Contrary Regulation of Bladder Cancer Cell Proliferation and Invasion by Dexamethasone-Mediated Glucocorticoid Receptor Signals

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Abstract

In patients with advanced bladder cancer, glucocorticoids are frequently given to reduce acute toxicity, particularly hyperemesis, during chemotherapy, as well as to improve cachectic conditions. However, it remains unclear whether glucocorticoids directly affect the development and progression of bladder cancer through the glucocorticoid receptor (GR) pathway. GR expression was first investigated in human bladder cancer lines and tissue microarrays. Then, the effects of dexamethasone on GR transcription, cell proliferation, apoptosis/cell-cycle, and invasion were examined in bladder cancer lines. Finally, mouse xenograft models for bladder cancer were used to assess the efficacy of dexamethasone on tumor progression. All the cell lines and tissues examined were found to express GR. Dexamethasone increased GR-mediated reporter activity and cell proliferation, and inhibited apoptosis in the presence or absence of cisplatin. In contrast, dexamethasone suppressed cell invasion, the expression of its related genes (MMP-2/MMP-9, IL-6, VEGF), and the activity of MMP-2/MMP-9, and also induced mesenchymal-to-epithelial transition. Additionally, dexamethasone increased IκBα protein levels and cytosolic accumulation of NF-κB. In xenograft-bearing mice, dexamethasone slightly augmented the growth of the inoculated tumors but completely prevented the development of bloody ascites, suggestive of peritoneal dissemination of tumor cells, and actual metastasis. In all these assays, dexamethasone effects were abolished by a GR antagonist or GR knock-down via RNA interference. Thus, GR activation resulted in promotion of cell proliferation via inhibiting apoptosis yet repression of cell invasion and metastasis. These results may provide a basis of developing improved chemotherapy regimens, including or excluding GR agonists/antagonists, for urothelial carcinoma.
Introduction

Dichotomous genetic pathways have been implicated in urothelial carcinoma of the urinary bladder, leading to the development of clinicopathologically distinct types of tumors: low-grade, mostly non-invasive; and high-grade, often invasive (1,2). Patients with low-grade tumors carry a lifelong risk of frequent (50-70%) recurrence, occasionally with grade/stage progression, while high-grade carcinomas are often life-threatening despite currently available aggressive treatment modalities, including radical cystectomy and systemic chemotherapy in the neoadjuvant, adjuvant, or salvage setting. Therefore, novel therapeutic options that prevent tumor recurrence and/or progression need to be developed.

Glucocorticoids are involved in almost every cellular, molecular, and physiologic network of the organism and represent one of the most commonly prescribed drugs often employed in the treatment of inflammatory and autoimmune disorders. Several glucocorticoids have also been clinically used as cytotoxic agents, predominantly for hematological malignancies (3). Conversely, there are only limited amounts of experimental evidence suggesting that glucocorticoids inhibit cell growth of solid tumors, such as prostate cancer (4). In bladder cancer (BC) cells, Zhang et al. (5,6) demonstrated in vitro evidence suggesting glucocorticoid-induced resistance to cytotoxic effects of cisplatin (CDDP), currently the most effective agent against urothelial carcinoma. Nonetheless, in patients with solid tumors, glucocorticoids are frequently given to reduce acute toxicity, particularly hyperemesis during chemotherapy, to protect normal tissue against the long-term effects of genotoxic drugs, and to improve cachectic conditions (7). Due to these benefits, a glucocorticoid is often included as comedication in the standard chemotherapy regimens for BC. Meanwhile, prolonged systemic use of glucocorticoids has been shown to increase the subsequent risk of BC, possibly due to immunosuppression (8).
Accordingly, it remains unanswered whether glucocorticoids directly affect the development and progression of BC, presumably through glucocorticoid receptor (GR), a member of the nuclear receptor superfamily that functions as a ligand-inducible transcription factor. Recently, we and others showed that signaling pathways of other steroid hormone receptors, such as androgen receptor (AR) and estrogen receptors, play an important role in BC progression (9-11). In this study, we aim to determine whether and how GR signals regulate the growth of BC.

Materials and Methods

Cell culture and chemicals

Human urothelial carcinoma cell lines UMUC3, TCC-SUP, 5637, and J82, and human embryonic kidney cell line 293T (all obtained from the American Type Culture Collection) were maintained in appropriate medium (Mediatech, Manassas; RPMI-1640 for 5637 and DMEM for others) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in phenol-red free medium supplemented with 5% charcoal-stripped FBS (CS-FBS) at least 18 hours before experimental treatment. Although cell lines were not authenticated by the authors, cells were immediately expanded after receipt and stored in liquid nitrogen and were not cultured for more than 5 months following resuscitation. We obtained dexamethasone (DEX) (Fig.1A), mifepristone (RU486), dihydrotestosterone (DHT), CDDP, and tissue necrosis factor (TNF)-α from Sigma. Supplementary Table S1 lists all pertinent information on primary antibodies.

