

Administration of Glucocorticoids to Ovarian Cancer Patients Is Associated with Expression of the Anti-apoptotic Genes *SGK1* and *MKP1/DUSP1* in Ovarian Tissues

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Abstract Purpose: To prevent chemotherapy-related side effects, synthetic glucocorticoids, for example, dexamethasone, are routinely administered to patients with ovarian cancer. However, preclinical data implicate glucocorticoids in suppressing chemotherapy-mediated apoptosis in epithelial tumors. The anti-apoptotic mechanisms underlying this increased survival have been shown to require up-regulation of prosurvival genes, including serum and glucocorticoid-regulated kinase 1 (*SGK1*) and map kinase phosphatase 1 (*MKP1*)/dual specificity phosphatase 1 (*DUSP1*). Despite abundant preclinical data, there are no correlative studies in patients. We therefore evaluated anti-apoptotic gene expression in tumor samples from patients randomized to dexamethasone or normal saline.

Experimental Design: Eighteen patients were randomized before exploratory laparotomy for suspected ovarian cancer. Dexamethasone or normal saline was administered i.v. following anesthesia. Ovarian and omental tumor samples were collected intra-operatively before and after infusion. Samples were analyzed for histology and glucocorticoid receptor expression by immunohistochemistry. *SGK1* and *MKP1/DUSP1* mRNA levels were determined using quantitative real-time PCR.

Results: Ten patients were evaluable. At 30 min postinfusion, tumor samples from five patients receiving dexamethasone revealed an average *SGK1* mRNA induction of 6.1-fold (SEM, ± 2.6) compared with only 1.5-fold (SEM, ± 0.4) in tumor samples from five patients receiving normal saline ($P = 0.028$). Average *MKP1/DUSP1* mRNA expression was increased by 8.2-fold (SEM, ± 2.9) following dexamethasone versus 1.1-fold (SEM, ± 0.4) following normal saline ($P = 0.009$). All samples expressed glucocorticoid receptor.

Conclusion: Glucocorticoid administration to patients is associated with rapid up-regulation of *SGK1* and *MKP1* expression in ovarian tumors. This finding supports the hypothesis that pharmacologic doses of glucocorticoids may decrease chemotherapy effectiveness in ovarian cancer patients through increased anti-apoptotic gene expression.

Although synthetic glucocorticoids have antitumor effects in lymphocytic malignancies, endogenous and synthetic glucocorticoids can promote cell survival in epithelial tumors, including breast (1) and ovarian cancers (2, 3). The anti-apoptotic effects associated with glucocorticoid treatment of

solid tumors have been shown (a) *in vivo* using human xenografts in severe combined immunodeficiency mice, (b) *in vitro* using tumor cell lines, and (c) *ex vivo* in excised primary human tumors treated *in vitro* with glucocorticoids followed by chemotherapy (2). Glucocorticoids also promote cell survival in normal mouse mammary epithelium, wherein they prevent lobulo-alveolar duct involution through inhibition of apoptosis (4). In cell culture and xenograft studies, the mechanisms by which glucocorticoids inhibit apoptosis require changes in the expression of genes whose protein products alter downstream pro- and anti-apoptotic signaling events. Until now, evidence that these genes are induced in patient tumors following glucocorticoid administration has not been reported.

Our laboratory has done several genomewide expression studies to identify and validate gene expression changes following glucocorticoid treatment of benign and malignant mammary epithelial cells (1). Two such genes, serum and glucocorticoid-regulated kinase 1 (*SGK1*) and map kinase phosphatase 1 [*MKP1*; also known as dual specificity phosphatase 1 (*DUSP1*)], were found to be required for anti-apoptotic signaling mediated by glucocorticoids. Knockdown

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Translational Relevance

Ovarian cancer is the fifth leading cause of cancer deaths in women in the United States. Glucocorticoids are widely used in conjunction with chemotherapy for ovarian cancer because they have potent anti-emetic effects and can prevent hypersensitivity reactions associated with paclitaxel therapy. However, in preclinical models, glucocorticoid-mediated activation of the epithelial tumor cell glucocorticoid receptor is associated with decreasing chemotherapy-induced apoptosis. Here, we show for the first time that i.v. administration of dexamethasone to patients is associated with the increased expression of two potent anti-apoptotic genes, serum and glucocorticoid-regulated kinase 1 and map kinase phosphatase 1/dual specificity phosphatase 1, in ovarian tumors. Our findings suggest that glucocorticoids may decrease chemotherapy-induced apoptosis in human epithelial cancers through the activation of anti-apoptotic signaling cascades.

of either *SGK1* or *MKP1* using small hairpin RNA reversed the cell survival afforded by glucocorticoid pretreatment (1). *SGK1* encodes a serine/threonine kinase that was originally identified as a glucocorticoid-induced gene in rat mammary epithelial tumor cells (5). The exact molecular mechanisms by which glucocorticoids inhibit apoptosis remain unknown, although the ability of *SGK1* to phosphorylate and inactivate the proapoptotic forkhead transcription factors (6), the *c-jun*-NH₂-kinases (1 and 2; ref. 7), and p27 (8) are likely important mechanisms. In addition, a fraction of *SGK1* localizes to the mitochondrial membrane and endoplasmic reticulum (9, 10), wherein it may be involved in the regulation of membrane potential during apoptotic stress. Furthermore, the catalytic domain of *SGK* is 55% homologous to the protein kinase B (Akt) kinase domain (5), suggesting that *SGK1* may play a role in cancer. Indeed, *SGK1* has been shown to be overexpressed in a substantial proportion of cancers, including breast (11) and prostate cancers.⁶ To our knowledge, there have been no publications to date describing *SGK1* expression levels in primary ovarian cancers.

MKP1/DUSP1 encodes a phosphatase that is induced following glucocorticoid receptor activation (1). Increased expression of *MKP1/DUSP1* may be linked to shortened progression-free survival time in ovarian cancer and has been shown to inhibit chemotherapy-induced apoptosis in ovarian cancer cells (12, 13). Induction of *MKP1/DUSP1* expression results in dephosphorylation and inactivation of extracellular regulated kinases 1,2 and *c-jun*-NH₂-kinases (1 and 2), ultimately contributing to increased cell survival (14).

