

Expression and role of functional glucocorticoid receptors in the human androgen-independent prostate cancer cell line, DU145

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ABSTRACT

We investigated the presence of glucocorticoid receptors (GR) as well as the role of glucocorticoids (Gc) in the control of proliferation of the androgen-independent prostate cancer cell line, DU145. We detected the presence of a specific high affinity binding site (K_d 2.3 nM) for [³H]dexamethasone ([³H]Dex) in the cytosolic preparations of DU145 cells; the density of these binding sites is significantly higher than that detected in HA22T/VGH and in HepG2, two hepatoma cell lines classically considered models for the study of GR. Immunocytochemistry studies confirmed the presence of GR in the cytosolic compartment of DU145 cells; GR undergo translocation to the nucleus following exposure to dexamethasone (Dex). The functional activity of GR present in DU145 cells was also studied by analyzing the potency of Dex in

inducing chloramphenicol acyltransferase (CAT) activity in DU145 cells transfected with a glucocorticoid/progesterone response element (GRE/PRE) tkCAT plasmid (GRE/PREtkCAT plasmid). The results have shown that Dex stimulates the transcriptional activity of GR in transfected DU145 cells with an EC_{50} of 9.65 nM and a maximal induction of sevenfold above basal levels. Finally, a dose-dependent (IC_{50} 3.14 nM) decrease of DU145 cell numbers was observed after their exposure to Dex for 4 days; this effect was counteracted by the presence of the steroid antagonist, RU486. In conclusion, the present data suggest a possible role of corticoids in the control of the growth of androgen-independent prostate cancer.

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INTRODUCTION

The prostate gland specifically requires androgens for its differentiation and development (Cunha *et al.* 1987) but the presence of a multihormonal environment is essential for the maintenance of its functions (Griffiths *et al.* 1991).

Alterations of the hormonal milieu surrounding the prostate may lead to modifications of the normal behavior of prostatic tissue and cause aberrations in prostatic cell proliferation. Glucocorticoids (Gc) are among the hormones which may affect prostatic activity by interacting with their specific intracellular receptors. In 1987, Chang *et al.* detected the presence of glucocorticoid receptor (GR) mRNA in the ventral prostate of the normal rat. Glucocorticoids are reported to modify the molecular processes associated with the programmed death of prostatic cells (Rennie *et al.* 1989). The expression

of GR mRNA appears to be influenced by circulating levels of androgens (Rennie *et al.* 1989) since the number of nuclear GR in the rat prostate increases after castration (Davies & Rushmere 1990).

The effects of Gc have been investigated in several rat prostatic tumor cell lines. Smith and coworkers (1985) demonstrated that Gc counteracts the increase of androgen receptors (AR) induced by steroid deprivation in an androgen-dependent cell line. Moreover, the typical glucocorticoid agonist, dexamethasone (Dex), inhibits the proliferation of the androgen-independent PAIII rat prostate adenocarcinoma cells (Koutsilieris *et al.* 1992).

Evidence also exists suggesting that GR are present in human prostatic carcinoma, an originally androgen-dependent tumor that may progress, in later stages, to a condition in which androgen

control is lost. Mohler *et al.* (1996) described the presence of GR both in the stroma and epithelium of human prostatic carcinoma as well as in samples of benign prostatic hyperplasia. The presence of GR was also shown in several human prostatic cancer cell lines (Nakhla & Rosner 1994, Reyes-Moreno *et al.* 1995), but a specific role for GR in the development and in the control of growth of prostatic carcinoma in humans still remains unclear.

Since the presence of GR could be of some relevance in the evolution of prostatic carcinoma, we have performed studies to elucidate the presence and the role of GR in DU145 cells, a cell line derived from a brain metastasis of a human androgen-independent prostate cancer (Stone *et al.* 1978, Harley-Asp & Gunnarsson 1982). This cell line shows the characteristics of prostatic cells and does not possess androgen receptors (Culig *et al.* 1993). We have also investigated the possible role of Gc in controlling the proliferation of this cell line.

