PRACE ORYGINALNE
położnictwo

Immunolocalization of ABC drug transporters in human placenta from normal and gestational diabetic pregnancies

Immunolokalizacja transporterów leków ABC w łożyskach ludzkich pochodzących z ciąż prawidłowych i powikłanych cukrzycą ciążową

Danuta Kozłowska-Rup[1], Piotr Czekaj[1], Danuta Plewka[1], Jerzy Sikora[2]

[1] Department of Cytophysiology, Chair of Histology and Embryology, School of Medicine in Katowice, Medical University of Silesia, Katowice, Poland
[2] Chair and Department of Perinatology and Gynecology, School of Health Sciences, Medical University of Silesia, Katowice, Poland

Abstract

Objectives: *ABC transporters, P-gp, MDR3, BCRP and MRP1, can bind both endo- and exogenous ligands. The latter include immunosuppressive, anticancer, sedative, anticonvulsant, antiparasitic and cardiovascular drugs, as well as HIV protease inhibitors and antibiotics. Protein transporters are also involved in tissue distribution of orally administered medicines in combination therapy for gestational diabetes mellitus (GDM) and could be used during GDM treatment. The distribution depends on transporter specificity, its expression and subcellular localization.*

The aim: *The aim of the study was to compare P-gp, MDR3, BCRP and MRP1 localization in placental tissues from normal and GDM diabetic pregnancies.*

Material and methods: *Tissue samples were taken from 10 normal and 10 GDM placentas. Immunohistochemical reactions were performed with the use of adequate monoclonal antibodies. Avidin-biotin-peroxidase complex method was used for the visualization of antigen-antibody complexes.*

Results: *P-gp, MDR3 and BCRP were found in all parts of normal human placenta i.e. the amniotic epithelium, cytotrophoblast, syncytiotrophoblast and decidual cells. P-gp and BCRP, but not MDR3 and MRP1, were also localized on the endothelial cells of fetal blood vessels in the chorionic plate, as well as stem and tertiary villi. MRP1 expression was observed in the cytotrophoblast and the syncytiotrophoblast. Its expression was very low or undetectable in the amniotic epithelium and the majority of decidual cells. Immunohistochemical reactions within the syncytiotrophoblast showed apical (P-gp, BCRP), apical and basal (MRP1) or diffuse (MDR3) distribution. The main changes observed in GDM placentas included weaker MRP1 and MDR3 positive reactions in the syncytiotrophoblast, slightly lower expression of P-gp in the decidual and amniotic epithelial cells, and MDR3 in the amniotic epithelium.*

Corresponding author:
Piotr Czekaj,
Department of Cytophysiology, Chair of Histology and Embryology, School of Medicine in Katowice,
Medical University of Silesia, Katowice,
Medyków 18, 40-752 Katowice; Poland
Phone: +48 32 2088374
Fax : +48 32 2526574
e-mail: pcz@sum.edu.pl.

Otrzymano: **12.06.2013**
Zaakceptowano do druku: **15.11.2013**

Conclusions: *Our results indicate that GDM-related changes in the environment of placental cells do not substantially influence tissue and subcellular location of ABC transporters. Nevertheless, the expression of P-gp, MDR3 and MRP1 may be lower in comparison to normal placentas. Basal syncytiotrophoblast transporters, MRP1 and MDR3, seem to be more sensitive to the influence of GDM than apical proteins, what may result in altered biodisposition of endogenous substrates and drugs.*

Key words: **ABC drug transporters / P-glycoprotein / breast cancer resistance protein BCRP / multidrug resistance protein MDR3 / multidrug resistance-associated protein MRP1 / human placenta / gestational diabetes mellitus /**

Streszczenie

Wstęp: *Białka transportowe ABC: P-gp, MDR3, BCRP i MRP1 są zdolne do wiązania ligandów, zarówno endo-, jak i egzogennych. Wśród nich znajdują się leki immunosupresyjne, przeciwnowotworowe, uspakajające, przeciwdrgawkowe, przeciwpasożytnicze i sercowo-naczyniowe, inhibitory proteazy HIV oraz antybiotyki. Transportery uczestniczą też w dystrybucji tkankowej doustnych leków stosowanych w leczeniu skojarzonym cukrzycy, które mogłyby mieć zastosowanie także w terapii cukrzycy ciążowej (GDM). Dystrybucja tych związków zależy m.in. od specyficzności transportera, jego ekspresji oraz lokalizacji subkomórkowej.*