We designed a proof-of-principle trial to test the hypothesis that glucocorticoid administration to patients is associated with the increased expression of the anti-apoptotic genes *MKP1/DUSP1* and *SGK1* in ovarian tumor tissues. Eighteen patients were randomized to receive either dexamethasone or normal saline during exploratory laparotomy for suspected ovarian cancer. Tumor samples were collected intra-operatively

immediately before and at specified time points after dexamethasone/normal saline administration. We found that i.v. administration of dexamethasone (20 mg) is associated with a rapid and significant up-regulation of the *SGK1* and *MKP1/DUSP1* prosurvival gene expression in ovarian tumor tissues. These findings may have important implications for the use of glucocorticoids in conjunction with chemotherapy and are to our knowledge the first report of the *in vivo* gene expression response to dexamethasone administration in human ovarian tissues.

Materials and Methods

Study design. After obtaining University of Chicago institutional review board approval, a randomized placebo-controlled trial was done. Eighteen patients were enrolled between July 2003 and September 2007 through the Gynecologic Oncology Clinic at the University of Chicago. All patients agreed to and signed an institutional review board–approved written informed consent before enrollment. Principal inclusion criteria were women ≥ 18 y of age, suspected of having ovarian or primary peritoneal cancer, and scheduled for exploratory laparotomy for definitive diagnosis and debulking surgery. Patients who were concurrently receiving glucocorticoid treatment were excluded from the study, as were pregnant patients, diabetics, and patients with an albumin of < 3.0 mg/dL. The patients were randomized to receive dexamethasone 20 mg i.v. or an equivalent volume normal saline injection, to be administered in a blinded fashion immediately following the initial intra-operative biopsy.

Human tissue sample collection and storage. Intra-operative biopsies were taken from suspected gross tumor sites before the infusion of dexamethasone or normal saline and where possible, given limitations of tissue availability and operative time, at 30 min, 2 h, and 4 h after infusion. These time points were designed to capture the peak predicted induction of *SGK1* and *MKP1/DUSP1* mRNA based on *in vitro* cell culture data (15, 16); surgery was not prolonged to obtain tumor samples at the later time points, and therefore, most patients only had two collection points. In the operating room, the tissue samples were immediately placed in a plastic cassette, submerged in Optimum Cutting Temperature embedding media (Tissue-Tec), snap frozen in liquid nitrogen, and transported in a portable liquid nitrogen tank to the Department of Pathology for barcoded storage without direct patient identifiers. All samples were then stored at -70°C until processing.

Cells and cell culture. The ovarian carcinoma cell line SKOV3 and the breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection. HeyA8 ovarian carcinoma cells were a generous gift of Dr. Ernst Lengyel, Department of Obstetrics and Gynecology at the University of Chicago. Cells were cultured in DMEM (BioWhittaker) and antibiotics (1% penicillin/streptomycin, Lonza) supplemented with 10% FCS (Atlanta Biologicals). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Drugs for cell line experiments. Pharmaceutical grade paclitaxel (Calbiochem) was dissolved in 100% methanol to make a stock solution, which was then diluted 1,000-fold in culture medium to obtain a desired concentration. Dexamethasone and RU486 (Sigma) were dissolved in 100% ethanol to make a stock solution of 10^{-3} mol/L, which was then diluted in culture medium to obtain the desired concentration of 10^{-6} or 10^{-7} mol/L, respectively. Where indicated, cells were treated with the proteasome inhibitor MG132 (Sigma) at $10\ \mu\text{mol/L}$ for 4 h before cell lysate harvest.

Apoptosis assays. Cells were trypsinized and seeded subconfluently at 1×10^5 cells/6 cm dish or on plastic chamber slides (Nalgenue Nunc). Cells were allowed to adhere overnight, rinsed twice with $1 \times$ PBS, and cultured for 24 h in serum-free medium. Vehicle (ethanol) or dexamethasone (10^{-6} mol/L) was added for 1 h before adding paclitaxel

⁶ El-Hamadi and S. Conzen, unpublished observation.

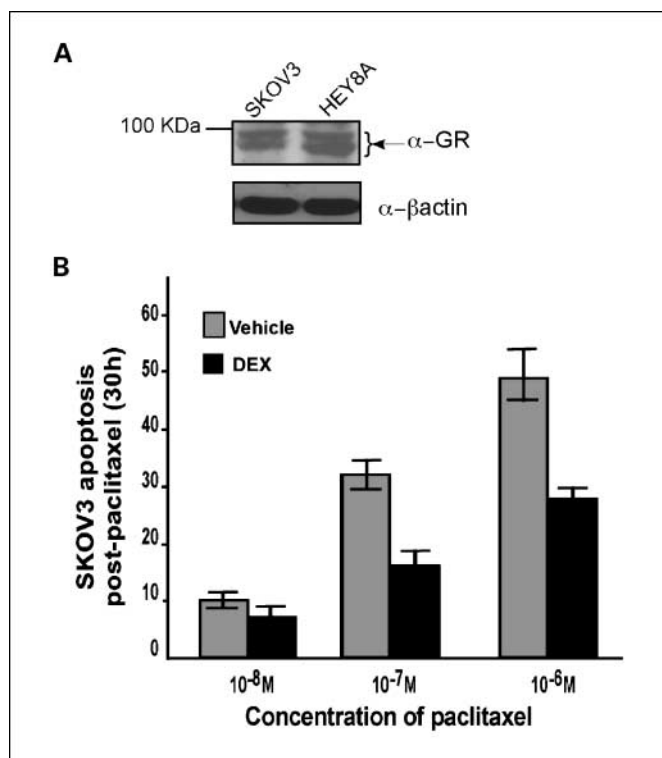


Fig. 1. Dexamethasone inhibits paclitaxel-induced apoptosis in ovarian cancer cells. **A**, Western blot analysis of the glucocorticoid receptor isoforms and β -actin was done using cell lysates from the ovarian cancer cell lines SKOV3 and HeyA8. **B**, SKOV3 cells were cultured in the absence of growth factors for 24 h and then treated with dexamethasone (10^{-6} mol/L) or ethanol for 1 h before paclitaxel at varying concentration of paclitaxel (10^{-6} , 10^{-7} , 10^{-8} mol/L). Apoptosis assays were done 30 h following chemotherapy treatment using 4',6'-diamidino-2-phenylindole staining and fluorescent microscopy to score the percentage of apoptotic cells. Experiments were done in triplicate. Bars, \pm SE (SEM).

