

EXPRESSION OF PROLACTIN RECEPTORS IN THE HUMAN EXTRAVILLOUS CYTOTROPHOBLAST CELL LINE HTR-8/SVNEO

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Abstract — Prolactin is a pituitary hormone that is widely produced at extrapituitary locations. One of the first described sites of its production is the decidualized endometrium, suggesting that it could be potentially significant for local processes such as immunomodulation, embryo implantation, and survival. In this study, expression of prolactin receptor protein is investigated by immunocytochemistry and Western blot in the HTR-8/SVneo cell line, often used as a model of first-trimester trophoblast. The obtained data enabled us to identify both long and intermediate forms of prolactin receptors, which were shown to be significant in PRL signaling in other tissues.

Key words: PRLR, human trophoblast, HTR-8/SVneo, immunocytochemistry

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INTRODUCTION

Prolactin (PRL) is, in addition to being synthesized and secreted by lactotrophic cells of the anterior pituitary gland, also produced by numerous other cells and tissues. It is one of the major proteins synthesized and secreted during decidualization of the human endometrium *in vivo* (Maslar and Riddick, 1979). Prolactin produced in the endometrium can thus act on adjacent cells, such as trophoblast cells, or on the PRL-secreting cell itself, acting in a paracrine or autocrine manner, respectively. The initial step in action of PRL is its binding to a specific membrane receptor, the PRL receptor (PRLR). The gene encoding human PRLR is located on chromosome 5 and contains at least 10 exons for an overall length exceeding 100 kb (Arden et al., 1990). Prolactin mediates its effect at the molecular level by inducing the homodimerization of prolactin receptors, which belong to the class 1 family of cytokine receptors.

Different membrane-bound isoforms of prolactin receptors, termed long, intermediate, and short (S1a and S1b) forms and Δ S1, have been identified *in*

in vivo in humans (Kline et al., 1999). These isoforms have different structure, but they share an identical extracellular ligand-binding domain. Prolactin receptor isoforms are co-expressed in human tissues, although the physiological significance of each of the isoforms remains to be elucidated. It has been suggested that the co-expression of different isoforms in the same type of cell may modulate the function of prolactin on target cells through formation of inactive receptor heterodimers. It is also considered that co-expression of the different receptor isoforms may be a mechanism for conveying multiprolactin receptor effects on target cells through a diversity of signalling cascades that may be receptor isoform-specific (Jabbour et al., 2001).

Expression of PRLR has been previously shown in decidua, chorionic cytotrophoblast, amniotic epithelium, and placenta at term (Maskant et al., 1996). The aim of the present work was to assess expression of PRLR in the HTR-8/Svneo immortalized cell line derived from first trimester extravillous trophoblast using immunocytochemistry and Western blot analysis.

MATERIALS AND METHODS

Materials

Medium RPMI 1640 and fetal calf serum (FCS) were from PAA Laboratories (Linz, Austria). Trypsin solution (0.25%) was from the Institute of Immunology and Virology (Belgrade, Serbia). The Vectastain Elite ABC kit and 3,3' diaminobenzidine (DAB) kit were from Vector Laboratories (Burlingame, CA, USA).

Acrylamide, N,N,N',N'- tetramethylethylenediamine (TEMED), ammonium sulfate, Ponceau S, penicillin, streptomycin, amphotericin B, and glycine were purchased from Sigma Chemical Company (St. Louis, MO, USA). Tris and 2-mercaptoethanol were from ICN Biomedicals, Inc. (Aurora, OH, USA). Roti-Mark prestained standards were from Carl Roth GmbH and Co. (Karlsruhe, Germany). The nitrocellulose transfer membrane Protran[®] was from the Schleicher and Schuell BioScience GmbH Whatman Group. Polyclonal goat anti-human PRLR (recognizing the extracellular domain of hPRLR) was from R and D Systems (UK). Biotinylated rabbit anti-goat IgG was from Vector laboratories.

