

EFFECT OF STEROIDS ON TRANSCRIPTION AND SECRETION OF GAL-1 BY THE HUMAN TROPHOBLAST CELL LINE *IN VITRO*

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Abstract - Galectin-1 (Gal-1) is a lectin with recently documented pro-invasive function in trophoblasts *in vitro*, whose regulation is currently insufficiently known. The potential involvement of steroid hormones, synthetic glucocorticoid dexamethasone (DEX), the sex steroid progesterone (PRG) and mifepristone (RU486) in the regulation of Gal-1 in the trophoblast-derived cell line HTR-8/SVneo was investigated. Gal-1 mRNA levels were assessed by real-time PCR. The effect on secretion of Gal-1 into the culture media was followed using the SELDI-TOF protein chip array. We present evidence that DEX and RU486 significantly reduced Gal-1 in the HTR-8/SVneo cell line at the mRNA level. In addition, trophoblast-derived HTR-8/SVneo cells were shown to secrete detectable Gal-1 protein, which was only slightly increased by PRG. The potential clinical relevance of these findings remains to be determined.

Key words: Galectin-1, trophoblast, HTR-8/SVneo cells, regulation, steroids

List of Abbreviations: ABC, avidin-biotinylated peroxidase complex; BCA, bicinchoninic acid; DEX, dexamethasone; ECL, enhanced chemiluminescence; ECM, extracellular matrix; FCS, fetal calf serum; Gal-1, galectin-1; IMAC, immobilized metal affinity chromatography; GC, glucocorticoid; GR, glucocorticoid receptor; PCR, polymerase chain reaction; PRG, progesterone; RU486, mifepristone; SDS-PAGE, sodium-dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight; SPA EAM, sinapinic acid energy absorbing matrix; sFCS, charcoal-stripped fetal calf serum

INTRODUCTION

Galectin-1 (Gal-1) belongs to the phylogenetically conserved galectin family with a shared affinity for N-acetyl lactosamine residues. It exists as a non-covalent homodimer, containing two carbohydrate recognition domains that can recognize and bind a wide range of glycoconjugates (Barondes et al., 1994; Leffler et al. 2004). Although Gal-1 has the charac-

teristics of a typical cytoplasmic protein, it can be secreted and found on the cell surface, as well as in the extracellular matrix (ECM) (Hughes, 1999; Cooper and Barondes, 1990). It acts both intracellularly (Park et al., 2001) and extracellularly through its lectin activity (Camby et al., 2006). This lectin has been shown to play a role in a variety of biological functions, including cell adhesion, migration, invasion, metastasis, apoptosis, as well as assembly and

remodeling of ECM (Hughes, 1999; Hsu and Liu, 2004). It has been documented to bind various ECM components – laminin, cellular fibronectin, thrombospondin, vitronectin and osteopontin, in a dose- and β -galactoside-dependent manner (Ozeki et al., 1995; Moiseeva et al., 2000; Moiseeva et al., 2003). In the human female reproductive tract, Gal-1 is abundantly expressed, including placenta and trophoblast-derived cell lines (Maqui et al., 1997; Vićovac et al., 1998; Bojić-Trbojević et al., 2008). We have recently shown that Gal-1 participates in trophoblast cell invasion *in vitro* (Kolundžić et al., 2011).

Human pregnancy is associated with elevated levels of glucocorticoids (GC) in both maternal and fetal sera (Dorr et al., 1989) and increasing progesterone (PRG). It has been suggested that glucocorticoids might chronically regulate placental protein expression during gestation (Ryu et al., 1999). In isolated human cytotrophoblast in primary culture, synthetic GC dexamethasone (DEX) regulates synthesis of extracellular matrix proteins, oncofetal fibronectin and laminin (Guller et al., 1993). This synthetic GC influences trophoblast invasion (Librach et al. 1994), as well as Gal-1 protein in the extravillous trophoblast cell line HTR-8/SVneo (Bojić-Trbojević et al., 2008). In addition, DEX was found to regulate Gal-1 in rat neonatal lung, at the mRNA and protein levels (Clerch et al., 1987). Therefore, the present study was intended to investigate whether previously described effects of DEX and PRG on the Gal-1 protein in a trophoblast cell line were due to a change in gene transcription, or to an altered secretion of the Gal-1 protein. The effect of mifepristone (RU486), which acts as an antagonist of the glucocorticoid receptor (GR) and as an antiprogesterin, was also assessed.

