Effects of sex hormones on regulation of ABCG2 expression in the placental cell line BeWo.

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ABSTRACT: PURPOSE: The aim of this study was to elucidate the effects of sex hormones that are secreted during gestation from the placenta on ABCG2 mRNA and protein expression levels by using the placental cell line BeWo. **METHODS:** We investigated the effects of estrogens (estrone, 17-β-estradiol and estriol) on the expression level of ABCG2 mRNA by RT-PCR. The expression level of ABCG2 protein was analyzed by Western blot analysis. We also investigated the localization of ABCG2 in BeWo cells by Western blot analysis of the plasma membrane fraction and by immunohistochemistry. **RESULTS:** It was found that all estrogens induce the expression of ABCG2 mRNA in a concentration-dependent manner. Furthermore, Western blot analysis showed that 17-β-estradiol induces the expression of ABCG2 protein. Western blot analysis of the plasma membrane fraction and immunohistochemistry showed that ABCG2 localized on only the apical side of BeWo cells and that 17-β-estradiol had no effect on the localization of ABCG2. In addition, progesterone suppressed the induction of ABCG2 expression by 17-β-estradiol at 1-10 μM. **CONCLUSION:** The expression of ABCG2 in the placenta is regulated by estrogen and progesterone during gestation.

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INTRODUCTION

Placenta has been viewed as a protective barrier and as a site for nutrient and waste exchange between the 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-resistant 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]**. It is known that ABCG2 transports a variety of substrates, including anticancer drugs, doxorubicin, methotrexate and SN-38, as well as endogenous hormones and nutrients **[2-5]**.

Recently, there have been some reports on regulation systems of ABCG2 expression. The structure and characteristics of the ABCG2 promoter have been described, and the presence of an estrogen response element in the ABCG2 promoter and estradiol-mediated increase in ABCG2 mRNA expression in T47D:A18 cells have been reported **[6]**. However, it has been reported that estrogen also down-regulates ABCG2 expression by a post-transcriptional mechanism in MCF-7 cells **[7]**. It has been shown that ABCG2 expression level in the human placenta, a major organ of sex hormone secretion, changes with advance of gestational age **[8]**. Furthermore, we previously reported that the expression level of ABCG2 decreases in the mid stage to end stage of gestation in the rat and that progesterone might be involved in the regulation of ABCG2 in the placenta (9).

It has been proposed that ABCG2 plays an important role in the blood-placental barrier (BPB) for the fetus. It is known that modulation of transporter expression and activity of steroids is a key component in the placenta. Trophoblast cells express several steroid receptors and are involved in many regulation systems **[10-12]**. However, the regulation system of ABCG2 in the placenta is not known, and the effect of estrogen and the combined effect of estrogen and progesterone on

ABCG2 expression level have not been investigated in detail. In this study, we used the human choriocarcinoma cell line BeWo as a model of human trophoblast cells and investigated the effects of sex hormones on regulation of ABCG2 in the placenta.

MATERIALS AND METHODS

Chemicals

Estrone, 17-β-estradiol, estriol and progesterone were purchased from Wako (Osaka, Japan). All other reagents were of the highest grade available and used without further purification.

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 Kaighn's modification (Sigma Aldrich Japan, Tokyo) supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin at 37°C under 95% air/5% CO_2. The cells were grown for 4-5 d, and after they reached confluency they were washed with PBS and harvested by exposure to a trypsin-EDTA solution and then passed into new flasks. Estrone, 17-β-estradiol, estriol and progesterone were dissolved in methanol and added to the cells at various concentrations for a period of 72 h. Methanol vehicle was used as a control.

