel, and the intact AR is present at approx 110 kDa. The relative levels of AR protein (shown in the lower panel) were calculated by scanning densitometry with NIH Image 1.61, and a ratio relative to the loading control (cytokeratin 8 for epithelial cells and vimentin for pf1SV1) was produced. (b) Relative stabilisation of AR in stripped medium containing steroid supplements. Twenty-four hours after transfection with pCEP4.AR, PNT1A cells were washed twice with R10dcc medium and cultured in fresh R10dcc medium for 1 h. At this point DHT, hydroxyflutamide or Casodex was added to a final concentration of 10^{-6} M. Six hours later the adherent cells were washed and harvested for western blot analysis. The results of densitometry of the western blot are shown in the histogram, normalised to a value of 1 for AR transfected cells maintained in R10dcc (the errors are shown in the first bar by the narrow bar). (c) Time course of AR loss after treatment of pf1SV1 cells with Casodex. Two days after transfection with pCEP4.AR, pf1SV1 cells were treated with Casodex (10^{-6} M) (lane 1) or DHT (10^{-6} M) (remaining lanes) for 12 h. All cells were then washed with PBS and harvested for protein and western blotting (lanes 1 and 2) either immediately or after treatment with Casodex (10^{-6} M in R10 medium) for 1 h (lane 3), 3 h (lane 4), 6 h (lane 5) and 12 h (lane 6). All protein samples were western blotted for AR protein detection (top panel) and stripped and reprobed for vimentin as before (lower panel). Quantification (shown in the bottom panel) was carried out as in (a). (d) Transcription of AR in Casodex-treated cells. One million PNT1A and pf1SV1 cells were transfected with 2 µg either pCEP4.CAT or pCEP4.AR and treated with 10^{-6} M DHT or 10^{-6} M Casodex as indicated for 12 h. Total cell RNA was extracted and analysed by Northern blotting as described in Materials and Methods. The blots were probed sequentially for AR (2.9 kb mRNA in the top panel) and GAPDH (1.3 kb mRNA in the bottom panel). Molecular sizing (not shown) was by comparison with standard RNA markers (Life Technologies).

manufacturer's instructions (Life Technologies). The RNA was quantified on a standard agarose gel before electrophoresis of 10 µg aliquots in a 1% agarose gel containing 2.2 M formaldehyde. A capillary blot with 20 × SSC was used to transfer the separated RNA on to HybondN+ (Amersham). RNA was immobilised with alkali fixation (0.05 M NaOH) for 5 min. Membranes were prehybridised with Rapid Hyb (Amersham) containing 200 µg denatured sonicated salmon sperm DNA/ml at 65 °C for 20 min. All AR probes were PCR-generated from pCEP4.AR using the following primers: forward 5'-ACCGTCAGATCTCTAGA AGC-3' (from the CMV promoter in pCEP4), reverse 5'-CACTGCCTTACACAACCTCCT-3' (from the AR gene). The PCR product was Qiagen-purified and radiolabelled with [³²P]dATP in a second PCR reaction using only the reverse primer. The final single-stranded antisense probe was purified by chromatography in a NAP5 column (Pharmacia). Blots were hybridised at 65 °C overnight and then washed by incubation in 2 × SSC, 0.1% SDS at room temperature for 2 × 10 min, followed by an incubation at 65 °C for 15 min in 1 × SSC, 0.1% SDS and finally two washes for 10 min at 65 °C in 0.1 × SSC, 0.1% SDS. AR mRNA expression was visualised by autoradiography. The expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA served as a loading control (the primers used to generate the antisense probe, exactly as described for the AR probe, were:

forward 5'-AAGGTGAAGGTCCGAGTC
AACG-3',
reverse 5'-CGGTTTTCCAGTAGTAGAG
ACGG-3').

Immunofluorescence

Slides were washed twice in PBS and then fixed in methanol/acetone (2 : 1) for 5 min, washed for 3 × 5 min in PBS and air dried. Cells were rehydrated with a drop of PBS, 0.1% bovine serum albumin (BSA) for 10 min and blocked for 30 min according to the ABC kit (Vector Laboratories, Burlingame, CA, USA), followed by washing for 2 × 10 min with PBS, 0.1% BSA and incubation in a humidified chamber at room temperature overnight with antihuman AR antibody clone 2F12 (Novocastra) at a dilution of 1/10 in PBS, 1% BSA. For cytokeratin 8 (clone M20, Sigma) and vimentin (V-4630 Sigma) controls, dilutions were 1/1000 and 1/10 respectively in PBS, 3% BSA for 1 h. The primary antibodies were washed off with two incubations for 10 min with PBS, 0.1% BSA and the secondary anti-mouse FITC conjugated antibody (Dako) was added for 2 h at a dilution of 1/20 in PBS, 3% BSA. Slides were washed four times in

PBS, 0.1% BSA and mounted in Citifluor (Agar Scientific, Stansted, Essex, UK).