Cell culture

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 trophoblast 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 and extravillous trophoblast *in vivo*. Cells were cultured in RPMI 1640 supplemented with 10% FCS and an antibiotic-antimycotic agent (penicillin 10000 IU/ml, streptomycin 10mg/ml, amphotericin B 25 µg/ml) under 5% CO₂ and 95% air at 37°C. Cells were grown in tissue culture flasks for propagation, or on glass coverslips for immunocytochemistry.

Human lymphocytes were isolated from whole blood on Lymphoprep[™] gradient (AXIS-SHIELD PoC AS, Norway). Briefly, 6 ml blood with EDTA was diluted with 0.9% NaCl (1:1) and centrifuged

on 3 ml Lymphoprep[™] to isolate lymphocytes. The lymphocyte-containing gradient layer was collected, after which cells were washed in 0.05 M phosphate buffered saline (pH 7.2) (PBS) and used to prepare cytopins. Cytopins of HTR-8/Svneo cells and lymphocytes were prepared by centrifugation of 2 x 10⁴ cells/80 µl PBS at 500 rpm for 2 min. Slides were air dried and fixed in absolute ethanol for 5 min.

Immunocytochemistry

Cytopin preparations of HTR-8/SVneo cells and lymphocytes and HTR-8/SVneo cell monolayers were washed with PBS and stained for PRLR by immunocytochemistry. Briefly, after blocking of nonspecific binding, cells were incubated with anti-PRLR (25 µg/ml) overnight at 4°C. After incubation with biotinylated secondary antibody for 30 min and avidin-biotinylated peroxidase complex (ABC) for 30 min, the reaction was visualized using DAB as chromogen. Between steps, sections were washed three times with PBS. Negative controls were performed routinely. Omission of the primary antibody and use of nonimmune serum, normal horse serum (Vector Laboratories, Burlingame, CA, USA), in place of specific antibody resulted in complete absence of staining. Slides were dehydrated, cleared in xylene, mounted, examined, and photographed using a Carl Zeiss Axio Imager 1.0 microscope (Jena, Germany) with a Canon A640 Digital Camera System (Japan).

SDS-PAGE and immunoblotting

Polyacrylamide electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed on 6% polyacrylamide gel and 4% stacking gel under reducing conditions (Laemmli, 1970; Beeley et al., 1991). The sample was prepared by boiling in 0.125 M Tris-HCl buffer containing 4% SDS, 20% glycerol, 0.1% bromphenol-blue, 10% 2-mercaptoethanol, and a protease inhibitor cocktail composed of [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride]-AEBSF, aprotinin, bestatin hydrochloride, [N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide]-E-64, leupeptin hemisulfate salt, and pepstatin A for 5 min (electrophoresis buffer). For electrophoresis, HTR-8/SVneo

trophoblast cells were lysed in electrophoresis buffer and used as the 1000 x g supernatant, which was loaded at 100 µg/lane. Roti-Mark prestained molecular weight markers composed of seven native proteins (245 kDa—myosin, 123 kDa—β-galactosidase, 77 kDa—serumalbumin, 42 kDa—ovalbumin, 30 kDa—carbonic anhydrase, 25.4 kDa—trypsin inhibitor, and 17 kDa—lysozyme) were used according to the manufacturers' instructions. The proteins separated by electrophoresis were transferred onto a nitrocellulose membrane. The transfer was performed at constant power (1.2 mA/cm²) for 1 h and 30 min. Successful transfer was confirmed by Ponceau S staining of the blots. Nonspecific binding sites on the membrane were blocked with 1% casein overnight at 4°C. After blocking, the membrane with immobilized antigens was incubated with goat anti-PRLR (0.5 µg/ml) overnight at 4°C with constant shaking. After five washes for 5 min each, the blotting membrane was incubated with the biotinylated secondary antibody for 30 min. After intensive washing, the membrane was incubated with avidin-biotinylated peroxidase complex (ABC) for 30 min. Bound conjugates were visualized using DAB/Ni as chromogen. Nonspecific binding was performed with omission of the primary antibody. Membranes were scanned by a Mustek 1200 UB plus scanner and analyzed with the ImageMaster TotalLab program ver. 2.01 (Amersham Biosciences, UK).