MATERIALS AND METHODS

Cell culture

The cell line DU145 was obtained from the American Type Culture Collection (Rockville, MD, USA). The hepatoma cell lines HA22T/VGH and HepG2 cells were kindly provided by Prof. P Barnaba, University of Rome, and by Dr F Visioli, University of Milan respectively. DU145 cells were routinely grown in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 5% fetal calf serum (FCS; Gibco, Paisley, Strathclyde, UK), glutamine (1 mM), and antibiotics (100 units/ml penicillin G sodium, 100 mg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO₂:95% air. HA22T/VGH and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Biochrom KG) supplemented with 10% FCS (Gibco), glutamine (1 mM), and antibiotics (100 units/ml penicillin G sodium, 100 mg/ml streptomycin sulfate) in a humidified atmosphere of 5% CO₂:95% air. Where described, to reduce possible effects of endogenous glucocorticoids found in calf serum, cells were maintained in media (RPMI 1640 for DU145 cells, DMEM for HA22T/VGH and HepG2 cells) supplemented with 10% charcoal-stripped FCS (CS-FCS).

Binding studies

Cells were plated at 1×10^6 cells/100 mm Petri dishes in complete culture medium (RPMI 1640–5% FCS for DU145 cells, DMEM–10% FCS for HA22T/VGH and HepG2 cells). The next day,

the medium was replaced with media supplemented with 10% CS-FCS. Cells were allowed to grow for 2 days. On the day of the experiments, the cells were collected, homogenized in TEDG buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 5 µg/ml antipain and leupeptin) and centrifuged for 1 h at $110\,000 \times g$ to obtain the cytosolic fraction. Protein concentration was determined using the Bradford assay (Bradford 1976).

The binding assays were performed using [³H]dexamethasone ([³H]Dex; specific activity (S.A.) 82.0 mCi/µmol; Amersham International, Amersham, Bucks, UK) as specific GR ligand; binding curves were carried out by incubating 100 µl (300–500 µg proteins) cytosolic fractions for 16 h in the presence of [³H]Dex (0.1 nM) and increasing concentrations (0.1–1000 nM) of Dex, testosterone, progesterone or estradiol used as competitors. Incubation was carried out at 4 °C to prevent receptor degradation and the possible metabolism of steroids. The unbound [³H]Dex was then removed by absorption with dextran-coated charcoal (0.5% Norit A, 0.05% dextran, 10 mM Tris, and 1 mM EDTA, pH 7.5) and the amount of radioactivity was measured in a counter for β emitters. The binding parameters (K_d , dissociation constant; B_{max} , maximal binding capacity; number of binding sites per cell) of [³H]Dex for GR were obtained from the analysis of the homologous competition curves. The potency (K_i) of competitors on the binding of [³H]Dex was also evaluated by computerized analysis of the heterologous competition curves.

The density of [³H]Dex binding sites per cell was evaluated from the B_{max} to derive the number of molecules of ligand needed to saturate the binding sites; this value was normalized for the number of cells equivalent to the amount of cytosolic protein incubated.

The cytosolic preparation of DU145 cells was used to perform binding studies using [³H]progesterone ([³H]Prog; S.A.: 110 µCi/µmol; Amersham International); binding curves were carried out as described before for [³H]Dex binding assays.

Immunocytochemistry

DU145 cells cultured on glass coverslips were treated with vehicle (ethanol) or Dex (10^{-6} M) for 24 h. Cells were then fixed with 4% paraformaldehyde, treated with 0.2% Triton X-100 and incubated in 5% normal goat serum (NGS) in Tris buffered saline (TBS). Cells were incubated with a monoclonal anti-human GR antibody (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:25 in 5% NGS for 1 h, washed in TBS and incubated for

1 h with a biotinylated anti-mouse immunoglobulin G. The reaction was developed using an ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Transient transfections and chloramphenicol acyltransferase (CAT) activity assay