RESULTS

Maximising efficiency of AR expression

To prevent potential transcriptional silencing of the transfected AR gene, a modified AR cDNA was constructed. First, the 5' untranslated sequence, which has been shown to encode a translational silencer sequence in mouse, was deleted and the non-optimal Kozak consensus sequence (Kozak 1989) surrounding the ATG start codon was modified. These modifications increased translational efficiency by up to fourfold in human cells. Secondly, the entire construct was sequenced and inserted into the mammalian expression vector pCEP4 (Invitrogen), which permits episomal replication and maintenance of the transfected gene.

Transient transfection was optimised for both the PNT1A and pf1SV1 cell lines with wild-type AR cDNA in pCEP4, using different combinations of commercially available liposomes (see Materials and Methods). This produced levels of expression of AR protein approximately equal to those of the AR₈₇₇ mutant in LNCaP, as estimated from western blotting (Fig. 2). As an average of 25% of PNT1 cells in each culture had been transfected, we estimate that the amounts of AR per cell are fourfold greater in the transient system than in the untransfected LNCaP cells – a conclusion borne out by exposure times for photomicroscopy of the immunofluorescence detection of AR (see below).

To verify that the translationally optimised AR cDNA constructs were producing active protein, an equilibrium steroid binding assay using the synthetic androgen mibolerone was carried out, essentially as described by Zhou *et al.* (1995). The slopes of the resultant Scatchard plots were virtually identical for the AR-transfected cells and for the mutant receptor in LNCaP, indicating that the affinity of the receptor for steroid was unaffected by the AR cDNA manipulation. Equilibrium binding affinity constants were within the range 0.25–0.4 nM as described by Zhou *et al.* (1995). The numbers of active receptors/cell as calculated from the same plot were more variable, but were two- to threefold greater in the transfected cells than in LNCaP cells, in agreement with the AR protein levels determined by western blotting.

AR protein was readily detectable in a western blot within 24 h of transfection (Fig. 2a: leftmost lane in each panel shows amounts after 48 h). However, after 12 h of treatment with 10⁻⁶ M Casodex, the AR was undetectable in both the wild-type and mutant AR-expressing cells (Fig. 2a,

FIGURE 3. Detection of AR protein expression by immunofluorescence. Prostate cells grown on sterile glass microscope slides were transfected with pCEP4.AR as described previously, and treated for 48 h in R10dcc medium (*a*, *e*, *i*). Duplicates were then treated for 6 h with 10^{-6} M DHT (*b*, *f*, *j*) or 10^{-6} M Casodex (*c*, *g*, *k*). Further cultures, maintained in R10 after transfection, were stained with monoclonal antibodies against cytokeratin 8 (*d*, *h*) or vimentin (*l*). A threefold longer exposure time was required for photography at $400\times$ magnification of the positive AR stain in LNCaP (indicated by the greater overall background fluorescence).

second lane). Comparisons of the relative stabilisation of transfected AR with DHT, Casodex and hydroxyflutamide in stripped (R10dcc) medium in a number of prostatic epithelial cells confirmed both the AR binding activity in the transfected cells and the specificity of Casodex action. For all the androgens and antiandrogens, the optimal concentrations were obtained by variation between 10^{-3} M and 10^{-10} M in preliminary experiments. A constant 1 μ M concentration for both agonists and antagonists was selected, as it showed optimal stabilisation and degradative responses in the model system (Waller 1998). In other systems the concentration of DHT required is somewhat lower (nM range) (Zhou *et al.* 1995). However, addition of 10^{-6} M DHT stabilised the AR, relative (2–2.5-fold) to both R10dcc alone and R10dcc+hydroxyflutamide, whereas Casodex reduced the levels of AR to between 10 and 50% of the original values within 6 h of its addition to the growth medium (Fig. 2b). The effects of Casodex were also observed in treated LNCaP cells, but the magnitude was considerably reduced. Maintenance of the LNCaP cells in R10+dcc also led (over a longer period) to a loss of cell viability, as reported by Saeed *et al.* (1997). Control transfections of PNT1, pf1SV1 and LNCaP cells with the pCEP4.CAT gene construct showed endogenous AR expression in only the LNCaP cells.