RT-PCR analysis

Total RNA was prepared from BeWo cells using an Isogen (Nippon Gene, Tokyo). Single-strand cDNA was made from 2 µg total RNA by reverse transcription (RT) using an Omniscript RT Kit. PCR was performed using Hot Star Taq PCR (QIAGEN) with ABCG2- and GAPDH-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 ABCG2 and GAPDH were designed on the basis of sequence data in the GenBank™ database (accession no.: **NM_004827** and **NM_002046**, respectively). The sequences of the specific primers were as follows: 5'-CAC CTT ATT GGC CTC AGG AA-3' (sense) and 5'-GAA ACA CTG GTT GGT CGT CA-3' (antisense) for hABCG2 and 5'-TGG AAA TCC CAT CAC CAT CT-3' (sense) and 5'-TTC TAG ACG GCA GGT CAG GT-3' (antisense) for GAPDH. The PCR products were subjected to electrophoresis on a 1% agarose gel and then visualized by ethidium bromide staining. ABCG2 mRNA data were expressed as ratios between the densitometric values (Scion Image software) of each gene expression. The PCR products were normalized to the amplified GAPDH, the internal reference gene.

Western blot analysis

Total protein extracts were prepared from BeWo cells. 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 5 min and sonicated for 15 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 3 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 then 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 (BXP-21) (Sigma) (dilution of 1:200) or mouse anti-actin monoclonal antibody (Chemicon) (dilution of 1:500) for 1 h at room temperature and then washed with PBS/T (3 x 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 x 10 min). The bands were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences Corp., Piscataway, NJ).

Preparation of membrane fraction

Vehicle- or 17-β-estradiol-treated BeWo cells were prepared for the fractionation procedure. The plasma membrane fraction was obtained by using a Plasma Membrane Protein Extraction Kit (BioVision, Mountain view, CA) according to the manufacturer's instructions.

Immunocytochemistry

BeWo cells were fixed in 10% formaldehyde and permeabilized in 0.1% Triton X-100 for 15 minutes. The cells were first incubated in a blocking buffer (10% FBS in PBS) for 60 minutes. Then the cells were incubated overnight at 4°C with monoclonal anti-breast cancer resistance protein (dilution of 1:20). The cells were subsequently incubated for 1 h at room temperature with rhodamine-conjugated donkey anti-mouse secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:400. Nuclei were stained with DAPI and the cells were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). The localization of ABCG2 protein was visualized by using a confocal microscope (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).

RESULTS

Effect of estrogen on expression level of ABCG2 mRNA

In the first part of this study, we investigated whether expression of ABCG2 in BeWo cells is changed by estrogen treatment. In all experiments, BeWo cells were treated with 0.01-10 μM estrogen in culture medium for 72 h, because of no difference was observed in a shorter period (data not shown). The expression level of ABCG2 mRNA was determined by semi-quantitative RT-PCR. We found that all estrogens, estrone, 17-β-estradiol and estriol, induced expression of ABCG2 mRNA in a concentration-dependent manner. The expression level of ABCG2 in cells treated with 17-β-estradiol or estriol at 1-10 μM was 1.4-1.6-fold higher than that in vehicle- treated cells (Figure 1).

Effect of 17-β-estradiol on expression level of ABCG2 protein

It is known that 17-β-estradiol is the strongest activator among the endogenous estrogens for the estrogen receptor activation pathway and that estrogen is involved in up-regulation of the expression of ABCG2 mRNA by genome reaction. We also examined the effect of 17-β-estradiol on the expression level of ABCG2 protein in BeWo cells by Western blot analysis. A single band for ABCG2 was observed at 60-70 kDa. The expression level of ABCG2 protein was increased by 2-4 fold in a 17-β-estradiol concentration-dependent manner (Figure 2).

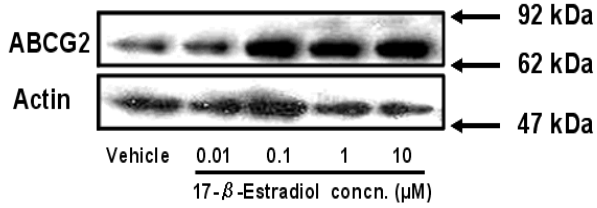

Figure 2: Effect of estradiol on expression of ABCG2 protein in BeWo cells. Cells were treated with culture medium containing 0.01-10 μM 17-β-estradiol for 72 h. Methanol vehicle was used as a control. ABCG2 protein levels were determined by Western blot analysis. Three μg of cell lysate was applied in each lane. Data shown are typical results from three independent experiments.

