Expression Level of ABCG2 in the Placenta Decreases from the Mid Stage to the End of Gestation

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The aim of this study was to elucidate the expression pattern of ABCG2 in the placenta from the mid stage to the end of gestation. rABCG2 expression was investigated in rats on the 14th gestation day (gd) and the 20th gd. Expression of the rABCG2 gene and expression of rABCG2 protein in the placenta were detected on gd 14 by RT-PCR and Western blot analysis respectively. The expression level of rABCG2 on gd 20 was less than that on gd 14. We investigated whether progesterone, secreted from the placenta, regulates the expression of ABCG2 in BeWo cells. Expression levels of the ABCG2 gene and protein in BeWo cells were decreased by progesterone treatment. We conclude that progesterone plays a role in reduction in the expression level of ABCG2 in the placenta with the advance of gestation from the mid stage to the end of gestation.

Key words: ABCG2; placenta; regulation; progesterone

The placenta has been viewed as a protective barrier and as a site for nutrient and waste exchange between mother and fetus. A group of transporters in the ATP binding cassette (ABC) superfamily, such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs), in the placenta limit the entry of various potentially toxic drugs and xenobiotics into the fetus. ABCG2 (also called breast cancer resistance protein or mitoxantrone-resistant protein) has also recently been found to be expressed in the placenta at a level as high as the expression levels of ABC transporters.1–3 It is known that ABCG2 transports a variety of substrates, including anticancer drugs, doxorubicin, methotrexate and SN-38, endogenous hormones, and nutrients.2–4

It has been proposed that ABCG2 plays an important role in the blood-placental barrier (BPB) for the fetus. Recently, it has been reported that the expression of P-gp, an efflux transporter functionally similar to ABCG2, in the rat placenta increases from half to the 18th day of pregnancy,5 but little is known about the expression and function of placental ABCG2 over the course of pregnancy. There have been several reports on ABCG2 gene expression and polymorphisms,7,8 and it has been shown that folate deprivation and anticancer drug treatment induce expression of ABCG2,9 but there have been few studies of ABCG2 expression at different stages of placental development.

Modulation of transporter expression and activity by steroids is one key factor in the placenta. In pregnant women, the placenta produces about 1 mmol of progesterone per day at 40 weeks of gestation.10 It has been shown that P-gp activity is modulated by progesterone.11 It is known that the expression of ABCG2 is up-regulated by estrogen.12 Although the placenta also produces a large amount of progesterone during gestation, the effect of progesterone on the expression of ABCG2 has not yet been investigated.

The aim of this study was to elucidate the expression pattern of ABCG2 in the placenta from the mid stage to the end of gestation and to determine the effect of progesterone on expression of ABCG2.

Materials and Methods

Chemicals. Progesterone was purchased from Wako (Osaka, Japan). All other reagents were of the highest grade available and were used without further purification.

Animals. Pregnant Wistar rats were obtained from Slc (Hamamatsu, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”. Placentas were collected from three dams on the 14th gestation day (gd) and the 20th gd. About 10 placentaes were obtained from each dam. Randomly selected placentaes were dissected free of the endometrium and fetal membranes and then snap-frozen in liquid nitrogen and stored at −80°C for RT-PCR and Western blot analysis.

Cell culture and hormone treatment. BeWo cells were obtained from Riken Cell Bank (Saitama, Japan). They were cultivated in nutrient mixture F-12 with Ham
Kaihnh’s modification (Sigma Aldrich Japan, Tokyo) supplemented with 15% fetal bovine serum and 1% penicillin–streptomycin at 37°C under 95% air/5% CO₂. The cells were grown for 4–5 d, and after they reached confluence they were washed with PBS and harvested by exposure to a trypsin–EDTA solution and then passed into new flasks. Progesterone was dissolved in methanol and added to the cells to a final concentration of 10μM for a period of 72 h. Methanol vehicle was used as a control.

**RT-PCR analysis.** Total RNA was prepared from rat placenta tissue and BeWo cells using Isogen (Nippon Gene, Tokyo). Single-strand cDNA was made from 2μg total RNA by reverse transcription (RT) using an Omniscript RT Kit. PCR was performed using Hot Start Taq PCR (Qiagen K.K., Tokyo) with rABCG2, hABC2, rGAPDH, and hGAPDH specific primers through 33–40 cycles of 94°C for 30 s, 51°C for 1 min and 72°C for 1 min. The primers specific to rABCG2, rGAPDH, hABC2 and hGAPDH, were designed on the basis of sequences in the GenBank™ database (accession nos.: AB094089, AF106860, NM_004827 and NM_002046 respectively). The sequences of the specific primers were as follows: the sense sequence was 5’-ACT GAC CCT TCC ATC TT-3’ and the antisense sequence 5'-ATG TCA CCT GAG CGT AC-3’ for rABCG2; the sense sequence was 5’-GCC AAA AGG GTC ATC ATC TC-3’ and the antisense sequence 5’-AAA GGT GGA GGA ATG GGA GT-3’ for rGAPDH; the sense sequence was 5’-CAC CTT ATT GGC CTC AGG AA-3’ and the antisense sequence 5’-GAA ACA CTG GTT GGT CGT CA-3’ for hABC2; and the sense sequence was 5’-TGG AAA TCC CAT CAC CAT CT-3’ and the antisense sequence 5’-TTG AAC TAG AGA GGT CAG GT-3’ for hGAPDH. The PCR products were subjected to electrophoresis on a 1% agarose gel and then visualized by ethidium bromide staining. rABCG2 or hABC2 mRNA data were expressed as ratios between the densitometric values (Scion Image software) of each gene expression. The PCR products were normalized to amplified rGAPDH or hGAPDH, the internal reference gene.