RESULTS

Expression of PRLR in HTR-8/SVneo cells was studied using immunocytochemistry. For that purpose, both cytopins (Figs. 1A and 1B) and cell monolayers were analyzed (Fig. 1D). In both, prolactin receptor expression was evident when compared to negative and positive controls in Figs. 1A and 1C, respectively. Staining for prolactin receptor protein can be seen in cytoplasmic and membrane locations (Figs. 1B and 1D). A similar staining pattern can be observed in peripheral blood lymphocytes, known to express PRLR (Fig. 1C). In order to identify the type of receptor expressed, biochemical analysis was performed.

Biochemical analysis of anti-PRLR reactive material was conducted on cell lysate preparations

of HTR-8/SV cells. Western blot analysis of proteins separated on 6% poly-acrylamide gel confirmed the presence of PRLR in HTR-8/SV cells, identifying a major band at estimated molecular weight of 98 kDa (Fig. 2). This band is likely to represent the mature glycosylated long form of the human PRLR. In addition there is a minor specific band of 77 kDa.

DISCUSSION

Cells of the HTR-8/SVneo line are widely used as one of the models of human extravillous trophoblast cells of the first trimester of pregnancy. They have not been previously studied with respect to expression of PRLR. In the present study, immunocytochemistry and Western blot analysis were used to investigate expression of PRLR protein in HTR-8/SVneo cell line. Prolactin receptor was demonstrated within the cytoplasm and associated with the cell membrane. A similar localization of PRLR was described in other cells and tissues, including chorion trophoblast cells at term of pregnancy (M a a s k a n t et al., 1996) and peripheral blood lymphocytes (used as a positive control here). Prolactin receptor protein was visible in membranes of both cytopins and cells grown in monolayers, and it apparently is not critically affected by trypsinization preceding preparation of cytopins. A major receptor species of 98 kDa and a minor 77-kDa band were identified by Western blot, which is consistent with the long and intermediate molecular forms described in other tissues (S h a o et al., 2008). When term placenta, deciduas, and amnion were analyzed previously (M a a s k a n t et al., 1996), six major molecular species of 95, 85, 63, less than 63, more than 30, and 30 kDa were detected using an antibody directed to the extracellular domain of the PRLR. Of these bands, the two 85–95-kDa bands were suggested to represent mature glycosylated forms of PRLR, while the other forms were postulated to be different PRLR-related proteins derived by partial degradation (M a a s k a n t et al., 1996). This result differs from our finding, since M a a s k a n t et al. did not identify an intermediate form in their preparations. This could be caused by intrinsic differences between the cell line used in this study and human tissues at term or may reflect change of isoform expression with advancing gesta-