Stable cell lines with GR-short hairpin RNA (shRNA)

To establish stable GR knock-down lines, UMUC3 and TCC-SUP were directly infected with GR-shRNA or control-shRNA lentiviral particles (Santa Cruz Biotechnology) in the presence of 5 µg/ml
polybrene (Millipore), as described for AR knock-down (12). After 48 hours of infection, the target cells were selected by 2 µg/ml puromycin (Sigma).

**Reporter gene assay**

Cells seeded in 24-well plates were co-transfected with 250 ng of MMTV-luc reporter plasmid DNA and 2.5 ng of PRL-TK-luc plasmid DNA, using GeneJuice (Novagen), as described previously (9,12), and cultured in medium supplemented with CS-FBS for 24 hours in the presence of DEX and/or RU486. The harvested cells were assayed for luciferase activity determined using a Dual-Luciferase Reporter Assay kit (Promega) and luminometer (TD-20/20, Turner BioSystems).

**Cell proliferation**

We used the MTT (thiazolyl blue) assay to assess cell viability, as described previously (9,12). Briefly, cells (3×10^3) seeded in 96-well plates were incubated with medium supplemented with or without CS-FBS containing ligands (DEX/RU486) and/or CDDP. After 4 days of treatment, we added 10 µl of MTT (Sigma) stock solution (5 mg/ml) to each well with 0.1 ml of medium for 4 hours at 37°C. Then, we measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

**Cell morphology**

Morphology of cells cultured with DEX and/or RU486 was assessed, using the NIH ImageJ software. Parameters included the area, perimeter, circularity, and roundness.

**Transwell assay**

Cell invasiveness was determined, using a Matrigel (30 µg; BD Biosciences)-coated transwell chamber (5.0 µm pore size polycarbonate filter with 6.5 mm diameter; Costar). Cells (1×10^5) in 100 µl of serum-free medium were added to the upper chamber of the transwell, while 600 µl of medium containing 5% FBS was added to the lower chamber. The media in both chambers contained ligands.
(DEX/RU486). After incubation for 36 hours at 37°C in a CO₂ incubator, invaded cells were fixed, stained with 0.5% crystal violet, and counted under a light microscope.

**Flow cytometry**

Cells (1×10⁶/10-cm dish) were cultured in medium supplemented with CS-FBS containing ligands (DEX/RU486) for 24 hours, harvested with trypsin, fixed in 70% ethanol, and stained with propidium iodide (PI) buffer. Cellular PI content was measured on a BD FACSCanto™ flow cytometer (BD Biosciences) equipped with an argon ion laser at 488 nm wavelength. Data were analyzed using FlowJo software (Tree Star).

**Gelatin zymography**

Cells (1×10⁶ cells/10-cm dish) were cultured in serum-free medium containing ligands (DEX/RU486) at 37°C in a CO₂ incubator for 24 hours. The conditioned medium was collected/centrifuged and electrophoresed in 8% polyacrylamide gels copolymerized with 1 mg/ml gelatin. After washing and overnight incubation at 37°C in a buffer containing 50 mM Tris, 5 mM CaCl₂, and 1 μM ZnCl₂, the gels were stained with 0.4% Coomassie blue.

**Reverse transcription (RT) and real-time polymerase chain reaction (PCR)**

Total RNA (0.5 μg) isolated from cultured cells, using TRIzol (Invitrogen), was reverse transcribed using 1 μmol/L oligo (dT) primers and 4 units of Ominiscript reverse transcriptase (Qiagen) in a total volume of 20 μl. Real-time PCR was then performed, using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (12). The primer sequences are given in Supplementary Table S2.

**Western blot and co-immunoprecipitation**

Whole cell protein extraction and western blot were performed, as described previously (12) with minor modifications. Separate cytoplasmic and nuclear protein fractions were obtained, using NE-PER
Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Protein (30 µg) was separated in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Specific antibody binding was detected, using horseradish peroxidase detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific).

