Evidence for androgen receptor gene expression and growth inhibitory effect of dihydrotestosterone on human adrenocortical cells

R Rossi¹, M C Zatelli¹, A Valentini¹, P Cavazzini², F Fallo³,
L del Senno⁴ and E C degli Uberti¹

¹Department of Biomedical Sciences and Advanced Therapies – Section of Endocrinology, ²Istituto di Anatomia Patologica, ⁴Centro di Studi Biochimici delle Patologie del Genoma Umano, Dipartimento di Biochimica e Biologia Molecolare, University of Ferrara, I-44100 Ferrara and ³Division of Endocrinology, University of Padova, Italy

(Requests for offprints should be addressed to E C degli Uberti, Department of Biomedical Sciences and Advanced Therapies – Section of Endocrinology, University of Ferrara, Via Savonarola 9, I-44100 Ferrara, Italy)

Abstract

Evidence for the expression of the canonic androgen receptor (AR) in human adrenal cortex has not been provided so far. The aim of the present study was to demonstrate the expression of the AR gene in normal and neoplastic adrenocortical human tissues and in the human adrenocortical cancer cell line, NCI-H295, and then to evaluate the effect of dihydrotestosterone (DHT) on human adrenocortical cell growth. An AR cDNA fragment with the expected size of 262 bp was detected by using reverse transcription (RT)-PCR in normal and neoplastic adrenocortical human tissues and in the neoplastic cell line, demonstrating that the gene for AR is indeed expressed in human adrenal cells.

In the human adrenocortical cancer cell line NCI-H295, DHT at physiological concentrations

produced a significant reduction in cell proliferation and inhibition of colony formation in soft agar. The inhibitory effect on adrenocortical cell growth was evident after both 24 and 48 h of treatment. The antiandrogens, cyproterone acetate and hydroxyflutamide, were capable of reversing the effects exerted by DHT. The androgen-induced growth inhibitory effect was also detected in primary culture of three non-functioning adrenocortical adenomas.

These findings show that the canonic AR is present in human adrenocortical cells and that androgens may have a role in the adrenal cortex by reducing cell proliferation.

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Introduction

Androgens exert a variety of effects upon mammalian tissues by controlling cellular growth and differentiation through a stereospecific high-affinity intracellular receptor and modification of gene expression (Mooradian *et al.* 1987, Berger & Watson 1989). Whereas androgenic effects associated with differentiation of the male phenotype occur in reproductive tract tissues, anabolic effects occur within non-reproductive tissues such as liver, kidney and muscle (Berger & Watson 1989).

Androgen receptor (AR) has been identified in the adrenal cortex of immature and adult Rhesus monkeys by immunocytochemistry (Hirst *et al.* 1992) and in adrenocortical tissue of adult rat by immunoblot assay (Bentvelsen *et al.* 1996). Furthermore, testosterone has been shown to reduce adrenal weight in castrated male rats (Rifka *et al.* 1978). These data, taken together, suggest that androgens can influence adrenocortical growth and function by a mechanism involving receptor-mediated pathways.

The aim of the present work was to investigate the expression of AR RNA in normal and neoplastic adrenocortical human tissues and in a human adrenocortical cancer cell line, NCI-H295. We also studied the effect of dihydrotestosterone (DHT) on human adrenocortical cell growth, assessing the response of these cells to androgens in terms of morphology and growth properties.

In order to examine whether the action of DHT on human adrenocortical cell growth is mediated by the androgen receptor, we attempted to antagonize it by the antiandrogens, cyproterone acetate and hydroxyflutamide.

Materials and Methods

Collection of adrenocortical tissues

Normal and neoplastic adrenocortical tissues were obtained from female and male patients undergoing surgery for functioning and non-functioning adrenocortical adenomas. No patient was receiving androgenic or steroid

treatment. Eleven benign tumours, including five non-functioning adenomas, four cortisol- and two aldosterone-producing adenomas were studied. Normal adrenal tissue was obtained from apparently normal areas of glands from patients undergoing surgery for non-functioning adenoma. After surgery, tissues were immediately frozen in liquid nitrogen under sterile conditions, stored at -80°C until they underwent RNA extraction, and then pulverized by a tissue dismembrator (Mikro-Dismembrator II, B Braun, Melsungen, Germany). For primary culture experiments, adrenocortical tissue samples were collected under sterile conditions in RPMI 1640 medium (without phenol red, with L-glutamine; Serva Feinbiochemica, Heidelberg, Germany) and processed within 30 min.

