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 pathway. Glucocorticoid receptor expression was first investigated in human bladder cancer lines and tissue microarrays. Then, the effects of dexamethasone on glucocorticoid receptor 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 glucocorticoid receptor. Dexamethasone increased glucocorticoid receptor–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, interleukin (IL)-6, VEGF], and the activity of MMP-2/MMP-9, and also induced mesenchymal-to-epithelial transition. In addition, 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 glucocorticoid receptor antagonist or glucocorticoid receptor knockdown via RNA interference. Thus, glucocorticoid receptor 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 glucocorticoid receptor agonists/antagonists, for urothelial carcinoma.

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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 noninvasive; 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, whereas 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 used in the treatment of inflammatory and autoimmune disorders. Several glucocorticoids have also been clinically used as cytotoxic agents, predominantly for hematologic 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 cells, Zhang and colleagues (5, 6) have shown in vitro evidence suggesting glucocorticoid-induced resistance to cytotoxic effects of cis-diaminedichloroplatinum (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). Because of these benefits, a glucocorticoid is often included as a medication in the standard chemotherapy regimens for bladder cancer. Meanwhile, prolonged systemic use of glucocorticoids has been shown to increase the subsequent risk of bladder cancer, possibly due to immunosuppression (8).

Accordingly, it remains unanswered whether glucocorticoids directly affect the development and progression of bladder cancer, 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 bladder cancer progression (9–11). In this study, we aim to determine whether and how glucocorticoid receptor signals regulate the growth of bladder cancer.

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; RPMI-1640 for 5637 and Dulbecco’s Modified Eagle’s Medium 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 treatments. 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 TNF-α from Sigma. Supplementary Table S1 lists all pertinent information on primary antibodies.

Stable cell lines with glucocorticoid receptor-shorn hairpin RNA

To establish stable glucocorticoid receptor knockdown lines, UMUC3 and TCC-SUP were directly infected with GR-short hairpin RNA (shRNA) or control-shRNA lentiviral particles (Santa Cruz Biotechnology) in the presence of 5 μg/mL polybrene (Millipore), as described for AR knockdown (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 cotransfected with 250 ng of mouse mammary tumor virus (MMTV)-luc reporter plasmid DNA and 2.5 ng of PRL-TK-luc plasmid DNA, using Genelucite (Novagen), as described previously (9, 12), and cultured in medium supplemented with CS-FBS for 24 hours in the presence of dexamethasone 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 × 10⁴) 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 dexamethasone 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 × 10⁵) in 100 μL of serum-free medium were added to the upper chamber of the transwell, whereas 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 mmol/L Tris, 5 mmol/L CaCl₂, and 1 μmol/L ZnCl₂, the gels were stained with 0.4% Coomassie blue.

Reverse transcription and real-time 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 carried out, using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (12). The primer sequences are given in Supplementary Table S2.

Western blot analysis and coimmunoprecipitation

Whole-cell protein extraction and Western blot analyses were conducted, as described previously (12) with minor modifications. Separate cytoplasmic and nuclear protein fractions were obtained, using the 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 blot analysis.

Apoptosis

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted 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 dexamethasone 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**

Bladder cancer lines (1 x 10⁶ 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 severe combined immunodeficient (SCID) mouse (NCl). Slow-releasing pellets [dexamethasone (0.5 mg/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 callipers and tumor weight was calculated by the following formula: tumor weight (mg) = tumor length (mm) x [tumor width (mm)]² x 0.5 (9). After 5 weeks of treatment, the mice were killed and all the tumors including metastases were harvested for histologic and immunohistochemical assessment.

**Bladder tissue microarray and immunohistochemistry**

Appropriate approval from the Institutional Review Board of the University of Rochester (Rochester, NY) was obtained before construction and use of the tissue microarray (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 conducted 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. Miyamoto) blinded to sample identity.