DU145 cells were transiently transfected with a plasmid containing a CAT reporter with a tk promoter and a glucocorticoid/progesterone response element (GRE/PRE) (GRE/PREtkCAT). DU145 cells were plated at a density of 3×10^4 cells/well in a 24-well plate in RPMI 1640 medium supplemented with 5% FCS. The following day, the medium was replaced with RPMI 1640 supplemented with 10% CS-FCS. Sixteen hours later, the cells were transfected with 1 μ g GRE/PREtkCAT/well using the Tfx-50 reagent (Promega, Madison, IL, USA) and treated with the test steroids in ethanol solution. The transcriptional activity induced by steroids was measured after the treatment of the transfected cells with increasing concentrations (5×10^{-10} – 10^{-6} M) of Dex, progesterone, testosterone or with vehicle (ethanol). After 48 h exposure to the steroids, the cells were harvested in 0.1% Triton X-100, 250 mM Tris-HCl, pH 7.5 and homogenized using slight sonication. The CAT activity was measured as described by Seed & Sheen (1988) using the phase-extraction method.

Cell growth studies

DU145 cells were plated at a density of 500–800 cells/cm² in 100 mm dishes in RPMI 1640 medium supplemented with 10% CS-FCS. After 3 days of culture, the following experiments were performed. Experiment 1: DU145 cells were treated daily with increasing concentrations of Dex. After 4 days, cells were harvested and counted in a hemocytometer. Experiment 2: DU145 cells were treated daily with Dex (10^{-8} M) either in the absence or in the presence of a glucocorticoid antagonist, RU486 (10^{-6} M). After 4 days of treatment, cells were harvested and counted in a hemocytometer.

In all experiments, cell viability was determined by dye exclusion after staining for 5 min at room temperature with Trypan Blue dissolved in PBS.

Data analysis and statistical evaluation

The binding parameters K_d and B_{max} were obtained from the analysis of equilibrium ligand binding data performed by means of the program LIGAND (Munson & Rodbard 1980). Statistical analysis of the concentration–response curves was performed

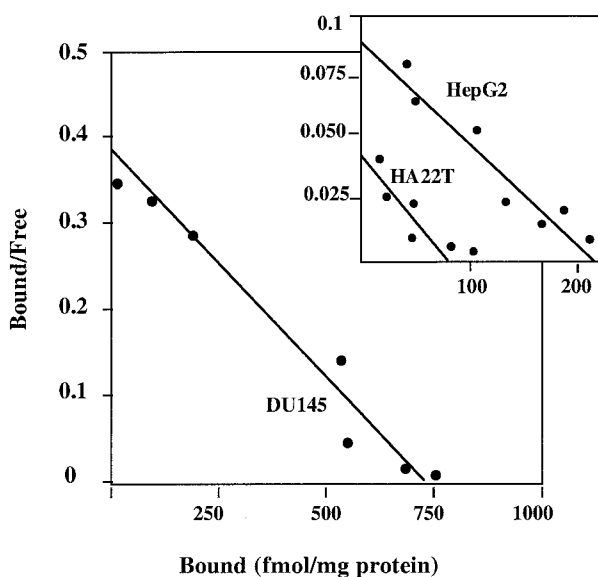


FIGURE 1. Scatchard plot representation of the binding of [³H]Dex to human prostate carcinoma DU145 cells and (inset) to human hepatoma HA22T/VGH and HepG2 cells. The K_d values were 2.3 nM, 1.8 nM and 8.1 nM respectively. Data are representative of 3 independent experiments.

by using the program ALLFIT (DeLean *et al.* 1978), which calculates the lower and upper 'plateau', the slope, the relative EC_{50} or the IC_{50} and allows the comparison of two or more curves. All the curves shown were computer generated.

The data from experiments involving cell proliferation were also analyzed according to Dunnett's test (Dunnett 1955) after one-way analysis of variance.

Every experiment was repeated three times with similar results.