NCI-H295 cell line culture

Human NCI-H295 adrenocortical tumour cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in complete RPMI 1640 medium (without phenol red and with L-glutamine; Serva Feinbiochemica) supplemented with 2% foetal calf serum (FCS; Mascia Brunelli, Milan, Italy), and containing sodium selenite (1 ng/ml, Sigma, St Louis, MO, USA), insulin (5 µg/ml, Novo Nordisk, Princeton, USA), transferrin (5 µg/ml, Sigma), dexamethasone (10^{-8} M, Sigma), 17- β -oestradiol (10^{-8} M, Sigma), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Mascia Brunelli), 100 µg/ml amphotericin (Squibb, New York, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To eliminate the influence of steroid hormones in the medium, in the hormone induction experiments, cells were switched to RPMI 1640 medium supplemented with 2% charcoal (1%)–dextran (0.1%)–treated FCS (CDT–FCS), sodium selenite (1 ng/ml), insulin (5 µg/ml), transferrin (5 µg/ml), and antibiotics 3 days before incubation with DHT (Sigma) alone, or with cyproterone acetate or hydroxyflutamide (Schering-Plough, Milan, Italy) alone, or DHT with one of these antiandrogens.

Growth curve studies

NCI-H295 cells ($5\text{--}7 \times 10^4$ cells/well) were plated in 24-multi-well plates (Falcon, Lincoln Park, NJ, USA). After overnight growth, cells were incubated in the absence or presence of DHT at concentrations ranging from 10^{-14} M to 10^{-8} M in medium without steroids and supplemented with 2% CDT–FCS. Cells were also tested with and without antiandrogens (10^{-8} M cyproterone acetate and 10^{-8} M hydroxyflutamide), with and without DHT. After a 24- or 48-h treatment, cells were harvested by trypsinization and counted using a haemocytometer in 12 different experiments. All experiments were carried out in quadruplicate. DHT, cyproterone acetate and hydroxyflutamide were added to the medium

in alcohol solution with a final concentration of ethanol $<0.1\%$ in both control and hormone-treated cultures. Viability of NCI-H295 cells was determined by tripan blue staining and the number of viable cells was always in the range 85–95%.

Primary culture of human adrenocortical cells

After surgical removal of three non-functioning adrenocortical adenomas, the tissue was finely minced with scissors and cells were dispersed by a collagenase digestion (1 mg/ml) in 6 ml complete RPMI 1640 medium as for NCI-H295 cells. The cell suspension was centrifuged at 250 g for 5 min and the cell pellet washed twice and then filtered through a double layer of nylon gauze (300 mesh). Cells were then seeded in monolayer cultures in 24-multi-well plates (Falcon) and grown in complete medium at 37°C in a humidified atmosphere of 5% CO_2 –95% air. After 24 h, the medium was replaced with fresh medium without steroids and with CDT–FCS. In this defined medium, treatment with 10^{-11} M DHT was performed, starting on day 3 of culture, and after 24 h of treatment the cells were harvested by trypsinization and counted using a haemocytometer. Each experiment was done in quadruplicate.

Isolation of RNA

Pulverized adrenocortical tissues and cultured carcinoma cells were lysed in guanidium isothiocyanate, and total RNA was purified by phenol–chloroform extraction and isopropanol–ethanol precipitation (RNAzol B solution, Biotech Lab Inc., Houston, TX, USA). To prevent further DNA contamination, RNA was treated for 5 min at 37°C with RNase-free DNase and, after 10 min at 70°C , it was again phenol–chloroform extracted. The amount of RNA was determined by optical density at 260 nm.