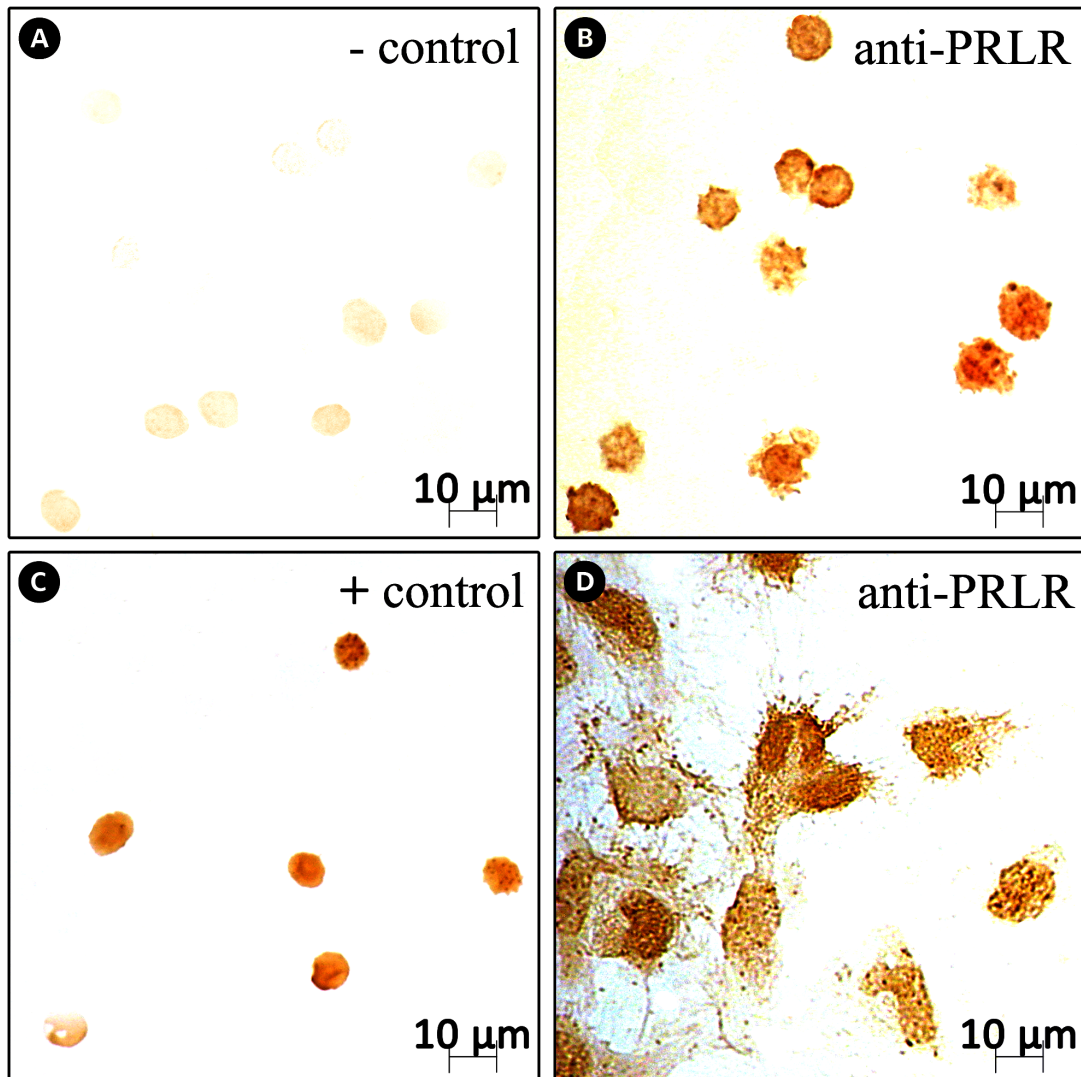


Fig. 1. Immunolocalization of PRLR in HTR-8/SVneo cells (A, B, D) and lymphocytes (C). Staining of cytopspins (A, B, C) and HTR-8/SVneo cell monolayer (D), nonspecific staining (A), and lymphocytes as positive control (C). The HTR-8/SVneo cells specifically express PRLR with strong and fairly uniform cytoplasmic and membrane staining in lymphocytes and HTR-8/SVneo cells (B, C, D). The scale bar represents 10 μm .

tional age, the studied cell line being representative of the first trimester as opposed to tissue after delivery. It is also possible that the lower-molecular-mass form of the two receptors in the range of 85-95 kDa (Maaskant et al., 1996) is in fact an intermediate form of the receptor. On the other hand, when isoform expression of PRLR in different human tissues was studied at the mRNA level, both long and intermediate forms were detected, at considerably

higher levels in the placenta than in any other tissue (Kline et al., 1999), which is in keeping with our finding at the protein level. In other human reproductive tissues such as the fallopian tube, both long (~97-kDa) and intermediate (64-97-kDa) PRLR forms were likewise recently identified (Shao et al., 2008), with molecular masses similar to the ones reported here. In addition, in fallopian tubes a short form of 51-64 kDa was also demonstrated,