RESULTS

Identification of GR in DU145 cells

Binding studies

Figure 1 shows the presence of specific binding sites for [³H]Dex in the cytosolic preparations of DU145 cells. Computer analysis of the data from homologous competition curves reveals the presence of a single class of high affinity binding sites (K_d 2.3 nM; B_{max} 711 fmol/mg cytosolic protein; 230 000 binding sites/cell). These results were then compared (Fig. 1) to those obtained using two hepatoma cell lines, HA22T/VGH and HepG2. In HA22T/VGH cells, binding studies revealed a K_d of 1.8 nM, and a B_{max} of 77 fmol/mg cytosolic

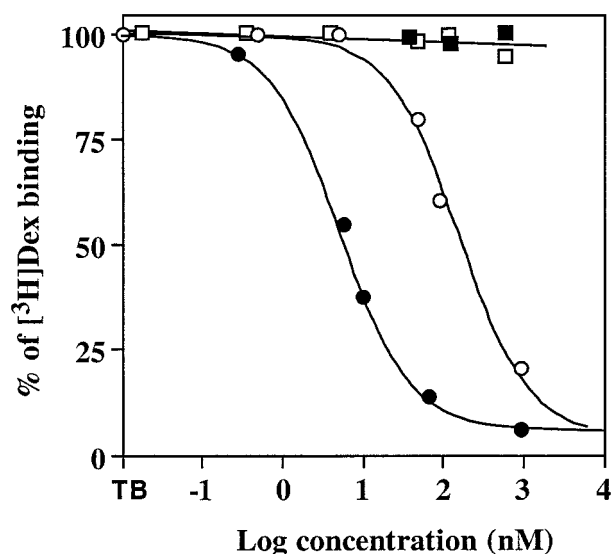


FIGURE 2. Competition of Dex (●), progesterone (○), testosterone (□) and estradiol (■) on the binding of [³H]Dex to cytosolic preparations of DU145 cells. TB, binding of [³H]Dex in the absence of competitors. K_i values of the various competitors were calculated using the program LIGAND. Data are representative of 3 independent experiments.

protein corresponding to 49 000 binding sites/cell, while in HepG2 cells K_d was 8.1 nM with a B_{max} of 208 fmol/mg cytosolic protein equivalent to 80 000 binding sites/cell.

Competition binding experiments were then performed to verify the specificity of the binding of [³H]Dex using progesterone, testosterone and estradiol as competitors. It appears from Fig. 2 that unlabeled Dex strongly competes with [³H]Dex for the binding sites present on DU145 cells. Testosterone and estradiol were completely ineffective in the range of concentrations tested. Progesterone showed a competition on [³H]Dex binding that was significantly lower than that of Dex. The calculated K_i for Dex was 5.25 nM, while that for progesterone was 95.9 nM, suggesting an aspecific interaction of progesterone on GR. On the other hand, a binding assay performed with [³H]Prog on cytosolic preparations of DU145 cells (data not shown) confirms the absence of progesterone binding sites in these cells.

Immunocytochemistry

Immunocytochemical experiments reported in Fig. 3A showed detectable staining for GR in DU145 cells. A positive immunoreactivity for GR was detected on the cytosolic compartment of approximately 95% of the cells. A translocation of the