MATERIALS AND METHODS

Materials

RPMI 1640 with or without phenol red, antibiotic/antimycotic solution and fetal calf serum (FCS) were obtained from PAA Laboratories (Linz, Austria). Trypan blue, acrylamide, N,N'-methylene-bis-acry-

lamide, N,N,N',N'-tetramethylenediamine, Ponceau S, glycine, protease inhibitor cocktail and RU486 were obtained from Sigma Chemical Company. Dexamethasone and PRG were from Galenika (Galenika AD, Belgrade, Serbia). SDS-PAGE protein standards were from Bio-Rad. Protran nitrocellulose transfer membrane was obtained from Schleicher&Schuell BioScience GmbH Whatman Group (Dassel, Germany). Reagents for enhanced chemiluminescence were from Pierce (Pierce Biotechnology, Rockford, IL, USA). Goat anti-Gal-1 was from R&D (Abingdon, UK), while biotinylated rabbit anti-goat IgG and avidin-biotinylated peroxidase complex (ABC) were obtained from Vector Laboratories (Burlingame, CA, USA). TRIreagent, primers, dNTPs, AmpliTaq Gold DNA polymerase were from Applied Biosystems (Carlsbad, USA). Hexamer primers, RevertAid reverse transcriptase were obtained from Fermentas (Lithuania) and KAPATMSYBR^{FAST} qPCR Universal Master Mix was from Kappa Biosystems (Boston, USA). All other reagents were of the best commercial grade available.

Cell culture

HTR-8/SVneo cells were established from human first-trimester explant cultures immortalized by SV40 large T antigen (Graham et al., 1993; Irving et al., 1995). HTR-8/SVneo cells were cultured in RPMI 1640 supplemented with 5% FCS (v/v) with antibiotic-antimycotic solution. For steroid treatments, cells were cultured in RPMI 1640 without phenol red supplemented with charcoal-stripped 5% FCS (sFCS) and antibiotic-antimycotic solution.

For SDS-PAGE and RT-PCR, HTR-8/SVneo cells were grown in tissue culture flasks in medium for steroid treatments. After 24 h, cells were further cultured in control or one of the media containing a) DEX, b) PRG, c) RU486, at final concentrations of 10 nmol/L and 1000 nmol/L. After 48 h treatment, culture media from experiments were collected and centrifuged for 5 min at 700 g to remove any cells, and the protein concentration was determined using the BCA assay (Pierce Biotechnology, Rockford, IL, USA). For RNA isolation, total RNA was collected

from the treated HTR-8/SVneo cells using TRIreagent according to manufacturer's instructions.

Real-time PCR

Quantitative real-time PCR was performed using SYBR Green chemistry in a 7500 Real Time PCR System (Applied Biosystems, Carlsberg, USA), as previously described (Kolundžić et al., 2011; Thijsen et al., 2008). Calculations were made using the comparative ddCt method (Livak and Schmittgen, 2001).

SDS-PAGE and immunoblotting

SDS-PAGE was performed on 12.5% polyacrylamide gel and 4% stacking gel under reducing conditions. Samples (20 µg) of collected unconditioned complete RPMI medium without phenol red and conditioned control culture medium were electrophoresed and transferred to nitrocellulose. Membrane was incubated with goat anti-Gal-1 antibody (1:1000) overnight at 4°C, with constant shaking. After incubation with biotinylated anti-goat secondary antibody, a Gal-1 band was detected in samples with Pierce ECL Western Blotting Substrate. Membrane was scanned with ImageScanner (Amersham Biosciences).

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry

HTR-8/SVneo cultured media were analyzed using ProteinChip IMAC30 Array (Bio-Rad Laboratories, Inc.). IMAC30 spots were activated by 5 µL of 0.1 mol/L CuSO₄, and incubated 10 min with shaking. After rinsing with distilled water and neutralization buffer (0.1mol/L sodium acetate, pH 4), spots were equilibrated with 5 µL of binding buffer (0.1mol/L sodium phosphate, pH 7.2, 0.5 mol/L NaCl and 10 mmol/L imidazole) before spotting the samples. Five µL of unconditioned media, conditioned media (control) or media after steroid treatments were applied on each spot on the ProteinChip IMAC30 arrays. Subsequent incubation was performed at room temperature in humid chamber for 2 h, with shaking. Each spot was washed with binding buffer and

distilled water and allowed to air-dry. Sinapinic acid dissolved in 0.5% trifluoroacetic acid and 50% acetonitrile was used as energy absorbing matrix (SPA EAM). Finally, SPA EAM was applied on each spot, allowed to air-dry at room temperature and protected from light. Protein array chips were read on Chipergen Protein Chip SELDI system and analyzed using ChipergenExpress Software 3.0 (Fremont, CA). Samples from three individual experiments were analyzed. The protein peaks were auto-identified in the mass/charge range from 14000 to 16000, and peak intensities were normalized by total ion current of mass/charge range from 1500 to 150000 using software.

