Differential glucocorticoid effects on proliferation and invasion of human trophoblast cell lines

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Abstract

Several clinical situations require continuous glucocorticoid (GC) treatment during pregnancy. A well-known deleterious side effect of such treatment is the higher incidence of growth-restricted fetuses, for which a too shallow trophoblast invasion is presently hypothesised as the underlying cause. This study investigated whether the synthetic GC triamcinolone acetonide (TA) influences proliferation, invasion and endocrine activity of human trophoblast. BeWo and JEG-3 choriocarcinoma cell lines both express GC receptors (western blotting) and were used as models for human trophoblast. JAR devoid cells of GC receptor were used as negative control. The cells were cultured for 48 h without (control) or with 0.5, 5 and 50 μ M TA. In the presence and absence of serum, proliferation was determined by cell counting and measuring the cell cycle regulating protein cyclin B1 (Western blotting); invasion was determined by a conventional Matrigel invasion assay and by measuring the secretion (ELISA) of matrix-metalloproteinases (MMP-2, MMP-9) into the culture medium; endocrine activity was assessed by measuring the levels of human chorionic gonadotropin (ELISA) into the culture medium. TA altered the number of viable and dead cells as well as cyclin B1 levels and, to a lesser extent, invasion of BeWo and JEG-3, with a strong influence of serum. BeWo and JEG-3 cells reacted differently and in most instances reverse. In the cell lines used as models of human trophoblast, TA alter some functions relevant to proliferation and invasion, and suggest that caution should be exercised when treating women with GCs during pregnancy.

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Introduction

In late human pregnancy, glucocorticoids (GC) play an important role in the preparation for birth, including involvement in lung and cardiac development of the fetus. Antenatal short-term corticosteroid therapy results in reduction of fetal mortality, respiratory distress syndrome and intraventricular haemorrhage in preterm babies (Sloboda *et al.* 2005). These benefits extend to a broad range of gestational age.

However, several clinical situations like asthma, allografts and systemic lupus erythematosus require continuous GC treatment throughout the gestational period (Ostensen 2001). A well-known deleterious side effect of GC treatment during pregnancy is the higher incidence of growth-restricted fetuses (Reinisch *et al.* 1978, Garvey & Scott 1981, Katz *et al.* 1990, Edwards *et al.* 1993, Seckl 1994) and an intrauterine programming of cardiovascular, metabolic and neuro-endocrine disorders in adult life (Seckl 2004). We recently identified a downregulation of trophoblast

glucose transporters by GC and hypothesised that this may contribute to the restricted fetal and placental growth observed with the GC treatment (Hahn *et al.* 1999). The pathogenesis of fetal growth restriction in the absence of GC treatment is more complex and the current consensus implicates too shallow trophoblast invasion into the maternal decidua in the first trimester of pregnancy as the underlying cause. Therefore, it is tempting to hypothesise that GC may affect trophoblast invasion. Moreover, the number of invasive trophoblast is closely linked to trophoblast proliferation, which determines the number of cells available for differentiation into an invasive phenotype. GC effects on trophoblast proliferation may also explain the correlation of maternal GC levels with the placental weight (Edwards *et al.* 1993), suggesting that the placenta can respond to maternal GC *in vivo*. In fact, high affinity, low capacity GC receptors have been identified in the human placenta (Speeg & Harrison 1979, Lageson *et al.* 1983, Robinson *et al.* 1988, Hahn *et al.* 1999).

Physiological cortisol levels in maternal plasma during human pregnancy are in the range of 1 µmol/l (Simmer *et al.* 1974), which is about 5–10 times higher than those in the fetus (Edwards *et al.* 1993). The majority of the circulating cortisol in the human fetuses is derived only relatively late during gestation from the fetal adrenal gland and not from maternal sources (Pasqualini *et al.* 1970). Transplacental transfer of maternal cortisol is limited because of the protective action of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD-2) (Krozowski *et al.* 1995). This enzyme is expressed in increasing amount throughout gestation in the placenta (Schoof *et al.* 2001), and predominantly in the trophoblast (Driver *et al.* 2001) as well as in many other fetal tissues (Stewart *et al.* 1994, Brown *et al.* 1996). It catalyses the conversion of active cortisol into inert cortisone (Albiston *et al.* 1994, Stewart *et al.* 1994). The physiological role of 11β-HSD-2 is to protect the mineralocorticoid receptor from activation by cortisol, which binds to the receptor with the same affinity as aldosterone (Arriza *et al.* 1987).

Synthetic GCs such as dexamethasone, however, are significantly less metabolised by 11β-HSD-2 than cortisol (Brown *et al.* 1996, Diederich *et al.* 1997). Therefore, these synthetic GCs readily cross the placenta. It is known that prenatal GC exposure reduces the birth weight in humans (Seckl 1994), and even small doses of dexamethasone given prenatally alter neuropsychological parameters such as emotionality (Trautman *et al.* 1995, Lajic *et al.* 1998).

The study investigated whether a synthetic GC influences proliferation and invasion of human trophoblast. The choriocarcinoma cell lines BeWo and JEG-3, both expressing GC receptors, have been used. In addition, JAR cells were employed as a negative control because they lack the GC receptors. The experiments were performed both in the absence and presence of fetal calf serum (FCS) to determine if serum influences the GC effects.

The synthetic GC triamcinolone-acetonide (TA) was used. It (1) specifically binds to the GC receptor in placental cytosol (Speeg & Harrison 1979) with higher affinity than dexamethasone (Dancis *et al.* 1980), which is also used for experimental studies into GC action, (2) is not inactivated by 11β-HSD-2 (Feinstein & Schleimer 1999), and (3) has proven to be effective in altering the placental function (Hahn *et al.* 1994, 1999).