**Statistical analyses**

Differences in variables with a continuous distribution were analyzed by Student t test. Differences in glucocorticoid receptor expression rates in human tissue samples were compared using Fisher 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**

**Glucocorticoid receptor expression**

We first examined the expression of glucocorticoid receptor in 4 human bladder cancer lines, UMUC3, TCC-SUP, J82, and 5637, by reverse transcription PCR (RT-PCR) and Western blot analysis. All the lines were found to express glucocorticoid receptor at both mRNA (Supplementary Fig. S1A) and protein (Supplementary Fig. S1B) levels. Silencing of glucocorticoid receptor expression in UMUC3-GR-shRNA and TCC-SUP-GR-shRNA was then confirmed (see Fig. 2F).

Next, we immunohistochemically stained for glucocorticoid receptor in the bladder TMA. Positive signals were detected typically in both nuclei and cytoplasms of epithelial cells (Supplementary Fig. S1C). All the nonneoplastic 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, glucocorticoid receptor 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 glucocorticoid receptor expression and gender, presence of muscle invasion (≤pT1 vs. >pT2), or status of lymph node involvement. Nonetheless, Kaplan–Meier analysis showed a trend to associate between weak or moderate positivity of glucocorticoid receptor and a risk of progression after radical cystectomy (\( P = 0.0925 \)). No tumor progression was seen in all 5 patients with strongly glucocorticoid receptor-positive tumors.

**Dexamethasone-enhanced glucocorticoid receptor transactivation**

Glucocorticoid receptor-mediated transcriptional activity was determined in the cell extracts with transfection of a luciferase reporter plasmid (MMTV-luc) and treatment of a synthetic glucocorticoid dexamethasone and/or a glucocorticoid receptor antagonist RU486. Dexamethasone 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 dexamethasone-induced glucocorticoid receptor transcription in these lines. As expected, dexamethasone effects on luciferase activity were significantly diminished in glucocorticoid receptor knockdown lines (Fig. 1C). Similar induction by dexamethasone was obtained in AR knockdown UMUC3 cells in which DHT failed to increase luciferase activity (Fig. 1D), excluding dexamethasone-mediated MMTV-luc activity via AR. Thus, these bladder cancer cell lines likely possess a functional glucocorticoid receptor.
Dexamethasone-mediated cell proliferation and apoptosis

To see if glucocorticoid affects bladder cancer cell proliferation, each line was cultured with dexamethasone and/or RU486 for 4 days, and cell viability was assessed by MTT assay. Dexamethasone 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 dexamethasone effect (Fig. 2A). We also assessed the effects of dexamethasone and/or RU486 on the growth of stable cell lines with or without CDDP (Fig. 2B). In the absence of CDDP, dexamethasone increased the growth of UMUC3-control/TCC-SUP-control to 26% (P = 0.0032)/36% (P = 0.0003), respectively, compared with mock treatment, and RU486 antagonized the dexamethasone effect. In addition, growth induction by dexamethasone 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 glucocorticoid receptor knockdown lines, only marginal effects of dexamethasone and/or RU486 (except TCC-SUP-GR-shRNA with no serum) were observed. These results are consistent with previous findings in fewer other bladder cancer lines (5) and further suggest that glucocorticoids promote bladder cancer cell proliferation/inhibit an antiapoptotic effect of CDDP through the glucocorticoid receptor pathway.

To investigate how dexamethasone stimulates cell proliferation, we conducted flow cytometry and TUNEL assay. Dexamethasone 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 dexamethasone effects (Fig. 2C). Significant reductions (all P < 0.01) in the G1 proportion were observed in UMUC3-GR-shRNA with respective treatments, compared with UMUC3-control-shRNA, but not in TCC-SUP-control-shRNA versus TCC-SUP-GR-shRNA. In UMUC3-GR-shRNA, dexamethasone still increased G1 fraction from 39% to 51% (P = 0.0140), which was blocked by RU486. Thus, dexamethasone appears to induce bladder cancer cell-cycle arrest at G1 phase. The effects of dexamethasone 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, dexamethasone decreased apoptotic indices by 53%/67% and 30%/67%, respectively, and RU486 blocked dexamethasone-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 dexamethasone diminished CDDP-induced apoptosis to the levels with mock (+ FBS) treatment (6%/7% in UMUC3/TCC-SUP; P = 0.0050/P = 0.0001). RU486 notably increased dexamethasone-inhibited apoptosis in control lines cultured with CDDP. Dexamethasone 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, dexamethasone-mediated glucocorticoid receptor signals likely prevent apoptosis of bladder cancer cells in the presence or absence of CDDP.