**Western blot analysis.** Total protein extracts were prepared from placenta tissue homogenates or BeWo cells. Tissue homogenates or cells were suspended in lysis buffer containing 1.0% Triton X-100, 0.1% SDS, and 4.5 M urea. The suspension was left to stand for 10 min at 4°C. Then it was centrifuged at 12,000 rpm for 15 min at 4°C, and the protein concentration in the clear supernatant was determined by the method of Lowry. The samples were denatured at 85°C for 5 min in loading buffer containing 50 mM Tris–HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% BPB, and 3.6 M urea, and separated on 4.5% stacking and 10% SDS polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose membranes (Trans-Blot; Bio-Rad) at 15 V for 90 min. The membranes were blocked with PBS containing 0.05% Tween 20 (PBS/T) and 10% non-fat dry milk for 1 h at room temperature. After being washed with PBS/T, the membranes were incubated with monoclonal anti-breast cancer resistance protein (Sigma) (dilution 1:200) or mouse anti-actin monoclonal antibody (Chemicon) (dilution 1:500) for 1 h at room temperature, and washed with PBS/T (3 × 10 min). The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4,000 and washed with PBS/T (3 × 10 min). The bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences Corp., Piscataway, NJ).

**Determination of progesterone concentration.** Serum progesterone was determined using an EIA kit, which was purchased from Oxford Biomedical Research (Oxford, MI). The assays were performed according to the manufacturer’s instructions. Standards were assayed in duplicate and unknowns were assayed in triplicate.

**MTT assay.** The assay relies on the production of a colored formazan by the action of mitochondrial enzymes on MTT. For the MTT assay, BeWo cells were seeded at a cell density of 5 × 10⁴ cells/ml on 96-well plastic plates. Following progesterone pretreatment (72 h), mitoxantrone was added at various concentrations, and cells were incubated for 24 h. At 4 h before the end of treatment, 10μl of PBS containing MTT solution (0.5%) was added. The MTT medium was then replaced with 0.2 ml dimethylsulfoxide, and absorbance was read at 590 nm. Absorbance measured in MTT assays was expressed as percentage of the control (defined as 100%).

**Results**

**Expression of rABCG2 mRNA in the rat placenta on gd 14 and gd 20**

In the first part of this study, we investigated whether expression of rABCG2 mRNA in the placenta changed during gestation. We used placentas obtained from rats on gd 14 and gd 20 and determined the expression of rABCG2 by semi-quantitative RT-PCR (Fig. 1A). A high expression level of rABC2 mRNA was observed on gd 14, but the expression level of rABCG2 on gd 20 was significantly lower than on gd 14 (Fig. 1B).

**Western blot analysis of rABCG2 in the rat placenta on gd 14 and gd 20**

We examined the expression of rABCG2 protein by Western blot analysis in placental samples obtained on gd 14 and gd 20. A band was detected in placental
samples at 100 kDa (Fig. 2). Although the molecular mass of rABCG2 has been reported to be 62 kDa, in rat brain capillaries, N-linked glycosylation of rABCG2 shifts the band to about 100 kDa. Consequently, the 100 kDa band was considered to correspond to rABCG2 in the placenta. The band in the placental samples obtained on gd 20 was detected only faintly.

Progesterone concentrations in rat serum

It is known that estrogen is involved in the up-regulation of ABCG2 expression. Although estrogen secretion from the placenta increases during gestation, the expression level of rABCG2 decreased from gd 14 to gd 20, suggesting that expression of rABCG2 might be regulated by other factors in the placenta.

We determined concentrations of progesterone, which is secreted from the placenta during gestation, in rat serum on gd 14 and gd 20. Progesterone concentrations in pregnant rat serum were significantly higher than in virgin rats. Furthermore, concentration of progesterone in gd 20 was 1.8-fold higher than on gd 14 (Fig. 3).

Fig. 1. Expression of rABCG2 mRNA in the Placenta.

Placenta samples were obtained from three dams on gd 14 and gd 20. Total RNA from each placental tissue was subjected to semi-quantitative RT-PCR using primer pairs specific to rABCG2 (A). Results were normalized against rGAPDH (B). Bars are means with SD for three different samples. *P < 0.05 compared to the control, using Student’s unpaired t test.

Fig. 2. Western Blot Analysis of rABCG2 Expression in the Placenta.

Placenta tissues were obtained from three dams on gd 14 and gd 20. Western blotting was performed using monoclonal anti-breast cancer resistance protein or mouse anti-actin monoclonal antibody. Ten or 20 µg protein was applied per lane.