Proliferation was determined by cell counting and measuring the expression level of cyclin B1, a cell cycle regulating molecule with peak expression at the G2/M interface. It has proven to be useful in determining the trophoblast mitotic activity (Genbacev *et al.* 1997). Invasion was assessed by a conventional Matrigel invasion assay and by measuring the amount of MMP-2 and MMP-9 secreted into the culture medium. Both play a key function in the trophoblast invasion (Staun-Ram *et al.* 2004). Because antenatal dexamethasone administration is associated, among other adverse

effects, with reduced placental hormone production (reviewed by Ogueh & Johnson 2000), measurements of potential TA effects on the secretion of human chorionic-gonadotropin-β (hCG-β) were included in the study.

Material and Methods

Cell culture

BeWo (CCL-98, American Type Culture Collection (ATCC) passage number, 191), JAR (HTB-144, ATCC passage number, 722) and JEG-3 (HTB-36, ATCC passage number, 124) choriocarcinoma cells were obtained from the (ATCC, Rockville, MD, USA). All cells were used up to ten in-house passages. The JAR and JEG-3 cells were cultured in Dulbecco's modified Eagle medium (Gibco), containing 10% FCS (HyClone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco), whereas BeWo cells were cultured in F-12 medium (Gibco) supplemented with 10% FCS, 1% penicillin/streptomycin and 2 mmol/l L-Gln (Gibco).

The cells were plated in six-well plates (Falcon Multiwell Cell Culture Plates, Becton Dickinson, NJ, USA) at a density of 4×10^4 (JAR, JEG-3) and 6×10^4 cells (BeWo) per well, and cultured for 24 h with FCS under a humidified 5% CO₂/95% air atmosphere at 37 °C. After this time, cells were incubated in the respective medium supplemented with 0.5, 5 and 50 µM triamcinolone acetonide (TA) (Volon A, Bristol-Myers Squibb, Vienna, Austria) dissolved in benzylalcohol in the presence and absence of 10% FCS for 48 h. After 48 h, all cultures were still subconfluent (-FCS, ~50%, +FCS, 80–90%), but extension of the culture period to 72 h had resulted in partial confluence (pilot experiments).

Determination of hCG-β

The amount of hCG-β secreted into the culture media was measured by an enzyme-immunoassay (Behring Diagnostics, Inc., Westwood, MA, USA), with intra- and interassay coefficients of variation of 6.4 and 9.2% respectively. Assay sensitivity was 1 mIU/ml.

Determination of cell number

A direct cell counting method was used. Cells were cultured under the conditions defined above. After 48 h, cells were detached by Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA) and cell aggregates dissociated by Accumax (Innovative Cell Technologies, Inc.). The number of cells was counted in the cell counter and analyser system CASY 1 (Schärfe System, Reutlingen, Germany) using a 150-µm capillary. Measurements with this system are based on the detection of changes in conductivity along an aperture during the flow of a cell-containing liquid.

The result is a size-distribution curve of the cells (Falkenhain *et al.* 1998), in which cell debris, dead and viable cells can be identified by their distinct diameters. This method has been recently used to demonstrate the effects of hyperglycaemia on chorio-carcinoma cells (Weiss *et al.* 2001).

Immunoblotting of GC receptor and cyclin B1 protein

Cells were lysed in buffer containing 0.01 mol/l Tris (pH 7.4), 1% SDS, 1 mmol/l Na-orthovanadate and complete protease inhibitor (Roche) mixed with an equal volume of Laemmli sample buffer (Sigma). Proteins (40 µg) of adherent cells were subjected to SDS-PAGE and immunoblotting as described (Weiss *et al.* 2001). The blotting membranes were incubated (overnight, 4 °C) with either a polyclonal anti-GC receptor antibody (1:500; P-20 Santa Cruz; Santa Cruz, CA) or a monoclonal anti-cyclin B1 antibody (1:500; Pharmingen, San Diego, CA). Anti-mouse IgG (H+L) horseradish peroxidase conjugate (1:1500; Southern Biotechnology Associates, Birmingham, AL, USA) was used as the secondary antibody. The immunolabelling was visualised using the chemiluminescence-based SuperSignal CL-HRP Substrate System (Pierce, Rockford, IL, USA). The high-performance chemiluminescence film, Hyperfilm ECL (Amersham) was developed and analysed by the ONE-Dscan program (Scanalytics, Billerica, MA, USA).

Matrigel invasion assay

Details of the assay were described by Cervar *et al.* (1996). Briefly, the cells were incubated overnight in the presence of 10 µCi/ml [³H]-thymidine (Amersham) in medium containing 10% FCS. Matrigel-coated (Biocoat, Becton Dickinson, Heidelberg, Germany) porous filters (8 µm pore size) were used as a barrier in a Boyden chamber (Albini *et al.* 1987) to test invasion of the cells. A total of 5×10^4 radiolabelled cells was added to the inside of the inserts and incubated for 48 h at 37 °C, 5% CO₂ atmosphere. TA at concentrations as above were added to the medium. The invasion index represents the proportion of cells that had invaded and traversed the extracellular matrix and was calculated as the amount of radioactivity in the lower wells expressed relative to the total radioactivity in upper and lower wells (Graham *et al.* 1993).

