Arch. Biol. Sci., Belgrade, 60 (1), 11-23, 2008 DOI:10.2298/ABS0801011B

STEROID HORMONES MODULATE GALECTIN-1 IN THE TROPHOBLAST HTR-8/SVNEO CELL LINE

ŽANKA BOJIĆ-TRBOJEVIĆ, MILICA BOŽIĆ, and LJILJANA VIĆOVAC

Department of Reproductive Biology, Institute for the Application of Nuclear Energy - INEP, University of Belgrade, 11080 Belgrade-Zemun, Serbia

Abstract — The effects of steroids on galectin-1 (gal-1) were studied in HTR-8/SVneo cells by immunocytochemistry, cell-based ELISA, the MTT proliferation test and the Matrigel™ invasion test. Dexamethasone (DEX), progesterone (PRG), and mifepristone (RU486) were used. Gal-1 was modulated in a steroid- and dose-dependent manner by DEX, which mildly but significantly stimulated production at low concentrations (0.1-10 nM), and inhibited it at 100 nM, while the effects of PRG and RU486 were opposite. HTR-8/SVneo cell invasion of Matrigel was significantly decreased in the presence of DEX and lactose. The obtained data support the proposed regulatory role of steroids in trophoblast gal-1 production.

Key words: Dexamethasone, galectin-1, HTR-8/SVneo, steroids, trophoblast

UDC 618.46:577.175.6:576

INTRODUCTION

Adequate extravillous trophoblast invasion is a crucial step in placental formation and for pregnancy outcome. It has been established that expression of a suitable repertoire of integrins, membrane glycoproteins involved in interactions with the extracellular matrix (ECM), is critical for differentiation into the invasive trophoblast (Aplin et al., 1999; Hohn and Denker, 2002). Galectins are β-galactoside-specific lectins associated with components of the ECM and counter receptors on the cell surface of mammalian cells (Janković and Čuperlović, 1996; Liu and Rabinovich, 2005). In other systems galectins have been shown to activate various cell types through cross-linkage of cell surface glycoproteins and to modulate cell adhesion and migration (Rabinovich, 1999). Prior to implantation, galectin-1 (gal-1) is synthesized in the trophectoderm of expanded blastocysts, suggesting a role in the attachment of the embryo to the uterine epithelium (Poirier et al., 1992). Gal-1 and gal-3 have, however, been found dispensable for survival and fertility of double mutant mice (Colnot et al., 1998). Gal-1 has been proposed to act as an optimizing molecule and is not required for absolute function, but for the most efficient function (Poirier, 2002). In humans, immunolocalization of gal-1 in the placental bed showed that it is expressed in the middle and distal cell columns of the anchoring villi giving rise to the extravillous trophoblast, but not in the fully invasive interstitially migrating cytotrophoblast (Bevan et al., 1994; Maqui et al., 1997; Vićovac et al., 1998).

The human placenta is a steroid- and glucocorticoid-responsive organ (Guller et al., 1993 a; Chan et al., 2003). The steroid hormones estrogen and progesterone (PRG) play important roles in preparation of the uterus for implantation and during early phases of pregnancy (Chan et al., 2003). Although elevated levels of glucocorticoids (GC) are also found in both maternal and fetal sera (Dorr et al., 1989), there are several clinical situations that require continuous GC treatment during pregnancy (Ostensen, 2001). While their precise role in pregnancy is not well defined, it has been postulated that GC are involved in uteroplacental adherence (Guller et al., 1993), fetal development and maturation (Ballard and Ballard, 1995), modulation

of inflammation in the placenta (Rosen et al., 1998) and chronic regulation of placental protein expression (Ryu et al., 1999). Steroid control of ECM protein expression in the human placenta has been studied extensively (Guller et al., 1993 a; Guller et al., 1995; Ryu et al., 1999). Thus, the synthetic GC dexamethasone (DEX) reduces expression of oncofetal fibronectin (onfFN), a major ECM protein synthesized by the cytotrophoblast, and laminin (LN) (Guller et al., 1993 a; Yoon et al., 1998). These two ECM glycoproteins have been identified as physiological ligands for gal-1 (Sato and Hughes, 1992; Ozeki et al., 1995). Gal-1 participates in several biological processes, including growth, differentiation and cancer metastasis (Hughes, 2001). The possible involvement of steroids in the regulation of expression of gal-1 in the human placenta has not been studied previously. However, treatment with DEX during postnatal rat lung development was found critical in regulation of gal-1 at the level of transcription, translation, and degradation (Clerch et al., 1987; Sandford et al., 1993). A putative steroid-binding site located at -210 is present in the gal-1 gene and regulation of transcription may be attributed to this (Gitt and Barondes, 1991). The present study was intended to investigate whether DEX affects gal-1 protein in a trophoblast cell line, human immortalized extravillous trophoblast cells, HTR-8/SVneo. To establish specificity of the effect of DEX, the synthetic steroid mifepristone (RU486), which acts as an antagonist of the glucocorticoid receptor (GR) but also exerts antiprogesterone action, and PRG were included in the study. We present evidence that steroids influence the production of gal-1 in the HTR-8/SVneo cell line, thus complementing other data that show coordinated down regulation by a synthetic glucocorticoid, DEX, of several molecular species relevant for cell migration and invasion, including ECM proteins and integrins. We also report that the process of Matrigel™ invasion *in vitro* by the first-trimester trophoblast cell line HTR-8/SVneo is sensitive to DEX and lactose, the inhibitory sugar for lectin-type interaction of galectins.

MATERIALS AND METHODS

Materials

Thiazolyl blue (MTT), bovine serum albumin (BSA), dexamethasone (DEX), antiglucocorticoid mifepristone (RU486), progesterone (PRG), gentamycin and lactose were from the Sigma Chemical Co. (USA). RPMI 1640 with and without phenol red were purchased from AppliChem GmbH (BioChemica, Germany). Fetal calf serum (FCS) was supplied by PAA Laboratories (Linz, Austria). Trypsin solution was obtained from the Institute of Immunology and Virology (Belgrade, Serbia). Horseradish peroxidase (HRP) and 3, 3', 5, 5' tetramethyl benzidine (TMB) were from ICN Biochemicals (Aurora, OH, USA). The Vectastain Elite ABC kit and 3, 3' diaminobenzidine (DAB) kit were from Vector Laboratories (Burlingame, CA, USA). Growth factor reduced Matrigel™ and laboratory plasticware were obtained from BD Biosciences (Becton Dickinson, USA), Costar (Corning Incorporated, NY, USA), and Sarstedt (Germany). All other chemicals were p. a. grade.

Antibodies

Antiserum to human placental gal-1 (pgal-1) was produced in our laboratory as described earlier (Čuperlović et al., 1995; Vićovac et al., 1998).

Monoclonal antibody against galectin-3 (mgal-3) was a gift from Dr. Margaret E. Huflejt (La Jolla Institute for Allergy and Immunology, San Diego, CA, USA). Monoclonal antibody to cytokeratin-18 was from DakoCytomation (Denmark). Monoclonal antibody against β1 integrin subunit was from Santa Cruz (CA).