A time course of Casodex effects (shown in Fig. 2c for pf1SV1) in all the cell types showed a progressive accumulation of AR breakdown products, implying a specific pattern of proteolysis. Random degradation of cell proteins and western loadings were controlled by reprobing the filters with antibodies against cytokeratin 8 (epithelial cells) and vimentin (fibroblasts) as appropriate. All of the data were consistent with enhanced AR protein turnover.

Extraction of total cell RNA and analysis of steady-state mRNA levels (Fig. 2d) indicated that the decrease in AR protein was not due to inhibition of transcription of the AR genes or a decrease in mRNA stability in the various cell types, as observed after primary culture of most prostate cells *in vitro*. At all stages, the loads of transfected plasmid DNA and transfection efficiencies were also carefully controlled and although the density/growth rates of the cell cultures affected the magnitude of the result in terms of overall signal, the AR degradative effect was identical.

Intracellular localisation of AR gene products

Both the normal (PNT1 and pf1SV1) transfected cells and the LNCaP cells were stained with an

anti-AR monoclonal antibody (Clone 2F12 from Novocastra) in the presence and absence of both androgens and Casodex. As shown in Fig. 3, all AR-positive cell types cultured in the absence of androgens (R10dcc) stained strongly for cytoplasmic AR (Fig. 3a, e, i), although the staining intensity was considerably greater in the wild-type AR-transfected cells (Fig. 3e, i). On addition of the potent androgen DHT, the pattern of staining changed dramatically as the wild-type AR translocated to the cell nucleus (Fig. 3f, j), whereas pancellular staining, only partly localised to the nucleus, was observed in LNCaP (Fig. 3b). After treatment with Casodex, the pattern again altered: with wild-type AR (Fig. 3g, k), the nuclear localisation of the receptor was confined to a punctate although still intense stain, which decreased over a 6-h treatment period. Also, after 6 h of treatment with Casodex, but not with DHT, the discrete architectural integrity of the cell nuclei in the AR-transfected cells was beginning to break down. Conversely, there was no similar nuclear punctate pattern observed in the overall staining of the LNCaP cells, although the stain did become rapidly more diffuse as the duration of the Casodex treatment period increased (Fig. 3c). As shown by the duplicate cultures stained with the phenotyping vimentin and cytokeratin antibodies, at this point there was no detectable change in overall cell morphology.

Detection of cell death in AR-transfected cells

To confirm that the AR-transfected cells and the LNCaP cells were responding to the addition of Casodex, AR-transfected cells were Casodex-treated for 6 h, followed by addition of the fluorescent dye DAPI (see Materials and Methods). Viable growing cells exclude the DAPI from their nuclei, whereas apoptotic or inviable cells accumulate DAPI, resulting in strong blue fluorescence when the still-living cultures are observed by inverted fluorescence microscopy under ultraviolet illumination. Measurements of cell viability in living cultures by addition of DAPI to the medium readily detects both apoptotic and necrotic cells, as shown in Fig. 4a in which the arrowed (panel A, red fluorescent) apoptotic cells, detected by AnnexinV binding) in an overgrown LNCaP culture, are already counterstained with DAPI (panel B, blue fluorescence).

The DAPI fluorescence patterns shown in Fig. 4b confirm that only the AR-transfected PNT1A and LNCaP cultures (panels B and D), and not PNT1A transfected either with CAT (panels A and C) or an

AR antisense construct (not shown) take up DAPI after Casodex treatment. Note that only a proportion of the AR-transfected PNT1A cells showed evidence of DAPI uptake (equivalent to the transfected proportion), whereas all the LNCaP cells were DAPI permeable after 6 h of Casodex treatment, even without transfection of the recombinant wild-type AR. There was some evidence that a proportion of LNCaP cells within the individual colonies displayed a more rapid response to the Casodex treatment. As this was observed only in the pCEP4.AR transfected cells, it is most likely that these cells corresponded to those in which the AR₈₇₇ mutant had been supplemented with the more Casodex-responsive wild-type AR from pCEP4.AR. This is shown in more detail in panels E and F of Fig. 4b in which the combined phase/fluorescence picture of AR-transfected PNT1A and LNCaP cells, after 2 h of treatment with Casodex, reveals a subset of strongly DAPI-permeable cells. Untransfected PNT1A cells or untreated PNT1A and LNCaP cells produced images identical to those in panels A and C of Fig. 4b, showing an average spontaneous cell death rate (probably a result of apoptosis) of less than 1%, as previously shown by Berthon *et al.* (1995).