Statistical analysis

Values expressed as fold change (mRNA expression) or percent of control (SELDI-TOF data) are given as mean ± SEM. Statistical analysis of the data was carried out with the Statistical Software Program version 5.0 (Primer of Biostatistic) using one-way ANOVA, followed by *post-hoc* analysis, with values considered significantly different when $p < 0.05$.

RESULTS

Identification of Galectin-1 as a secreted protein: in vitro studies

Gal-1 regulation by steroid treatment was investigated by examining mRNA expression (Fig. 1). Gal-1 mRNA level was not changed with 10 nmol/L DEX, but was significantly decreased to less than 50% of control level after treatment with 1000 nmol/L of DEX, which suggests that the Gal-1 gene could be regulated partially at transcriptional level by DEX. Both concentrations of PRG induced minor reductions of Gal-1 mRNA level. Treatment with mifepristone significantly decreased the Gal-1 mRNA level, at both concentrations used.

The SELDI-TOF approach was used to analyze the spectrum of secreted proteins and Gal-1 in particular, with or without treatment with steroid hormones. Proteins were trapped onto IMAC30 arrays

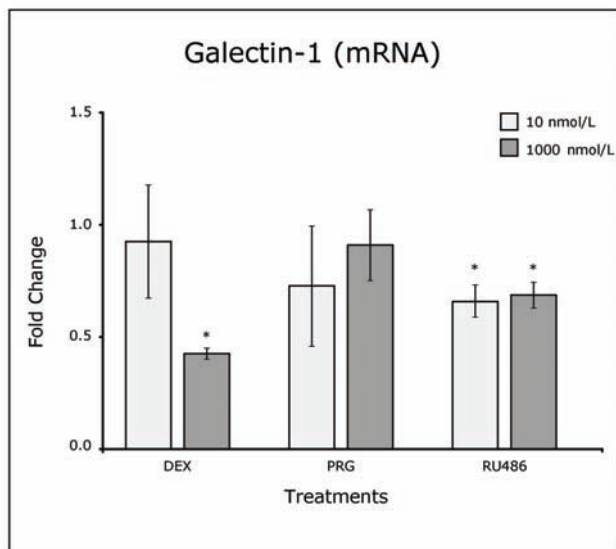


Fig. 1. Effect of DEX, PRG and RU486 on Gal-1 mRNA expression in HTR-8/SVneo cell line. Cells were maintained for 48 h in complete RPMI medium without (control) or with steroids (10 and 1000 nmol/L). Galectin-1 mRNA was normalized to β -actin as endogenous control. Data are represented as fold change of control; $n=3$.

and the resulting proteomic spectra were analyzed. A 14.6 kDa protein was consistently detectable in the media of culture samples (Fig. 2A) as opposed to the zero time medium with sFCS (unconditioned medium in Fig. 2A). To identify this 14.6 kDa protein, the control unconditioned medium and the medium from untreated cells were separated by one-dimensional gel electrophoresis followed by Western blot (Fig. 2B) using anti-Gal-1 antibody. A band of ~14 kDa consistent with Gal-1 molecular mass was obtained in medium from the cell cultures only, indicating that Gal-1 under the conditions employed in the study is secreted into the culture medium. The influence of the steroid and mifepristone treatments on the relative intensity of the 14.6 kDa peak was investigated using the SELDI-TOF approach. The spectra of media from the control and treated cells are shown in Fig. 3A. The 14.6 kDa peak was internally normalized for each of the runs, and the data from three experiments were analyzed statistically (Fig. 3B). Only treatment with 10 nmol/L of PRG significantly stimulated the secretion of Gal-1, to 120% above the control value.