Determination of proMMP-2 and proMMP-9 levels

ProMMP-2 and proMMP-9 levels were measured in all cell culture supernatants by ELISA (Biotrak, Amersham) following the instructions of the manufacturer. They measured the precursor of MMP-2 (proMMP-2), i.e. free proMMP-2 and that complexed with TIMP-2, but not the

active form of MMP-2 (Fujimoto *et al.* 1993) and proMMP-9 (free and complexed to TIMP 1; not active MMP-9; Fujimoto *et al.* 1994). The range of detection for proMMP-2 is 1.5–24 ng/ml (sensitivity of 0.37 ng/ml) and for proMMP-9 1–32 ng/ml (sensitivity of 0.6 ng/ml).

Statistical analysis

The data expressed as mean $\pm$ s.d. were analysed with standard methods for descriptive values and with multi-factor ANOVA to compare the size of the effect. Significant overall results were further examined with Dunnett's *post hoc* test for comparison with the control and least significant difference tests (LSD) for all other pairwise comparisons. A significance level of 0.05 was used for all the tests. For all calculations, SPSS (SPSS, Chicago, IL, USA) was used.

Results

GC receptor expression

Bewo and JEG-3 cells expressed GC receptors, as identified by western blotting, whereas JAR cells did not (Fig. 1). The single band detected in the western blots corresponded to the molecular mass of about 85 kDa, which is similar to the molecular mass of the human GC receptor (UniProt entry name, GCR_HUMAN; accession number, P04150).

Effects on secretion of hCG-β

All three cell lines secreted varying levels of hCG-β with BeWo > JAR > JEG-3 (not shown). In the absence of FCS, TA did not affect hCG-β production in any of the three cell lines. In the presence of serum, elevated ($P < 0.05$, ANOVA) hCG-β levels were found in JEG-3 with a peak increase of +147% ($P < 0.05$) at a concentration of 5 µM TA (Fig. 2).

Effects on proliferation and cyclin B1

In the absence of serum, cell counts of BeWo cells decreased ($P < 0.01$, ANOVA) when the cells were cultured with 0.5 µM TA (14%, $P < 0.05$) and 5 µM TA (20%, $P < 0.01$), whereas those of JEG-3 cells increased ($P < 0.05$, ANOVA) by about 24% ($P < 0.01$) when the cells were cultured with 50 µM TA. In the presence of FCS, the effects, if any, were reverted, i.e. TA increased the cell number in BeWo ($P < 0.05$, ANOVA) by 46% ($P < 0.001$) at 50 µM, and, reduced the cell number in JEG-3 (37%, $P < 0.01$), at 0.5 and 5 µM. The latter effect was abolished by 50 µM TA. The GC receptor-negative JAR cells were not affected by the presence of TA in FCS (Fig. 3). In the presence of FCS, the number of dead BeWo and JEG-3 cells increased in a dose-dependent manner

Figure 1 Detection of glucocorticoid receptor in cell lysates. Western blotting showed the presence of glucocorticoid receptors in BeWo and JEG-3 and its absence in JAR choriocarcinoma cells that were used as models for first trimester human trophoblast.

($P < 0.05$, ANOVA) when TA was added. This effect was not found in the absence of FCS (data not shown).

To gain insight into TA effects on the cell cycle, cyclin B1 levels were measured. In the absence of serum, cyclin B1 expression levels decreased ($P < 0.05$, ANOVA) in BeWo cells reaching a maximum of 80% at

50 μM TA ($P < 0.01$). The presence of FCS reverted this effect, i.e. TA induced an increase ($P < 0.05$, ANOVA) in cyclin B1 levels (70% at 50 μM , $P < 0.01$). In JEG-3 cells, a non-significant trend towards increasing (–FCS) or decreasing (+FCS) cyclin B1 levels was noted that paralleled the effects on the cell number. Cyclin B1 levels in JAR were not affected (ANOVA) significantly (Fig. 4).

Effects on Matrigel invasion and on secretion of proMMP-2 and proMMP-9

In the BeWo cells, 50 μM TA produced a stimulatory effect on invasion by 27 and 37% ($P < 0.05$) in the presence and absence of FCS respectively. Invasion of JEG-3 cells was reduced by 50 μM TA under both conditions by about 20%, but only in the presence of FCS the effect reached significance ($P < 0.05$). Invasion of JAR cells did not respond to TA administration (Fig. 5).

In all the three cell lines, proMMP-9 levels in the culture supernatants were below the sensitivity of the

Figure 2 Effects of TA on hCG- β secretion after 48 h in absence or presence of FCS. TA (5 μM) decreased hCG- β secretion of JAR cells by about 25% ($P < 0.01$, panel A). In the presence of FCS (panel B) TA led to more hCG- β in the culture medium of BeWo cells (+23%) and of JEG-3 (+147%) ($P < 0.05$) at a concentration of 5 μM . Data (mean $\pm$ s.d.) are expressed relative to untreated control (=100%). * $P < 0.05$; ** $P < 0.01$ vs control. Control, TA 0.5, TA 5, TA 50 μM .

Figure 3 Effects of TA on cell number after 48 h in the absence and presence of FCS. BeWo, JAR and JEG-3 cells were cultured in serum-free medium (panel A) containing 0.5, 5 and 50 μM of TA. A stimulatory effect of 50 μM TA was found in JEG-3 (–FCS) and BeWo (+FCS) cells with maximum effects of 24 ($P < 0.01$) and 46% ($P > 0.01$) respectively. The cell number decreased at the lower TA doses in BeWo (–FCS) and JEG-3 (+FCS). This effect was abolished at a concentration of 50 μM . Data represent the mean ($\pm$ s.d.) of quadruplicate samples and are expressed relative to untreated control (=100%). * $P < 0.05$, ** $P < 0.01$ vs control. Control, TA 0.5, TA 5, TA 50 μM .