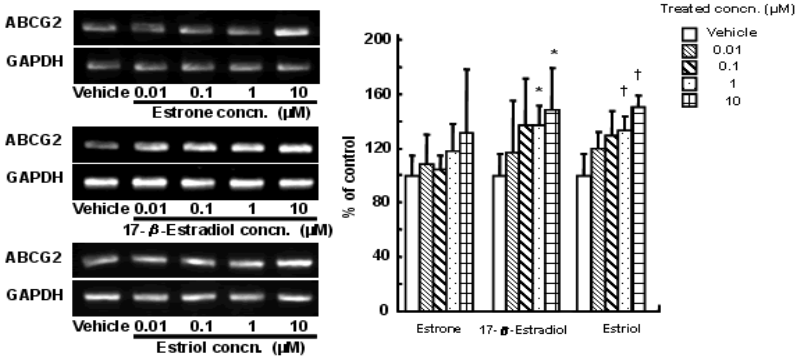

Figure 1: Effect of estrogen on expression of ABCG2 in BeWo cells. Cells were treated with culture medium containing 0.01-10 μM estrone, 17-β-estradiol or estriol for 72 h. Methanol vehicle was used as a control. ABCG2 mRNA levels were determined by semi-quantitative RT-PCR. *P<0.05, +P<0.05 compared to each vehicle, using Student's unpaired t test.

Effect of 17-β-estradiol on localization of ABCG2 in BeWo cells

Figures 1 and 2 show the 17-β-estradiol-induced expression level of ABCG2. However, it is known that 17-β-estradiol has an effect on retrieval of some proteins from the plasma membrane to the intracellular domain. We therefore investigated whether ABCG2 induced by 17-β-estradiol changes its localization in BeWo cells. We prepared a plasma membrane fraction from BeWo cells and determined the expression of ABCG2 by Western blot analysis (Figure 3).

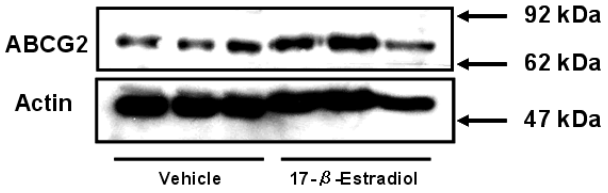

Figure 3: Expression level of ABCG2 protein in plasma membrane fraction of BeWo cells. Cells were treated with culture medium containing 10 μM 17-β-estradiol for 72 h. Methanol vehicle was used as a control. Membrane fractions were prepared as described in *Materials and Methods*, and the expression levels of ABCG2 were determined by Western blot analysis. One μg of plasma membrane fraction was applied in each lane. Data shown are typical results from three independent experiments.

The expression level of ABCG2 protein in the plasma membrane fraction from 17-β-estradiol-treated cells was higher than that in the membrane fraction from vehicle-treated cells. We also performed immunocytochemistry to determine the localization of ABCG2. A red signal (rhodamine, ABCG2) was observed at the plasma membrane (Figure 4A and 4C). In Z-sectioned images, a red signal was observed at the apical side of the cells and no red signal was observed at the same depth as that of the blue signal (DAPI, nucleus), suggesting that ABCG2 protein was expressed only in the apical membrane of BeWo cells (Figures 4B and 4D). There was no difference between the localization of ABCG2 in vehicle-treated cells and that in 17-β-estradiol-treated cells.

