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Glucocorticoid Receptor Function Suppresses Insulin-like Growth Factor 1 Activity in Human KLE Endometrial-like Cells

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Abstract. *We analysed the glucocorticoid receptor (GR) regulation on the expression of insulin-like growth factor 1 (IGF-1), type I IGF receptor (IGF-1.R), IGF-binding protein 3 (IGFBP-3), urokinase-type plasminogen activator (uPA) and uPA receptor (uPA.R) mRNA in human KLE endometrial-like cells. We documented that KLE cells express IGF-1, IGF-1.R, uPA and IGFBP-3 mRNA, however not uPA.R mRNA. Exogenous administration of dexamethasone inhibited the proliferation of KLE cells without inducing apoptosis. The inhibition of dexamethasone on KLE cell proliferation was neutralized by exogenous administration of IGF-1. Furthermore, dexamethasone suppressed the expression of IGF-1 mRNA and IGF-1.R mRNA as well as the IGF-1 bioavailability in KLE cell culture media, but it did not alter the expression of uPA mRNA and IGFBP-3 mRNA in KLE cells. Since the peritoneal fluid of women with endometriosis is known to contain IGF-1, which stimulates the proliferation and inhibits the apoptosis of endometrial-like cells, it is conceivable that GR-mediated down-regulation of IGF-1 bioavailability may be of clinical relevance for endometriosis.*

The pathogenesis of endometriosis implicates the growth of endometrial tissue outside its normal location within the uterus, producing a benign gynaecological condition frequently associated with pelvic pain and infertility. The evolution of endometriosis depends upon sex steroid hormones and growth factors, such as the insulin-like growth factors (IGFs) (1,2).

IGF-1 is present in peritoneal fluid (PF) of women with and without laparoscopic evidence of endometriosis and is co-eluted with N-terminal truncated forms of IGF-binding protein 3 (IGFBP-3) and urokinase-type plasminogen activator (uPA) in protein fractions of PF analysed by isoelectric focusing. Hence it was proposed that uPA-mediated proteolysis of IGFBP-3 increases IGF-1 bioavailability locally and generates N-terminal truncated forms of IGFBP-3 with intrinsic mitogenic activity for endometrial cells, such as human KLE endometrial-like cells (3-9).

Recently, the IGF-1/type I IGF receptor (IGF-1.R) bioregulation system was shown to inhibit the adriamycin-induced apoptosis of KLE cells, suggesting that IGF-1 can optimise the survival of endometrial-like cells grown ectopically onto pelvis peritoneum (10).

Since glucocorticoid receptor (GR) function regulates directly and/or indirectly the activity of several growth factors in various cell lines (11-13) and physiological or neoplastic tissues (14-19), we characterized the GR regulation of IGF-1, IGF-1.R, IGFBPs, uPA and uPA receptor (uPA.R) mRNA expression in KLE endometrial-like cells. Indeed, the GR function suppressed IGF-1 activity in KLE cells.

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Key Words: KLE cells, glucocorticoid receptor, insulin-like growth factor 1, type I insulin-like growth factor receptor, insulin-like growth factor-binding protein 3, urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor.

Materials and Methods

Cell culture systems. The KLE cell line was from the American Type Culture Collection (ATCC; Bethesda, MD, USA). Cells were grown in 75-cm^2 culture flasks using Dulbecco's modified Eagle medium-F12 (DMEM-F12; Gibco/BRL) containing 0-10% fetal bovine serum (FBS, Gibco, Cat# 10108-165). Cells were plated at a cell density of 5 x 10^4 cells/well in 24-well plates and grown with DMEM-F12 medium containing various concentrations of BS, depending on the experiment.

Trypan blue exclusion assays (cell counts). KLE cells were plated at a cell density of 2.5 x 10^4 cells in 24-well plates and grown with DMEM/F-12 containing 5% FBS (Gibco, Cat# 10108-165) and 100μU/mL//100μg/mL of Penicillin//Streptomycin solution (Gibco, Cat# 15070-063). Cells were then exposed to a variety of factors in a dose- and time-dependent manner and the number of living cells was counted by hematocytometer (12,13). IGF-1 activity was determined in KLE-conditioned media by ELISA assays (R&D systems) (20).