To further investigate the molecular mechanisms of DEX/GR-induced cell proliferation, we conducted Western blot analysis 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 bladder cancer cell lines cultured with dexamethasone and/or RU486. However, dexamethasone upregulated the expression of p27 and p21 and downregulated that of cleaved caspase-3 in a dose-dependent manner, and RU486 blocked the dexamethasone effect. The effects of dexamethasone and RU486 on the expression of p21, p27, and cleaved caspase-3 were modest or marginal in glucocorticoid receptor knockdown lines (Fig. 2F). In addition, dexamethasone significantly decreased the levels of glucocorticoid receptor expression.

Dexamethasone-suppressed cell invasion

The effects of glucocorticoid on the invasiveness of bladder cancer cells were assessed, using a transwell invasion assay. Dexamethasone treatment resulted in significant decreases (50%–52%) in the invasive properties of both control lines, and RU486 clearly abolished the dexamethasone effect (Fig. 3A). In glucocorticoid receptor knockdown lines, dexamethasone did not show significant suppressive effects on cell invasion. These data suggest that glucocorticoids inhibit bladder cancer cell invasion through the glucocorticoid receptor pathway.

Using real-time RT-PCR, we then assessed the effects of glucocorticoid/glucocorticoid receptor on the expression of the molecules that play a key role in tumor invasion. Consistent with the results of the transwell assay, dexamethasone decreased the levels of MMP-2, MMP-9, interleukin (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 glucocorticoid receptor knockdown lines, inhibitory effects of dexamethasone 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. Dexamethasone reduced their levels in control lines, but not in control lines cultured with RU486 or in GR-shRNA lines (Fig. 3C).

Dexamethasone-induced mesenchymal-to-epithelial transition

We perceived that dexamethasone-treated bladder cancer 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 measured by gelatin zymography to assess their enzymatic activity. Dexamethasone reduced their levels in control lines, but not in control lines cultured with RU486 or in GR-shRNA lines (Fig. 3C).
Figure 2. Dexamethasone (DEX) effects on cell proliferation. A, cell viability of UMUC3/TCC-SUP cultured with 0 to 1,000 nmol/L dexamethasone ± 1 μmol/L 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 3 independent experiments. *, P < 0.05 (vs. mock treatment); #, P < 0.05 (vs. respective doses of dexamethasone treatment only). B, cell viability of UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 μmol/L, with 5% FBS) in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L 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 3 independent experiments. *, **, P < 0.05 (vs. mock treatment); #, ##, P < 0.05 (vs. dexamethasone treatment only). GR, glucocorticoid receptor.
Figure 2. (Continued) D, UMUC3/TCC-SUP-control-shRNA/GR-shRNA cultured with 5% FBS, no serum, or CDDP (7 μmol/L, with 5% FBS) for 4 days in the presence of ethanol (mock), 100 nmol/L dexamethasone, and/or 1 μmol/L RU486 were analyzed for apoptotic index (percentage of TUNEL-positive cells in 1,000 cells). Each value represents the mean (±SD) from at least 3 independent experiments. *, **, P < 0.05 (vs. mock treatment); #, P < 0.05 (vs. dexamethasone treatment only). E, UMUC3/TCC-SUP cultured with 0 to 1,000 nmol/L dexamethasone/1 μmol/L RU486 for 24 hours were analyzed on Western blot analysis using an antibody to glucocorticoid receptor (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 nmol/L dexamethasone, and/or 1 μmol/L RU486 were analyzed on Western blot analysis, using an antibody to glucocorticoid receptor, p27, p21, cleaved caspase-3, or β-actin. GR, glucocorticoid receptor.
and RU486 antagonized the dexamethasone effects (Fig. 4A).