Both biotinylated goat anti-rabbit IgG and biotinylated horse anti-mouse IgG were from Vector laboratories and used at 1/200. The Alexa Fluor® 488 F(ab')2 fragment of goat anti-mouse IgG (H+L) secondary antibody was from Molecular Probes and used at 1/1000.

Sheep anti-rabbit IgG-HRP conjugates were produced in our laboratory according to the method of Nakane and Kawaoi (1974).

Cell cultures

The HTR-8/SVneo trophoblast cell line was kindly provided by Dr Charles H. Graham (Queen's

University, Kingston, ON, Canada). This cell line was established from extravillous cells derived from human first trimester placenta immortalized by SV40 large T antigen (Graham et al., 1993; Irving et al., 1995). These cells exhibit a high proliferation index and share various phenotypic similarities with the parental HTR-8 cells, including *in vitro* invasive abilities. The cells were cultured in RPMI 1640 supplemented with 10% FCS and gentamycin under 5% CO_2 and 95% air at 37°C if not specified otherwise.

Cells were grown in tissue culture flasks for propagation; in 96-well plates to study the effects of DEX, RU486, and PRG; on glass coverslips for immunocytochemistry or in Boyden chambers for invasion assay.

Immunocytochemistry

HTR-8/SVneo cells ($2x10^5$) were cultured on glass coverslips in RPMI 1640 medium. Cells were rinsed with phosphate-buffered saline (PBS 0.05 M, pH 7.2), air-dried, and fixed with 4% paraformaldehyde-0.05% Triton X-100 for 30 min at room temperature (RT). Endogenous peroxidase activity was blocked with 0.3% H_2O_2. Nonspecific binding was reduced using a protein block (Vectastain Elite ABC kit) for 20 min. Cells were further incubated with pgal-1 (1/400 dilution) or mgal-3 (1/10) for 2 h at RT and stained using the Vector ABC kit with DAB as chromogen. Slides were dehydrated, mounted and examined under a Reichert-Jung microscope with a Leica DC150 Digital Camera System (Wetzlar, Germany). Omission of primary antibody and use of non-immune serum (Vector, CA, USA) resulted in complete absence of staining.

Steroid measurement

The levels of cortisol and progesterone were measured in control medium containing 10% FCS using commercial radioimmunoassays (INEP, Belgrade, Serbia) and were not detected.

Treatment with DEX, RU486, PRG and lactose

HTR-8/SVneo cells were seeded at $2x10^4$cells/well in 96-well plates and cultured in humidified 5% CO_2/95% air at 37°C, in their respective medium. After 24 h, HTR-8/SVneo cells were briefly rinsed with PBS and cultured further in control or one of the treatment media containing a) DEX , b) RU486 or c) PRG at final concentrations of 0.1-100 nM d) DEX in the presence of RU486, e) PRG in the presence of RU486 at final equimolar concentrations of 1 nM and 100 nM, or f) 50 mM, 100 mM and 200 mM lactose. After 48 h, HTR-8/SVneo cells were washed once with PBS and used for determination of viable cell number or fixed with ice-cold methanol/acetone (1:1) at RT for 10 min, dried and stored at 20°C until use.

Cell based ELISA (CELISA)

HTR-8/SVneo cell fixed monolayers were rehydrated with PBS (3x5 min), blocked with 1% BSA (200 µL/well) for 1 h at RT, and incubated for 2 h at RT with pgal-1, 50 µL/well (1/200 in PBS-1% BSA). After incubation, plates were washed (5x5 min) with PBS containing 0.05% Tween-20 (PT) and incubated for 2 h at RT with sheep anti-rabbit-IgG-HRP 50 µL/well (1/500 in PBS-1% BSA). Wells were washed (5x5 min) with PT and incubated with 50 µL/well of substrate (0.003% H_2O_2) and 50 µL/well of chromogen (0.05% TMB). The reaction was stopped with 0.2M H_2SO_4 (100 µL/well). Absorbance was measured at 450 nm using a Microplate reader (LKB). Absorbances measured in the solid phase assay on cell layers were normalized to cell number. For assessment of non-specific binding, nonimmune rabbit IgG was used in place of anti-galectin-1. All experiments were repeated at least twice, $n \geq 12$.

Determination of viable cell number

Cell viability was investigated using the MTT test (Hanisch et al., 1993). HTR-8/SVneo cells were plated in 96-well plates and incubated with DEX, RU486, PRG and lactose, as described above. After treatment, 100 µL of MTT in the respective medium (1 mg/mL in RPMI 1640) was added to each well. After incubation for 2 h at 37°C, medium was replaced by 1-propanol (100 µL/well) and the plates were vigorously shaken to ensure complete solubilization of the blue formazan. Absorbance was measured at 570 nm and the cells were quantified using the standard curve obtained with $5x10^3$, $1x10^4$,

$2x10^4$, $4x10^4$, $6x10^4$ or $8x10^4$ cells/well.

In vitro invasion assay

The possible effect of lactose or DEX on HTR-8/SVneo cell invasion was investigated by the method previously described by Librach et al. (1991). Briefly, transwell inserts (8 μm pore size) were coated with 20 μL of growth-factor-reduced Matrigel™ (1:2 dilution) in RPMI 1640 medium and incubated at 37°C for 30 min. HTR-8/SVneo cells ($5x10^4$) were seeded onto the Matrigel™ coated transwell chambers in 200 μL of complete RPMI 1640 (or 0.1% BSA/RPMI 1640, as needed) in the presence of lactose (100 mM) or DEX (1 nM). 500 μL of complete medium supplemented with the same concentrations of lactose or DEX was added to the lower chambers. Control groups of cells were grown in the media without lactose or DEX. After incubation for 24 h in a humidified atmosphere at 37°C, media were removed and the cells were gently washed twice with PBS. Cells from the inner side of inserts were wiped out with a cotton swab. Cells from the bottom side of the filter membrane were fixed with 4% paraformaldehyde-0.05% Triton X-100 for 30 min at RT. The membranes were cut out from the inserts with a scalpel blade. Membranes were immunostained with monoclonal anti-cytokeratin-18 antibody (as described above) and mounted on microscope slides with the lower surface facing up. Cytokeratin-18-stained cell bodies and processes were analyzed under a light microscope. In each case, 60 random fields were counted and the results were expressed as a percentage of the control. The experiments were performed five times, with n = 3 in each experiment.

Confocal immunofluorescence microscopy

HTR-8/SVneo cells in an *in vitro* invasion assay, intended to be analyzed by confocal microscopy, were grown as described above. After 24 h of incubation, however, filter membranes with invaded cells were not wiped with a cotton swab. The cells were gently washed twice with PBS and fixed with 4% paraformaldehyde-0.05% Triton X-100 for 30 min at RT. Membranes were cut out from the inserts with a scalpel blade. Cells were immunostained with primary anti-integrin β1 antibody at 1/100 dilution for 1 h at RT, than stained with Alexa Fluor® 488 secondary antibody for 30 min. Slides were mounted in ProLong Gold antifade reagent with DAPI (Molecular Probes, USA) and examined under a Carl Zeiss 510 laser-scanning microscope. The emission wavelength was set at 488-543 nm and images were captured with a 40x/1.3 oil immersion Plan-Neofluar objective. Negative staining was performed by incubating cells with nonimmune serum in place of the specific antibody and by isotype-matched control IgG incubation.