Fig. 3. Progesterone Concentrations in Rat Serum.

Sera were obtained from three dams on gd 14 and gd 20. Virgin rat serum was used as a control. Progesterone concentrations were determined by EIA assay. Bars are means with SD for three different samples. *P < 0.05 compared to the control, using Student’s unpaired t test.

Effect of progesterone treatment on expression of hABCG2 in BeWo cells

Since a large amount of progesterone is secreted from the placenta during gestation, we examined whether
progesterone affects the expression of hABCG2. We used BeWo cells as a model for the human placenta. BeWo cells were treated with progesterone in culture medium for 72 h before each experiment. The expression level of hABCG2 mRNA was not changed by pretreatment with progesterone at a concentration of 1 μM, but was reduced to 70% of the control level by pretreatment with 10 μM of progesterone (Figs. 4A, 4B).

Western blot analysis demonstrated that the protein level of hABCG2 was also decreased by treatment with 10 μM progesterone (Fig. 5).

**Effect of progesterone on the viability of mitoxantrone-treated cells**

Mitoxantrone, which is widely used as an anti-cancer drug, is a typical substrate of ABCG2. We examined the effect of progesterone treatment on the viability of mitoxantrone-treated cells.

Progesterone treatment significantly decreased the viability of mitoxantrone-treated cells as compared to that of control cells. This suggests that the expression level of hABCG2 was decreased by progesterone treatment (Fig. 6). This result is consistent with the effect of progesterone on the expression levels of hABCG2 mRNA and protein.

**Discussion**

Syncytiotrophoblasts, which form the surface of the placental villi, play an essential role in restricting drug import through the BPB to the fetus. ABCG2 is expressed on the apical side of the syncytiotrophoblast layer, a maternal-facing membrane in contact with the maternal environment, and acts as an efflux pump for
various compounds. During gestation, ABCG2 in the placenta is thought to transport estrogen-sulfate to the maternal side in order to prevent exposure of the fetus to xenobiotics. Therefore, placental ABCG2 plays an important role in the growth and development of the fetus.

Recently, gender differences in the expression of ABCG2 have been observed in rats and mice. In the male rat kidney, estradiol suppresses and testosterone induces expression of rABCG2 mRNA. Moreover, hABCG2 has an estrogen response element in its promoter region, and expression of it in T47D:A18 cells is induced by estrogen treatment. On the other hand, estrogen has been shown to induce post-transcriptional down-regulation of ABCG2 in estrogen receptor-positive cell lines. It is known that the expression of ABCG2 is regulated by steroids, but ABCG2 expression at different stages of placental development has not yet been found.

The expression levels of rABCG2 mRNA and protein in the placenta on gd 20 were significantly lower than on gd 14 (Figs. 1 and 2). This suggests that expression of rABCG2 decreases by physiological alteration during gestation. Although estrogen secretion from the placenta increases with the progress of gestation, expression levels of both rABCG2 mRNA and protein decreased during gestation. In addition, since not only the protein level of rABCG2 but also its mRNA level decreased, it is thought that the decrease in rABCG2 was not caused by post-transcriptional down-regulation of rABCG2 induced by estrogen. These findings suggest that expression of rABCG2 in the placenta is suppressed by other factors in pregnancy.

The expression level of P-gp, which acts as a barrier in BPB like ABCG2, increases with the progress of gestation. On the other hand, although rABCG2 plays a role as a protective barrier for the fetus, the expression levels of rABCG2 mRNA and protein in the placenta decreased from gd 14 to gd 20 in the rats. It is thought that ABCG2 in the BPB plays a role not only as an efflux pump to restrict the transport of xenobiotics, but also in the control of the amount of nutrient permeation for the fetus with growth.

Progesterone is one of the major steroids required for all aspects of female reproductive function, including sexual behavior, mammary gland development, ovulation, implantation, and maintenance of pregnancy. The concentration of progesterone in rat serum increased during gestation, reaching peak levels of 300 nm, and 10 μM of progesterone was needed to suppress hABCG2 expression in BeWo cells. This suggests that the significant decrease in rABCG2 in the rat is not solely a down-regulation effect of progesterone, but that several regulation factors exist in the placenta. Further investigation is needed to clarify this in detail.

It is known that the genomic actions of progesterone are mediated through activation of its nuclear receptor. Several studies have shown that the progesterone receptor (PR) has two isoforms, PR-A and PR-B, and that these two isoforms have different transcriptional activities. PR-A acts as a dominant inhibitor of various other steroid receptors. It has been reported that PR is expressed in the rat placenta, but that both PR-A and PR-B are in JEG-3 cells, a subclone of BeWo cells. Taking all of these facts into consideration, it is thought that progesterone might be involved in the inhibition of other steroid receptors that induces ABCG2 in the placenta through PR-A activation.

In summary, the expression of rABCG2 mRNA and protein in the placenta decreased from the mid stage to the end of gestation. Furthermore, the expression levels of hABCG2 mRNA and protein in BeWo cells were decreased by progesterone treatment. These results suggest that progesterone is involved in the regulation of ABCG2 expression in the placenta.

References