Figure 4 Immunoblots of cyclin B1 after 48-h culture (top panel). Representative experiments of BeWo, JAR and JEG-3 cells incubated with TA (0.5, 5 and 50 μM) in serum-free medium are shown. Note that levels cannot be compared between cell lines. Effects of TA on cyclin B1 protein after 48 h in the absence and presence of FCS (bottom panel). Incubation with 50 μM TA without FCS (panel A) led to a decrease of cyclin B1 protein levels in BeWo cells (−80%, $P < 0.05$) and increase in JEG-3 cells (107%, $P = 0.05$). In FCS presence (panel B) cyclin B1 levels increased in BeWo cells by 70% ($P < 0.001$). Results represents mean $\pm$ S.E.M. of triplicate samples and are expressed relative to control (=100%). * $P < 0.05$, ** $P < 0.01$ vs control. Control, TA 0.5, TA 5, TA 50 μM.

assay. ProMMP-2 was detectable only in the supernatant of BeWo and JAR cells. BeWo cells produced about ninefold more proMMP-2 than JAR cells cultured in serum-free medium. In both cell lines, addition of FCS increased proMMP-2 secretion by about threefold. In the absence of FCS, the TA treatment led to an increase ($P < 0.01$, ANOVA) in proMMP-2 levels in BeWo and JAR cells (Table 1).

Summary of results

BeWo cells responded best to TA (Table 1). Although it did not alter the hCG- β secretion, it profoundly changed cell proliferation. In the absence of FCS, proliferation was reduced, whereas cell number and cyclin B1 levels increased in the presence of FCS, as reflected. Invasion was only stimulated by 50 μM TA, regardless of the absence or presence of serum. TA only increased the proMMP-2 secretion in the absence FCS.

JEG-3 cells responded better in the presence of FCS than in its absence. TA increased hCG levels only in the presence of FCS. The cell number was higher after TA treatment, which was paralleled by a tendency to higher cyclin B1 expression levels (not significant) in the absence of FCS. Upon addition of FCS, these effects were reversed. Invasion was reduced under both conditions, but was significant only in the presence of FCS.

JAR cells, which lack the GC receptor, did not respond to TA treatment, apart from the increase in proMMP-2 levels in the absence of FCS.

Discussion

The effects of GC treatment on the fetus have received considerable interest (reviewed Seckl 1997), but less is

known about the effects of GC on human placenta and trophoblast cells. The aim of this study was to investigate the effects of TA, a synthetic GC, on proliferation and invasion of three choriocarcinoma cell lines. BeWo, JEG-3 and JAR are models of trophoblasts, which secrete the major placental peptide hormone hCG (Hochberger *et al.* 1991, Taylor *et al.* 1991, Mausitz *et al.* 2000). They differ in their proliferative activity and degree of differentiation. All three cell lines show invasive behaviour in invasion assays (Graham *et al.* 1994, Grummer *et al.* 1994).

GC and mineralocorticoid receptors (Driver *et al.* 2001) have been identified in the human placenta, in particular in the trophoblast (Lageson *et al.* 1983, Driver *et al.* 2001). Because these choriocarcinoma cells are well known for their potential of phenotypic drifts during passage in culture, it was necessary to confirm earlier data (Speeg & Harrison 1979) using a different technique. Two of the choriocarcinoma cell lines (BeWo and JEG) express GC receptor, whereas no detectable levels of GC receptor were identified in the JAR cells. Moreover, 11 β -HSD-2 activity (Gomez-Sanchez *et al.* 1996) and functional mineralocorticoid receptors (Listwak *et al.* 1996) were identified in JEG-3 cells. JAR cells were used as negative control in this study to identify a potential involvement of the mineralocorticoid receptor in GC effects, because GCs can also transactivate this receptor (Grossmann *et al.* 2004). The absence of major GC effects on JAR cells is in line with the absence of GC receptors in this particular choriocarcinoma cell line. The TA-induced proMMP-2 secretion may be the result of mineralocorticoid receptor activation, although conclusive information is pending.

Figure 5 Effect of TA on invasion into Matrigel after 48 h in the absence and presence of FCS. TA stimulated invasion in a dose-dependent manner in BeWo cells ($P < 0.05$, panel A), the decreasing effect on JEG-3 cells was not significant. In FCS presence (panel B) 50 μM TA increased invasion of BeWo cells (27%, $P < 0.01$) and decreased invasion of JEG-3 cells (23%, $P < 0.05$). Data represent the invasion index (mean $\pm$ s.d.) of quadruplicate wells. * $P < 0.05$, ** $P < 0.01$ vs control. Control, TA 0.5, TA 5, TA 50 μM .

The TA doses were chosen because they have previously resulted in dose-dependent effects on term trophoblast hCG- β and GLUT1 levels (Hahn *et al.* 1999). The lowest TA dose (0.5 μM) corresponds to the TA concentration in blood resulting after injection of 40 mg TA, a dose recommended by the manufacturer for therapy in humans (Mollmann *et al.* 1986).