To link these results to mesenchymal-to-epithelial transition (MET), we assessed expression levels of mesenchymal (e.g., N-cadherin, vimentin, snail) and epithelial (e.g., E-cadherin, β-catenin) markers by Western blot analysis. In glucocorticoid receptor–positive lines, dexamethasone up-/downregulated the expression of epithelial/mesenchymal markers, respectively, compared with mock treatment (Fig. 4B). These dexamethasone-mediated changes in their levels were marginal or less significant in cells with RU486 treatment and/or glucocorticoid receptor silencing. In addition, basal levels of these epithelial markers were lower in GR knockdown lines than in GR-positive controls.

**Dexamethasone-induced disruption of NF-κB**

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

We then conducted coimmunoprecipitation to test if dexamethasone affects protein–protein interactions (Fig. 5B). Dexamethasone induced the interaction between NF-κB and IkBα in control UMUC3 cells, and RU486 or GR-shRNA diminished the dexamethasone effect. Interestingly, glucocorticoid receptor was also pulled down by NF-κB, but not by IkBα.

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

**Dexamethasone 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.
Bladder cancer cells (UMUC3-GR-shRNA/control-shRNA) were implanted subcutaneously into the flanks of SCID mice, and after 2 weeks slow-releasing dexamethasone or placebo pellets were injected into mice. Control glucocorticoid receptor–positive tumors in dexamethasone-treated mice were larger/heavier than other tumors at 5 weeks of treatment \[e.g., 20%/23% \text{(vs. placebo-control cells)}; \text{Fig. 6A}\]. Similarly, placebo/glucocorticoid receptor knockdown tumors were slightly \(10\%\) lighter than placebo/control glucocorticoid receptor–positive tumors, and DEX/GR knockdown tumors were slightly \(9\%\) heavier than placebo/GR knockdown 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 dexamethasone-treated mice.

Histologic examination of the tumors revealed invasion into the skeletal muscle in all groups of mice except the control-shRNA/DEX group \(\text{Fig. 6B}\). Harvested tumor specimens were also assessed for cell proliferation \(\text{(Ki-67)}\), apoptosis \(\text{(TUNEL)}\), and angiogenesis or metastatic ability \(\text{(MMP-9/VEGF/CD34)}\). Dexamethasone treatment in control glucocorticoid receptor–positive tumors led to marginal changes in proliferation but decreased apoptosis and angiogenesis/metastasis-related factors, compared with DEX/GR-shRNA, placebo/control-shRNA, placebo/GR-shRNA, or placebo/metastasis \(\text{Fig. 6C}\). Metastatic tumors seen in placebo-treated mice were likely derived from GR-shRNA expressing cells based on weak glucocorticoid receptor signals. It was also noted that glucocorticoid receptor levels were significantly reduced in dexamethasone-treated UMUC3-control-shRNA tumors. These \textit{in vivo} data suggest that dexamethasone stimulates bladder cancer cell proliferation yet represses tumor invasion and metastasis.

**Discussion**

It appears that the status of glucocorticoid receptor expression has never been examined in human bladder cancer \(\text{[11]}\). Our immunohistochemical study in 24 cystectomy specimens showed that: (i) glucocorticoid receptor was detected in all cases of benign urothelium/urothelial carcinoma; (ii) glucocorticoid receptor expression tended to be weaker in tumor than in benign; and (iii) strong glucocorticoid receptor expression tended to correlate with better prognosis. These results may suggest a protective/inhibitory role of glucocorticoid receptor 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 hematologic malignancies \(\text{[3]}\). In contrast, limited amounts of experimental evidence have suggested inhibitory effects of glucocorticoids on cell growth of solid tumors. In a previous \textit{in vitro}...
study using bladder cancer lines (5), dexamethasone inhibited CDDP-mediated apoptosis, suggesting glucocorticoid-induced chemotherapy resistance. Using 2 bladder cancer lines expressing a functional glucocorticoid receptor, we here show that dexamethasone promoted cell proliferation, which was restored by a glucocorticoid receptor antagonist and/or glucocorticoid receptor knockdown. The stimulatory effects of dexamethasone were more significant when cultured with CDDP or in serum-free conditions. In addition, dexamethasone-induced cell growth was confirmed, using mouse xenograft models that showed larger tumor sizes in dexamethasone-treated mice than in mock-treated and/or glucocorticoid receptor knockdown cell-bearing mice. Thus, glucocorticoid receptor signals are likely associated with bladder cancer cell growth. Previous (5) and our current results may therefore imply that clinical use of glucocorticoids as comedication can be harmful to patients with bladder cancer.