Cel pracy: *Porównanie lokalizacji białek transportowych P-gp, MDR3, BCRP i MRP1 w tkankach łożysk ludzkich pochodzących z ciąży prawidłowej i powikłanej cukrzycą ciążową.*

Materiał i metody: *Z 10 łożysk pochodzących z ciąż prawidłowych i 10 łożysk z ciąż powikłanych cukrzycą ciążową pobrano wycinki tkankowe, z których uzyskano skrawki parafinowe. Wykonano reakcje immunohistochemiczne z zastosowaniem odpowiednich przeciwciał monoklonalnych oraz dokonano wizualizacji odczynów z zastosowaniem kompleksu awidyna-biotyna-peroksydaza.*

Wyniki: *Zidentyfikowano P-gp, MDR3 i BCRP we wszystkich warstwach prawidłowego łożyska ludzkiego, m.in.: w nabłonku owodni, cytotrofoblaście, syncytiotrofoblaście oraz w komórkach doczesnowych. P-gp i BCRP zlokalizowano także w śródbłonku płodowych naczyń krwionośnych w płycie kosmówkowej oraz w pniach kosmówkowych i kosmkach trzeciorzędowych. Dla odmiany, ekspresja MDR3 i MRP1 w śródbłonku była słaba lub nieoznaczalna. Ekspresję MRP1 zaobserwowano w cytotrofoblaście i syncytiotrofoblaście. W komórkach nabłonka owodni i większości komórek doczesnowych ekspresja ta była bardzo niska lub nieoznaczalna. W syncytiotrofoblaście reakcje immunohistochemiczne lokalizowały się w części szczytowej (P-gp, BCRP), szczytowej i podstawnej (MRP1) lub miały charakter dyfuzyjny (MDR3). Najistotniejszymi zmianami obserwowanymi w łożyskach GDM były: słabsza pozytywna reakcja dla MRP1 i MDR3 w syncytiotrofoblaście, niższa ekspresja P-gp w doczesnej i w nabłonku owodni oraz MDR3 w nabłonku owodni.*

Wnioski: *Przeprowadzone obserwacje wskazują, że procesy zachodzące w środowisku komórek łożyskowych w GDM nie powodują zasadniczych zmian w lokalizacji tkankowej i subkomórkowej transporterów ABC. Tym niemniej, ekspresja P-gp, MDR3 i MRP1 może być obniżona w stosunku do łożysk prawidłowych. Transportery typowe dla części podstawnej syncytiotrofoblastu: MDR3 i MRP1 wydają się bardziej wrażliwe na wpływ GDM niż transportery apikalne, co może skutkować zmianami w biodyspozycji substratów endogennych i ksenobiotyków.*

Słowa kluczowe: **transportery ABC / glikoproteina P / białko oporności raka sutka BCRP / białko oporności wielolekowej MDR3 / białko związane z opornością wielolekową MRP1 / łożysko ludzkie / cukrzyca ciążowa /**

Skróty:
ABC – nadrodzina białek wiążących ATP; P-gp – glikoproteina P; BCRP – białko oporności raka sutka; MDR3 – białko oporności wielolekowej 3; MRP1 – białko związane z opornością wielolekową; GDM – cukrzyca ciążowa.

Abbreviations:
ABC, ATP-Binding Cassette Superfamily; P-gp, P-glycoprotein; BCRP, Breast Cancer Resistance Protein; MDR3, Multidrug Resistance Protein 3; MRP1, Multidrug Resistance-Associated Protein 1; GDM, Gestational Diabetes Mellitus

Introduction

The placental barrier between maternal and fetal blood circulations is an important factor limiting the influx of endogenous compounds that are essential for proper fetal development, and those products of cell metabolism and xenobiotics which might pose a threat for the developing organism. The syncytiotrophoblast, with underlying basement membrane, and the endothelium of the fetal capillaries, lying on its own basement membrane, constitute the main components of the placental barrier. The syncytiotrophoblast is formed by fusion of mononuclear cytotrophoblast cells. The apical surface of the syncytiotrophoblast is in direct contact with maternal blood flow.