(10^{-6} , 10^{-7} , 10^{-8} mol/L) for 30 h. The cells were then fixed by adding formaldehyde at a final concentration of 7% to each well for 30 min. The fixative was aspirated, the wells allowed to air dry, and cells were then stained with a $1 \mu\text{mol/L}$ 4',6'-diamidino-2-phenylindole/ $1 \times$ PBS solution as described previously (17). A Nikon Eclipse E800 microscope with UV illumination at $600\times$ magnification was used to count at least 200 4',6'-diamidino-2-phenylindole-stained cells in several fields to determine the percentage of apoptotic cells per experimental condition. The apoptosis assays were done three independent times to calculate the average percentage of apoptosis and the SE (SEM).

Quantitative real-time PCR. Total RNA was extracted (Qiagen All-Prep DNA/RNA Mini kit) from tissue samples weighing ~ 30 mg. cDNA was then reverse transcribed from 0.5 μg of total RNA with Taqman reverse transcription reagents (Applied Biosystems). Reverse transcription was done in the GeneAmp PCR System 9700 (Applied Biosystems), beginning with an incubation period of 10 min at 25°C , followed by a reverse transcription period of 30 min at 48°C , and ending with reverse transcription inactivation by 95°C for 5 min. Quantitative real-time PCR was carried out in the ABI Prism 7700 (Applied Biosystems) thermal cycler/detector in the following sequence: 2 min at 50°C and 10 min at 95°C , 40 cycles consisting of 15 s at 95°C and 1 min at 60°C , 15 min at 95°C , 20 min at 60°C , and 15 min at 95°C . The following primers were used: SGK1, 5'AGGCCATCCTTCTCTGTTT 3' (forward) and 5'TTCACTGCTCCCTCAGTCT 3' (reverse); MKP1/DUSP1, 5'-CCTGACAGCGCGGAATCT-3' (forward) and 5'-GATTTCCACCGGGCCAC-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GAGTCAACGGATTGGTCGT-3' (forward) and 5'-TTGATTTTGGAGGGATCTCG-3' (reverse). GAPDH was amplified as an internal control. The samples were loaded in triplicate, and the

results of each sample were normalized to GAPDH expression. Within-patient fold changes were compared between treatment arms using the Wilcoxon rank-sum test, and $P < 0.05$ was considered statistically significant. For the cell line experiments, total RNA was extracted at 1 and 4 h following the addition of vehicle (ethanol), dexamethasone (10^{-6} mol/L) or dexamethasone/RU486 (10^{-7} mol/L). Quantitative real-time PCR was done as above using primers for SGK1, MKP1/DUSP1, and GAPDH, all of which resulted in a single PCR product. Relative quantification of gene expression and SDs were calculated according to the standard curve method, as described by Applied Biosystems.⁷ Based on the $\Delta\Delta\text{Ct}$ approach, differences between treatment groups were evaluated using an ANOVA model with treatment group, gene (target or reference), and their interaction. The reported P value for the difference between treatment groups is based on a linear contrast incorporating standard curve based estimate of PCR efficiencies, as previously described (18).

Western blot analysis. Cells were trypsinized and seeded subconfluently at 6×10^5 cells per 6-cm dish. Cells were allowed to adhere overnight, rinsed twice with $1 \times$ PBS, and cultured for 24 h in serum-free medium. Dexamethasone (10^{-6} mol/L) was added for 1, 4, 16, or 24 h. In some samples, MG-132 ($10 \mu\text{mol/L}$) was added for 4 h before harvest. Equal amounts of protein in each experimental condition were denatured in $2 \times$ Laemmli buffer and separated on 9% SDS-PAGE gels. Proteins were transferred to nitrocellulose and stained with Ponceau S Red dye to confirm equal protein loading. The membranes were blocked in 5% milk and incubated with anti-SGK1 C-terminus antibody (1:500 dilution; SGK1 CT, Assay Designs), anti-MKP1/DUSP1 antibody (1:200 dilution; C19, Santa Cruz Biotechnology), or anti-glucocorticoid receptor antibody (1:200 dilution; PA1-510A, Affinity Bioreagents) in $1 \times$ TBS-Tween (0.1%), followed by incubation with goat anti-rabbit horse radish peroxidase-conjugated (1:5,000) or goat anti-mouse peroxidase-conjugated (1:5,000) secondary antibodies (Santa Cruz Biotechnology). The membranes were treated with enhanced chemiluminescent staining (Amersham) per manufacturer's instructions before exposure to film. Blots were also probed with a mouse anti- β -actin antibody (1:20,000; Sigma) as a loading control.

For tissue samples, ~ 50 mg of frozen tissue was pulverized with an ice cold stainless steel mortar and pestle and homogenized using a 25 g needle in 200 μL of cold lysis buffer containing protease and phosphatase inhibitors [50 mmol/L Tris (pH 7.5), 120 mmol/L sodium chloride, 2% Triton X-100, 10 mmol/L EDTA, 25 mmol/L sodium fluoride, 1 mmol/L benzamide, 40 mmol/L β -glycerol phosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{mol/L}$ N-Acetyl-Leu-Leu-Nle-CHO, 10 $\mu\text{mol/L}$ N-Acetyl-Leu-Leu-Met-CHO, 0.1 mmol/L sodium orthovanadate, protease inhibitor cocktail (Sigma; P8340), phosphatase inhibitor cocktail (Sigma; P2850), Complete Tablet (Roche), and 1% NP40]. Lysates were then centrifuged at 3,500 rpm for 30 min at 4°C to remove debris and protein concentrations measured by Bradford assay before the addition of Laemmli sample buffer. Equal amounts of cell lysates were run on 9% SDS-PAGE gels, transferred to nitrocellulose, and probed with primary and secondary antibodies, as described above.

Histopathologic examination. Frozen tissue samples were sectioned, prepared for H&E staining, and analyzed by a pathologist (B.D.) for tissue diagnosis and percent of tumor involvement. For immunohistochemistry, ovarian and omental tissue samples were formalin-fixed and embedded in paraffin. Sections ($5 \mu\text{m}$ thick) were adhered to positively charged slides, dewaxed in xylene, and hydrated through graded ethanol. Heat-induced antigen retrieval was then done using 10 mmol/L sodium citrate (pH 6.0) incubation in a microwave for 10 min. The endogenous peroxidase activity was blocked by immersing the slides in 1% H_2O_2 v/v methanol for 30 min. After 30 min of blocking in 5% milk-PBS, slides were incubated with either a 1:20 dilution of anti-glucocorticoid receptor monoclonal antibody (NCL-GCR, Novocastra;

⁷ Applied Biosystems User Bulletin 2, October 2001.

ref. 19) or a 1:20 dilution of a nonimmune mouse immunoglobulin G (DAKO) as a control. Antibodies were incubated at 4°C overnight. The DakoCytomation Envision + System-HRP (3,3'-diaminobenzidine) reaction reagent was used for immunodetection. The slides were counterstained with hematoxylin and mounted using Micromount (Micromount-Surgipath). Slides were then examined on an Olympus microscope (original magnification, $\times 100$ and $\times 200$).