Statistical analysis

The obtained data were analyzed statistically with the Statistical Software Program, version 5.0 (Primer of Biostatistic, McGraw-Hill Companies, Inc., New York, NY, USA) using the non-parametric Mann-Whitney Rank Sum Test and one-way analysis of variance (ANOVA), as required, with values considered significantly different when $p< 0.05$.

RESULTS

Immunolocalization of gal-1 and gal-3 in the HTR-8/SVneo extravillous cell line

The expression of gal-1 and gal-3 was previously described in trophoblast *in vivo* and choriocarcinoma cell lines. Figure 1A illustrates the presence of gal-1 in HTR-8/SVneo cells as evidenced by immunocytochemistry. Strong and uniform expression of gal-1 is visible at intracellular and cell membrane domains. HTR-8/SVneo cells did not express gal-3 (Fig. 1B).

Steroid hormone effects on trophoblast cells

This study aimed to assess possible effects of steroid hormones on gal-1 production by HTR-8/SVneo cells.

A) Cell viability

The effects of DEX, RU486 and PRG on viable HTR-8/SVneo cell number are illustrated in Fig. 2A. Viable cell number was not changed after treatment with DEX, except for a significant decrease to 88.1% of the control after culture with 10 nM of

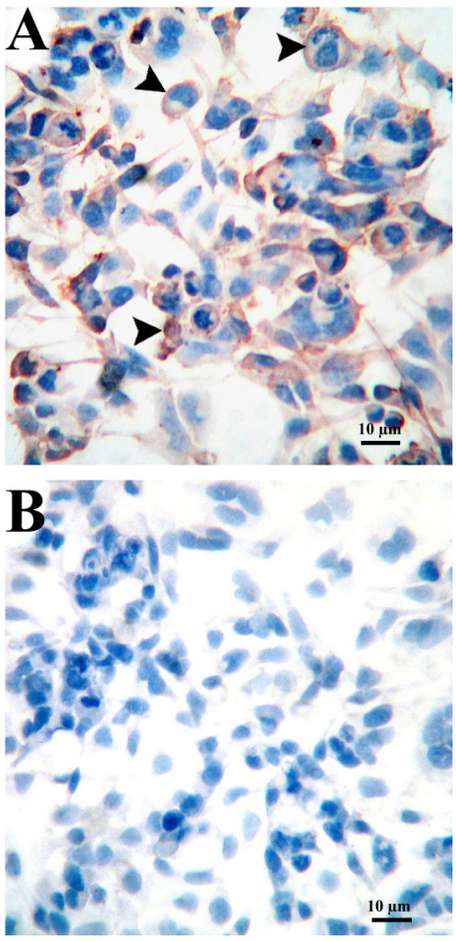

Fig. 1. Immunolocalization of gal-1 (A) and gal-3 (B) in HTR-8/SVneo cells. Cells were cultured on glass and the monolayers were immunostained as described in Materials and Methods. Gal-1 staining is intracellular and associated with the cell membrane domain (arrowhead). Gal-3 is not expressed. Scale bar 10 µm.

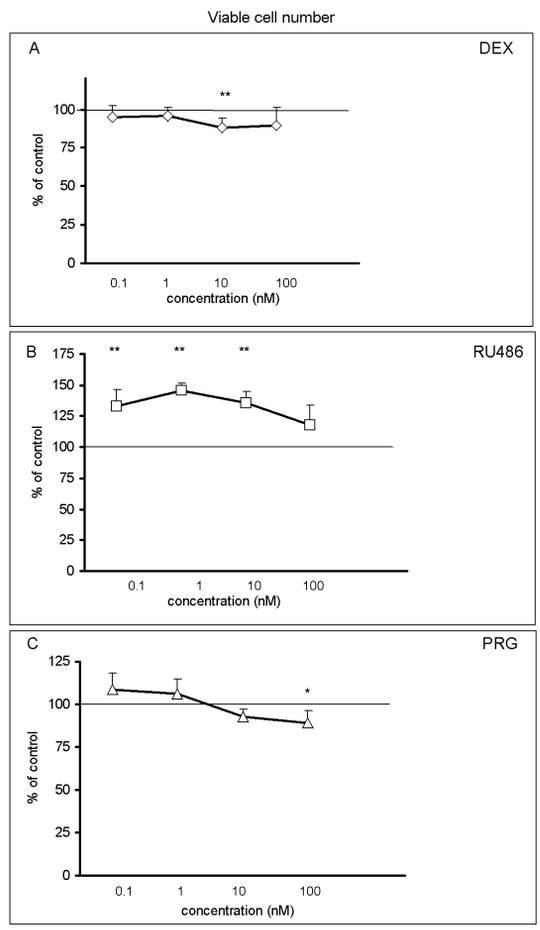

Fig. 1. Effects of DEX (A), RU486 (B), and PRG (C) on viabilty of HTR-8/SVneo cells. Values are given as a percentage of the control (means ± SD); n = 12. Differences vs. control significant at p<0.05 (*), p<0.005 (**).

DEX alone. RU486 significantly increased (p<0.005) viable cell number up to 140% in the lower concentration range, from 0.1-10 nM (Fig. 2B). A dose dependent effect was also observed for PRG (Fig. 2C). Thus, the viable cell number increased slightly in the lower concentration range (0.1 and 1 nM), but decreased at the higher concentration of 100 nM (p<0.05). Co-treatment of DEX and PRG with the antagonist RU486 at equimolar concentrations showed that the effect of combined treatments was consistent with the specific antagonism of RU486 to both DEX and PRG (Fig. 3). Thus, the slight inhibition of viable HTR-8/SVneo cell number induced by DEX was altered to a significant stimulation (Fig. 3A). On the other hand, the slight stimulatory effect of PRG was turned into a significant decrease during co-treatment at 1 nM (Fig. 3B).

B) Gal-1 expression

After cell-based ELISA, the relative levels of gal-1 expression were determined, normalized for the effects of hormones on viable HTR-8/SVneo cell number, and expressed as a percentage of the control value (Fig. 4A). DEX increased the levels of gal-1 by 7-15% at concentrations from 0.1-10 nM, significantly at 0.1 and 1 nM. The higher DEX dose of 100 nM induced a small decrease in gal-1. On the other hand, PRG induced the opposite effect,