GC treatment stimulated the hCG secretion of human placental trophoblasts (Ringler *et al.* 1989, Hahn *et al.* 1999) and a transcriptional activation of hCG- β gene by GC was suggested. In JEG-3 cells TA also stimulated hCG- β secretion in FCS presence. The effects were significant at a concentration of 5 μM and became smaller, hence insignificant, upon addition of 50 μM TA. It was reported that dexamethasone up-regulated blood cell GR-mRNA levels at a concentration of 10 nM, whereas incubation with 10 μM resulted in toxic effects on blood cells with a decreased amount of total mRNA samples (Vedder *et al.* 1999). This could be an explanation for the bimodal effect of TA not only on hCG- β secretion, but also on proliferation and proMMP-2 secretion, since TA increased the number of dead JEG-3 cells.

Cell proliferation was determined by direct cell counting. The classical thymidine incorporation method was not a suitable option here, because thymidine kinase, the rate-limiting enzyme, is regulated by GCs (Osborne *et al.* 1979). TA effects on proliferation of BeWo and JEG-3 cells were converse and showed a strong influence of serum. In both cell lines the direction of the effects was reversed upon addition of serum, i.e. a TA-induced stimulation become an inhibition and vice versa. In BeWo cells these changes were paralleled by changes in cyclin B1 expression levels. Moreover, when BeWo and JEG-3 cells were cultured in FCS presence the number of dead cells increased suggesting some toxic effect of TA, which can also induce apoptosis (Schmidt *et al.* 2004). The parallel increase in the number of both viable and dead cells in BeWo may be attributable to the polyclonic nature of the carcinoma cells, and hence, reflect a different response of different BeWo subclones. In FCS absence the opposite effect was observed.

Cyclins and cyclin-dependent kinases (cdks) are subunits of cell cycle dependent protein kinases that regulate key events during the progression of the cell cycle (reviewed in Sanchez & Dynlacht 2005). Cyclins are selectively expressed in different phases of the cell cycle. Cyclin B1 is a mitotic cyclin, its expression is normally low in G_0/G_1 , increases in S and is maximal during G_2/M . Cyclin B1 is rapidly degraded at the end of mitosis, and is required for cells to enter and exit mitosis. In JEG-3 cells changes in cell count and cyclin B1 levels did not correlate. A reason could be that cyclin B1

Table 1 ProMMP-2 (ng/ 10^6 cells; mean $\pm$ s.d.) levels in culture medium of BeWo and JAR cells after 48-h incubation without (control) or with TA.

		BeWoGC-R+		JARGC-R-	
TA concentration (μM)		-FCS	+FCS	-FCS	+FCS
Control		414 $\pm$ 16	1164 $\pm$ 139†	47 $\pm$ 2	154 $\pm$ 9†
TA	0.5 (μM)	592 $\pm$ 51*	1229 $\pm$ 67†	55 $\pm$ 3*	136 $\pm$ 23†
	5 (μM)	682 $\pm$ 50*	1601 $\pm$ 184*†	53 $\pm$ 0*	135 $\pm$ 14†
	50 (μM)	958 $\pm$ 59*	1110 $\pm$ 58†	82 $\pm$ 1*	133 $\pm$ 14†

* $P < 0.01$ vs control. † $P < 0.01$ vs FCS.

changes precede changes in cell number, which would have been identified by a time course analysis over a longer culture period. Alternatively, a block at the G₀/G₁ transition does not allow cells to complete the cell cycle (JEG-3; +FCS). Without further detailed measurements of other cell cycle regulating proteins and a cell cycle analysis the underlying mechanism cannot be identified.

All three cell lines invade into extracellular matrix (Graham *et al.* 1994, Grummer *et al.* 1994, present study), because of their ability to secrete matrix metalloproteinases (Fisher *et al.* 1989, Bischof *et al.* 1995). Among these, MMP-2 and MMP-9 are involved in trophoblast invasion into the extracellular matrix of maternal decidua.

BeWo and JEG-3 cell invasion responded differently to TA treatment; in BeWo the highest TA dose induced invasion, whereas reduced it in JEG-3 cells similar to cytotrophoblasts (Librach *et al.* 1994, Morrish *et al.* 1998). Synthetic GCs inhibit MMP production (elastase, collagenase, stromelysin), either directly by affecting transcription (Werb 1978, Frisch & Ruley 1987), or indirectly by inhibiting interleukin-1 production (Butler *et al.* 1989). Interleukin-1 β is a strong regulator of invasion. Its secretion is reduced upon GC treatment (Librach *et al.* 1994). It is unknown, however, if it is involved in TA-effect on invasion in JEG-3 cells.

The absence of correlation of TA effects on the invasion and those on proMMP-2 and proMMP-9 expression is in line with the complexity of mechanisms governing trophoblast invasion and may reflect GC effects on other invasion regulating systems such as the plasminogen activator inhibitor (Ma *et al.* 2002). Moreover, activity of MMP-2 is regulated by several mechanisms, including gene expression, modifications of the balance between inactive pro-enzyme and active enzyme and by inhibition of MMP-2 activity by naturally occurring tissue inhibitors of metalloproteinases (for review, Mauviel 1993, Ries & Petrides 1995, Yu *et al.* 1996).

The experiments were carried out in the presence and absence of 10% serum. Both culture conditions allow studying the effect of external stimuli on the cells at different phases of the cell cycle. Whereas in the absence of FCS, cells become arrested in G₀, presence of serum maintains the cells in the cell cycle. Serum completely changed TA effects on hCG- and MMP-2-secretion and on proliferation, but not on invasion. This suggests that TA effects on invasion are elicited through cell-signalling pathways that are not altered by FCS or some of its components such as cytokines and immune mediators. These can modulate GC-induced activity of the GC receptor (Arzt *et al.* 2000).