For immunoprecipitation, whole cell lysates in 500 µl were precleared with 15 ml of protein A/G beads (Santa Cruz Biotechnology) for 30 minutes at 4°C. After centrifuging, supernatant was incubated with an antibody overnight at 4°C followed by addition of 25 µl A/G agarose beads for 2 hours. The beads were washed and the proteins were separated by SDS-PAGE for western blotting.

**Apoptosis**

The terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) assay was performed on cell-burdening coverslips and rehydrated sections from paraffin-embedded mouse xenograft tumors, using the DeadEnd™ Fluorometric TUNEL System (Promega), followed by counterstaining for DNA with 4’,6-diamidino-2-phenylindole (DAPI). Apoptotic index was determined in the cells visualized by fluorescence microscopy.

**Immunofluorescent staining**

Cells plated onto 22 mm square coverslips in 6-well plates were cultured in medium with CS-FBS containing DEX and/or TNF-α for 24 hours. Culture medium was then aspirated, and the adherent cells were fixed by 4% paraformaldehyde for 10 minutes at room temperature. After being washed with 0.1 M glycine for 20 minutes at room temperature, the coverslips were kept in 1% Triton X-100 for 20 minutes at 37°C and blocked with blocking buffer for 1 hour at 37°C. Primary antibodies were incubated at 4°C overnight, and Alexa 488- or 568-conjugated secondary antibodies (Invitrogen) were added for 45 minutes at 37°C. Fluorescence images were acquired with an Olympus FV1000 confocal microscope.
Mouse xenograft models

BC lines (1×10^6 cells in 100 μl per site) resuspended in Matrigel (BD Biosciences) were inoculated subcutaneously into the right (GR-shRNA) and left (control-shRNA) flanks of 7-week-old male SCID mouse (NCI). Slow-releasing pellets (DEX [0.5 mg per mouse] or placebo, Innovative Research of America) were injected with a precision trochar when the sizes of all tumors in each group reached 40 mm³. Tumors were measured using calipers and tumor weight was calculated by the following formula: tumor weight (mg) = tumor length (mm) × [tumor width (mm)]^2 × 0.5 (9). After 5 weeks of treatment, the mice were killed and all the tumors including metastases were harvested for histological and immunohistochemical assessment.

Bladder tissue microarray (TMA) and immunohistochemistry

Appropriate approval from the Institutional Review Board of the University of Rochester was obtained prior to construction and use of the TMA. Bladder TMA was constructed from 24 cystectomy specimens, as described previously (12,13). These patients included 19 men and 5 women, with a mean follow-up after the surgery of 11.4 months (range: 3-24). All cases were histologically diagnosed as high-grade urothelial carcinoma, including 5 ≤pT1, 19 ≥pT2, 12 pN0, and 12 pN+ tumors. None of the patients had received radiotherapy or systemic chemotherapy with or without glucocorticoids preoperatively.

Immunohistochemical staining was performed on the sections (5 μm thick) from the bladder TMA and xenograft tumors, as described previously (9,12). Briefly, tissues were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide. Samples were incubated overnight at 4°C with a primary antibody and then with a broad spectrum secondary antibody (Invitrogen). The stains were manually scored by one pathologist (H.M.) blinded to sample identity.

Statistical analyses
Differences in variables with a continuous distribution were analyzed by Student’s t-test. Differences in GR expression rates in human tissue samples were compared using Fisher’s exact test. Progression-free survival rates were calculated by the Kaplan-Meier method and comparison was made by log-rank test. $P<0.05$ was considered to be statistically significant.

**Results**

**GR expression**

We first examined the expression of GR in four human BC lines, UMUC3, TCC-SUP, J82, and 5637, by RT-PCR and western blotting. All the lines were found to express GR at both mRNA (Supplementary Fig.S1A) and protein (Supplementary Fig.S1B) levels. Silencing of GR expression in UMUC3-GR-shRNA and TCC-SUP-GR-shRNA was then confirmed (see Fig.2F).

Next, we immunohistochemically stained for GR in the bladder TMA. Positive signals were detected typically in both nuclei and cytoplasms of epithelial cells (Supplementary Fig.S1C). All the non-neoplastic and neoplastic bladders as well as metastases showed at least weak signals in urothelial cells. Strong signals were found in 5/24 (21%) primary tumors, 0/4 (0%) metastatic lymph nodes, and 8/18 (44%) corresponding benign tissues. Thus, GR expression tended to be weaker in urothelial carcinoma than in benign urothelium ($P=0.0916$). There were no statistically significant correlations between the intensity of GR expression and gender, presence of muscle invasion ($\leq$pT1 vs. $\geq$pT2), or status of lymph node involvement. Nonetheless, Kaplan-Meier analysis showed a trend to associate between weak or moderate positivity of GR and a risk of progression after radical cystectomy ($P=0.0925$). No tumor progression was seen in all 5 patients with strongly GR-positive tumor.