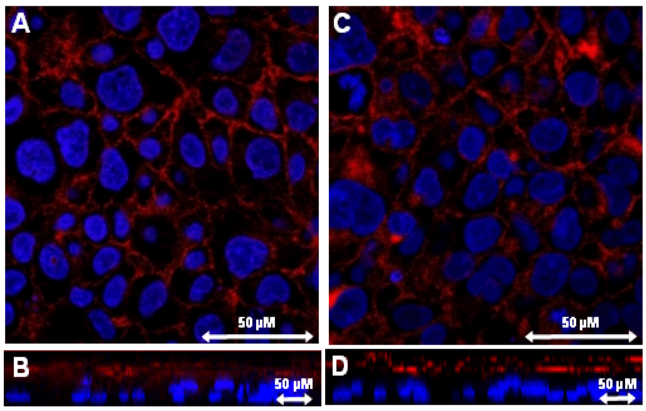

Figure 4: Immunohistochemistry of ABCG2 in BeWo cells. Cells were treated with culture medium containing 10 μM 17-β-estradiol for 72 h (C, D). Methanol vehicle was used as a control (A, B). Localization of ABCG2 was determined using an antibody against ABCG2 (red). Nuclei were stained with DAPI (blue). B and D show Z-sectioned images.

Effect of progesterone on induction of the expression of ABCG2 by 17-β-estradiol

Estrogen is not the only major hormone that the placenta secretes; progesterone is also secreted from the placenta during gestation. Moreover, we previously reported that the expression level of ABCG2 in BeWo cells is decreased by progesterone treatment. However, the combined effect of estrogen and progesterone on ABCG2 expression has not been investigated in detail. We examined the effect of progesterone on induction of the expression of ABCG2 by 17-β-estradiol. We cultured BeWo cells in medium containing 10 μM of 17-β-estradiol and various concentrations of progesterone. Western blot analysis showed that progesterone had a suppressive effect on the induction of ABCG2 expression by 17-β-estradiol. The level of expression of ABCG2 induced by 17-β-estradiol was decreased to about the same level as that in vehicle-treated cells in the presence of progesterone at concentrations of 1-10 μM (Figure 5).

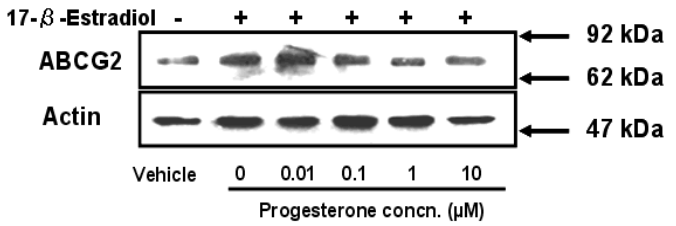

Figure 5: Combined effect of progesterone and 17-β-estradiol on expression of ABCG2 protein. Cells were treated with culture medium containing 10 μM 17-β-estradiol and various concentrations of progesterone for 72 h. Methanol vehicle was used as a control. Three μg of cell lysate was applied in each lane. Data shown are typical results from three independent experiments.

DISCUSSION

Syncytiotrophoblasts, which form the surface of the placenta villi, play an essential role in restriction of drug import through the BPB to the fetus. ABCG2 is expressed in the apical side of the syncytiotrophoblast layer, a maternal-facing membrane in contact with the maternal environment, and acts as an efflux pump of various compounds (13). During gestation, ABCG2 in the placenta is thought to transport estrogen-sulfate to the maternal side for protection of the fetus from xenobiotics. Therefore, placental ABCG2 plays an important role in fetus growth and development.

It has recently been shown that ABCG2 has an estrogen response element in its promoter region and that its expression in T47D:A18 cells is induced by estrogen treatment **[6]**. On the other hand, estrogen has been shown to induce post-transcriptional down-regulation of ABCG2 in estrogen receptor-positive cell lines **[7]**. Furthermore, gender differences in the expression of ABCG2 have been observed in rats and mice. In the male rat kidney, 17-β-estradiol suppresses and testosterone induces expression of rABCG2 mRNA **[14]**. Therefore, it is known that the expression of ABCG2 is regulated by steroids, but the regulation system of ABCG2 expression in the placenta, which secretes various hormones, has not been revealed in detail.