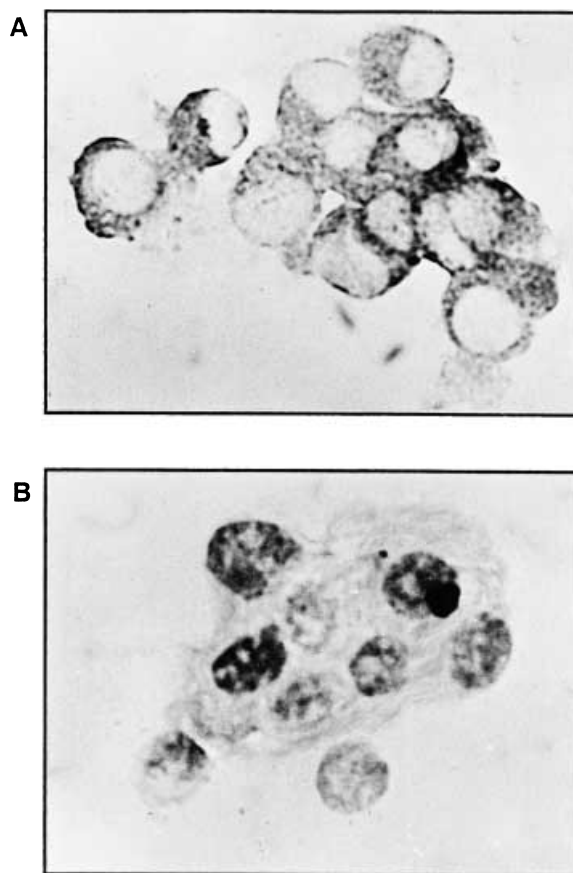


FIGURE 3. (A) Immunocytochemical staining for GR in the cytosolic compartment of DU145 cells; (B) nuclear translocation of GR immunoreactivity after Dex treatment.

activated GR to the nucleus was detected after a 24-h exposure to 10^{-6} M Dex (for 24 h) (Fig. 3B).

Transcriptional activity of GR present in DU145 cells

The transcriptional activity of GR present in DU145 cells is summarized in Fig. 4. The figure illustrates the dose-response curve of the CAT activity elicited in DU145 cells transfected with the GRE/PREtkCAT plasmid (see Materials and Methods) after exposure to increasing concentrations of Dex, progesterone, testosterone and estradiol. The results clearly show that only Dex is able significantly to increase CAT activity in transfected DU145 cells (EC_{50} 9.65 nM and a maximal induction of sevenfold over basal levels). Progesterone, testosterone and estradiol were unable to induce CAT activity at the concentrations used.

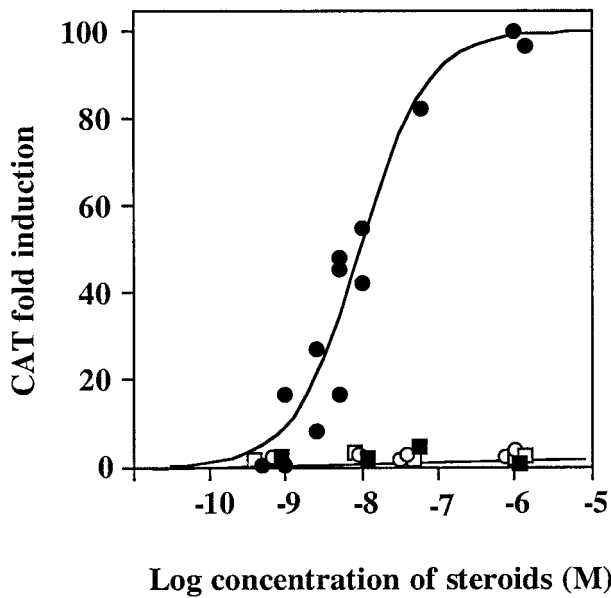


FIGURE 4. Transcriptional activity of Dex (●), progesterone (○), testosterone (□) and estradiol (■) in transfected DU145 cells. Data are representative of 3 independent experiments.

Cell growth studies

Figure 5 shows the effect of exposure to increasing concentrations of Dex on the number of DU145 cells grown in culture in steroid-free medium for 4 days. In these conditions, DU145 cells undergo 4–5 replications. However, the treatment with Dex produced a significant and dose-dependent decrease in the number of DU145 cells. The maximal inhibition of growth (50% of control untreated cells) was obtained at concentrations of 1–10 μM Dex. Computerized analysis of these data reveals a relative IC₅₀ of 3.14 nM. No cell detachments or cell death, indicative of a possible toxicity of the steroid, were observed during the treatments.

Figure 6 shows that the inhibitory effect of Dex (10⁻⁸ M) was completely counteracted by the presence of the GR antagonist, RU486 (10⁻⁶ M). No effect was observed after the treatment of cells with the antagonist alone.