**DEX-enhanced GR transactivation**

GR-mediated transcriptional activity was determined in the cell extracts with transfection of a
luciferase reporter plasmid (MMTV-luc) and treatment of a synthetic glucocorticoid DEX and/or a GR antagonist RU486. DEX increased luciferase activity in UMUC3 (23.6-fold), TCC-SUP (14.9-fold), J82 (1.4-fold), and 5637 (3.1-fold), compared with respective mock treatments (Fig.1B). RU486 showing marginal agonist activity could block DEX-induced GR transcription in these lines. As expected, DEX effects on luciferase activity were significantly diminished in GR knock-down lines (Fig.1C). Similar induction by DEX was obtained in AR knock-down UMUC3 cells where DHT failed to increase luciferase activity (Fig.1D), excluding DEX-mediated MMTV-luc activity via AR. Thus, these BC cell lines likely possess a functional GR.

**DEX-mediated cell proliferation and apoptosis**

To see if glucocorticoid affects BC cell proliferation, each line was cultured with DEX and/or RU486 for 4 days, and cell viability was assessed by MTT assay. DEX increased cell growth in a dose-dependent manner (up to 41%/66% increases in UMUC3/TCC-SUP, respectively), and RU486 at least partially antagonized the DEX effect (Fig.2A). We also assessed the effects of DEX and/or RU486 on the growth of stable cell lines with or without CDDP (Fig.2B). In the absence of CDDP, DEX increased the growth of UMUC3-control/TCC-SUP-control to 26% ($P=0.0032$)/36% ($P=0.0003$), respectively, compared to mock treatment, and RU486 antagonized the DEX effect. Additionally, growth induction by DEX was found to be more significant (all $P<0.0001$) when cultured in serum-free conditions (31%/202% increase in UMUC3-control/TCC-SUP-control) or with CDDP (48%/44% increase in UMUC3-control/TCC-SUP-control). In GR knock-down lines, only marginal effects of DEX and/or RU486 (except TCC-SUP-GR-shRNA with no serum) were observed. These results are consistent with previous findings in few other BC lines (5) and further suggest that glucocorticoids promote BC cell proliferation/inhibit an anti-proliferative effect of CDDP through the GR pathway.

To investigate how DEX stimulates cell proliferation, we performed flow cytometry and TUNEL assay. DEX treatment for 24 hours led to significant increases in G1 phase cell population in control
UMUC3 (57%→78%, P=0.0139) and TCC-SUP (46%→53%, P=0.0269) lines, and RU486 abolished the DEX effects (Fig.2C). Significant reductions (all P<0.01) in the G1 proportion were observed in UMUC3-GR-shRNA with respective treatments, compared to UMUC3-control-shRNA, but not in TCC-SUP-control-shRNA vs. TCC-SUP-GR-shRNA. In UMUC3-GR-shRNA DEX still increased G1 fraction from 39% to 51% (P=0.0140), which was blocked by RU486. Thus, DEX appears to induce BC cell cycle arrest at G1 phase. The effects of DEX on apoptosis were then assessed in these lines cultured with or without FBS and CDDP for 4 days (Fig.2D). In control UMUC3 and TCC-SUP lines with/without FBS, DEX decreased apoptotic indices by 53%/67% and 30%/67%, respectively, and RU486 blocked DEX-induced apoptosis. CDDP (with FBS) significantly increased the index in control UMUC3 (5%→19%; P=0.0052) or TCC-SUP (8%→37%; P<0.0001), and DEX diminished CDDP-induced apoptosis to the levels with mock (+ FBS) treatment (6%/7% in UMUC/TCC-SUP, P=0.0050/P=0.0001). RU486 notably increased DEX-inhibited apoptosis in control lines cultured with CDDP. DEX slightly/significantly reduced CDDP-induced apoptosis in UMUC3-GR-shRNA (22%→17%, P=0.0572)/TCC-SUP-GR-shRNA (31%→25%, P=0.0239). Thus, DEX-mediated GR signals likely prevent apoptosis of BC cells in the presence or absence of CDDP.