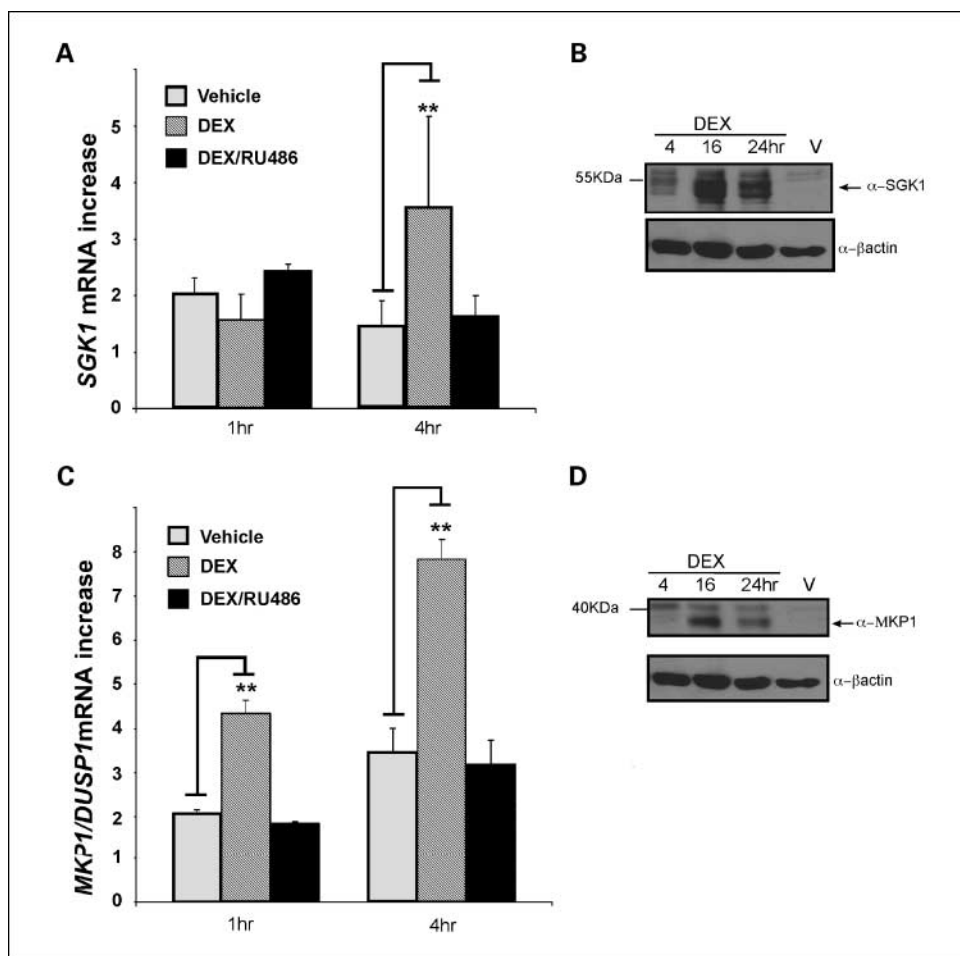
Results

Dexamethasone suppresses paclitaxel-induced apoptosis in ovarian cancer cells. Previous preclinical studies have shown that glucocorticoid pretreatment suppresses chemotherapy-induced apoptosis in many cancer cell lines. Glucocorticoid receptor expression by Western analysis was first confirmed in SKOV3 and HeyA8 cancer cells with β -actin expression evaluated as a loading control (Fig. 1A). We next determined whether or not dexamethasone pretreatment was associated with decreased paclitaxel-induced apoptosis in SKOV3 cells. Cells were cultured in the absence of growth factors for 24 hours and then treated with either dexamethasone (10^{-6} mol/L) or ethanol for 1 hour before treatment with varying concentrations of paclitaxel (10^{-6} , 10^{-7} , 10^{-8} mol/L). Apoptosis was measured at 30 hours using 4',6-diamidino-2-phenylindole staining to detect condensed chromatin by fluorescence microscopy (Fig. 1B). A reduction in apoptosis (37% and 42%) relative to control pretreatment was observed

with dexamethasone (10^{-6} mol/L) pretreatment in the 10^{-7} and 10^{-6} mol/L paclitaxel concentrations, respectively. This finding suggests that dexamethasone suppresses paclitaxel-induced cell death in glucocorticoid receptor expressing ovarian cancer cells.

Glucocorticoid receptor activation is associated with increased expression of the anti-apoptotic genes *SGK1* and *MKP1/DUSP1* in SKOV3 and HEYA8 ovarian cancer cells. Previous cell culture studies have shown that glucocorticoid receptor activation in mammary epithelial and breast cancer cell lines results in the up-regulation of both *SGK1* and *MKP1/DUSP1* gene expression and that expression of these genes is required for glucocorticoid receptor-mediated cell survival (1). To determine whether or not glucocorticoid receptor activation induces *SGK1* in ovarian cancer cell lines, we measured *SGK1* mRNA by quantitative real-time PCR at 1 and 4 hours following treatment with either dexamethasone alone or dexamethasone combined with the selective glucocorticoid receptor modulator RU486 (10^{-7} mol/L). In SKOV3 cells, we found that treatment with dexamethasone significantly increased *SGK1* mRNA expression by 2.5-fold at 4 hours in comparison with vehicle ($P = 0.016$) and that the increase in expression was partially reversed by the concomitant treatment with RU486 (Fig. 2A). This implies that dexamethasone is acting through the glucocorticoid receptor because RU486 is a selective glucocorticoid receptor modulator. We next used Western blot analysis to examine *SGK1* protein expression at 4, 16, and 24 hours following dexamethasone