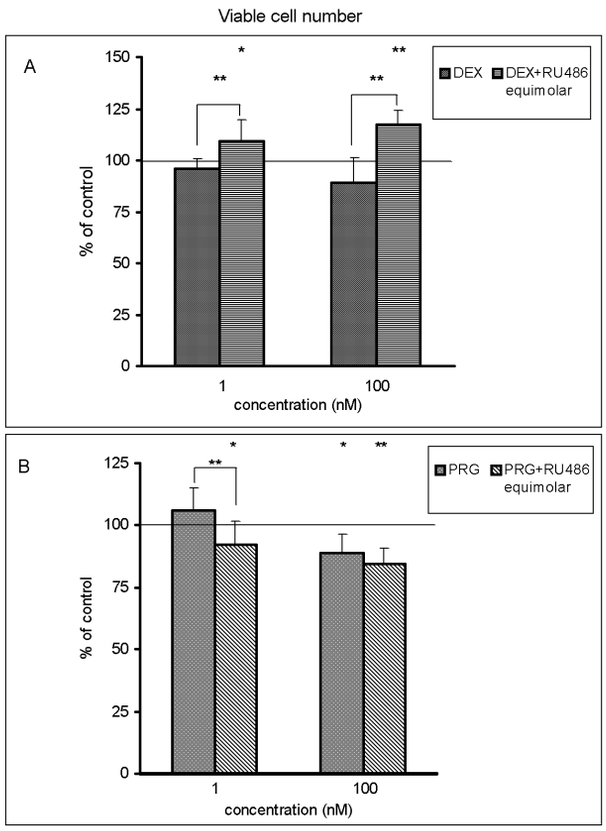

Fig. 3. Effects of co-treatment on cell viability of HTR-8/SVneo cells with equimolar concentrations of DEX + RU486 (A) and PRG + RU486 (B). Values are given as a percentage of the control (means ± SD); n = 12. Differences vs. control, or DEX vs. DEX + RU486, PRG vs. PRG + RU486, significant at $p<0.05$ (*), $p<0.005$ (**).

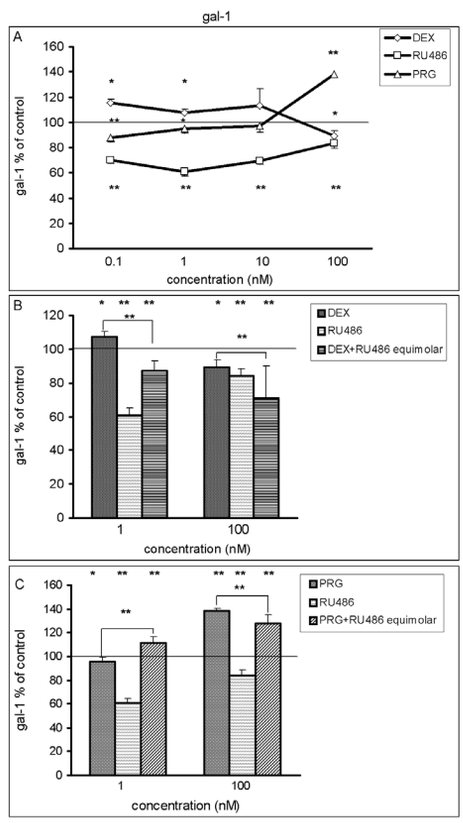

Fig. 4. A) Gal-1 protein in HTR-8/SVneo cell line after treatment with DEX, RU486, or PRG. Cells were maintained for 48 h in RPMI 1640 medium without (control) or with DEX, RU486, and PRG (0.1-100 nM). Data (means ± SD) were corrected for the cell number after each experiment. Differences vs. control significant at $p<0.05$ (*), $p<0.005$ (**), n = 12. B) Gal-1 protein after co-treatment of HTR-8/SVneo cells with equimolar concentrations of DEX and RU486. Cells were cultured in the absence (control) or presence of the indicated treatments. Data are corrected for cell number and presented as mean ± SD. Differences vs control or DEX vs DEX + RU486 (n = 12) significant at $p<0.05$ (*), $p<0.005$ (**). C) Gal-1 protein after co-treatment with equimolar concentrations of PRG and RU486. HTR-8/SVneo cells were cultured without (control) and with the indicated treatments. Data are corrected for cell number and presented as means ± SD. Differences vs. control or PRG vs. PRG + RU486 (n = 12) significant at $p<0.05$ (*), $p<0.005$ (**).

slightly decreasing gal-1 production in the concentration range of 0.1-10 nM, which was the reverse of the stimulation of 38% at a concentration of 100 nM. The most pronounced effect occurred with RU486, which inhibited gal-1 in the whole concentration range studied, down to 40% of control value with 1 nM. The observed suppression was significant with 0.1-10 nM. When RU486 was added at equimolar concentrations to either DEX- or PRG-treated of HTR-8/SVneo cell cultures, the effects on gal-1 were modulated as shown in Fig. 4 (panel B for DEX, panel C for PRG). Thus, co-treatment with 1 nM of DEX and 1 nM of RU486 induced a small, but significant inhibition of gal-1, i.e., addition of mifepristone prevented the stimulatory effect of DEX, inducing a net inhibitory effect. On the other hand, the presence of DEX reduced the inhibitory effect of 1 nM RU486 (down to 61%) to the intermediate value of 87%. These results are in keeping with the DEX effect on gal-1 production mediated by the glucocorticoid receptor (GR). Co-treatment of HTR-8/SVneo cells with PRG and RU486, however,

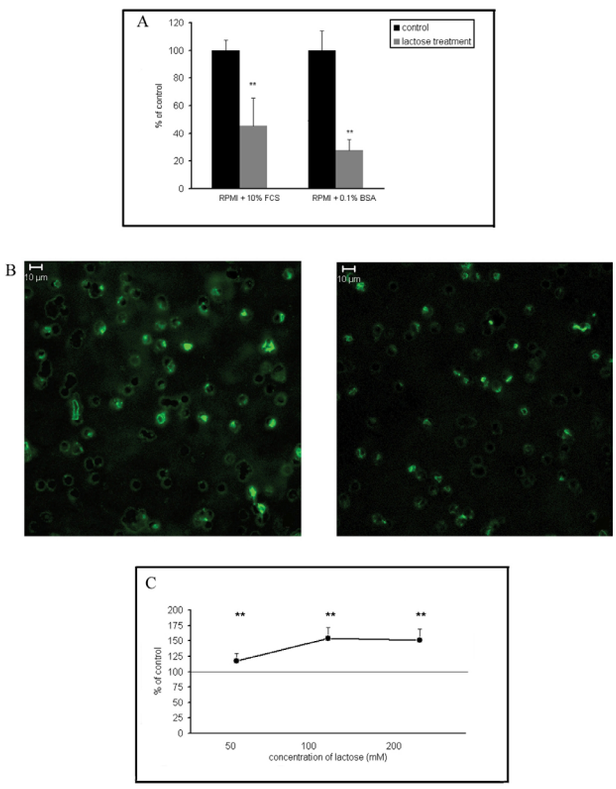

Fig. 5. A) Effect of 100 mM lactose on HTR-8/SVneo cell invasion. The invasion assay was performed using Matrigel coated inserts either in complete RPMI 1640 media or in RPMI 1640 media supplemented with 0.1% BSA, with or without 100 mM lactose. Data (means ± SD) are presented as a percentage of the control, differences vs control significant at $p<0.05$ (*). B) Effect of 100 mM lactose on HTR-8/SVneo invasion as evidenced by confocal microscopy at the pore level (intact invasion chamber after 24 h of culture). Cells were stained for integrin β1, control-left, lactose-right; scale bar 10 µm. C) Number of viable HTR-8/SVneo cells after treatment with 50, 100 and 200 mM of lactose. Data (means ± SD) are presented as a percentage of the control, differences vs. control significant at $p<0.005$ (**), n = 12.

did modulate overall gal-1 production, which was more consistent with the effect of PRG alone than with the effect of RU486 alone.