To further investigate the molecular mechanisms of DEX/GR-induced cell proliferation, we performed western blot for detecting the expression of cell cycle- and apoptosis-related molecules (Fig.2E). No significant changes in the expression of cyclins (D1/D2/D3) and cyclin-dependent kinases (2/4/6) were seen in BC lines cultured with DEX and/or RU486. However, DEX up-regulated the expression of p27 and p21 and down-regulated that of cleaved caspase-3 in a dose-dependent manner, and RU486 blocked the DEX effect. The effects of DEX and RU486 on the expression of p21, p27 and cleaved caspase-3 were modest or marginal in GR knock-down lines (Fig.2F). In addition, DEX significantly decreased the levels of GR expression.

**DEX-suppressed cell invasion**
The effects of glucocorticoid on the invasiveness of BC cells were assessed, using a transwell invasion assay. DEX treatment resulted in significant decreases (50-52%) in the invasive properties of both control lines, and RU486 clearly abolished the DEX effect (Fig.3A). In GR knock-down lines, DEX did not show significant suppressive effects on cell invasion. These data suggest that glucocorticoids inhibit BC cell invasion through the GR pathway.

Using real-time RT-PCR, we then assessed the effects of glucocorticoid/GR on the expression of the molecules that play a key role in tumor invasion. Consistent with the results of the transwell assay, DEX decreased the levels of MMP-2, MMP-9, IL-6, and VEGF by 46%/43%, 47%/66%, 33%/61%, 30%/48% in control UMUC3/TCC-SUP lines, respectively (Fig.3B). In RU486-treated control and GR knock-down lines, inhibitory effects of DEX on the expression of these 4 genes were not significant. MMP-2 and MMP-9 expression was also measured by gelatin zymography to assess their enzymatic activity. DEX reduced their levels in control lines, but not in control lines cultured with RU486 or in GR-shRNA lines (Fig.3C).

**DEX-induced mesenchymal-to-epithelial transition (MET)**

We perceived that DEX-treated BC cells appeared to be larger and rounder than mock-treated cells (Fig.4A). Using the ImageJ software, the area, perimeter, circularity, and roundness of the cells were compared among different treatments. DEX increased these parameters in GR-positive lines, compared with those in mock-treated lines, and RU486 antagonized the DEX effects (Fig.4A).

To link these results to MET, we assessed expression levels of mesenchymal (e.g. N-cadherin, vimentin, snail) and epithelial (e.g. E-cadherin, β-catenin) markers by western blots. In GR-positive lines, DEX up-/down-regulated the expression of epithelial/mesenchymal markers, respectively, compared with mock treatment (Fig.4B). These DEX-mediated changes in their levels were marginal or less significant in cells with RU486 treatment and/or GR silencing. Additionally, basal levels of these epithelial markers were lower in GR knock-down lines than in GR-positive controls.
DEX-induced disruption of NF-κB

In prostate cancer, DEX could reduce GR-positive cell growth via inhibiting NF-κB activation (4,14). We therefore studied the effects of DEX on NF-κB in BC cells. Western blotting showed increases in the level of IκBα, a natural cytoplasmic inhibitor of NF-κB, but not in NF-κB levels, in both of DEX-treated GR-positive lines (Fig.5A). There were no significant increases in IκBα levels by DEX in cells with RU486 and/or GR-shRNA.

We then performed co-immunoprecipitation to test if DEX affects protein-protein interactions (Fig.5B). DEX induced the interaction between NF-κB and IκBα in control UMUC3 cells, and RU486 or GR-shRNA diminished the DEX effect. Interestingly, GR was also pulled down by NF-κB, but not by IκBα.

To further assess whether increased IκBα prevented nuclear translocation of NF-κB, we examined subcellular localization of NF-κB in TCC-SUP by western blot (Fig.5C) and immunofluorescence (Fig.5D). NF-κB localized predominantly to the cytoplasmic compartment in both mock- and DEX-treated cells. DEX reduced nuclear NF-κB expression, which was prevented by RU486. In particular, DEX blocked nuclear translocation of NF-κB induced by TNF-α. GR localized to the cytoplasm of mock-treated cells and translocated to the nucleus in DEX-treated cells. In TCC-SUP-GR-shRNA, no significant effects of DEX and/or RU486 on subcellular localization of NF-κB were seen (figure not shown).