Fig. 2. *SGK1* and *MKP1/DUSP1* mRNA and protein analysis following dexamethasone in SKOV3 cells. **A**, ovarian cancer cells SKOV3 were treated with vehicle (ethanol), dexamethasone (10^{-6} mol/L) or dexamethasone/RU486 (10^{-7} mol/L), quantitative real-time PCR analysis of *SGK1* mRNA was done at 1 and 4 h after treatment, and expression levels were normalized to *GAPDH* mRNA. Average expression levels of *SGK1* mRNA in the cells treated with vehicle, dexamethasone, or dexamethasone/RU486 are shown along with SD. **, significant increase in *SGK1* mRNA at 4 h in dexamethasone compared with vehicle-treated cells ($P < 0.05$). **B**, Western blot analysis of the expression of *SGK1* isoforms and β -actin in SKOV3 cells treated with dexamethasone (10^{-6} mol/L) for 4, 16, and 24 h, or vehicle alone for 24 h. All cells were cultured with the proteasome inhibitor MG132 ($10 \mu\text{mol/L}$) for 4 h before harvesting. **C**, *MKP1/DUSP1* mRNA quantitative real-time PCR analysis. **, significant increase in *MKP1/DUSP1* mRNA levels at 1 h ($P < 0.01$) and 4 h ($P < 0.05$) compared with vehicle alone. **D**, the corresponding *MKP1/DUSP1* Western blot. β -actin was used as a loading control.



treatment (Fig. 2B) and found that steady-state protein levels increased, peaking at 16 hours and persisting through 24 hours (compared with vehicle treatment for 24 hours). We did a similar experiment using HeyA8 ovarian cancer cells (Fig. 3A) and found that dexamethasone also significantly increased *SGK1* mRNA expression at 1 and 4 hours by 1.6- and 3.8-fold respectively, in comparison with vehicle ($P = 0.009$ and 0.011 , respectively). Interestingly, in HeyA8 cells, RU486 did not significantly inhibit the effect of dexamethasone treatment on *SGK1* mRNA induction (Fig. 3A, *black columns*), consistent with a gene target and cell-specific effect of RU486 on glucocorticoid receptor modulation. In HeyA8 cells, Western analysis (Fig. 3B) confirmed that *SGK1* protein expression increased 1 hour following dexamethasone treatment and persisted through 24 hours.

Parallel experiments were done to examine *MKP1/DUSP1* steady-state mRNA and protein expression levels following dexamethasone treatment of both ovarian cancer cell lines. In SKOV3 cells, quantitative real-time PCR showed an average dexamethasone-associated 2.2-fold increase in *MKP1/DUSP1* mRNA over vehicle alone (*hatched columns*; $P = 0.01$); the dexamethasone-associated increase in gene expression was reversed in the presence of RU486 (Fig. 2C, *black columns*). Similar to the *SGK1* protein expression, *MKP1/DUSP1* protein expression peaked at 16 hours and persisted through 24 hours,

whereas increased protein was barely detected in cells treated with vehicle alone for 24 hours (Fig. 2D). These results suggest that dexamethasone is associated with a significant glucocorticoid receptor-dependent increase in *SGK1* and *MKP1/DUSP1* mRNA and protein expression in SKOV3 ovarian cancer cells. In HeyA8 cells, an average 3.9-fold increase in *MKP1/DUSP1* mRNA was detected at 1 hour and a 2.3-fold increase was seen at 4 hours following dexamethasone treatment in comparison with vehicle-treated HeyA8 cells ($P = 0.005$ and 0.017 , respectively; Fig. 3C). In the case of *MKP1/DUSP1*, concomitant RU486 treatment partially reversed the effect of dexamethasone on mRNA induction. Western analysis also confirmed a significant increase in *MKP1/DUSP1* protein expression at 16 and 24 hours following dexamethasone treatment of HeyA8 cells (Fig. 3D).

SGK1 and MKP1/DUSP1 mRNA expression is significantly increased in primary ovarian and omental tissues from patients treated with dexamethasone. Based on the previously published and ovarian cell culture data above, we did a small proof-of-principle trial in patients with suspected ovarian cancer undergoing exploratory laparotomy. Patients routinely receive 10 to 20 mg of dexamethasone before chemotherapy as part of standard anti-emetic regimens; this dose range leads to an average plasma dexamethasone concentration of $0.8 \mu\text{mol/L/L}$ (21). The equivalent plasma