It is enlarged by numerous microvilli and has approximately 13 m² by the end of pregnancy. The thickness of the placental barrier changes over the course of pregnancy, decreasing to 4-5 μm towards the end. Syncytiotrophoblast plasma membrane is not only structurally polarized because of the existence of the brush border on its apical surface, but it is also functionally differentiated as a consequence of specific, apical or basolateral distribution of enzymes, hormone receptors and transporters [1, 2, 3, 4]. Transport proteins are also present in the plasma membrane of amniotic epithelial cells and endothelial cells of blood vessels [5, 6]. In general, they ensure mother-to-fetus transfer of nutrients, and metabolic products in the opposite direction. Furthermore, some protein transporters, involved in the efflux of endogenous ligands behind the placental barrier, can bind exogenous compounds and protect the fetus against xenobiotics [6, 7].

The ATP-Binding Cassette (ABC) superfamily represents a group of placental proteins that transport their substrates across the plasma membrane against the concentration gradient, with the use of ATP as the energy source. Glycoprotein P (P-gp), Multidrug Resistance Protein 3 (MDR3), and Breast Cancer Resistance Protein (BCRP) are known as the efflux transporters, while Multidrug Resistance-Associated Protein 1 (MRP1) as the efflux-influx transporter [5, 7, 8, 9]. P-gp mRNA and protein expression in the syncytiotrophoblast has been known to decrease during pregnancy. In addition, transplacental passage of drugs that are P-gp substrates is much higher at 38-40 weeks of gestation than in earlier pregnancy. Thus, P-gp-dependent protection may be the weakest in the late stages of fetal development [10, 11]. Whilst P-gp expression is diminished, MRP1 and MRP2 expression continues to increase throughout pregnancy [12]. Among other MRP proteins, MRP3 expression remains unchanged up to the end of the third trimester. In turn, MDR5 decreases at the same time. BCRP shows high expression during pregnancy, achieving maximal protein level in the middle of the gestation period. It should be noted that the placental BCRP mRNA level is more than one hundred times higher than in other tissues. It is believed that different expression levels of ABC transporters during pregnancy allow for the continuous functioning of compensatory mechanisms for fetal protection at various stages of intrauterine development [13].

The expression of placental ABC transporters is probably regulated by steroids, growth factors and cytokines secreted during pregnancy [10, 13]. In case of P-gp and BCRP, it may depend on the polymorphisms of ABCB1 and ABCG2 genes, respectively [11, 13, 14]. Secretion of regulatory factors changes significantly in pregnancy-associated disorders. The incidence of both, diabetes and gestational diabetes mellitus (GDM), continues to increase among pregnant women. GDM has been estimated to develop in 3-5% of pregnant women and, if untreated, can lead to severe complications [15]. As ABC proteins are able to transfer numerous chemical substances (organic and inorganic ions, proteins, steroids, thyroxin, vitamins and prostaglandins), and therapeutics (immunosuppressive, anticancer, sedative, anticonvulsant, antiparasitic and cardiovascular drugs, HIV protease inhibitors, antibiotics and oral hypoglycemic agents), across the cytoplasmic membrane, understanding the role of ABC transporters in tissue distribution of these compounds in the mother, placenta and fetus, may be crucial for evaluating therapy efficiency in diabetes and other accompanying diseases in pregnancy [16, 17, 18]. Drug transfer across the placenta depends, among others, on specificity (type), expression and localization of placental ABC transporters. As a consequence of various localizations and specific functions, the directions of drug passage can be changed, determining fetal drug exposure. The knowledge could be crucial for optimizing the therapeutic strategy for pregnant mother and protecting the fetus.

Data on proper tissue and cellular location of ABC transport proteins in the human placenta are scarce. Hence, the direction of drug passage and transporter functional role during pregnancy have not been fully explained [19]. Changes in the localization of placental transporter proteins in GDM-complicated pregnancy are yet to be elucidated. In our study we used the immunohistochemical method to determine tissue and cellular localization of ABC transporter proteins: P-gp, MDR3, BCRP and MRP1, in human placenta from physiological and GDM-complicated pregnancies.

Material and methods

Human placentas

Human full-term placentas were obtained after elective, uncomplicated caesarian sections from the Perinatology and Gynecology Ward, Central Clinical Hospital, Medical University of Silesia, Katowice. Ten healthy women (control group) and ten subjects with diet-managed GDM (study group), with no symptoms of infection, were recruited. All participants gave their informed consent for the study.