DEX increased tumor size but inhibited invasion/metastasis in mouse xenograft models

Finally, we used mouse xenograft models to investigate whether glucocorticoid regulates bladder tumor growth in vivo. BC cells (UMUC3-GR-shRNA/control-shRNA) were implanted subcutaneously into the flanks of SCID mice, and after 2 weeks slow-releasing DEX or placebo pellets were injected into mice. Control GR-positive tumors in DEX-treated mice were larger/heavier than other tumors at 5 weeks of treatment [e.g. 20%/23% (vs. placebo-control cells)] (Fig.6A). Similarly, placebo/GR knock-
down tumors were slightly (10%) lighter than placebo/control GR-positive tumors, and DEX/GR knock-down tumors were slightly (9%) heavier than placebo/GR knock-down tumors. When the mice were killed, bloody ascites, suggestive of peritoneal dissemination of the tumors, and actual metastatic tumors in the peritoneum were identified in 7 (88%) and 4 (50%) of 8 placebo-treated mice, respectively, but in none of DEX-treated mice. Histological examination of the tumors revealed invasion into the skeletal muscle in all groups of mice except the control-shRNA/DEX group (Fig.6B). Harvested tumor specimens were also assessed for cell proliferation (Ki-67), apoptosis (TUNEL), and angiogenesis or metastatic ability (MMP-9/VEGF/CD34). DEX treatment in control GR-positive tumors led to marginal changes in proliferation but decreased apoptosis and angiogenesis/metastasis-related factors, compared to DEX/GR-shRNA, placebo/control-shRNA, placebo/GR-shRNA, or placebo/metastasis (Fig.6C). Metastatic tumors seen in placebo-treated mice were likely derived from GR-shRNA expressing cells based on weak GR signals. It was also noted that GR levels were significantly reduced in DEX-treated UMUC3-control-shRNA tumors. These in vivo data suggest that DEX stimulates BC cell proliferation yet represses tumor invasion and metastasis.

Discussion

It appears that the status of GR expression has never been examined in human BC (11). Our immunohistochemical study in 24 cystectomy specimens showed that: 1) GR was detected in all cases of benign urothelium/urothelial carcinoma; 2) GR expression tended to be weaker in tumor than in benign; and 3) strong GR expression tended to correlate with better prognosis. These results may suggest a protective/inhibitory role of GR signals in bladder tumorigenesis and tumor progression. Further study including larger patient cohorts with longer follow-up are needed to validate these preliminary findings.
It has been shown that glucocorticoids induce apoptosis and inhibit proliferation in lymphoid cells, leading to their clinical use as cytotoxic agents for hematological malignancies (3). In contrast, limited amounts of experimental evidence have suggested inhibitory effects of glucocorticoids on cell growth of solid tumors. In a previous in vitro study using BC lines (5), DEX inhibited CDDP-mediated apoptosis, suggesting glucocorticoid-induced chemotherapy resistance. Using two BC lines expressing a functional GR, we here demonstrated that DEX promoted cell proliferation, which was restored by a GR antagonist and/or GR knock-down. The stimulatory effects of DEX were more significant when cultured with CDDP or in serum-free conditions. In addition, DEX-induced cell growth was confirmed, using mouse xenograft models that showed larger tumor sizes in DEX-treated mice than in mock-treated and/or GR knockdown cell-bearing mice. Thus, GR signals are likely associated with BC cell growth. Previous (5) and our current results may therefore imply that clinical use of glucocorticoids as comedication can be harmful to BC patients in terms of tumor cell proliferation.

Anti-proliferative effects of glucocorticoids via induction of cell cycle arrest and apoptosis have been shown in lymphomas as well as in other malignancies including osteosarcoma, cervical carcinoma, and thyroid medullary carcinoma (3,15-17). Similarly, DEX enhanced cell cycle at the G1 phase in two GR-positive BC lines, which correlated with increased levels of the CDK inhibitors p27 and p21 but not cyclins (D1/D2/D3) or CDKs (2/4/6). Nonetheless, we found glucocorticoid/GR-induced BC cell proliferation in vitro and in vivo. In contrast to previous observations in non-bladder cells, DEX strongly inhibited apoptotic cell death, along with down-regulation of cleaved caspase-3 expression, in BC lines and prevented CDDP-induced apoptosis. Because cell line specific mechanisms for GR-mediated growth arrest have been demonstrated (15), further analyses are required to elucidate the involvement of cell-cycle regulatory proteins in BC cells.

To our knowledge, no studies have assessed the effects of glucocorticoids on BC cell invasion. Using a transwell assay, we showed that DEX suppressed cell invasion of GR-positive lines, but not GR knock-down lines, and that RU486 abolished the DEX-induced invasion. In mouse xenograft
models for BC, DEX successfully prevented the development of metastasis. Furthermore, invasion/metastasis-related molecules, including MMP-2, MMP-9, IL-6, and VEGF, as well as microvessel density, were down-regulated in DEX-treated cells/tumors, compared to mock-treated and/or GR knock-down cells/tumors. Thus, opposite to the effect on cell proliferation, glucocorticoids likely have an inhibitory role in BC cell invasion and metastasis through the GR pathway.