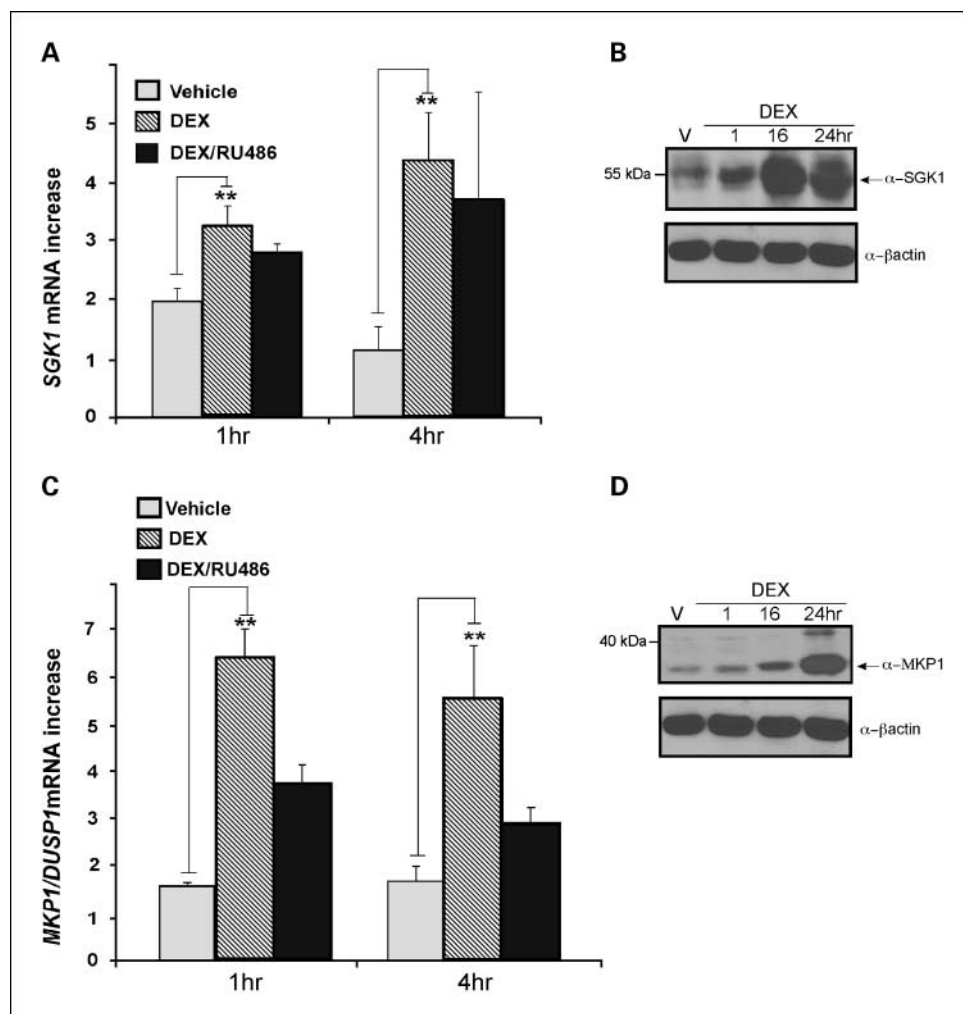


Fig. 3. *SGK1* and *MKP1/DUSP1* mRNA and protein analysis following dexamethasone in HeyA8 ovarian cancer cells. **A**, HeyA8 cells were treated with vehicle (ethanol), dexamethasone (10^{-6} mol/L), or dexamethasone/RU486 (10^{-7}), and quantitative real-time PCR analysis of *SGK1* mRNA was done at 1 and 4 h, followed by normalization of expression levels to *GAPDH* mRNA. Average expression levels of *SGK1* mRNA in the cells treated with vehicle, dexamethasone, or dexamethasone/RU486 along with SD. **, significant increase in *SGK1* mRNA at 1 ($P < 0.01$) and 4 ($P < 0.05$) h in dexamethasone versus vehicle-treated cells. **B**, Western blot analysis with the expression of *SGK1* and β -actin in HeyA8 cells treated with dexamethasone (10^{-6} mol/L) for 1, 16, and 24 h, or vehicle for 24 h. All cells were cultured with the proteasome inhibitor MG132 ($10 \mu\text{mol/L}$) for 4 h before cell harvesting. **C**, *MKP1/DUSP1* mRNA by quantitative real-time PCR analysis. **, a significant increase in *MKP1* levels at 1 h ($P < 0.01$) and 4 h ($P < 0.05$). **D**, corresponding *MKP1/DUSP1* Western blot, with β -actin as a loading control.

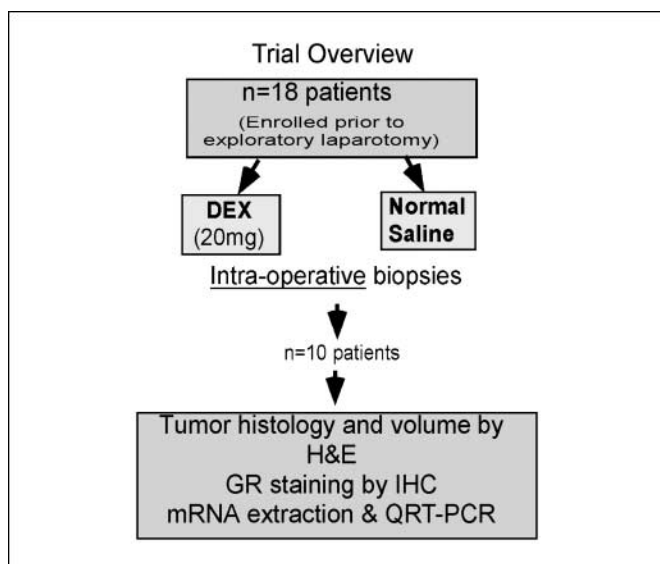


Fig. 4. Human subjects' protocol schema.

cortisol level is about 1,000 $\mu\text{g}/\text{dL}$, that is, ~ 50 -fold higher than an average peak endogenous cortisol level of 23 $\mu\text{g}/\text{dL}$ (22). Moreover, the level achieved pharmacologically with dexamethasone is significantly higher than the peak stress-induced cortisol levels reported following general anesthesia and subsequent abdominal surgery (39 $\mu\text{g}/\text{dL}$; ref. 23). Therefore, we predicted that the administration of i.v. dexamethasone (20 mg) to patients would result in a significantly higher glucocorticoid level compared with control patients receiving normal saline, despite the stress of general anesthesia and exploratory surgery.

The clinical trial schema is depicted in Fig. 4. Eighteen patients were enrolled and randomized to receive dexametha-

sone or normal saline during exploratory laparotomy for suspected ovarian cancer. No toxicities related to dexamethasone/normal saline administration were reported. We attempted to collect primary ovarian and/or omental metastasized tumor tissue samples intra-operatively, immediately before and after dexamethasone/normal saline in all enrolled patients. However, 8 of the 18 randomized patients were unevaluable because there was not enough tissue available during surgery for experimental studies; the remaining 10 patients had adequate tissue samples at baseline and at least at 30 minutes following dexamethasone/normal saline administration. Of these 10 patients, four dexamethasone-treated patients and one normal saline-treated patient had extended operative times, as well as enough tissue to be sampled for collection beyond the initial 30-minute time point; these additional samples were also analyzed for gene expression by quantitative real-time PCR, and the data for *SGK1* and *MKP1/DUSP1* gene expression are reported from individual dexamethasone-treated patients in Supplementary Fig. S1.

Patient and tumor characteristics are shown in Supplementary Table S1. Ages ranged from 44 to 82 years old; four patients were self-described as African-American and six were of European-American ancestry. The tissue biopsy diagnoses and the percentage of glucocorticoid receptor staining by immunohistochemistry are also shown. All tissues exhibited at least 10% of cells positive for glucocorticoid receptor staining. Figure 5A to C show the predominantly nuclear glucocorticoid receptor expression in representative tumor samples. Figure 5D shows the negative control with a nonimmune mouse immunoglobulin G primary antibody, followed by the anti-mouse secondary antibody used in Fig. 5A to C.