MTT proliferation assays. This colorimetric assay has been used for either proliferation or complement-mediated cytotoxicity assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a

substance that is cleaved by active mitochondria of living cells to yield a dark blue formazan product the intensity of which is proportional to the number of living cells present in the culture. The KLE cells were plated in 96-well plates 24 h prior to treatment with the appropriate drug at various concentrations and for different periods of time. After 4 doubling times of exposure to the drugs, the cells were incubated with 10% MTT (Sigma M-5655) added directly to the medium for 4 h at 37˚C. The medium was then aspirated and the cells were solubilized with the organic solvent dimethylsulfoxide (DMSO). Absorbance was determined in a VERSA max microplate reader (Molecular Devices Corporation) at 540 nm and results are presented as the percent of OD in the treated wells *versus* the controls.

Flow cytometry. Apoptosis was assessed by flow cytometry analysing DNA content in Annexin V-FITC and propidium iodide (PI)-stained cells (TACS Annexin V-FITC Apoptosis Detection Kit, Cat#TA4638, R&D Systems) with FACSCalibur (Becton Dickinson) cytometry. Following harvesting, KLE cells were washed, incubated for 15 min with Annexin V-FITC and PI and analysed by flow cytometry, as previously described (21).

Isolation of RNA and quantitative PCR. The KLE endometrial-like cell line was grown in 25-cm^2 flasks at 37˚C in 5% CO_2 in DMEM (Gibco, Cat #: 31330-038), supplemented with 10% FBS, 100 μU/ml, 100 μg/mL Penicillin/Streptomycin (Gibco Cat#: 15070-063), and incubated for 48 h. After the 48-h incubation period, the media was changed and 100 nM or 200 nM of dexamethasone was added to the experimental flasks for 24 h and 48 h. Parallel experiments using 95% ethanol as the vehicle were performed in the control flasks. After a 48-h incubation with dexamethasone or with vehicle, the cells were detached with Trypsin/EDTA solution (Gibco Cat #:25300-054) and collected by centrifugation. Total RNA from each flask was extracted using Tri-Reagent TR (MRI Cat #: TR-118), quantified and subjected to rt-PCR.

The oligonucleotide sequences of the primers, used in the PCR amplification of IGF-1, IGF-1.R, uPA, uPA.R and IGFBP3, were selected using the Primerfinder Program based on sequences obtained from the genebank and the specificity of the resulting primers was examined by a BLAST search. In order to detect changes in gene expression we performed relative quantitative reverse transcription (RQRT-PCR) analysis of total RNA using the Quantum RNA 18 S Internal Standards kit (Ambion, Austin, TX, USA; # 1716). The reverse transcriptase (rt) reaction was carried out using SuperScript II RNAse H$^-$ as suggested by the manufacturer (Invitrogen Corp. Cat. #: 18064-014). Briefly, 3.0 μg of total RNA was mixed in thin-walled tubes with 0.5 mM dNTPs (Invitrogen Corp. Cat#: 10297018), 5 μM Random Hexamer Primer (MBI Cat#:K1612) and filled to 12 μl with depc-treated ddH_2O. The reaction was then heated to 65˚C for 5 min and quick-chilled on ice water. The rt buffer containing 200 U/μl of superscript reverse transcriptase was then added and mixed. Fifty microliters of mineral oil was added to minimize evaporation and cross-contamination and the reactants were incubated at 42˚C for 50 min. The PCR mix for the amplification of IGF-1, IGF-1.R, uPA, uPA.R and IGFBP3 consisted of 0.05 units Taq Polymerase (Invitrogen Corp. Cat#: pri10342-020), 1x PCR Buffer, 200 μM of each dNTP (Invitrogen Corp. Cat#: 10297018), 1.6 mM $MgCl_2$ and 4 μL of Primer: Competimers Mix at a ratio of 2:8. The cycle parameters for the PCR of the IGF-1 and IGF-1.R target fragments were: one cycle 94˚C: 4 min, followed by 35 cycles at 94˚C: 45sec, 54˚C: 45 sec, 72˚C: 45sec and a final cycle at 72˚C for 4 min. The cycle parameters for the PCR of the uPA and IGFBP-3 fragments were: 95˚C: 6 min followed by 39 cycles at 95˚C: 45 sec, 56˚C: 45 sec, 72˚C: 30 sec and a final cycle at 72˚C for 4 min.