Glucocorticoids are known to interfere with the transcriptional activity of several transcription factors, including NF-κB. DEX has also been shown to reduce the growth of GR-positive prostate cancer cells mainly via inhibiting NF-κB activation and the production of NF-κB-dependent cytokines such as IL-6 (4,14). In BC, the association of NF-κB activity with cell invasion, as well as the transcriptional regulation of MMPs through the NF-κB pathway, has been reported (18). We therefore assessed the effect of DEX on NF-κB in BC and found an increase in IκBα level, but not in NF-κB level, in DEX-treated GR-positive cells as well as blockade of TNF-α-induced nuclear translocation of NF-κB by DEX. Co-immunoprecipitation further showed DEX-enhanced interactions between NF-κB and IκBα and between GR and NF-κB but not IκBα. The latter suggests that GR may directly function as a co-repressor of NF-κB. Thus, NF-κB inactivation and IL-6 down-regulation induced by DEX may be a central mechanism involved in GR-mediated inhibition of BC cell invasion.

During our preliminary experiments, we found changes in the morphology of DEX-treated BC cells. These might imply DEX-induced MET which was reported in a mink lung epithelial cell line (19). Indeed, epithelial-to-mesenchymal transition has been implicated in drug resistance and invasion/metastasis in urothelial carcinoma (20). In addition to morphological changes compatible with MET, we showed that GR activation correlated with increased/decreased expression of epithelial/mesenchymal markers, respectively. These findings indicate that DEX/GR induce MET in BC cells, which could be an underlying mechanism of GR-mediated suppression of tumor progression.

As mentioned above, glucocorticoids have been widely used as comedication in patients with advanced BC. However, there was no molecular evidence indicating that glucocorticoids function
directly through the GR pathway in BC cells and exert a stimulatory or inhibitory effect on tumor growth. The current study in BC demonstrates that GR signals correlate positively with cell proliferation and negatively with cell invasion and metastasis. The former appeared to result in a significant reduction in cytotoxic effects of CDDP. Further analyses employing various GR agonists/antagonists will facilitate improving chemotherapy regimens for urothelial carcinoma. Based on the current results, ideal GR ligands would be those showing marginal stimulatory effects on cell proliferation without reducing the cytotoxic activity of anti-cancer drugs yet significant inhibitory effects on cell invasion either alone or in combination with other agents. Additional assessments of the relationship between glucocorticoid use and tumor phenotype in clinical samples are also required to directly address the hypothesis we tested in cell line models.

References


Figure legends

Figure 1. DEX effects on GR transactivation. A, Chemical structure of DEX. BC cells (B, UMUC3/TCC-SUP/J82/5637; C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA; D, UMUC3-control-shRNA/AR-shRNA) transfected with MMTV-luc were cultured for 24 hours in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486. Luciferase activity is presented relative to that of mock treatment in each control line. Each value represents the mean(+SD) from at least three independent experiments.

Figure 2. DEX effects on cell proliferation. A, Cell viability of UMUC3/TCC-SUP cultured with 0-1000 nM DEX ± 1 µM RU486 for 4 days was assayed with MTT, and growth induction is presented relative to cell number with mock treatment. Each value represents the mean(+SD) from at least three independent experiments. *P<0.05 (vs. mock treatment). #P<0.05 (vs. respective doses of DEX treatment only). B, Cell viability of UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 µM, with 5% FBS) in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486 for 4 days was assayed with MTT, and growth induction is presented relative to cell number with mock treatment in each cell line/condition (serum or CDDP). Each value represents the mean(+SD) from at least three independent experiments. *, **P<0.05 (vs. mock treatment). #, ##P<0.05 (vs. DEX treatment only). C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486 for 24 hours were used for flow cytometry analysis. Each value for G1 arrest represents the mean(+SD) from at least three independent experiments. *, **P<0.05 (vs. mock treatment). #P<0.05 (vs. DEX treatment only). D, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 µM, with 5% FBS) for 4 days in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486 were analyzed for apoptotic index (percentage of TUNEL-positive cells in 1000 cells). Each value represents the mean(+SD) from at least three independent experiments. *, **P<0.05 (vs. mock
treatment). $P<0.05$ (vs. DEX treatment only). E, UMUC3/TCC-SUP cultured with 0-1000 nM DEX ± 1 µM RU486 for 24 hours were analyzed on western blotting, using an antibody to GR (95+90 kDa), cyclin D1 (36 kDa), cyclin D2 (31 kDa), cyclin D3 (31 kDa), CDK2 (33 kDa), CDK4 (30 kDa), CDK6 (36 kDa), p27 (27 kDa), p21 (21 kDa), or cleaved caspase-3 (19+17 kDa). β-Actin (43 kDa) served as an internal control. F, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured for 24 hours in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486 were analyzed on western blotting, using an antibody to GR, p27, p21, cleaved caspase-3, or β-actin.