TA is not a substrate for 11 β -HSD-2, and thus can bind to GC and mineralocorticoid receptor. The differences in receptor expression levels between BeWo and JEG-3 cells (Speeg & Harrison, 1979) may explain quantitative differences among the cell lines, but are unlikely to account for the opposite reactions. The actions of steroid

hormones not only depends on the presence of hormone receptors in the cytosol/nucleus and of hormone response elements on the promoter of target genes, but also requires the recruitment and coordinated action of co-activators/co-repressors (Katzenellenbogen *et al.* 1996). Differential endowment with these factors may be one of the reasons for the differential response of BeWo and JEG-3 to TA. Alternatively, the response of the cells on GC may also depend on their differentiation state. BeWo choriocarcinoma cells are regarded as poorly differentiated ones, whereas JEG-3 have a higher degree of differentiation, i.e. they contain several syncytial elements (Babalola *et al.* 1990, Aplin *et al.* 1992, Mitchell *et al.* 1995). Term trophoblasts that also contain a proportion of multi-nucleated syncytia *in vitro* responded differently to the GC than mononucleated first trimester cytotrophoblasts (Guller *et al.* 1994).

Collectively, TA, a synthetic GC, is able to influence proliferation and, to a lesser extent, invasion of choriocarcinoma cells that serve as models for human trophoblast. Although the effects induced by the GC depend on the cell line and on the presence of FCS, these data suggest that GC treatment *in vivo* may have the potential to adversely affect trophoblast development and differentiation. Therefore, we recommend that care must be exercised when women are given synthetic GCs during gestation. Further, investigations with trophoblast cells from different periods of gestation are necessary to identify how GC treatment affects the placenta *in vivo*. Our study is a first step to explore physiological processes during placental and trophoblast development that are influenced by GCs.

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References

- Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kotzowski JM & McEwan RNA 1987 Rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Research* **47** 3239–3245.
- Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS 1994 Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular Cellular Endocrinology* **105** R11–R17.
- Aplin JD, Sattar A & Mould AP 1992 Variant choriocarcinoma (BeWo) cells that differ in adhesion and migration on fibronectin display conserved patterns of integrin expression. *Journal of Cell Science* **103** 435–444.
- Arriza JL, Weinberger C, Cerelli G, Glazer TM, Handelin BL, Houseman DE & Evans RM 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237** 268–275.