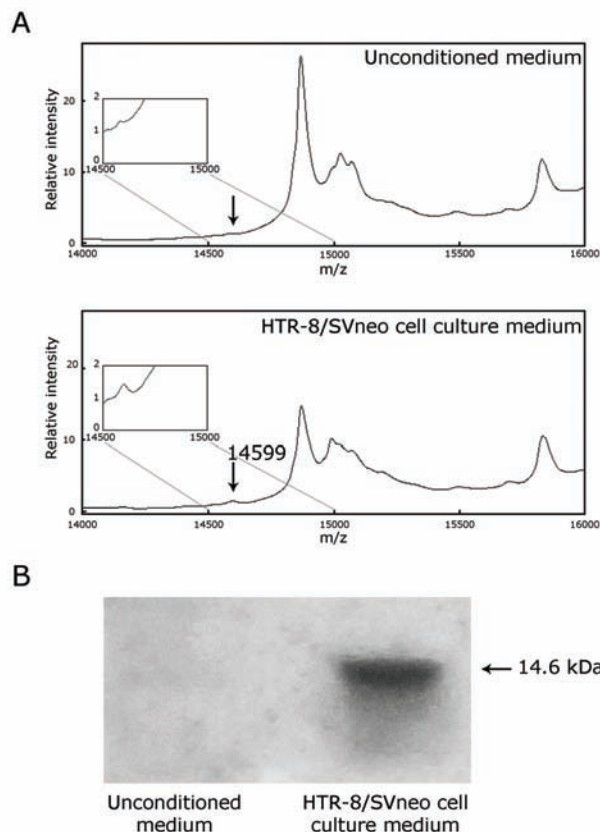


Fig. 2. Secretion of Gal-1 by HTR-8/SVneo cell line. SELDI-TOF spectra of unconditioned complete RPMI medium and conditioned HTR-8/SVneo medium after 48 h of culture (A). Spectra are presented as relative peak intensity (y-axis) versus mass to charge ratio (x-axis). Galectin-1 corresponding peak is indicated with an arrow. (B) Western blot analysis of Gal-1 protein in complete RPMI medium (left) and conditioned HTR-8/SVneo cell culture medium (right). The 14.6 kDa band Gal-1 present in conditioned medium is indicated with an arrow.

DISCUSSION

Recent proteomic studies by other authors have strongly implicated downregulated Gal-1 in early pregnancy loss, a clinical condition characterized by defective trophoblast invasion (Liu et al., 2006). Our *in vitro* findings have also shown that the availability of Gal-1 is positively correlated with trophoblast cell invasiveness (Kolundžić et al., 2011). For this reason, investigation of natural or pharmacologic factors that have a potential to influence Gal-1 may bear clinical