**Figure 3.** DEX effects on cell invasion. A, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured in the Matrigel-coated transwell chamber for 36 hours in the presence of ethanol (mock), 100 nM DEX, and/or 1 µM RU486 were used for invasion assay. The number of invaded cells in five random fields was counted under a light microscope (10× objective). Each value represents the mean(+SD) from three independent experiments. $*P<0.01$ (vs. mock treatment). $^#P<0.01$ (vs. DEX treatment only). B, Real-time RT-PCR for MMP-2, MMP-9, IL-6, and VEGF were performed in UMUC3/TCC-SUP-control-shRNA/GR-shRNA treated with ethanol (mock), 100 nM DEX, and/or 1 µM RU486 for 24 hours. Expression of each specific gene was normalized to that of GAPDH. Transcription amount is presented relative to that of mock treatment in each cell line. Each value represents the mean(+SD) from at least three independent experiments. $*P<0.05$ (vs. mock treatment). $^#P<0.05$ (vs. DEX treatment only). C, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours were analyzed by SDS-PAGE gelatin zymography for MMP-2 (72 kDa) and MMP-9 (92 kDa).

**Figure 4.** DEX effects on MET. A, UMUC3/TCC-SUP cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours were assessed for cell morphology (area, perimeter, circularity, roundness), using the ImageJ software. Each value represents the mean(+SD) from data in at least 50 cells. $*P<0.05$ (vs. mock treatment). $^#P<0.05$ (vs. DEX treatment only). B, UMUC3/TCC-SUP-control-shRNA/GR-
shRNA cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours were analyzed on western blotting, using an antibody to E-cadherin (128 kDa), N-cadherin (130 kDa), β-catenin (92 kDa), vimentin (57 kDa), or snail (29 kDa). Tubulin (55 kDa) served as an internal control.

**Figure 5.** DEX effects on NF-κB. A, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours were analyzed on western blotting, using an antibody to NF-κB (65 kDa) or IκBα (37 kDa). Tubulin (55 kDa) served as an internal control. B, UMUC3-control-shRNA/GR-shRNA were cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours. Cell lysates were immunoprecipitated with anti-NF-κB or IκBα antibody and then immunoblotted for GR, NF-κB, or IκBα. C, TCC-SUP was cultured with 100 nM DEX and/or 1 µM RU486 for 24 hours. Separate cytoplasmic and nuclear protein fractions were analyzed on western blotting, using an antibody to GR or NF-κB. β-Actin (43 kDa) and histone 3 (15 kDa) served as internal controls for cytoplasmic and nuclear proteins, respectively. D, TCC-SUP cultured for 24 hours in the presence of ethanol (mock), 100 nM DEX, and/or 20 ng/ml TNF-α were analyzed on immunofluorescence, using an antibody to GR or NF-κB. DAPI was used to visualize nuclei.

**Figure 6.** DEX effects on tumor progression in mouse xenograft models. A, UMUC3-GR-shRNA/control-shRNA were implanted subcutaneously into the right/left flanks of male SCID mice, and treatment (injection of DEX or placebo pellet) began when estimated tumor volume reached 40 mm³. Tumor volume (n=8 tumors in each group) was monitored for 5 weeks (left upper panel, mean values). The tumors were then harvested and weighed [left lower panel, mean(+SD)]. Bloody ascites and metastatic tumor (arrow) are seen in mock-treated mice (right panels). B, Histology (H&E staining) of harvested xenograft tumors. Tumor invasion into the skeletal muscle (arrowheads) is seen in all groups of mice except the control-shRNA/DEX group. C, GR/MMP-9/VEGF/Ki-67/CD34 immunohistochemistry and TUNEL analysis in harvested tumors. Means(+SDs) of the percentage of...
Ki-67-positive cells, micro-vessel density (MVD, number of vessels highlighted by CD34 staining/high-power field), and the percentage of TUNEL-positive cells are shown.
Figure 3

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Figure 4

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Figure 5

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