- Arzt E, Kovalovsky D, Igaz LM, Costas M, Plazas P, Refojo D, Paez-Pareda M, Reul JM, Stalla G & Holsboer F 2000 Functional cross-talk among cytokines T-cell receptor and glucocorticoid receptor transcriptional activity and action. *Annals of the New York Academy of Sciences* **917** 672–677.
- Babalola GO, Coutifaris C, Soto EA, Kliman HJ, Shuman H & Strauss JF 3rd 1990 Aggregation of dispersed human cytotrophoblastic cells: Lessons relevant to the morphogenesis of the placenta. *Developmental Biology* **137** 100–108.
- Bischof P, Martelli M, Campana A, Itoh Y, Ogata Y & Nagase H 1995 Importance of metalloproteinases (MMP) in human trophoblast invasion. *Early Pregnancy Biology and Medicine* **1** 263–269.
- Brown RW, Chapman KE, Kotelevtsev Y, Yau JL, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ *et al.* 1996 Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2. *Biochemical Journal* **313** 1007–1017.
- Butler LD, Layman NK, Riedl PE, Cain RL, Shellhaas J, Evans GF & Zuckerman SH 1989 Neuroendocrine regulation of *in vivo* cytokine production and effects: I *in vivo* regulatory networks involving the neuroendocrine system interleukin-1 and tumor necrosis factor- α . *Neuroimmunology* **24** 143–153.
- Cervar M, Puerstner P, Kainer F & Desoye G 1996 Endothelin-1 stimulates the proliferation and invasion of first trimester trophoblastic cells *in vitro*—a possible role in the etiology of pre-eclampsia? *Journal of Investigative Medicine* **44** 447–453.
- Dancis J, Jansen V & Levitz M 1980 Placental transfer of steroids: effect of binding to serum albumin and to placenta. *American Journal of Physiology* **238** E208–E213.
- Diederich S, Hanke B, Oelkers W & Bahr V 1997 Metabolism of dexamethasone in the human kidney: nicotinamide adenine dinucleotide-dependent 11 β -reduction. *Journal of Clinical Endocrinology and Metabolism* **82** 1598–1602.
- Driver PM, Kilby MD, Bujalska I, Walker EA, Hewison M & Stewart PM 2001 Expression of 11 β -hydroxysteroid dehydrogenase isozymes and corticosteroid hormone receptors in primary cultures of human trophoblast and placental bed biopsies. *Molecular Human Reproduction* **7** 357–363.
- Dupont E, Rheaume E, Simard J, Luu-The V, Labrie F & Pelletier G 1991 Ontogenesis of 3 β -hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase in the rat adrenal as revealed by immunocytochemistry and *in situ* hybridisation. *Endocrinology* **129** 2687–2692.
- Edwards CR, Benediktsson R, Lindsay RS & Seckl JR 1993 Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* **341** 355–357.
- Falkenhain A, Loren TH, Behrendt U & Lehmann L 1998 *Dead cell estimation – a comparison of different methods, New Developments and New Applications in Animal Cell Technology*.
- Feinstein MB & Schleimer RP 1999 Regulation of the action of hydrocortisone in airway epithelial cells by 11 β -hydroxysteroid dehydrogenase. *American Journal of Respiratory Cell Molecular Biology* **21** 403–408.
- Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang GY, Tarpey J & Damsky CH 1989 Adhesive and degradative properties of human placental cytotrophoblast cells *in vitro*. *Journal of Cell Biology* **109** 891–902.
- Frisch SM & Ruley HE 1987 Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. *Journal of Biological Chemistry* **262** 16300–16304.
- Fujimoto N, Hosokawa N, Iwata K, Shinya T, Okada Y & Hayakawa T 1994 A one-step sandwich enzyme immunoassay for inactive precursor and complexed forms of human matrix metalloproteinase 9 (92 kDa gelatinase/type IV collagenase gelatinase B) using monoclonal antibodies. *Clinica Chimica Acta* **231** 79–88.
- Fujimoto N, Mouri N, Iwata K, Ohuchi E, Okada Y & Hayakawa T 1993 A one-step sandwich enzyme immunoassay for human matrix metalloproteinase 2 (72-kDa gelatinase/type IV collagenase) using monoclonal antibodies. *Clinica Chimica Acta* **221** 91–103.
- Garvey D & Scott J 1981 Placental and fetal contraindications of dexamethasone administration to pregnant rats. *Experientia* **37** 757–759.
- Genbacev O, Zhou Y, Ludlow JW & Fisher SJ 1997 Regulation of human placental development by oxygen tension. *Science* **277** 1669–1672.
- Gomez-Sanchez EP, Cox D, Foecking M, Ganjam V & Gomez-Sanchez CE 1996 11 β -hydroxysteroid dehydrogenases of the choriocarcinoma cell line JEG-3 and their inhibition by glycyrrhetic acid and other natural substances. *Steroids* **61** 110–115.
- Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG & Lala PK 1994 Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor- β . *Experimental Cell Research* **214** 93–99.
- Graham CH, Hawley TS, Hawley RG, Mac Dougall JR, Kerbel RS, Khoo N & Lala PK 1993 Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental Cell Research* **206** 204–211.
- Grossmann C, Scholz T, Rochel M, Bumke-Vogt C, Oelkers W, Pfeiffer AF, Diederich S & Bahr V 2004 Transactivation via the human glucocorticoid and mineralocorticoid receptor by therapeutically used steroids in CV-1 cells: a comparison of their glucocorticoid and mineralocorticoid properties. *European Journal of Endocrinology* **151** 397–406.
- Grummer R, Hohn HP, Mareel MM & Denker HW 1994 Adhesion and invasion of three human choriocarcinoma cell lines into human endometrium in a three-dimensional organ culture system. *Placenta* **15** 411–429.
- Guller S, Markiewicz L, Wozniak R, Burnham JM, Wang EY, Kaplan P & Lockwood CJ 1994 Developmental regulation of glucocorticoid-mediated effects on extracellular matrix protein expression in the human placenta. *Endocrinology* **134** 2064–2071.
- Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JM, Holsboer F, Dohr G & Desoye G 1999 Placental glucose transporter expression is regulated by glucocorticoids. *Journal of Clinical Endocrinology and Metabolism* **84** 1445–1452.
- Hahn T, Graf R, Oney T & Desoye G 1994 Effects of the synthetic glucocorticoid triamcinolone acetonide on vasoactive hydrolases of the human placenta *in vitro*. *Placenta* **15** 377–388.
- Hochberger A, Sibley C, Pixley M, Sadovsky Y, Strauss B & Boime I 1991 Choriocarcinoma cells increase the number of differentiating human cytotrophoblasts through an *in vitro* interaction. *Journal of Biological Chemistry* **266** 8517–8522.
- Katz VL, Thorp JM Jr & Bowes WA Jr 1990 Severe symmetric intrauterine growth retardation associated with the topical use of triamcinolone. *American Journal of Obstetrics and Gynecology* **162** 396–397.
- Katzenellenbogen JA, O'Malley BW & Katzenellenbogen BS 1996 Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Molecular Endocrinology* **10** 119–131.
- Krozowski Z, McGuire JA, Stein-Oakley AN, Dowling J, Smith RE & Andrews RK 1995 Immunohistochemical localization of the 11 β -hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. *Journal of Clinical Endocrinology and Metabolism* **80** 2203–2209.
- Lageson JM, Spelsberg TC & Coulam CB 1983 Glucocorticoid receptor in human placenta: studies of concentration and functional differences of preterm and term tissue. *American Journal of Obstetrics and Gynecology* **145** 515–523.
- Lajic S, Wedell A, Bui TH, Ritzen EM & Holst M 1998 Long-term somatic follow-up of prenatally treated children with congenital adrenal hyperplasia. *Journal of Clinical Endocrinology and Metabolism* **83** 3872–3880.
- Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y, Quigley JP, French DL & Fisher SJ 1994 Interleukin-1 β regulates human cytotrophoblast metalloproteinase activity and invasion *in vitro*. *Journal of Biological Chemistry* **269** 17125–17131.