Physiological relevance of gal-1 in the HTR-8/SVneo trophoblast cell line

This was investigated by studying cell invasion under conditions that were expected to alter the availability of functional gal-1 for extracellular

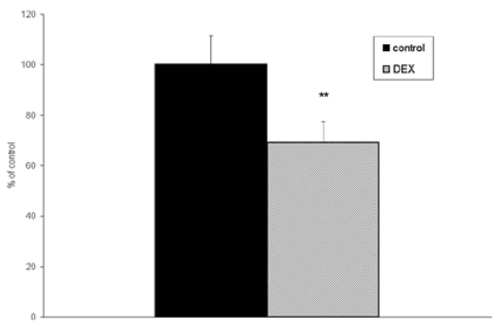

Fig. 6. Effect of 1 nM DEX on HTR-8/SVneo invasion. Invasion assay was performed for 24 h in medium with 10% FCS. Data (means ± SD) from the representative experiment are presented as percent of control, differences vs. control significant at $p<0.05$ (*), n = 3.

interactions, such as DEX treatment or an excess of competing sugar.

Invasion assay

Using inserts coated with Matrigel™ gel in media containing an excess of lactose (100 mM) supplemented with either 10% FCS or 0.1% BSA, invasion was compared to the corresponding control (Fig. 5A). Significant inhibition of invasion was observed both in the FCS containing medium (down to 45%) and in serum free medium (down to 28%). Confocal microscopy of randomly chosen fields of the insert membrane showed occupied pores without (Fig. 5B, left) and with (Fig. 5B, right) lactose. The cells were stained for the membrane protein, integrin subunit β_1. To rule out the possibility that decreased HTR-8/SVneo invasion results from reduced cell viability after treatment with lactose, an MTT assay was performed in parallel, with three different concentrations of lactose (Fig. 5C). Lactose significantly increased the number of viable extravillous trophoblast cells (by 17-54%) at all three concentrations. The observed inhibitory effect of lactose on invasion of HTR-8/SVneo cells supports the possible involvement of lectin mediated mechanisms in trophoblast cell invasion. Invasion by HTR-8/SVneo cells was also inhibited after treatment with 1 nM of DEX (down to 69%) (Fig. 6). This confirmed the inhibitory effect of DEX on cell invasion of trophoblast cells demonstrated for primary cytotrophoblast by Libra ch et al. (1991), indicat-

ing further the suitability of this cell line as a model for the early pregnancy invasive trophoblast.

DISCUSSION

As the possible involvement of steroids in the regulation of expression of gal-1 in the human placenta has not been studied previously, we aimed to determine whether the glucocorticoid DEX influences its expression in trophoblast cells. The HTR-8/SVneo cell line used was derived from the human invasive extravillous trophoblast (Graham et al., 1993) and has a phenotype and physiological characteristics consistent with those of normal first-trimester invasive trophoblast cells (Irving et al., 1995). HTR-8/SVneo cells have already been used in models of EVT migration (Gleeson et al., 2001; Chakraborty et al., 2003) and invasion (Huber et al., 2006).

Maintenance of utero-placental attachment during human pregnancy depends on adhesive interactions between the cytotrophoblast and ECM involving integrins and possibly galectins, among other molecules. In addition to being present in JAr and JEG-3 choriocarcinoma cells (Bojić-Trbojević et al., 2005), we have shown here that HTR-8/SVneo cells express gal-1 in the cytoplasm and the cell membrane domain. Other galectin family members also exhibit dual localization, being observed in both extracellular (cell-surface and medium) and intracellular (cytoplasm and, in some cases, the nucleus) compartments (Hughes, 1997). On the other hand, gal-3, another galectin family member identified in the trophoblast *in vivo*, was not found in HTR-8/SVneo cells. Similarly, gal-3 was not expressed in JAr cells, a choriocarcinoma derived trophoblast cell line, but was present in the JEG-3 cell line (Božić, 2003).

There are several lines of evidence to implicate GCs in uteroplacental adherence and chronic regulation of placental protein expression, including ECM proteins (Guller et al., 1993a,b; Guller et al., 1995; Ryu et al., 1999). Thus, DEX was shown to reduce the expression of onfFN and LN (Guller et al., 1993; Yoon et al., 1998). Since these ECM glycoproteins have been identified as physiological ligands for gal-1 (Sato and Hughes, 1992; Ozeki et al., 1995), we hypothesized that GCs may also affect its production. Treatment of rats with DEX was found to be critical in the regulation of gal-1 in the neonatal lung at the levels of transcription, translation and degradation (Clerch et al., 1987; Sandford et al., 1993). Physiological cortisol levels in maternal plasma during pregnancy are elevated and are believed to reach 1 μM (Simmer et al., 1974). The transplacental transfer of maternal cortisol is partly regulated by 11 β-hydroxysteroid dehydrogenase (11 β-HSD-2) (Krozowski et al., 1995), which catalyzes conversion of active cortisol to inert cortisone (Albiston et al., 1994; Stewart et al., 1994). The extravillous trophoblast, however, is assumed to be exposed to rather high concentrations of GCs within the uterine environment. The GC receptor is functional in normal trophoblast (Karalis et al., 1996) and has been identified in isolated cytotrophoblast (Robinson et al., 1988). In HTR-8/SVneo cells, both cortisol and DEX significantly enhanced TGF-beta effects on PAI-1 and suppressed PAI-2 (Ma et al., 2002) suggesting the presence of a functional GC receptor in this cell line. Since GC treatment profoundly affected several placental genes, including ECM proteins (Guller et al., 1993 a) and integrins (Guller et al., 1995), the possible involvement of a full agonist of GR, DEX, in the regulation of gal-1 in HTR-8/SVneo cells was studied here. It has also been proposed that cortisol might be a local antiprogestin (Karalis et al., 1996), that placental PRG might act by binding to GR, and that cortisol would then compete with PRG for the GR. Therefore, both RU486, which has antiglucocorticoid and antiprogesterone action, and PRG were included in this study. The chosen steroid doses have already been shown to affect ECM protein synthesis in term trophoblast (Guller et al., 1993 a; Guller et al., 1995; Ryu et al., 1999). Also, the same concentration range of GCs drastically inhibited production of IL-1β and invasion by first trimester human trophoblast (Librach et al., 1994).

Since steroid effects on gal-1 production were determined over a 48-h interval, during which time the cells would normally divide, it was important to

establish whether DEX, RU486 and PRG influenced viable cell number. The data obtained over a wide range of concentrations (0.1-100 nM) showed that DEX only slightly decreased the number of viable HTR-8/SVneo cells. On the other hand, RU486 had a pronounced stimulatory effect on proliferation, while PRG slightly stimulated viable cell number at low doses. Another synthetic GC, triamcinolone-acetonide, was also shown to influence proliferation of choriocarcinoma cells, dose-dependently increasing BeWo cell number and decreasing the number of viable JEG-3 cells in the presence of FCS (Mandl et al., 2006). The antagonist nature of RU486 was confirmed in co-treatment of the HTR-8/SVneo cell line with either DEX or PRG at equimolar concentrations. The inhibitory effect of DEX on viable cell number was blocked by RU486, suggesting action through the GC receptor. Moreover, RU486 diminished the stimulatory effect of PRG as well.