DISCUSSION

By using both immortalised prostate epithelial cells (PNT1A) and fibroblasts (pf1SV1) derived from normal tissue, efficiently transfected with a wild-type AR gene, we have demonstrated distinct mechanistic differences in the AR response to a major non-steroidal antiandrogen Casodex, when compared with the AR₈₇₇ mutant in the hormone dependent LNCaP tumour cell line. Although the Casodex-mediated enhanced AR turnover was present in all prostate cell types transfected with wild-type AR, the intracellular location and nature of this response was different from that in the widely used LNCaP cell line. This is clearly shown in Fig. 3, in which the diffuse nature of the AR immunostain in both DHT and Casodex-treated LNCaP cells and the apparent failure of most of the mutant AR₈₇₇ to translocate to the nucleus are in direct contrast to the results obtained with the wild-type AR-transfected cells, in which the intracellular localisation and trafficking of the wild-type AR in response to the natural substrate DHT is consistent with the proposed mechanism of steroid action.

Cytoplasmic HSPs are normally complexed with AR in the absence of androgens such as DHT. Both wild-type and the AR₈₇₇ mutant in LNCaP are

capable of binding HSPs, but the addition of Casodex, unlike addition of DHT, is less able to release the mutant AR from HSP sequestration (Veldscholde *et al.* 1992b, Kallio *et al.* 1994), leading to impaired nuclear translocation. Casodex-treated LNCaP cells are therefore unable to transactivate AR-responsive genes, as the receptor is not correctly targeted to the AR-responsive genes. However, as the AR₈₇₇ is nevertheless degraded, the protease(s) responsible for turnover of transfected AR, as for other proteins such as β -galactosidase, are probably present in both the nuclear and cytoplasmic compartments (Zhou *et al.* 1995, Tsuneoka & Mekada 1992). Zhou *et al.* (1995) used a series of site directed mutant ARs expressed in COS cells and elegantly showed that neither nuclear localisation nor dimerisation influenced AR degradation.

Antagonist-induced degradation of steroid receptors is not, however, a novel observation. Two antioestrogens – ICI 164,384 and ICI 182,780 – have been shown to reduce intracellular oestrogen receptor (ER) content by inhibiting receptor dimerisation (Fawell *et al.* 1990), destroying nucleo-cytoplasmic shuttling of ER (Dauvois *et al.* 1993) and increasing receptor turnover (Dauvois *et al.* 1992). ICI 182,780 induced a punctate staining pattern, similar to that observed in our AR-transfected prostate cells, in the nuclei of COS-1 cells transfected with a wild-type ER. Loss of ER protein expression *in vivo* after treatment with ICI 182,780 has also been documented previously (McClelland *et al.* 1996). The mechanism was explained in more detail by exploiting a novel oestrogen-independent cell line MCF-7:2A, which expresses both a functional wild-type ER and a mutant ER that can no longer bind ligands (Pink & Jordan 1996). Treatment of MCF-7:2A with ICI 182,780 caused a rapid loss of only the wild-type ER protein. Interestingly, as autoregulation of the endogenous wild-type ER transcript was lifted, ER mRNA actually increased, which was reflected in an accumulation of mutant ER protein over the following 2 days (Pink & Jordan 1996). No such upregulation of transcription of the wild-type AR was observed in the transient expression model.

Examination of the structures of the various ligands (Fig. 1) reveals that, whereas ICI 182,780 closely resembles oestrogen, Casodex is apparently different from the natural ligand DHT. This may explain the observation that Casodex is capable of targeting for degradation both wild-type AR and the mutant AR₈₇₇. In contrast, to date only wild-type ER has been shown to be degraded by ICI 164,384 and ICI 182,780 treatment (Dauvois *et al.* 1992, McClelland *et al.* 1996). The AR model

FIGURE 4.

reported here is the currently the most representative *in vitro* model available for the prostatic response to antisteroid receptor agents.