Diagnostic criteria for GDM

Diagnostic criteria for GDM were in accordance with the Polish Gynecological Society standards of medical care in management of women with diabetes [20]. The study group included patients with onset and first recognition of glucose intolerance during pregnancy, although no known risk factors for GDM were recognized at that time. Fasting plasma glucose level <100mg/dl was evaluated in all patients at the first prenatal visit. Afterwards, between 24 and 28 weeks of gestation, oral glucose tolerance test (OGTT) was performed. The test involved drinking a solution containing 75 g glucose by fasting individuals, and measurements of the blood glucose concentration at fasting, and after 1 and 2 hours. The diagnostic criteria for GDM from the WHO guidelines were applied for OGTT evaluation. The test was "positive" when at least one of the following values was found: fasting blood glucose level ≥ 100 mg/dl (5.5 mmol/l), 1-hour blood glucose level ≥180 mg/dl (10.0 mmol/l) or 2-hour blood glucose level ≥140mg/dl (7.8 mmol/l). Before the final diagnosis, a 24-hour glycemic profile and glycated hemoglobin (HbA1c) levels were evaluated. GDM type G1 was diagnosed in each patient from the study group if blood glucose concentration was 60-90 mg/dl (3.3-5.0 mmol/); maximal glucose level one hour after a meal was <120mg/dl (<6.7 mmol/l); glucose concentration between 2.00 and 4.00 hours was >60 mg/dl (3.3 mmol/l); mean 24-hour glycemic value was 95 mg/dl (5.3 mmol/l); and HbA1c level was <6.1%. Then, dietary treatment was implemented in order to achieve normoglycemia. Therapy effectiveness was monitored by measuring fasting plasma glucose levels (once a week), a 24-hour glycemic profile (once a month), and HbA1c levels (every 6 weeks).