There is little information available about the regulation of gal-1 protein, and less still about the factors responsible for its induction. TGF-β, PDGF and angiotesin II have been discussed as possible mediators of increased gal-1 in vascular smooth muscle cells (Moiseeva et al., 1999). Gal-1 in retinal pigment epithelial cells is moderately upregulated after treatment with hepatocyte growth factor, both at the mRNA and at the protein level (Alge et al., 2006). A pronounced increase of gal-1 expression was demonstrated in nasal polyps of allergic patients after treatment with GC budenoside (Delbrouck et al., 2002). Ours is the first study demonstrating that gal-1 in HTR-8/SVneo cells is susceptible to steroid treatment at the protein level. The effect is dose-dependent, as gal-1 was increased after treatment with 0.1-10 nM of DEX, but reduced slightly at 100 nM. DEX has previously been shown to diminish onfFN and LN in term cytotrophoblasts (Guller et al., 1993a). However, GC-dependent reduction of protein synthesis was not generalized, since GC treatment profoundly increased hCG synthesis in cytotrophoblast (Ringler et al., 1989), suggesting a regulatory role of GC in the maintenance of pregnancy. The GC antagonist RU486, on the other hand, markedly reduced gal-1 production in extravillous HTR-8/SVneo cells in the whole concentration range. The effect could be reversed by DEX at an equimolar concentration, which strongly suggested mediation by GR, as RU486 blocks GC action at the receptor level (Brogden et al., 1993) and can suppress GR itself in the first-trimester human placenta (Chan et al., 2003). This effect was detectable from 12 h after administration and lasted at least 48 h (Chan et al., 2003). However, no data are available regarding the possibility of the same action of RU486 in HTR-8/SVneo cells. On the other hand, neither exposure of trophoblast cells to DEX in concentrations as high as 1 μM nor treatment with PRG at 1-100 nM resulted in a decrease in GR (Robinson et al., 1988).

Compared to DEX, PRG induced an opposed effect, namely a dose-dependent reduction of gal-1 at 0.1-10 nM, which was transformed into stimulation upon treatment with 100 nM. It was previously found that PRG did not affect the levels of onfFN in term cytotrophoblast (Guller et al., 1993b). Co-treatment with equimolar PRG and RU486 showed that the effect is more consistent with that of PRG alone. It is well known that mifepristone has a high affinity for human uterine PR, interrupting the progression of pregnancy. However, the most prominent steroid receptor in the first trimester placenta is GR, followed by PR (Chan et al., 2003). The dramatic inhibitory effect of RU486 on gal-1 suggests there is a possibility for other complementary mechanisms during its action in induced termination of pregnancy. Our data indicate that gal-1 can be modulated by GCs, but possibly also by other steroids, which is particularly interesting in the case of PRG, given the high physiological concentrations in pregnancy. Galectin(s) may be implicated in some of the physiological or pathological conditions involving the trophoblast. Expression of gal-1 in the placental bed material of human pregnancy (Vićovac et al., 1998) and increase of gal-1 in gestational trophoblast diseases (Božić et al., 2004) support this possibility for gal-1 in human trophoblast. In BeWo cells, gal-1 binding to a TF disaccharide ligand was correlated with cell proliferation (Jeschke et al., 2006), as well as with modulation of hormone production (Jeschke et al., 2004). The putative extracellular function of galectins in cell migration has been

described in other systems, such as cancer metastasis (Hittelet et al., 2003) and optical nerve growth (Mahathappa et al., 1994). This has been particularly well documented in cancer (Danguy et al., 2002). The pattern of expression of gal-1 in placental bed material of human pregnancy (Vićovac et al., 1998) leaves open the question of a role for it and other family members in the trophoblast. Proteomic analysis has recently shown that down regulation of gal-1 expression can be linked to early pregnancy loss (Liu et al., 2006). We have made preliminary investigations regarding the potential involvement of gal-1 and DEX in invasion by HTR-8/SVneo cells. The presence of lactose in culture media, as an inhibiting sugar for gal-1 lectin activity, significantly reduced Matrigel invasion, at the same time increasing the viable cell number. This line of investigation will provide more information in the future, but our current finding indicates that galectin - ECM interactions seem to play a role in trophoblast invasion. In addition, we found DEX to inhibit invasion of Matrigel by HTR-8/SVneo down to 69% of the control value, as previously shown for isolated first trimester trophoblast cells (Librach et al., 1994). This effect of GCs was accompanied by a decrease in production of IL-1β and inhibition of trophoblast MMP-9 release and activity (Librach et al., 1994), with the most effective DEX concentration being 100 nM, as observed here. Interestingly, Librach and co-workers also reported that PRG, which had no significant effect of its own on cytotrophoblast invasion, was able partially to block inhibition by corticosteroids.

Taken together, our data on steroid induced modulation of gal-1 complement previous findings suggesting an important role for glucocorticoids in implantation and point to a new action of PRG relevant to the maintenance of pregnancy.

Acknowledgment — Rabbit antiserum to galectin-1 was kindly donated by Dr. Miroslava Janković, INEP-Zemun, Serbia. The authors thank Dr. Marija Radojčić for critical reading and advice. This work was supported by research grant 143059 from the Ministry of Science, Republic of Serbia.

REFERENCES

Albiston, A.L., Obeyesekere, V.R., Smith, R.E. and *Z.S. Krozowski* (1994). Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell Endocrinol.* **105**, R11-R17.

Alge, C.S., Priglinger, S.G., Kook, D., Schmid, H., Haritoglou, C., Welge-Lussen, U. and *A. Kampik* (2006). Galectin-1 influences migration of retinal pigment epithelial cells. *Investigative Opthalm. Vis. Sci.* **47**, 415-426.

Aplin, J.D., Haigh, T., Jones, C.J.P., Church, H.J. and *L. Vićovac* (1999). Development of cytotrophoblast columns from expanded first-trimester human placental villi: role of fibronectin and integrin α5β1. *Biol. Reprod.* **60**, 828-838.

Ballard, P.L. and *R.A. Ballard* (1995). Scientific basis and therapeutic regimens for use of antenatal glucocorticoids. *Am. J. Obstet. Gynecol.* **173**, 254-262.

Bevan, B.H., Kilpatrick, D.C., Liston, W.A., Hirabayashi, J. and *K. Kasai* (1994). Immunohistochemical localization of a β-D-galactoside-binding lectin at the human maternofetal interface. *Histochem. J.* **26**, 582-586.

Bojić-Trbojević, Ž., Janković, M. and *L. Vićovac* (2005). Influence of IGF-I on adhesion, proliferation and galectin-1 production in JAr and JEG-3 choriocarcinoma cell lines. *Arch. Oncol.* **13**, 7-11.

Božić, M. (2003). MS Thesis, Phenotype characterization of invasive trophoblast of the gestational trophoblastic diseases *in situ* and *in vitro*- expression of galectin-1 and galectin-3. University of Belgrade, Faculty of Biology (in Serbian).