RT-PCR

Using 1st Strand cDNA Synthesis Kit (Boehringer, Mannheim, Germany), 1 µg total RNA from cultured cells and adrenocortical tissues was reverse-transcribed at 25°C for 10 min and then at 42°C for 60 min in a 20-µl solution containing 20 U AMV Reverse Transcriptase, 50 U RNase inhibitor and 100 pmol AR reverse primer. cDNA product (4 µl RT reaction) was amplified with 1 U *Thermo aquaticus* (Taq) polymerase (Boehringer) and 100 pmol reverse and forward primers in 25 µl reaction mixture containing 200 µmol dNTPs, 50 mmol KCl, 1 mmol MgCl_2 , 0.01% gelatin and 0.1% Triton X-100. The PCR reaction was performed under mineral oil for 30 cycles of amplification. Each cycle consisted of 94°C for 60 s, 55°C for 60 s, 72°C for 60 s. After the last cycle, samples were incubated for an additional 10 min at 72°C

to ensure that the final extension step was complete. The cycling reaction was performed in the DNA thermal cycler from Violet.

Oligonucleotide primers used for AR cDNA were 5'GTGGATGGGCTGAAAATC3' (sense) and 5'AGATCATCTCTGTGCAAGT3' (antisense). These are located in exons 7 and 8 of the human AR gene and are expected to amplify an AR cDNA fragment of 262 bp in size. The nucleotide sequence corresponds to the C-terminal sequence of the protein in the hormone binding domain.

After amplification, PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

Northern blot analysis and probe labelling

RNA from tissues and cultured adrenocortical carcinoma cells were separated onto denaturing agarose gel, stained with ethidium bromide, transferred to a Gene Screen Plus Filter (NEN, Dupont, Boston, MA, USA) and hybridized essentially as described previously (degli Uberti *et al.* 1991, Sambrook *et al.* 1989). Human AR and GAPDH cDNA plasmids were ³²P-labelled by the Random Primed DNA labelling kit (Boehringer). Filters were then exposed to X OMAT films at -80 °C with Dupont intensifying screens. The levels of AR and GAPDH mRNA in each sample were measured using densitometric analysis of the autoradiographic signals.

Immunocytochemistry

In order to visualize the AR protein in adrenocortical carcinoma cell lines, the monoclonal mouse antibody F39.4.1, directed against a fragment of the N-terminal domain of the androgen receptor (aminoacids 301–320) was used, as previously described (Rossi *et al.* 1996a).

NCI-H295 cells were grown in monolayer (approximately 10⁵ cells/well) for 24 h in chamber slides (Nunc Inc., Naperville, IL, USA) in 2% CDT-FCS medium and then incubated with and without 10⁻¹¹ M DHT. After 24 h, cells were washed in saline and, after removal from chamber, were fixed in 3.7% paraformaldehyde for 10 min, rinsed in PBS and transferred to cold methanol and acetone (-20 °C) for 3 and 5 min, respectively. After rinsing in PBS, slides were stored at -20 °C until required for immunostaining. The antibody was used at 1:200 dilution in PBS. The avidin-biotin detection system was used. All the antisera (normal serum, biotinylated antibody against the primary antibody with 40% normal human serum, and avidin-biotinylated horseradish peroxidase complex) were supplied by commercial kit (Vector Laboratories Inc., Burlingame, CA, USA).

Assay for anchorage-independent growth

Agar (Difco) was suspended in water to a final concentration of 2%, autoclaved, and cooled to 50 °C. This agar

solution (330 µl) was added to twofold concentrated CDT-FCS medium (1 ml) with the hormonal treatment, to make a final volume of 2 ml/well and an agar concentration of 0.6%. Trypsinized NCI-H295 cells were counted and 2 × 10⁵ cells were suspended in 50 µl and added to each agar solution (containing hormonal treatment) and immediately poured into 6-multi-well plates. After 10 days of incubation, colonies of more than 100 µm in diameter were randomly scored in triplicate.

Statistical analysis

Data are expressed as the mean ± s.e.m. Statistical analysis of the data was performed using the unpaired and paired Student's *t*-test and the non parametric Wilcoxon's test, as appropriate.