Antiandrogens currently offer an effective treatment for hormone-responsive prostate cancer, particularly against disseminated disease. The development of androgen-independent disease is inevitably fatal. While the simple inhibition of AR function may provide a ready explanation for the effectiveness of Casodex, the mechanism appears to be more complex and positive in its effects on cells in the prostate. Involution of the prostate (of both normal and tumour phenotype) is a rapid event the mechanism of which has been studied to some extent *in vivo* and *in vitro* (Isaacs *et al.* 1994, Westin *et al.* 1993, 1995). The variations in nuclear morphology seen in Fig. 3 after Casodex treatment are reminiscent of the initial stages of apoptosis, which we have already shown can occur in the untransfected immortalised cell model (Berthon *et al.* 1995). A chemical model of apoptosis in LNCaP using lovastatin has implicated a caspase cascade (Marcelli *et al.* 1998), but androgen deprivation models have proven more difficult to reproduce in the LNCaP cell line, in which maintenance in stripped medium initiates but does not complete the apoptotic program (Saeed *et al.* 1997).

The cell death observed after Casodex treatment (Fig. 4) confirms that the downstream pathways for the antiandrogen response are intact in the immortalised prostate cells, but also indicates that the active pathway is distinct from the apoptotic pathways potentially inhibited by the immortalising SV40 large T antigen.

Loss of androgen sensitivity in prostate tumours is believed to arise partly by mutation of the AR gene (Culig *et al.* 1993a,b, Suzuki *et al.* 1993, Gaddipati *et al.* 1994, Elo *et al.* 1995, Taplin *et al.* 1995, Visakorpi *et al.* 1995, Yeh & Chang 1996, Koivisto *et al.* 1998), although only a few tumour cell line studies have been published (Visakorpi *et al.* 1995, Koivisto *et al.* 1998). Unlike some other

antiandrogens, Casodex is clearly able to inhibit the growth of both wild-type and mutant AR₈₇₇-containing cells, and targets the degradation of the AR in both types of cell. Other antiandrogens may also induce degradation of wild-type AR and induce the same conformational changes, but Casodex has been shown to induce a different conformational change in AR₈₇₇ *in vitro* compared with the related compound hydroxyflutamide (Kuil & Mulder 1994, 1995, Kallio *et al.* 1994). Our data confirm that addition of hydroxyflutamide does not stabilise the wild-type AR to the same extent as DHT and does not induce AR turnover with the same kinetics as Casodex (Fig. 2).

Interestingly, attempts to develop a stable constitutive wild-type AR expression model in prostate have not been successful, as these cells have undergone deletion or mutation of the transgene in the small minority of surviving lines (AS Waller and NJ Maitland, unpublished observations). Several normal breast epithelial cell lines show the same effect after transfer of an exogenous ER cDNA (Zajchowski *et al.* 1993), and reduced growth rates after AR transfection into androgen-independent prostate cancer cell lines such as PC3 have been reported (Yuan *et al.* 1993). Efficient and biologically relevant expression of wild-type or various mutant ARs in the transient system reported here should nevertheless permit mechanistic studies of the response in the human prostate to both native androgens and a variety of inhibitors, with the aim of a more refined treatment for prostate cancer, and an understanding of its molecular basis.

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FIGURE 4. Use of DAPI stain to detect Casodex-induced toxicity in AR-transfected cells. (a) Specificity of DAPI staining to detect inviable cells in cell culture. The panels show LNCaP cells grown to high density and stained with DAPI in the culture medium without permeabilisation (blue fluorescence in panel B). The cultures were then washed briefly and stained with AnnexinV (see Materials and Methods) to detect apoptotic cells (red fluorescence in panel A). (C) Phase image of the cells. (D) Combined image in which the co-localisation of the intact apoptotic cells and a subset of the DAPI permeable cells (indicated by the arrows) is shown. (b) DAPI stain of AR- and CAT-transfected PNT1A and LNCaP cells. Subconfluent monolayers of PNT1A (panels A, B, E) and LNCaP (panels C, D, F) cells were transfected with pCEP4.CAT (panels A,C) or pCEP4.AR (panels B, D, E and F) plasmids. After 24 h, 10^{-6} M Casodex was added to the confluent monolayers of PNT1A cells shown in panels A and B, and 10^{-6} M DHT to cells shown in panel E. The LNCaP cells (which grew as multilayered colonies) shown in panels C and F received 10^{-6} M DHT and cells shown in panel D 10^{-6} M Casodex. Two hours (panels E and F) or 6 h (panels A–D) after addition of steroid, DAPI stain (see Materials and Methods) was added to the culture medium and fluorescent images were captured at 250 × magnification (panels A–D) and 400 × (panels E, F) 15 min later.

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