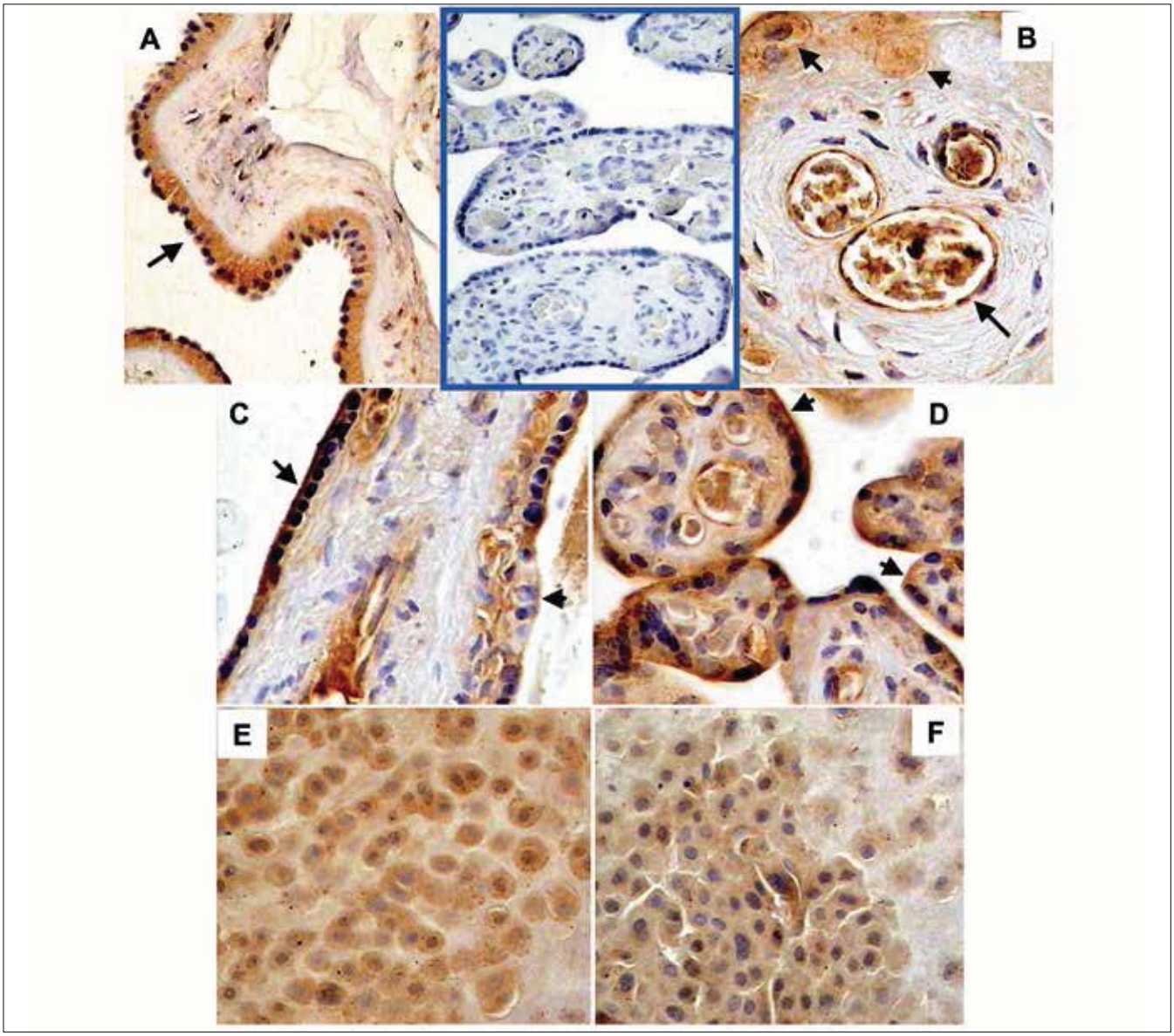

Figure 1. P-gp immunolocalization in normal and GDM human placentas at term.
Microphotographs show P-gp detection in human placenta. A – in the amniotic epithelium (GDM); B – in extravillous cytotrophoblast cells and fetal blood vessel endothelial cells in the chorionic plate (Control). Arrows point to the amniotic epithelium, cytotrophoblast and endothelium. C – shows P-gp expression at the apical syncytiotrophoblast plasma membrane in stem villi (Control); D – in tertiary villi (GDM). Arrows point to the places of both, high and low expression. E and F – show a difference in P-gp expression between control and GDM decidual cells, respectively. Negative control is shown in the blue frame. Magnification: A, E, F and blue frame – 200x; B, C, D – 400x.

Table I. Antibodies used for ABC transporters immunolocalization.

Primary antibody	Dilution	Secondary antibody	Dilution
Anti-P-Glycoprotein (MDR), Clone F4 (Sigma)	1:500	Anti-Mouse IgG biotinylated antibody (Vector)	1:200
Anti-MDR3 P-Glycoprotein, Clone P3II-26 (Sigma)	1:100		
BCRP/ABCG2 antibody [BXP-21] (Abcam)	1:200		
MRP1 (QCRL-1) (Santa Cruz Biotechnology)	1:500		