Results

AR RNA in human adrenocortical cells and tissues

Figure 1 shows the presence of AR RNA in human adrenocortical tissues and in the adrenocortical carcinoma cell line NCI-H295. RT-PCR analysis (Fig. 1a), revealed a band with the expected size of 262 bp in normal and adenomatous adrenocortical tissues, and in hyperplastic prostate and in the AR cDNA plasmid, which were included as positive controls. This band was also detected in the adrenocortical carcinoma cell line, NCI-H295, incubated for 24 h in medium supplemented with 2% FCS. Because primers were derived from sequences of exons 7 and 8 of AR cDNA, this band was not the result of genomic contamination. AR RNA transcripts revealed by RT-PCR analysis were also studied by Northern blot analysis. As indicated in Fig. 1b, a signal of approximately 11 kb, as expected for the AR mRNA (Lubahn *et al.* 1988, Faber *et al.* 1991), was detected by hybridization with AR cDNA probe in RNA isolated from adrenocortical tissues.

Detection of AR protein in human adrenocortical carcinoma cells

Figure 2 shows immunocytochemistry staining for AR protein in NCI-H295 cells grown in the absence and presence of 10⁻¹¹ M DHT, using the monoclonal antibody, F39.4.1. In untreated cells, weak staining was present in the cytoplasm and in the nucleus, with few strongly positive nuclei. In androgen-treated cells, the number of positive nuclei markedly increased. No immunostaining was observed when the primary antibody was replaced by diluent or by non-immune serum.

Effect of DHT on NCI-H295 cell growth

Figure 3 shows the effect of androgen on the growth of NCI-H295 cells. DHT significantly (*P*<0.05) reduced

Figure 1 AR RNA in human adrenocortical tissues and in the human adrenocortical carcinoma cell line NCI-H295. (a) RT-PCR products obtained from RNA of three normal (N1–N3) and three adenomatous (A1–A3) adrenocortical tissues and from the human adrenocortical carcinoma cell line, NCI-H295 (NCI). A hyperplastic prostate (Pr) and the AR cDNA plasmid (pl) are included as positive controls. A molecular size DNA marker (M) and an AR-specific fragment of 262 bp in size, predicted by the AR cDNA plasmid sequence (pl) are indicated. RNA was isolated and reversed with an AR reverse primer, as described in Materials and Methods. Aliquots of RT reaction mixtures were amplified with AR primers, separated in agarose gel and analysed by u.v. examination. The unlabelled well (C) represents the negative control and contains the reaction product without the cDNA. (b) Northern blot analysis of total RNA isolated from two normal (N1, N2) and two adenomatous (A1, A2) adrenocortical tissues. RNA was denaturated with formaldehyde, fractionated in denaturing agarose gel, analysed by blot hybridization with ³²P-labelled AR and GAPDH cDNA plasmid, as described in the Methods section.

cell proliferation at concentrations ranging from 10^{-12} to 10^{-8} M, with a $49.56 \pm 14.8\%$ mean inhibition after 24 h of treatment and with a $51.2 \pm 5.5\%$ mean inhibition after 48 h of treatment. The greatest inhibition of growth was apparent with DHT 10^{-9} M ($P < 0.01$) after 24 h of treatment. Testosterone reduced NCI-H295 cell prolifer-

ation by the same rate and to the same degree of significance as was found for DHT (data not shown). After 24 h treatment, the antiandrogen, cyproterone acetate, 10^{-8} M did not change NCI-H295 cell growth, but was capable of reversing the androgen-induced inhibition of proliferation (Fig. 4A). Comparable results were

Figure 2 Visualization of AR protein in the human adrenocortical carcinoma cell line, NCI-H295. Cells were grown for 24 h on a chamber glass slide and cultured in the absence (- DHT) or presence of 10^{-11} M DHT (+ DHT). Cells were immunostained for AR with monoclonal antibody F39.4.1 and visualized by the indirect conjugated peroxidase technique using methyl green for nuclear counterstaining, as described in Materials and Methods. Original magnification, $\times 400$.