DISCUSSION

The present results clearly indicate, for the first time, the presence of high affinity and high capacity binding sites for the synthetic glucocorticoid Dex in the cytosolic preparation of DU145 cells. The specificity of the binding was ascertained by competition experiments with different steroids

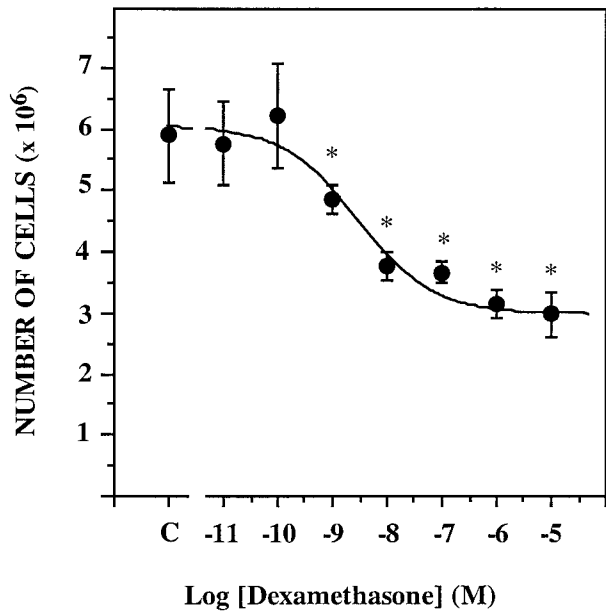


FIGURE 5. Number of DU145 cells grown in culture in the presence of Dex. Cells cultured in CS-FCS were exposed to different concentrations of Dex; after a 4-day treatment, cells were harvested and counted in a hemocytometer. Each value is the mean (± s.e.m.) of 3 independent experiments. *P<0.05 compared with control untreated cells (C).

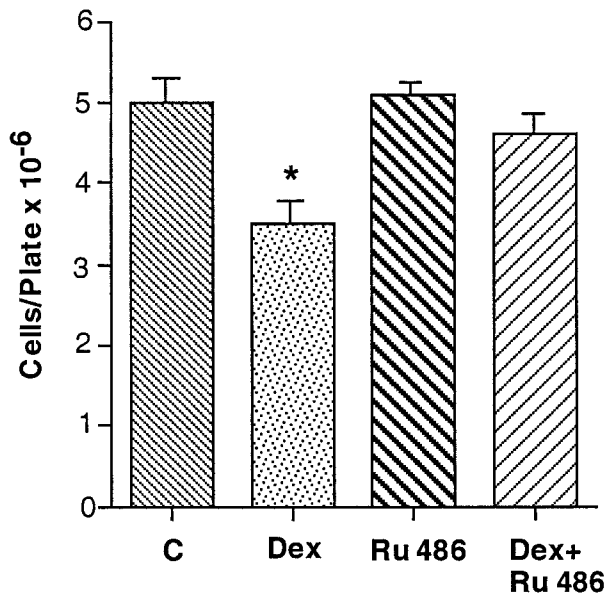


FIGURE 6. Effect of RU486 on the inhibition of DU145 cell proliferation induced by Dex; each value is the mean (± s.e.m.) of 3 independent experiments. *P<0.05 compared with control untreated cells (C).

(progesterone, testosterone and estradiol). Among the steroids tested only progesterone was found to compete weakly with [³H]Dex binding, suggesting a non-specific interaction of progesterone on GR at high concentrations. In fact, binding experiments performed with [³H]Prog and the inability of progesterone to induce transcriptional activity of the GRE/PRE-containing reporter gene exclude the presence of progesterone receptors in these cells. The affinity of Dex for binding sites present in DU145 cells is similar to that found in the rat prostate (Davies & Rushmere 1990) and in the two human hepatoma cell lines HA22T/VGH and HepG2, classically considered models for the study of GR. Moreover, in the present study we have compared, with the same methodology, the presence of GR in DU145 cells with that found in HA22T/VGH and HepG2 cells. We found that the amounts of GR expressed in DU145 cells are much greater than those observed in the two hepatoma cell lines just mentioned. The significance of such high presence of GR, obtained also by immunocytochemical studies performed using specific antibody directed to human GR, remains unclear.

Finally, the ability of Dex to activate the transcriptional activity of a transfected reporter gene (GRE/PREtkCAT) clearly shows that the binding sites present in DU145 cells are actually functional GR. This is also supported by immunocytochemical experiments showing that the activation of GR by Dex brings about the translocation of GR from the cytosolic to the nuclear compartment.