It was shown in this study that the expression level of ABCG2 mRNA was increased by estrone, 17-β-estradiol and estriol treatment in a concentration-dependent manner (Figure 1).

It was also shown that 17-β-estradiol treatment increased the expression level of ABCG2 protein (Figure 2). This finding suggests that the expression of ABCG2 is regulated by estrogen through the estrogen receptor-activated pathway in the ABCG2 promoter region and that there is no post-transcriptional down-regulation pathway by estrogen in the placenta.

17-β-estradiol has various effects on some transporter regulation systems **[15-17]**. Multidrug resistance protein 2 in the sinusoidal membrane of the rat liver was retrieved to the intracellular domain by 17-β-estradiol treatment **[18]**. Therefore, it is also possible that the systems by which ABCG2 is regulated by estrogen in the placenta also a number of pathways. We investigated whether ABCG2 protein induced by 17-β-estradiol changes its localization in BeWo cells. Western blot analysis showed that the expression level of ABCG2 on the plasma membrane was increased by 17-β-estradiol treatment (Figure 3). Although only one μg of plasma membrane fraction showed a large amount of actin as a house-keeping protein, this was because of the membrane skeleton was concentrated at the process of the fractionation. We also found that ABCG2 was not expressed on the intracellular domain and was expressed only on the apical membrane in both vehicle-treated and 17-β-estradiol-treated cells, suggesting that its localization was not changed by 17-β-estradiol treatment (Figure 4). These results suggest that estrogen has only an inductive effect by genomic reaction and no other effect on ABCG2 expression in the placenta.

We showed that the expression level of ABCG2 was increased by estrogen in BeWo cells, and it is known that estrogen secretion from the placenta increases with advance of gestation **[19]**. On the other hand, we previously reported that the expression level of ABCG2 decreases from the mid stage to the end of gestation in the rat placenta **[9]**. Furthermore, it has been shown that the expression level of ABCG2 in the human placenta decreases from gestation days 90-120 to 60-90 **[8]**. Therefore, we hypothesized that the placenta has regulation systems other than estrogen that suppress ABCG2 expression. We investigated the effect of progesterone, which has

been shown to down-regulate ABCG2 **[9]**, on induction of ABCG2 expression by 17-β-estradiol. The induction of ABCG2 expression by 17-β-estradiol was suppressed by combined treatment with progesterone (Figure 5). It has been reported that progesterone receptor (PR)-A and PR-B are expressed on JEG-3 cells **[20]**, a subclone of BeWo cells. Several studies have shown that PR-A acts as a dominant inhibitor of various other steroid receptors **[21-22]**. These findings suggest that progesterone inhibited the effect of 17-β-estradiol at the estrogen-estrogen receptor pathway by PR-A activation. However, the concentration of each hormone used in this study is higher than the *in vivo* concentration during gestation **[23]**. It has also been reported that folate deprivation down-regulates the expression level of ABCG2 in MCF-7 cells **[24]**. Since folate is an essential nutrient for the fetus, folate might be involved in the regulation of ABCG2 expression in the placenta. Taken together, these findings indicate that estrogen and progesterone secreted during gestation are involved in the regulation system of ABCG2 in the placenta but that they are not the only factors involved in the regulation of ABCG2 expression and that other regulation systems and/or pathways exist. Further detailed investigation is needed to reveal the relationship between the role and regulation mechanism of ABCG2 in placenta.

In conclusion, estrogen induces the expression of both mRNA and protein in BeWo cells. In addition, ABCG2 is localized at the apical membrane of BeWo cells and 17-β-estradiol treatment has no effect on its localization. These findings suggest that estrogen has no post-transcriptional effect on the expression of ABCG2 but increases the expression level of ABCG2 by genomic reaction in the placenta. Moreover, progesterone suppresses the induction of ABCG2 expression by 17-β-estradiol. Taking these facts into consideration, sex hormones are involved in the regulation of ABCG2 expression in the placenta during gestation.

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