Having established that glucocorticoid receptor was expressed in the primary tumor samples, we next extracted RNA, reverse transcribed to cDNA, and measured steady-state levels of *SGK1*

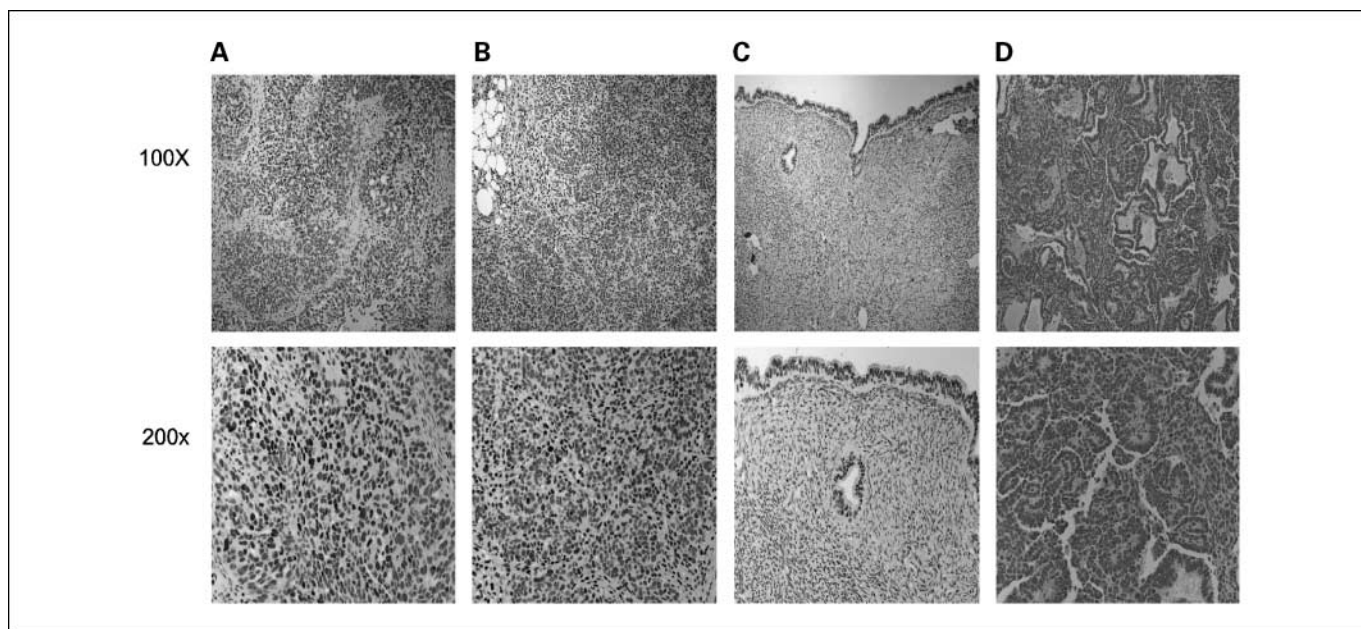


Fig. 5. Glucocorticoid receptor immunohistochemistry of representative tissue samples from enrolled patients. A, ovarian papillary serous carcinoma; B, omental metastasis of an ovarian carcinoma; C, benign serous cyst adenoma; D, primary nonimmune mouse immunoglobulin G antibody (negative control). Top and bottom, different magnifications (original magnification, $\times 100$ and $\times 200$, respectively).

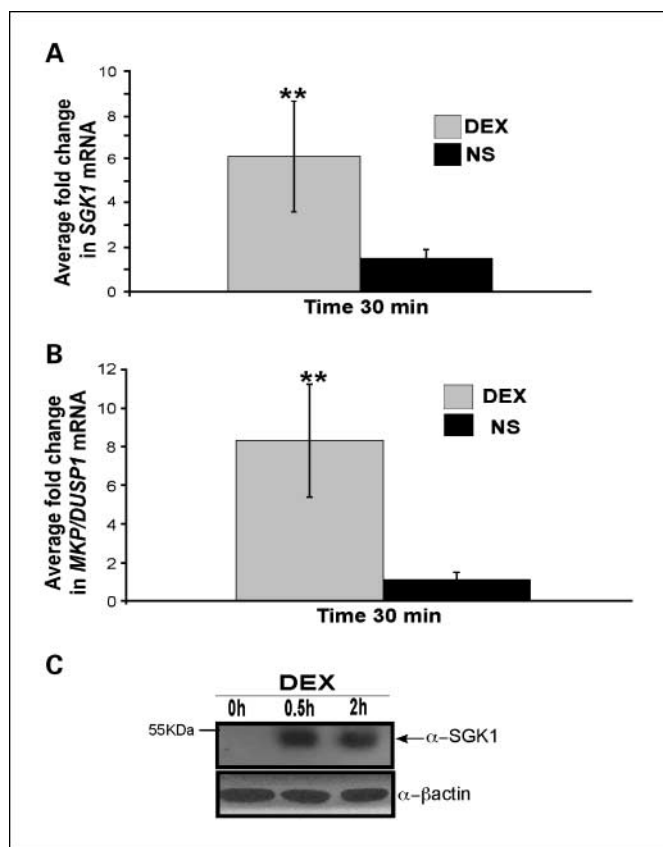


Fig. 6. Dexamethasone administration to patients is associated with increased expression of *SGK1* and *MKP1/DUSP1* mRNA in tumor samples. **A**, quantitative real-time PCR analysis of *SGK1* mRNA was done on tumor samples obtained immediately before dexamethasone 20 mg or normal saline administration and 30 min after infusion. Expression levels were normalized to *GAPDH* mRNA as an internal control. The average-fold change relative to baseline in dexamethasone and normal saline-treated patient tissues together with the SE (SEM; bars). **, significant increase in *SGK1* mRNA levels at 30 min in patients treated with dexamethasone ($P = 0.028$). **B**, *MKP1/DUSP1* mRNA expression from the same samples as in **A**. **, significant increase in *MKP1/DUSP1* mRNA levels at 30 min in patients treated with dexamethasone compared with normal saline ($P = 0.009$). **C**, Western blot analysis of *SGK1* and β -actin in tumor tissue samples obtained at 0, 0.5, and 2 h from a patient randomized to receive dexamethasone.

and *MKP1/DUSP1* expression using quantitative real-time PCR. Steady-state mRNA levels were analyzed for the pretreatment samples and for the samples taken at 30 minutes (and later, if available) following dexamethasone or normal saline administration in all 10 patients. In tumor samples from the five evaluable patients randomized to treatment with i.v. dexamethasone, *SGK1* mRNA steady-state levels showed an average-fold increase of 6.1 ± 2.6 [SE (SEM)] compared with baseline pretreatment expression levels (Fig. 6A). This was significantly higher than the average 1.5-fold increase ± 0.4 SEM in tumor *SGK1* mRNA observed in patients treated with normal saline alone for 30 minutes ($P = 0.028$). Similarly, we found an average-fold increase in *MKP1/DUSP1* mRNA of 8.2 ± 2.9 SEM in dexamethasone-treated patients compared with the average-fold increase of 1.1 ± 0.4 SEM (Fig. 6B) observed in patients treated with normal saline ($P = 0.009$).