The levels of IGF-1, IGF-1.R, uPA, uPA.R and IGFBP-3 expressed in KLE cells were quantified relative to the level of 18 S rRNA by using the Quantum 18 S RNA Internal Standards Kit (Ambion Cat # 1617). The optimal ratio of competimer to 18 S primer was found to be 2:8 and both the IGF-1 and IGF-1.R co-amplified 18 S rRNA within a linear range between 25 and 35 cycles as did the uPA.R fragments; the uPA and IGFBP-3 required 39 cycles. The RT-PCR products were then analyzed using the Kodak EDAS 290 Electrophoresis Documentation & Analysis System Software.

Statistical analysis. Differences of group means were assessed for statistical significance using the one-tailed multiple comparison procedure of the Dunnett test only when treatments were compared with control values. When single comparisons were made, the Student's *t*-test was used. Analysis is based on triplicate determinations.

Results

GR function in KLE cells. Incubation of KLE cells with increasing concentrations of dexamethasone reduced the number of living KLE cells as detected by trypan blue exclusion (maximum inhibition=36%±2.75 of controls at 250 nM dexamethasone for 48 h; X±SE; $p<0.05$) and MMT assays (maximum inhibition= 26%±1.75 of controls at 250 nM dexamethasone; X±SE; $p<0.05$). Dexamethasone did not produce apoptosis of KLE cells, as assessed by flow cytometry (Figure 1). Therefore, dexamethasone decreases the number of living KLE cells by inhibiting KLE proliferation (Table I).

In addition, incubation of KLE cells with dexamethasone suppressed the IGF-1 content (ng/ml) of the KLE cell culture media (maximal suppression of 69%±4.25; X±SE; $p<0.05$) by 250 nM dexamethasone for 48 h, as measured by ELISA assay, while the exogenous administration of IGF-1 stimulated the proliferation of KLE cells (maximal stimulation of 38%±4.75; X±SE; $p<0.05$) (Table I). Notably, the dexamethasone inhibition of the growth of KLE cells was neutralized by exogenous administration of IGF-1 (Table I).

GR regulation of mRNA expression of the IGF-1, IGF-1.R, IGFBP-3, uPA and uPA.R in KLE cells. Exogenous administration of dexamethasone (100 nM and 250 nM) for 24 h and 48 h significantly suppressed the expression of IGF-1 mRNA in KLE cells [100 nM dexamethasone: 24 h=0.8±0.03; 48 h=0.38±0.01 and 250 nM dexamethasone: 24 h=0.72±0.01; 48 h=0.25±0.03; X±SE; $p<0.05$ *vs.* baseline] (Figure 2).

In addition, dexamethasone (100 nM and 250 nM) for 24 h and 48 h significantly suppressed the expression of IGF-1.R mRNA in KLE cells [100 nM dexamethasone: 24

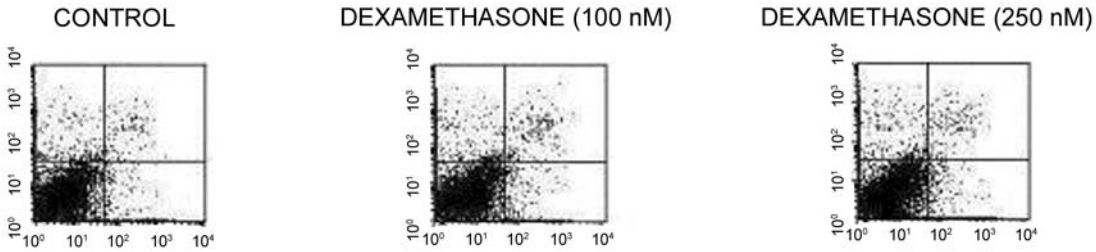

Figure 1. *Analysis of DNA content in Annexin V-FITC and propidium iodide (PI)-stained KLE cells. Administration of dexamethasone (100 nM and 250 nM for 48 h) did not produce apoptosis of KLE cells.*