Antiproliferative 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, dexamethasone enhanced cell cycle at the G1 phase in 2 glucocorticoid receptor–positive bladder cancer 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/glucocorticoid receptor–induced bladder cancer cell proliferation in vitro and in vivo. In contrast to previous observations in nonbladder cells, dexamethasone strongly inhibited apoptotic cell death, along with downregulation of cleaved caspase-3 expression, in bladder cancer lines and prevented CDDP-induced apoptosis. Because cell line–specific mechanisms for glucocorticoid receptor–mediated growth arrest have been shown (15), further analyses are required to elucidate the involvement of cell-cycle regulatory proteins in bladder cancer cells.

To our knowledge, no studies have assessed the effects of glucocorticoids on bladder cancer cell invasion. Using a transwell assay, we showed that dexamethasone suppressed cell invasion of glucocorticoid receptor–positive lines, but not glucocorticoid receptor knockdown lines, and that RU486 abolished the dexamethasone–induced apoptosis. In mouse xenograft models for bladder cancer, dexamethasone 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 downregulated in dexamethasone-treated cells/tumors, compared with mock-treated and/or glucocorticoid receptor knockdown cells/tumors. Thus, opposite to the effect on cell proliferation, glucocorticoids likely have an inhibitory role in bladder cancer cell invasion and metastasis through the glucocorticoid receptor pathway.

Glucocorticoids are known to interfere with the transcriptional activity of several transcription factors, including NF-κB. Dexamethasone has also been shown to reduce the growth of glucocorticoid receptor–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 bladder cancer, the association of NF-κB activity with cell invasion, as well as the transcriptional regulation of matrix metalloproteinases through the NF-κB pathway, has been reported (18). We therefore assessed the effect of dexamethasone on NF-κB in bladder cancer and found an increase in IκBα level, but not in NF-κB level, in dexamethasone-treated glucocorticoid receptor–positive cells as well as blockade of TNF-α-induced nuclear translocation of NF-κB by dexamethasone. Co-immunoprecipitation further showed dexamethasone-enhanced interactions between NF-κB and IκBα and between glucocorticoid receptor and NF-κB but not IκBα. The latter suggests that glucocorticoid receptor may directly function as a corepressor of NF-κB. Thus, NF-κB inactivation and IL-6 downregulation induced by dexamethasone may be a central mechanism involved in glucocorticoid receptor–mediated inhibition of bladder cancer cell invasion.

During our preliminary experiments, we found changes in the morphology of dexamethasone-treated bladder cancer cells. These might imply dexamethasone-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 morphologic changes compatible with MET, we showed that glucocorticoid receptor activation correlated with increased/decreased expression of epithelial/mesenchymal markers, respectively. These findings indicate that DEX/GR induce MET in bladder cancer cells, which could be an underlying mechanism of glucocorticoid receptor–mediated suppression of tumor progression.

As mentioned earlier, glucocorticoids have been widely used as comedication in patients with advanced bladder cancer. However, there was no molecular evidence indicating that glucocorticoids function directly through the glucocorticoid receptor pathway in bladder cancer cells and exert a stimulatory or inhibitory effect on tumor growth. The current study in bladder cancer has shown...
that glucocorticoid receptor 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 using various glucocorticoid receptor agonists/antagonists will facilitate improving chemotherapy regimens for urothelial carcinoma. On the basis of the current results, ideal glucocorticoid receptor 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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