If sufficient tissue sample was available, we also did Western blot analysis of *SGK1* (Fig. 6C) and *MKP1/DUSP1* (data not shown). Figure 6C shows a representative blot illustrating increased *SGK1* protein expression in samples of a papillary

serous carcinoma from a patient with biopsies available at 30 minutes and 2 hours following dexamethasone administration. However, unlike *SGK1*, *MKP1/DUSP1* protein could not be convincingly detected from primary human tissues by Western blot analysis, possibly because of the inhibitory effect of the Optimum Cutting Temperature embedding medium on *MKP1/DUSP1* immunodetection or because of the relatively rapid degradation of *MKP1/DUSP1* after tumor collection. Although protein analysis of these tumors was limited, the quantitative real-time PCR results strongly suggest that *SGK1* and *MKP1/DUSP1* gene expression is consistently and significantly induced in human ovarian and omental tissues following the administration of a pharmacologic dose of 20 mg of dexamethasone.

Discussion

To our knowledge, this is the first reported measurement of gene expression in human solid tumor tissues following the administration of glucocorticoids to patients. Previously published preclinical studies have suggested that glucocorticoids lead to a significant inhibition of chemotherapy-mediated apoptosis in ovarian cancer cell lines in association with changes in gene expression (2, 3). For example, the anti-apoptotic effects of glucocorticoids have been shown in xenografted human cancer cell lines, as well as in ovarian cancer cell lines and freshly isolated primary ovarian carcinomas (2). Our current experiments in the ovarian cancer cell line SKOV3 support these previous findings with paclitaxel treatment. Furthermore, as previously shown in breast cancer cell lines (1), we now show that SKOV3 and Hey8 ovarian cancer cell lines treated with pharmacologically equivalent doses of dexamethasone show increased expression of two prosurvival genes, *SGK1* and *MKP1/DUSP1*.

To take these intriguing results from a preclinical to a translational level, we next investigated whether or not *SGK1* and *MKP1/DUSP1* gene expression is in fact induced in association with dexamethasone treatment in human ovarian tissues. By doing a prospective proof-of-principle trial in patients with suspected ovarian cancer undergoing exploratory laparotomy for an abnormal imaging test, we show for the first time that *SGK1* and *MKP1/DUSP1* are significantly increased *in vivo* following pharmacologic dexamethasone administration. These results support the hypothesis that glucocorticoid receptor activation results in increased anti-apoptotic gene expression in patient tumors and may therefore be detrimental to chemotherapy-induced apoptosis (24). These findings also raise concern over whether or not the widespread use of dexamethasone as a glucocorticoid receptor agonist could be detrimental to the long-term outcome of a patient for whom maximal tumor kill is critical.

At the current time, dexamethasone is one of the most widely administered glucocorticoids. However, the development of selective glucocorticoid receptor modulators beyond RU486 could eventually provide an alternative to dexamethasone treatment. For example, selective glucocorticoid receptor modulators that have anti-inflammatory benefits and yet lack effects on induction of anti-apoptotic gene expression could be screened for in large-scale analyses (25). Ideally, selective glucocorticoid receptor modulators that have anti-emetic activity similar to that afforded by conventional glucocorticoids,

although not up-regulating *SGK1* and *MKP1/DUSP1*, might be selected for further clinical development. Several selective glucocorticoid receptor modulators are currently under development for anti-inflammatory and anti-emetic indications (25).

This report also illustrates the potential power of using serially collected frozen human tissues for corroborating *in vitro* experimental findings in human tumor cell lines. Although induction of the prosurvival genes was shown 30 minutes following dexamethasone, a lack of patient samples from later time points was a limiting factor in doing more extensive time course assessments in the human subjects' tissues. However, tissue samples taken from three dexamethasone-treated patients were available for analysis and showed an increase in gene expression that persisted through 4 hours (Supplementary Fig. S1). The very rapid nature of *SGK1* mRNA induction (occurring at 30 minutes in all five patient samples) was somewhat unexpected. *In vitro*, HeyA8 cells showed a similar time course of *SGK1* mRNA induction with a peak expression occurring at 1 hour. However, SKOV3 cells showed a slower induction of *SGK1* mRNA occurring only at 4 hours, with no significant increase evident before 4 hours (data not shown). The relatively rapid *in vivo* increase in gene expression following systemic administration of dexamethasone agrees with previous experiments in Sprague-Dawley rats, wherein an increase in *SGK1* mRNA was noted in kidney and colon tissue samples at 30 minutes following i.p. dexamethasone administration (26).

Results from these ovarian cancer cell line experiments also show that the anti-apoptotic effects seen with dexamethasone persisted for at least 30 hours following paclitaxel treatment. Furthermore, xenograft studies done with MDA-MB-231 breast cancer cells (27) and OV2008 ovarian cancer cell lines (2) suggested that apoptosis is diminished in tumors from mice treated with dexamethasone and then paclitaxel. Taken together, these results suggest that the up-regulation of prosurvival genes by dexamethasone in patient tissues is immediate and that the relative decrease in apoptosis of cancer cells is manifested days following chemotherapy treatment.

In this study, we were not able to directly answer the question of whether increased *SGK1* and *MKP1/DUSP1* gene expression ultimately leads to suppression of chemotherapy-

induced apoptosis in patients. Determining the physiologic relevance of gene expression changes in patients' tumors following administration of pharmacologic doses of glucocorticoids will require a prospective treatment trial with clinical outcomes. In such a trial, patients could be randomized to glucocorticoids or placebo with standard chemotherapy and response rates and duration of response determined. However, because of the much-needed anti-inflammatory effects of dexamethasone, this type of prospective clinical trial may not be feasible until appropriate selective glucocorticoid receptor modulators are available that decrease the side effects of chemotherapy without inducing anti-apoptotic genes or until new chemotherapies are developed that do not require dexamethasone premedication (e.g., nanoparticle paclitaxel). In addition, it is likely that dexamethasone and the newer selective glucocorticoid receptor modulators will have cell type-specific effects on tumor epithelium and stromal cells that will cause combinatorial effects on tumor growth.

In summary, this study shows for the first time that specific and reproducible gene expression changes occur in human ovarian tumors over time, following systemic administration of glucocorticoids; these gene expression changes were initially predicted by global gene expression studies on cultured cell lines. Given the additional preclinical evidence that induction of *SGK1* and *MKP1/DUSP1* gene expression in epithelial tumor cell lines inhibits chemotherapy-induced tumor cell apoptosis, the current results suggest that glucocorticoid-mediated anti-apoptotic signaling should be considered in the context of treating patients with epithelial cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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