Božić, M., Petronijević, M., Milenković, S., Atnacković, J., Lazić, J. and *L. Vićovac* (2004). Galectin-1 and galectin-3 in the trophoblast of the gestational trophoblastic disease. *Placenta* **25**, 797-802

Brogden, R.N., Goa, K.L. and *F. Faulds* (1993). Mifepristone: a review of its pharmacodynamic properties, and therapeutical potential. *Drugs* **45**, 384-409.

Chakraborty, C., Barbin, Y.P., Chakrabarti, S., Chidiac, P., Dixon, S.J. and *P.K. Lala* (2003). Endothelin-1 promotes migration and induces elevation of [Ca2+]i and phosphorylation of MAP kinase of a human extravillous trophoblast cell line. *Mol. Cell Endocrinol.* **201**, 63-73.

Chan, C.C.W., Lao, T.T., Ho, P.C., Sung, E.O.P. and *A.N.Y. Cheung* (2003). The effect of mifepristone on the expression of steroid hormone receptors in human decidua and placenta: a randomized placebo-controled double-blind study. *J. Clin.Endocrinol. Metab.* **88**, 5846-5850.

Clerch, L.B., Whitney, P.L. and *D. Massaro* (1987). Rat lung synthesis, degradation and activation. *Biochem. J.* **245**, 683-690.

Colnot, C., Fowlis, D., Ripoche, M.A., Bouchaert, I. and *F. Poirier* (1998). Embryonic implantation in galectin 1/galectin 3 double mutant mice. *Dev. Dyn.* **211**, 306-313.

Čuperlović, M., Janković, M., Pfeifer, M. and *W.E.G. Müller* (1995). Isolation and characterization of a β-galactoside-binding protein (14kD) from rat liver nuclei. *Cell. Physiol. Biochem.* **5**, 33-44.

Danguy, A., Camby, I. and *R. Kiss* (2002). Galectins and cancer. *Biochim. Biophys. Acta* **1572**, 285-293.

Delbrouck, C., Doyen, I., Belot, N., Decaestecker, C., Ghanooni, R., de Lavareille, A., Kaltner, H., Choufani, G., Danguy, A., and *G. Vandenhoven* (2002). Galectin-1 is overexpressed in nasal polyps under budenoside and inhibits eosinophil migration. *Lab. Invest.* **82**, 147-158.

Dorr, H.G., Heller, A. and *H.T. Versmold* (1989). Longitudinal study of progestins, mineralocorticoids, and glucocorticoids throughout human pregnancy. *J. Clin. Endocrinol. Metabol.* **68**, 863-868.

Gitt, M.A. and *S.H. Barondes* (1991). Genomic sequence and organization of two members of a human lectin gene family. *Biochemistry* **30**, 82-89.

Gleeson, L.M., Chakraboty, C., McKinnon, T. and *P.K. Lala* (2001). Insulin-like growth factor-1 stimulates human trophoblast migration by signaling through alpha5 beta1 integrin via mitogen activated protein kinase pathway. *J. Clin. Endocrinol. Metab.* **86**, 2484-2493.

Graham, C.H., Hawley, T.S., Hawley, R.C., MacDougall, J.R., Kerbel, R.S., Khoo, N. and *P.K. Lala* (1993). Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp. Cell Res.* **206**, 204-211.

Guller, S., Wozniak, R., Krikun, G., Burnham, J.M., Kaplan, P. and *C.J. Lockwood* (1993a). Glucocorticoid suppression of human placental fibronectin expression: implications in uterine-placental adherence. *Endocrinol.* **133**, 1139-1146.

Guller, S., LaCroix, N.C., Krikun, G., Wozniak, R., Markiewicz, L., Wang, E.Y., Kaplan, P., and *C.J. Lockwood* (1993b). Steroid regulation of oncofetal fibronectin expression in human cytotrophoblasts. *J. Steroid Biochem. Mol. Biol.* **46**, 1-10.

Guller, S., Wozniak, R., Kong, L. and *C.J. Lockwood* (1995). Opposing actions of transforming growth factor-β and glucocorticoids in the regulation of fibronectin expression in the human placenta. *J. Clin. Endocrinol. Metabol.* **80**, 3273-3278.

Hanisch, F.G., Dressen, F. and *H. Uhlenbruck* (1993). Quantitative micro-adhesion assay on polystyrene matrices. In: *Lectins* and *Glycobiology* (Eds. H.J. Gabius and S.Gabius), 411-417, Springer-Verlag, Berlin-Heidelberg.

Hittelet, A., Legendre, H., Nagy, N., Bronckart, Y., Pector, J.C., Salmon, I., Yeaton, P., Gabius, H.J., Kiss, R. and *I. Camby* (2003). Upregulating of galectins-1 and -3 in human colon cancer and their role in regulating cell migration. *Int. J. Cancer* **103**, 370-379.

Hohn, H.P., and *H.W. Denker* (2002). Experimental modulation of cell-cell adhesion, invasiveness and differentiation of trophoblast cells. *Cells Tissues Organs* **172**, 218-236.

Huber ,V., Saleh, L., Bauer, S., Husslein, P. and *M. Knöfler* (2006) TNFalpha-mediated induction of PAI-1 restricts invasion of HTR-8/SVneo trophoblast cells. *Placenta* **27**, 127-136.

Hughes, R.C. (1997). The galectin family of mammalian carbohydrate-binding molecules. *Biochem. Soc. Trans.* **25**, 1194-1198.

Hughes, R.C. (2001). Galectins as modulators of cell adhesion. *Biochimie* **83**, 667-676.

Irving, J.A., Lysiak, J.J., Graham, C.H., Hearn, S., Han, V.K., and *P.K. Lala* (1995). Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. *Placenta* **16**, 413-433.

Janković, M., and *M. Čuperlović* (1996) Animal soluble beta-galactoside binding lectins-galectins. *Archives of Biological Sciences Belgrade*, **48**, 65-77.

Jeschke, U., Karsten, U., Wiest, I., Schulze, S., Kuhn, C., Friese, K. and *H. Walzel* (2006). Binding of galectin-1 (gal-1) to the Thomsen-Friedenreich (TF) antigen on trophoblast cells and inhibition of proliferation of trophoblast tumor cells *in vitro* by gal-1 or an anti-TF antibody. *Histochem. Cell Biol.* **126**, 437-444.

Jeschke, U., Reimer, T., Bergemann, C., Wiest, I., Schulze, S., Friese, K. and *H. Walzel* (2004). Binding of galectin-1 (gal-1) on trophoblast cells and inhibition of hormone production of trophoblast tumor cells *in vitro* by gal-1. *Histochem. Cell Biol.* **121**, 501-508.

Karalis, K., Goodwin, G., and *J.A., Majzoub* (1996). Cortisol blockade of progesterone: a possible mechanism involved in the initiation of human labor. *Nat. Med.* **2**, 556-560.

Krozowski, Z., MaGuire, J.A., Stein-Oakley, A.N., Dowling, J., Smith, R.E., and *R.K. Andrews* (1995). Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. *J. Clin. Endocrin. Metab.* **80**, 2203-2209.