- Listwak SJ, Gold PW & Whitfield HJ Jr 1996 The human mineralocorticoid receptor gene promoter: its structure and expression. *Journal of Steroid Biochemistry and Molecular Biology* **58** 495–506.
- Ma Y, Ryu JS, Dulay A, Segal M & Guller S 2002 Regulation of plasminogen activator inhibitor (PAI)-1 expression in a human trophoblast cell line by glucocorticoid (GC) and transforming growth factor (TGF)-beta. *Placenta* **23** 727–734.
- Mauschitz R, Cervar M, Hahn T, Purstner P & Desoye G 2000 Self-regulation of the endothelin receptor system in choriocarcinoma cells. *Biochimica Biophysica Acta* **1502** 224–234.
- Mauviel A 1993 Cytokine regulation of metalloproteinase gene expression. *Journal of Cell Biochemistry* **53** 288–295.
- Mitchell AM, Yap AS, Payne EJ, Manley SW & Mortimer RH 1995 Characterization of cell polarity and epithelial junctions in the choriocarcinoma cell line JAR. *Placenta* **16** 31–39.
- Mollmann HW, Barth J, Haack D, Gruener A, Stroband D, Gyselby G, Rohdewald P & Derendorf H 1986 Comparative studies on the pharmacokinetics and duration of effect of intra-articular application of glucocorticoid crystal suspensions. *Acta Rheumatologica* **11** 55–60.
- Morrish DW, Dakour J & Li H 1998 Functional regulation of human trophoblast differentiation. *Journal of Reproductive Immunology* **39** 179–195.
- Osborne CK, Monaco ME, Kahn CR, Huff K, Bronzert D & Lippman ME 1979 Direct inhibition of growth and antagonism of insulin action by glucocorticoids in human breast cancer cells in culture. *Cancer Research* **39** 2422–2428.
- Ostensen M 2001 Drugs in pregnancy. Rheumatological disorders. *Best Practice and Research Clinical Obstetrics and Gynaecology* **15** 953–969.
- Pasqualini JR, Nguyen BL, Uhrich F, Wiquist N & Diczfalusi E 1970 Cortisol and cortisone metabolism in the human foeto-placental unit at midgestation. *Journal of Steroid Biochemistry* **1** 209–219.
- Reinisch JM, Simon NG, Karow WG & Gandelman R 1978 Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science* **202** 436–438.
- Ries C & Petrides PE 1995 Cytokine regulation of matrix metalloproteinase activity and its regulatory dysfunction in disease. *Biological Chemistry Hoppe Seyler* **376** 345–355.
- Ringler GE, Kallen CB & Strauss JF 1989 Regulation of human trophoblast function by glucocorticoids: dexamethasone promotes increased secretion of chorionic gonadotropin. *Endocrinology* **124** 1625–1631.
- Robinson BG, Emanuel RL, Frim DM & Majzoub JA 1988 Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta. *PNAS* **85** 5244–5248.
- Sanchez I & Dynlacht BD 2005 New insights into cyclins, CDKs and cell cycle control. *Seminars in Cell & Developmental Biology* **16** 311–321.
- Schmidt S, Rainer J, Ploner C, Presul E, Riml S & Kofler R 2004 Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. *Cell Death and Differentiation* **11** (Supplement 1) S45–S55.
- Schoof E, Girstl M, Frobenius W, Kirschbaum M, Repp R, Knerr I, Rascher W & Dotsch J 2001 Course of placental 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase mRNA expression during human gestation. *European Journal of Endocrinology* **145** 187–192.
- Seckl JR 1994 Glucocorticoids and small babies. *Quarterly Journal of Medicine* **87** 259–262.
- Seckl JR 1997 Glucocorticoids fetoplacental 11 beta-hydroxysteroid dehydrogenase type 2 and the early life origins of adult disease. *Steroids* **62** 89–94.
- Seckl JR 2004 Prenatal glucocorticoids and long-term programming. *European Journal of Endocrinology* **151** (Suppl 3) U49–U62.
- Simmer HH, Tulchinsky D, Gold EM, Frankland M, Greipel M & Gold AS 1974 On the regulation of estrogen production by cortisol and ACTH in human pregnancy at term. *American Journal of Obstetrics and Gynecology* **119** 283–296.
- Sloboda DM, Challis JR, Moss TJ & Newnham JP 2005 Synthetic glucocorticoids: antenatal administration and long-term implications. *Current Pharmaceutical Design* **11** 1459–1472.
- Speeg KV Jr & Harrison RW 1979 The ontogeny of the human placental glucocorticoid receptor and inducibility of heat-stable alkaline phosphatase. *Endocrinology* **104** 1364–1368.
- Staun-Ram E, Goldman S, Gabarin D & Shalev E 2004 Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reproductive Biology and Endocrinology* **2** 59.
- Stewart PM, Murry BA & Mason JI 1994 Human kidney 11 beta-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform. *Journal of Clinical Endocrinology and Metabolism* **79** 480–484.
- Taylor RN, Newman ED & Chen S 1991 Forskolin and methotrexate induce an intermediate trophoblast phenotype in cultured human choriocarcinoma cells. *American Journal of Obstetrics and Gynecology* **164** 204–210.
- Trautman PD, Meyer-Bahlburg HF, Postelnik J & New MI 1995 Effects of early prenatal dexamethasone on the cognitive and behavioral development of young children: results of a pilot study. *Psychoneuroendocrinology* **20** 439–449.
- Weiss U, Cervar M, Puerstner P, Schmut O, Haas J, Mauschitz R, Arian G & Desoy G 2001 Hyperglycaemia *in vitro* alters the proliferation and mitochondrial activity of the choriocarcinoma cell lines BeWo, JAR and JEG-3 as models for human first-trimester trophoblast. *Diabetologia* **44** 209–219.
- Werb Z 1978 Biochemical actions of glucocorticoids on macrophages in culture. Specific inhibition of elastase, collagenase and plasminogen activator secretion and effects on other metabolic functions. *Journal of Experimental Medicine* **147** 1695–1712.
- Yu AE, Hewitt RE, Kleiner DE & Stetler-Stevenson WG 1996 Molecular regulation of cellular invasion-role of gelatinase A and TIMP-2. *Biochemistry and Cellular Biology* **74** 823–831.

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