Table I. *Glucocorticoid receptor function in KLE cells.*

	Percent (%) of change from baseline levels		
	Cell count	MMT assay	IGF-1 content (ng/ml)
Dexamethasone			
15.62 nM	1±1.8	-1±2.5	- 1±2.2
31.25 nM	-3±2.5	-1±4.5	-5±3.5
62.50 nM	-22±5.5*	-8±4.8	-20±4.7*
125.00 nM	-28±2.2*	-22±1.9*	-58±3.75*
250.00 nM	-36±2.75*	-26±1.75*	-69±4.25*
IGF-1			
10 ng/ml:	15±4.5*		
25 ng/ml:	38±4.75*		
Dexamethasone (100 nM) plus IGF-1 (25 ng/ml)	5±2.5		

*=$p<0.05$

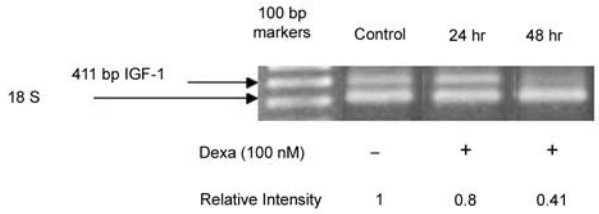

Figure 2. *Glucocorticoid receptor (GR) function down-regulates insulin-like growth factor 1 (IGF-1) mRNA expression in KLE cells. An example of the effects of dexamethasone (100 nM for 24 h and 48 h) on IGF-1 mRNA expression in KLE cells.*

h=0.83±0.02; 48 h=0.41±0.05 and 250 nM dexamethasone: 24 h: 0.76±0.02; 48 h: 0.33±0.03; X±SE; $p<0.05$ *vs.* baseline] (Figure 3).

However, dexamethasone did not affect the expression of IGFBP-3 mRNA in KLE cells [100 nM dexamethasone: 24 h=0.98±0.037; 48 h=1.02±0.06 and 250 nM dexamethasone: 24 h: 0.97±0.015; 48 h: 1.03±0.08; X±SE; $p>0.1$ *vs.* baseline] (Figure 4).

In addition, dexamethasone did not affect the uPA mRNA in KLE cells [100 nM dexamethasone: 24 h=1.02±0.05; 48 h=1.03±0.08 and 250 nM dexamethasone: 24 h: 1.06±0.07; 48 h: 1.06±0.12; X±SE; $p>0.1$ *vs.* baseline] (Figure 5).

Under our experimental conditions, we could not detect uPA.R mRNA expression in KLE cells (Figure 6).

Discussion

A growing body of evidence implicates growth factors both as autocrine/paracrine regulators of eutopic endometrial function and as mediators of ectopic growth of endometrial cells onto pelvic peritoneum (1,2,20-26). In particular, sex steroid hormones regulate IGFs expression, which in turn interact with other steroid hormone-inducible growth factors, such as epidermal growth factor (EGF), transforming growth factor beta 1 (TGFβ1) and interleukin 6 (IL-6) to regulate the growth and differentiation of human endometrial cells (1,24-26).

In addition, IGF-1 activity is present in the PF of women with and without laparoscopic evidence of endometriosis and its bioavailability is, at least partly, increased in the PF of women with laparoscopic evidence of endometriosis by uPA-mediated hydrolysis of IGFBP-3 (2,6,9). Recently, we detected a 5-fold up to 10-fold increase of uPA and IGFBP-3 mRNA expression in endometriotic lesions as compared with that of homologous eutopic endometrium, indicating that the key players of IGF-1 bioavailability in PF (uPA-orchestrated hydrolysis of IGFBP-3) are present in endometriotic lesions (27).

Herein we confirmed the presence of functional GR in KLE cells, an endometrial-like cell line successfully used in the past as an *in vitro* model for studying the response of endometrial cells to PF mitogens (5,6,8). Moreover, the GR function in KLE cells decreased IGF-1 bioactivity, both by down-regulating the expression of IGF-1 and IGF-1.R mRNA and by suppressing IGF-1 content in KLE cell culture media.