Figure 3 Effect of DHT on NCI-H295 cell growth: number of NCI-H295 cells grown for 24 and 48 h without (—) and with DHT at concentration ranging from 10^{-14} to 10^{-8} M. Cells were seeded at approximately 6×10^4 per well in 24-multi-well plates, cultured for 24 or 48 h with and without DHT and then collected and counted in a cell counter. The mean values $\pm$ S.E.M. in hormone-treated cells were compared with those of untreated cells and expressed as a percentage of control values in at least 12 independent experiments in quadruplicate. * $P < 0.05$, ** $P < 0.01$, compared with untreated cells.

obtained using 10^{-8} M hydroxyflutamide (Fig. 4B), which was capable of reversing the growth inhibition induced by 10^{-9} and 10^{-11} M DHT.

Effect of DHT on growth of human adrenocortical cells in primary culture

The effect of androgen on human adrenocortical cell proliferation in primary culture was also tested. After 24 h incubation with 10^{-11} M DHT, a significant inhibition of

Figure 4 Effect of cyproterone acetate (CA) and hydroxyflutamide (Flu-OH) on NCI-H295 cell growth. (A) Cell number in samples of NCI-H295 cells grown for 24 h with and without DHT (10^{-11} M), in the absence and presence of CA (10^{-8} M). The mean values $\pm$ S.E.M. in hormone-treated cells were compared with those of untreated cells and expressed as a percentage of control values in at least six independent determinations. * $P < 0.05$ compared with untreated cells. (B) Cell number in samples of NCI-H295 cells grown for 24 h with and without DHT (10^{-11} and 10^{-9} M), in the absence and presence of Flu-OH (10^{-8} M). The mean values $\pm$ S.E.M. in hormone-treated cells were compared with those of untreated cells and expressed as a percentage of control values in at least six independent determinations. * $P < 0.05$, ** $P < 0.01$, compared with untreated cells.

cell growth was found in three different experiments, with a mean percentage inhibition of 26.5% compared with control cells ($P < 0.05$).

Anchorage-independent growth inhibition by DHT

Figure 5 shows the effect of DHT on anchorage-independent growth of NCI-H295 cells. After a 10-day period of growth, control cells formed large colonies in soft

Figure 5 Effect of DHT on anchorage-independent growth of NCI-H295 cells. The percentage of colonies greater than 100 μm in diameter was determined after 10 days of growth in soft agar without hormones (control) and in the presence of 10^{-11} M DHT (+ DHT) in six independent experiments. * $P < 0.01$ compared with untreated cells. Original magnification, $\times 100$.

agar whereas, in the presence of 10^{-11} M DHT, the growth potential of NCI-H295 cells was consistently reduced. In control samples, 50.3% of colonies showed a diameter greater than 100 μm . In presence of androgen, colonies were less dense and only 21.9% of them reached a size greater than 100 μm ($P < 0.01$). Cyproterone acetate again counteracted the DHT-induced reduction of anchorage-independent growth (data not shown).

Discussion

In the present study, we have demonstrated the presence of the canonic AR RNA in human adrenocortical tissues, indicating that the gene for AR is, indeed, expressed in human adrenal cortex. AR expression has been confirmed in the human adrenocortical cancer cell line, NCI-H295, in which the AR protein has also been demonstrated by the use of a specific monoclonal antibody, F39.4.1 (Lubahn *et al.* 1988). These observations are in accordance with previously reported findings in other mammalian adrenal tissues. A relatively high level of a specific high-affinity androgen-binding protein (Calandra *et al.* 1978, Rifka *et al.* 1978) has been previously determined by ligand-binding assays in the adrenal gland of rodents. More recently, the presence of AR protein has been demon-

strated, by immunocytochemistry and immunoblot, in the adrenal gland of the Rhesus monkey (Hirst *et al.* 1992) and rat (Bentvelsen *et al.* 1996). However, different observations were made by Ruizeveld de Winter *et al.* (1991) who were unable to visualize AR protein in human adrenal specimens using the monoclonal antibody F39.4.1. Such a finding may reflect a level of AR that was too low to be detected immunohistochemically in this tissue.