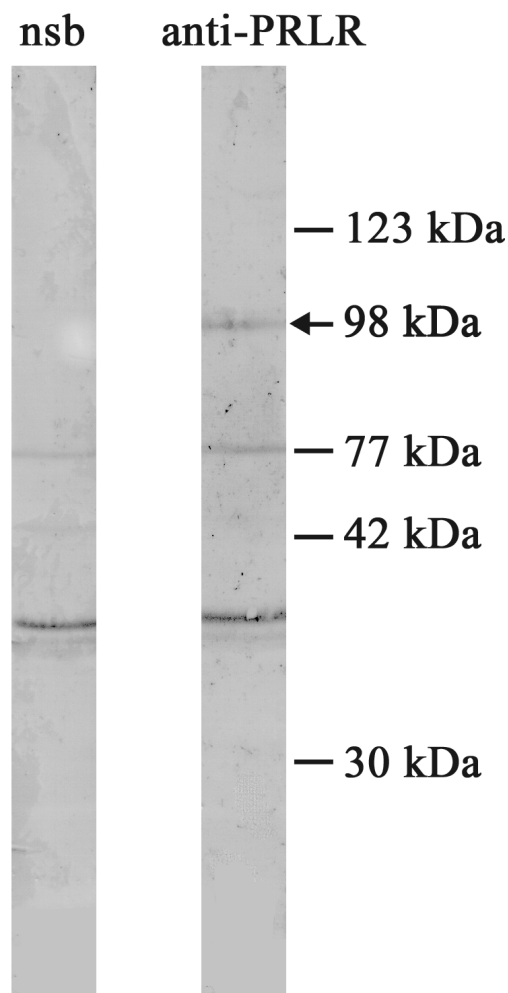


Fig. 2. Western blot analysis of molecular forms of PRLR expressed by HTR-8/SVneo cells. Proteins from cell lysate (100 μ g/lane) were resolved by electrophoresis on 6% polyacrylamide gel under reducing conditions. Membranes with immobilized proteins were probed with anti-PRLR antibody (0.5 μ g/ml). Positions of bands corresponding to molecular weight standards are indicated by their masses, that of a major PRLR band of 98 kDa is shown by an arrow and its estimated mass, while a minor PRLR band at 77 kDa is already marked for one of the standards.

one which was not observed in HTR-8/SVneo cell lysates. Previous studies have shown that the number of isoforms and levels of PRLR expression vary between tissues, suggesting a differing physiological role. It was also demonstrated that homo- and heterodimerization of PRLR variants can be inde-

pendent of PRL (Qazi et al., 2006). The decidua was the first documented extrapituitary site producing PRL (Riddick et al., 1978; Golander et al., 1978). The placenta was also suggested as a site of PRL production (Okamura et al., 1989; Tomoda et al., 1983), but this was not confirmed in later studies. On the other hand, it was shown that the placental syncytiotrophoblast transcribes and translates the PRLR (Masksant et al., 1996). The sources of the ligand for this receptor may be multiple, PRL from the decidua (Ali et al., 1991) or the placental lactogen, which is produced in large amounts by trophoblast.

Based on these findings, it can be concluded that the HTR-8/SVneo cell line is representative of the trophoblast *in vivo* in its expression of PRLR. At least two forms of PRLR are expressed, a long form and an intermediate one, both of which have been shown to be significant for PRL signal transduction in other systems.

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ЕКСПРЕСИЈА РЕЦЕПТОРА ЗА ПРОЛАКТИН У ТРОФОБЛАСТНОЈ ЂЕЛИЈСКОЈ ЛИНИЈИ НТR-8/SVNEO

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Пролактин је хипофизни хормон кога продукују и многа друга ткива у организму. Децидуализовани ендометријум је ткиво за које је међу првима доказано да продукује пролактин, и постоји могућност његовог утицаја на локална ткива и ћелије, што може бити од значаја за имунокомпетентне ћелије и трофобласт. У овој студији испитивана је

експресија пролактинског рецептора у трофобласту коришћењем имуноцитохемије и Western blot анализе на НТR-8/Svneo ћелијској линији, једном од модела за трофобласт ране трудноће. Добијени резултати документују експресију дуге и средње форме пролактинског рецептора, за које је потврђен сигнални значај на другим ткивима.