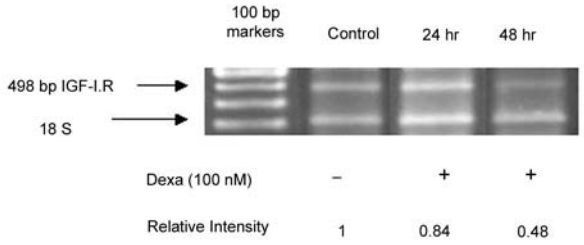

Figure 3. *Glucocorticoid receptor (GR) function down-regulated insulin-like growth factor 1 receptor (IGF-1.R) mRNA expression in KLE cells. An example of the effects of dexamethasone (100 nM for 24 h and 48 h) on IGF-1. R mRNA expression in KLE cells.*

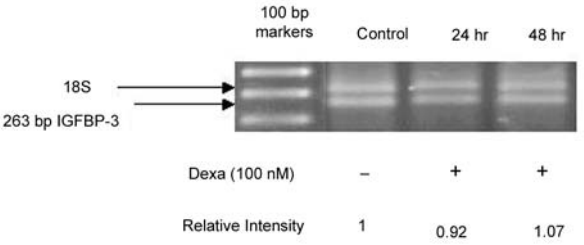

Figure 4. *Glucocorticoid receptor (GR) function did not regulate the expression of insulin-like growth factor-binding protein 3 (IGFBP-3) mRNA in KLE cells. An example of the effects of dexamethasone (100 nM for 24 h and 48 h) on IGFBP-3 mRNA in KLE cells.*

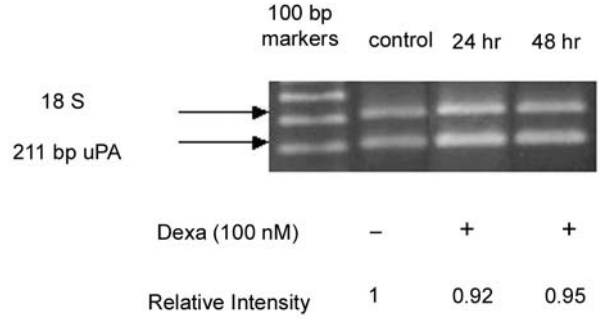

Figure 5. *Glucocorticoid receptor (GR) function did not regulate the expression of urokinase-type plasminogen activator (uPA) mRNA in KLE cells. An example of the effects of exogenous dexamethasone (100 nM for 24 h and 48 h) on uPA mRNA in KLE cells.*

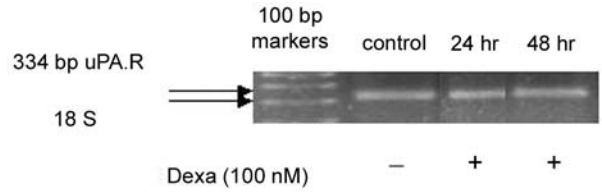

Figure 6. *Under our experimental conditions, we did not detect the expression of urokinase-type plasminogen activator receptor (uPA.R) mRNA in KLE cells.*

GR function did not alter the uPA mRNA and IGFBP-3 mRNA expression of KLE cells, therefore it is conceivable that, at least in this *in vitro* system, GR function directly regulates IGF-1 bioactivity without affecting the uPA/IGFBP-3 bioregulation system. Since uPA.R plays a pivotal role in activating pro-uPA, the absence of uPA.R expression in KLE endometrial-like cells suggests that the activation of uPA in human endometrium and endometriotic lesions is achieved by the uPA.R of stromal cells. However, lack of uPA.R may reflect just the phenotype of the KLE cell line, having no relevance to uPA.R mRNA expression of endometrial cells in eutopic and ectopic endometrium.

In the past, the role of GR in endometriosis and endometrial-like cells was indirectly assumed by studies focusing on the RU 486 actions on endometriosis (28) and on endometrial cancer cell lines, including KLE cells (29). This initial assumption for RU 486 actions on endometrial-like cells has been recently modified by the clinical evidence that long-term administration of RU 486 can produce massive endometrial hyperplasia, apparently caused by prolonged and unopposed estrogen mileu in human endometrium (30), and *in vitro* studies showing that the RU 486 inhibition of the proliferation of endometrial-like cells is mediated by its antioxidant properties rather than by its anti-hormone receptor properties (31).

Nevertheless, herein we showed that GR function suppressed IGF-1 expression and inhibited KLE cell growth. Therefore, it is conceivable that administration of dexamethasone may have a role in the treatment of endometriosis, since IGF-1 is present in PF of women with endometriosis and acts both as a stimulator of endometrial-like cell growth and as an inhibitor of apoptosis of endometrial-like cells. Hence, investigation into the potential role of GR-mediated suppression of IGF-1 bioavailability in endometriotic lesions and the PF of women with endometriosis is warranted.

Acknowledgements

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