The increase in nuclear AR positivity induced by androgen treatment is consistent with the nuclear localization of the receptor in the human cells. Similarly, previous results (Husmann *et al.* 1990, Sar *et al.* 1990) have shown that, in rat ventral prostate and epididymis, nuclear immunostaining of AR that is lost after castration rapidly recovers after injection of androgen. These authors have suggested that the androgen-free receptor undergoes a marked increase in immunoreactivity after hormone binding or receptor stabilization, as later proposed by Kempainen *et al.* (1992). In addition, Jenster *et al.* (1992), studying the relationships between various structural domains and their different functions in the AR protein, showed that, in the absence of ligand, the wild-type AR expressed in COS-1 cells was distributed over nucleus and cytoplasm, and that the addition of hormone directed all ARs to the nucleus.

Although the action of sex steroid hormones in the adrenal cortex is not entirely clear, it is well known that androgens influence adrenal function, morphology and steroidogenesis. Experimental data have shown that androgen treatment induces an atrophic effect in male rat adrenal cortex, and a trophic action on the zona reticularis in female guinea pigs (Toscano *et al.* 1990).

To date, the mechanism of action of androgen on human adrenocortical growth and differentiation is not well defined. In agreement with previous data from Rifka *et al.* (1978), who demonstrated an androgen-induced decrease in rat adrenal weight, our results show that DHT is capable of reducing adrenocortical adenoma and carcinoma cell proliferation. These findings, together with the androgen-induced reduction of colony formation in the NCI-H295 cell line, provide evidence for an inhibitory role of androgen on the growth of the human adrenocortical gland. The inhibitory effect of DHT that we observed with doses ranging from 10^{-12} to 10^{-8} M was not associated with any induction of a cytotoxic action, as demonstrated by tripan blue staining, and was counteracted by preadministration of both the antiandrogens, cyproterone acetate and hydroxyflutamide. Hydroxyflutamide, in particular, is a pure antiandrogen because, unlike cyproterone acetate, it fails to promote AR dimerization and DNA binding, and lacks any agonistic activity (Wong *et al.* 1993). As neither of these androgens affected the NCI-H295 proliferation rate, but both blocked DHT-induced growth inhibition, we conclude that androgen, in our model, acts by binding its receptor. These results, together with the finding that human adrenocortical cells express the canonic AR gene at RNA and protein level, are consistent with the concept that androgens may act on the human adrenal gland by a receptor-mediated mechanism.

It is well known that sex steroid receptors have an important role in the control of the proliferation of sex steroid target cells and, in agreement with our results, several studies have shown that physiological doses of synthetic androgens, via interaction with AR, inhibit cell proliferation, colony formation in soft agar, and c-myc RNA levels in the LNCaP prostate cancer cell line (Wolf *et al.* 1991). Recent data have also demonstrated an AR-mediated androgen-induced inhibition of cell proliferation in the MCF7 cell line transfected with a full human androgen receptor vector (Szelei *et al.* 1997). In addition, we have recently demonstrated that androgens, via the canonic androgen receptor, reduce cell proliferation of human thyroid follicular cells (Rossi *et al.* 1996a, b).

DHT may regulate human adrenocortical cell growth via an AR-mediated mechanism, by influencing the expression of genes involved in cell proliferation; however, in the human adrenal gland, the signal transduction pathway from androgen binding to the ultimate cellular response currently remains to be elucidated. Androgen

may act on target cells by an autocrine or paracrine mechanism, or may modulate the sensitivity of androgen-responsive cells to stimulatory and inhibitory growth factors. We cannot exclude the possibility that, in the adrenal gland as was observed in the LNCaP cell line, DHT may increase the endogenous level of TGF- β 1 (Kim *et al.* 1996), which can interfere with the stimulatory effect of autocrine growth factors such as epidermal growth factor and basic fibroblast growth factor (Schuumans *et al.* 1988, Kasser *et al.* 1995).

In conclusion, our results demonstrate that DHT may induce an inhibitory effect on the growth of the human adrenocortical gland. However, further studies will be required, first to elucidate the exact mechanism of action of DHT on adrenocortical cell growth and, secondly, to investigate a potential role of androgens or their analogues in the treatment of AR-positive adrenocortical carcinomas.

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