Our data are in agreement with previous reports showing that another androgen-independent human prostate cancer cell line, PC3, expresses high affinity binding sites for Gc (Reyes-Moreno *et al.* 1995). Therefore, the detection of GR in two different cell lines of human androgen-independent prostate cancer, DU145 and PC3, appears strongly indicative of the presence of GR in tumors *in situ*. This appears to be confirmed by preliminary results from immunohistochemical studies performed in our laboratory on specimens of human prostatic carcinoma (A Poletti, E Scaccianoce, D Dondi, P Montironi & M Motta, unpublished observations).

The present results show that Dex exerts a significant and dose-dependent inhibitory effect on the growth of DU145 cells. This effect is completely counteracted by the antagonist RU486. Since, as stated below, these cells do not express progesterone receptors, the antagonistic effect of RU486 can be linked only to its ability to interact with the GR. These data further confirm the results obtained by Koutsilieris *et al.* (1992) on PAIII cells and by Reyes-Moreno *et al.* (1995) on PC3 cells. Our

results on DU145 cells and those obtained on PC3 cells (Reyes-Moreno *et al.* 1995) may suggest that, in androgen-independent prostatic cancers, a functional GR could mediate an inhibitory action on the local mechanisms controlling tumoral progression.

If the presence of GR and the inhibitory effect of Gc on cell proliferation of androgen-independent prostatic cell lines appear to be a rather constant finding of this and other laboratories, the role of Gc in androgen-dependent cell lines, on the other hand, remains rather controversial. For example, while Smith *et al.* (1984) showed that triamcinolone acetonide was able to inhibit the proliferation of R3327-G8-A1 cells (derived from the Dunning rat prostate adenocarcinoma), Wilding *et al.* (1989) demonstrated that the same steroid did not modify the growth of LNCaP cells. In the same model, Montgomery *et al.* (1992) also reported that Gc did not have any effect on prostatic specific antigen (PSA) expression; finally, Cleutjens *et al.* (1997) found that treatment with Dex did not affect the cell growth of LNCaP stably transfected with a GR expression plasmid. Burnstein *et al.* (1995) reported that Dex induces a down-regulation of androgen receptor mRNA in COS-1 cells co-transfected with androgen and GR cDNAs; this effect seems to be dependent on GR. A similar result was reported in the breast cancer cell line, T-47D, where a glucocorticoid-mediated down-regulation of androgen receptor mRNA was observed (Hall *et al.* 1992). It is possible that GR may directly control the transcription activity of the AR gene.

On the basis of the data reported here, the hypothesis may be put forward that GR may be involved in the mechanisms controlling tumor progression when tumor growth has lost androgen control. Preliminary studies from our laboratory, performed by immunohistochemical techniques on human prostatic cancer tissues (A Poletti, E Scaccianoce, D Dondi, P Montironi & M Motta, unpublished observations) have indeed shown that a high expression of GR is correlated with a much lower level of AR. This could be explained as an attempt by the tumor to reduce the cellular proliferation in a situation of androgen-independent growth. In fact, work presented here and that by other authors (Reyes-Moreno *et al.* 1995) have demonstrated the inhibitory effect of corticoids on the proliferation of different androgen-independent prostatic cancer cell lines. Further studies are needed to understand if the presence of, or an increased concentration of, GR could assume relevance in cancer growth.

It is known that approximately 20% of patients with advanced prostatic carcinoma are treated with,

and respond to, high doses of corticosteroids (Drago *et al.* 1984, Jubelirer & Hogan 1989). The responses have been attributed to the elimination of adrenal androgens obtained through the inhibitory action of Gc on the hypothalamic–pituitary–adrenal axis (Labrie *et al.* 1988). This mechanism cannot be operational when the tumor has reached the status of androgen-independence. The presence of specific binding sites for Gc in the androgen-independent prostatic cancer reported in the present paper may suggest a direct local effect of Gc.

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