Librach, C.L., Feigenbaum, S.L., Bass, K.E., Cui, T.Y., Verastas, N., Sadovsky, Y., Quigley, J.P., French, D.L., and *S.J. Fisher* (1994). Interleukin -1β regulates human cytotrophoblast metalloproteinase activity and invasion *in vitro*. *J. Biol. Chem.* **269**, 17125-17131.

Librach, C.L., Werb, Z., Fitzgerald, M.L., Chiu, K., Corwin, N.M., Esteves, R.A., Grobelny, D., Galardy, R., Damsky, C.H. and *S.J. Fisher* (1991). 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J. Cell Biol.* **113**, 437-439.

Liu, A.X., Jin, F., Zhang, W.W., Zhou, T.H., Zhou, C.Y., Yao, W.M., Oian, Y.L. and *H.F. Huang* (2006). Proteomic analysis of the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biol. Reprod.* **75**, 414-420.

Liu, F.T. and *G.A. Rabinovich* (2005). Galectins as modulators of tumour progression. *Nat. Rev. Cancer* **5**, 29-41.

M.a, Y, Ryu, J.S., Dulay, A., Segal, M., and *S. Guller* (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.

Mahathappa, N.K., Cooper, D.N.W., Barondes, S.H. and *G.A. Schwarting* (1994). Rat olfactory neurons can utilize endogenous lectin, L-14, in a novel adhesion mechanism, *Development* **120**, 1373-1384.

Mandl, M., Ghaffari-Tabrizi, N., Haas, J., Nöhammer, G. and *G. Desoye* (2006). Differential glucocorticoid effects on proliferation and invasion of human trophoblast cell lines. *Reproduction* **132**, 159-167.

Maqui, E., Van den Brule, F.A., Castronovo, V., and *J.M. Foidart* (1997). Changes in the distribution pattern of galectin-1 and galectin-3 in human placenta correlates with the differentiation pathways of trophoblasts. *Placenta* **18**, 433-439.

Moiseeva, E.P., Spring, E.L., Baron, J.H., and *D.P. deBono* (1999). Galectin 1 modulates attachment, spreading and migration of cultured vascular smooth muscle via interactions with cellular receptors and components of extracellular matrix. *J. Vasc. Res.* **36**, 47-58.

Nakane, P.K., and *A. Kawaoi* (1974). Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.* **22**, 1084-1091.

Ostensen, M. (2001). Drugs in pregnancy. Rheumatological disorders. *Best Practice Res. Clin. Obstet. Gynecol.* **15**, 953-969.

Ozeki, Y., Matsui, T., Yamamoto, Y., Funahashi, M., Hamako, J. and *K. Titani* (1995). Tissue fibronectin is an endogenous ligand for galectin-1. *Glycobiology* **5**, 255-261.

Poirier, F. (2002). Roles of galectins *in vivo*. *Biochem. Soc. Symp.* **69**, 95-103.

Poirier, F., Timmons, P.M., Chan, C-T. J., Guenet, J-L., and *P.W.J. Rigby* (1992). Expression of the L14 lectin during mouse embryogenesis suggests multiple roles during pre- and post-implantation development. *Development* **115**, 143-155.

Rabinovich, G.A. (1999). Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. *Cell Death Differ.* **6**, 711-721.

Ringler, G.E., Kallen, C.B. and *J.F. Strauss III* (1989). Regulation of human trophoblast function by glucocorticoids: dexamethasone promotes increased secretion of chorionic gonadotrophin. *Endocrinol.* **124**, 1625-1631.

Robinson, B.G., Emanuel, R.L., Frim, D.M. and *J.A. Majzoub* (1988). Glucocorticoid stimulates expression of cortocotropin–releasing hormone gene in human placenta. *Proc. Natl. Acad. Sci. USA* **85**, 5244-5248.

Rosen, T., Krikun, G., Ma, Y., Wang, E.Y., Lockwood, C.J. and *S. Guller* (1998). Chronic antagonism of nuclear factor-κB activity in cytotrophoblasts by dexamethasone: a potential mechanism for antiinflamatory action of glucocorticoids in human placenta. *J. Clin. Endocrinol. Metabol.* **83**, 3647-3652.

Ryu, J.S., Majeska, R.J., Ma, Y., LaChapelle, L. and *S. Guller* (1999). Steroid regulation of human placental integrins: suppression of α_2 integrin expression in cytotrophoblast by glucocorticoids. *Endocrinol.* **140**, 3904-3912.

Sandford, G.L., Owens, M.A. and *B.M. Odusanya* (1993). Differential influence of dexamethasone on the activity and synthesis of β-galactoside-specific lectin (galaptin) during postnatal lung development. *Exp. Lung Res.* **19**, 91-104.

Sato, S. and *R. Hughes* (1992). Binding specificity of a baby hamster kidney lectin for H type I and II chains, polylactosamine glycans and appropriately glycosylated forms of laminin and fibronectin. *J. Biol. Chem.* **267**, 6983-6990.

Simmer, H.H., Tulchinsky, D., Gold, E.M., Frankland Greipel, M. and *A.S. Gold* (1974). On the regulation of estrogen production by cortisol and ACTH in human pregnancy at term. *Am. J. Obstet. Gynecol.* **119**, 283-296.

Stewart, P.M., Murray, B.A., and *J.I. Mason* (1994). Human kidney 11 beta-hydroxysteroid dehydrogenase is a high-affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform. *J. Clin. Endocrin. Metab.* **79**, 480-484.

Vićovac, L., Janković, M. and *M. Čuperlović* (1998). Galectin-1 and -3 in cells of the first trimester placental bed. *Hum. Reprod.* **13**, 730-735.

Yoon, D.Y., Ma, Y., Krikun, G., Lockwood, C.J., LaChapelle, L. and *S. Guller* (1998). Glucocorticoid effects in the human placenta: evidence that dexamethasone-mediated inhibition of fibronectin expression in cytotrophoblasts involves a protein intermediate. *J. Clin. Endocrin. Metab.* **83**, 632-637.

СТЕРОИДНИ ХОРМОНИ МОДУЛИРАЈУ ГАЛЕКТИН-1 У HTR-8/SVNEO ТРОФОБЛАСТНОЈ ЋЕЛИЈСКОЈ ЛИНИЈИ

ЖАНКА БОЈИЋ-ТРБОЈЕВИЋ, МИЛИЦА БОЖИЋ и ЉИЉАНА ВИЋОВАЦ

Одељење за биологију репродукције, Институт за примену нуклеарне енергије - ИНЕП, Универзитет у Београду, 11080 Београд-Земун, Србија

Испитиван је ефекат стероида дексаметазона (DEX), прогестерона (PRG) и мифепристона (RU486) на галектин-1 (gal-1) у HTR-8/SVneo ћелијама трофобластног порекла користећи имуноцитохемију, ELISA тест на ћелијама, MTT тест пролиферације и тест инвазије Matrigela™. Gal-1 је модулиран стероид-специфично и зависно од дозе. DEX је благо, али значајно стимулисао gal-1 у ниским концентрацијама од 0.1-10 nM, а инхибирао са 100 nM, док су ефекти PRG и RU486 били супротни DEX. Инвазија Matrigela је значајно умањена у присуству DEX и лактозе као инхибиторног шећера за gal-1. Добијени подаци указују на значај стероида за продукцију gal-1 у трофобласту.