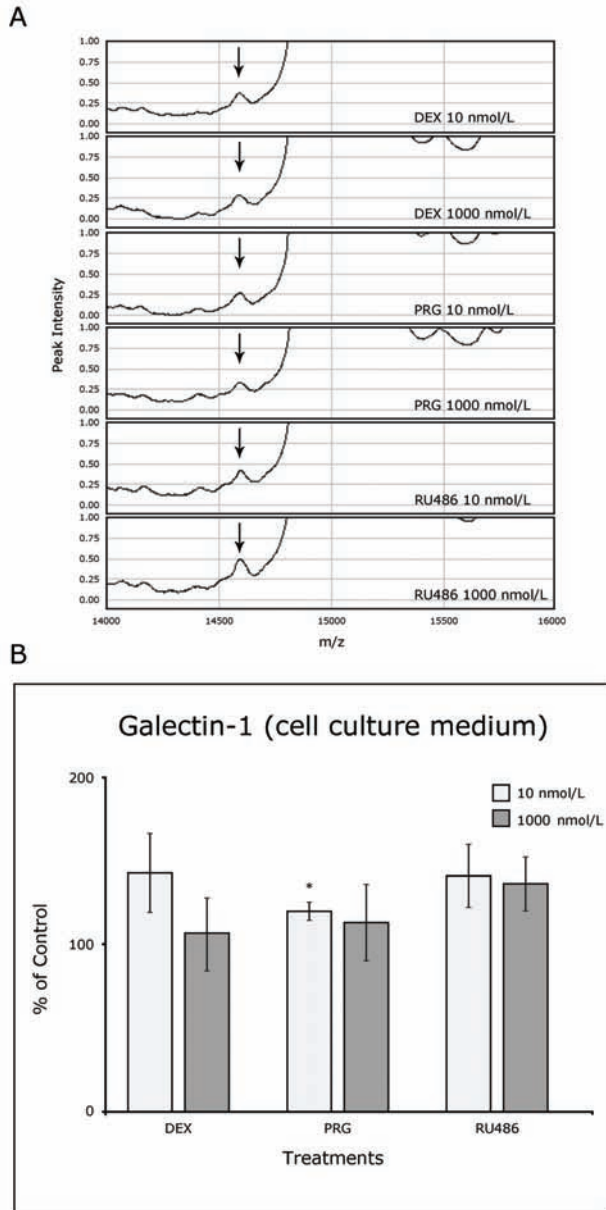


Fig. 3. Effect of DEX, PRG and RU486 on secreted Gal-1. (A) Representative SELDI-TOF spectra of HTR-8/SVneo culture medium after 48 h treatment with 10 and 1000 nmol/L DEX, PRG and RU486. Spectra are presented as relative peak intensity (y-axis) vs mass to charge ratio (x-axis). The peak corresponding to Gal-1 is indicated with an arrow. (B) Relative change in Gal-1 secretion by the HTR-8/SVneo cell line. Peak intensity for each steroid dose is expressed as a percentage of control (untreated HTR-8/SVneo cell medium), and data are represented as mean \pm SEM from three individual experiments.

relevance. The present study demonstrated that steroid hormones could influence transcription (DEX) and secretion (PRG) of Gal-1 in the trophoblast cell line HTR-8/SVneo. We have previously shown that total cellular Gal-1 in the same cells was mildly altered upon treatment with DEX and PRG, which, in case of DEX, was accompanied by a reduction of trophoblast cell invasion (Bojić-Trbojević et al., 2008). The effect of DEX on cellular Gal-1 was dose-dependent and cell-type specific (Bojić-Trbojević et al., 2008; Bojić-Trbojević et al., 2010). The glucocorticoid DEX was shown here to reduce significantly the transcription of Gal-1 at higher concentration (1000 nmol/L). Indeed, a biphasic effect was observed previously with concentrations 0.1-10 nmol/L inducing mild stimulation, and a higher concentration of 100 nmol/L significantly inhibiting Gal-1 (Bojić-Trbojević et al., 2008). Published data indicate that the induction of Gal-1 at the transcriptional level is regulated, at least in part, by different factors (Chiariotti et al., 2004). In the promoter region of the human Gal-1 gene, specific binding sequences for glucocorticoids, heat (environmental) shock, metals, and other factors have been identified, suggesting the role of these factors in the transcriptional control of the Gal-1 gene (Gitt and Baronides, 1991). There is evidence that sex steroids also regulate *LGALS1* expression in the human endometrium during the menstrual cycle and decidualization in pregnancy (von Wolff et al., 2005). Glucocorticoid regulation of trophoblast Gal-1 is in keeping with several lines of evidence, implicating GCs in uteroplacental adherence and chronic regulation of placental protein expression, including ECM proteins (Ryu et al., 1999; Guller et al., 1993; Guller et al., 1995). Treatment of rats with DEX was found to be critical in the regulation of Gal-1 in the neonatal lung at the mRNA and protein levels (Clerch et al., 1987; Sandford et al., 1993).

The placenta is a known source of PRG, important for the maintenance of pregnancy. Our previous results have shown that PRG effect was inverse to that of DEX (Bojić-Trbojević et al., 2008), which was confirmed herein. Unlike DEX, which strongly reduced Gal-1 mRNA, PRG had no effect. On the other hand, mifepristone, which has both antiglucocorticoid and

antiprogestone properties, also induced a significant decrease in Gal-1 mRNA. Previous study has shown the lack of effect of RU486, a GR and progesterone antagonist, on the expression of connexins Cx40, Cx43 and Cx45 in HTR-8/SVneo cells (Cervellati et al., 2011). Furthermore, in non-trophoblastic cell types mifepristone exerts GR agonist behavior, and could act as a partial agonist of the progesterone receptor (Petersen et al., 2008; Yague et al., 2009; Spitz, 2003; Wardell et al., 2010 34). The inhibitory effect of RU486 on trophoblast Gal-1 suggests that other complementary mechanisms, other than anti-progestin action on the endometrium, could be involved in RU486-induced termination of pregnancy.

This study is the first to use WB and SELDI-TOF to detect Gal-1 secreted by the HTR8/SVneo cell in a culture medium. Similar findings have been documented for other cell types (Gieseke et al., 2010). Galectins in general are known to be secreted by a non-classical pathway (Nickel et al., 2005) but the regulation of this process has not been studied in detail. Our data here show that the secretion of Gal-1 from HTR-8/SVneo cells was significantly increased by PRG upon treatment with these steroids, as evidenced by the SELDI-TOF analysis of conditioned media. A more quantitative approach is needed to get a full insight into this process. The potential clinical relevance of these findings remains to be determined.

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