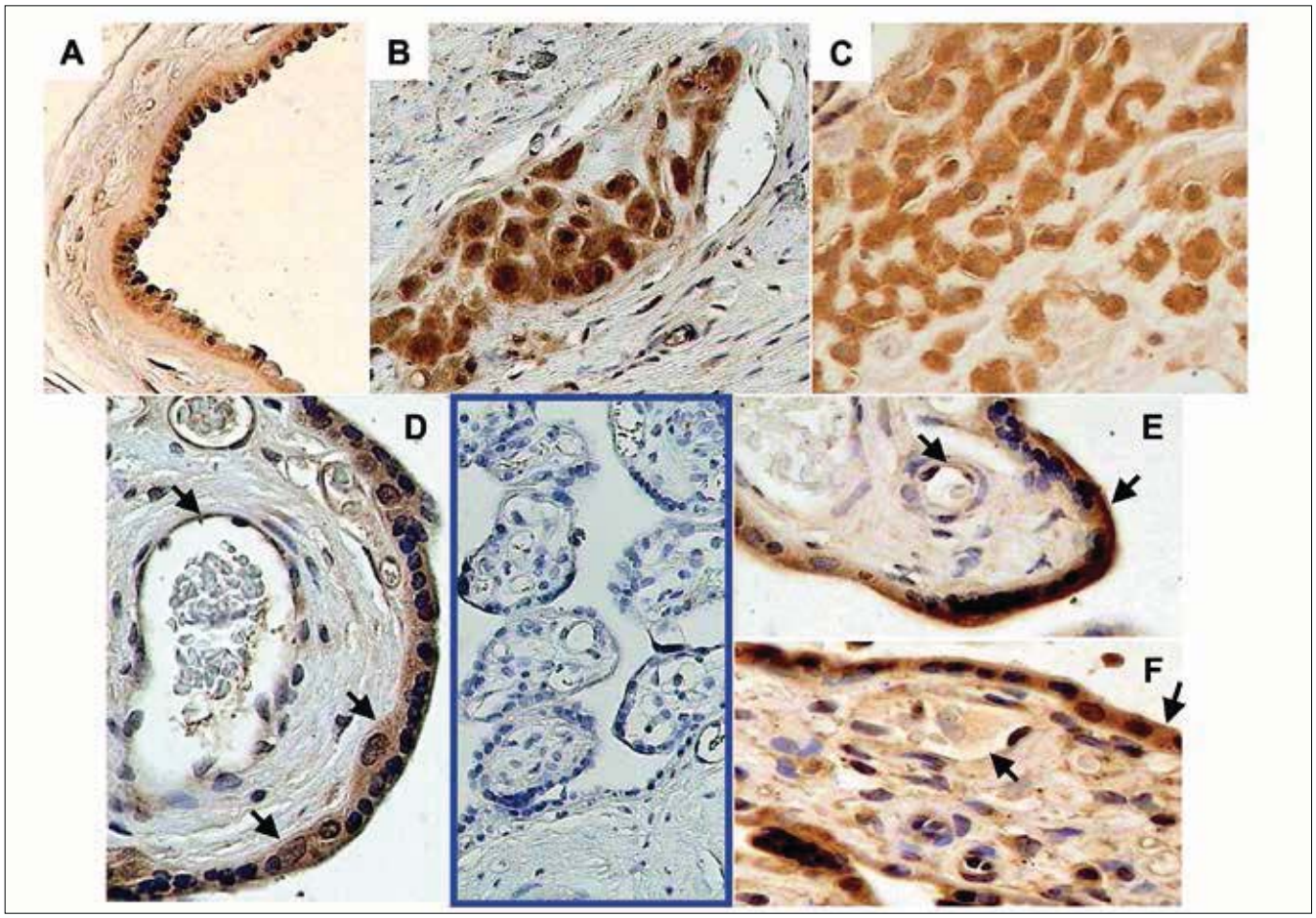

Figure 2. MDR3 immunolocalization in normal and GDM human placentas at term.
MDR3 protein detection in: A – amniotic epithelium (Control); B – cytotrophoblast cells in the chorionic plate (GDM); C – decidual cells (GDM). The expression in B and C locations was comparable in controls and GDM. D – Diffuse reaction with anti-MDR3 antibody in syncytiotrophoblast and cytotrophoblast cells (arrows) in the tertiary villous (Control). E – apical and F – diffuse reaction in tertiary villi of GDM placenta (arrows). Note almost no positive reaction in the endothelial cells both, in control and GDM placentas (arrows). Negative control is framed in blue. Magnification: A, B, C and blue frame – 200x; D, E, F – 400x.

Immunohistochemistry

The tissue sections were taken from the pericentral part of the placenta. Tissue samples were fixed overnight in 10% neutral-buffered formalin, dehydrated by standard procedure, and finally embedded in paraffin. The obtained paraffin blocks were cut into 5 μm slices. To perform immunohistochemical reactions (IHC), sections were dewaxed and rehydrated. Antigen retrieval was performed by incubating tissue sections in target retrieval solutions in water bath at 95°C for 30 min. and cooling for at least 30 min. Afterwards, the slices were washed for 5 min. in distillated water and phosphate buffered saline containing 0.05% Tween 20 (PBS-T).

To avoid non-specific binding of antibodies, tissue slices were incubated for 30 min. with PBS-T containing normal horse serum. Then, they were incubated overnight (at 4°C) with anti-human mouse monoclonal antibodies shown in Table 1**.** Mouse IgG (Vector Laboratories) were used as the negative control. Endogenous peroxidase activity was blocked for 10 min. with 0.3% H_2O_2. Biotinylated secondary horse anti-mouse antibodies (Vector laboratories) were applied for 30 min. at room temperature.

Avidin-biotin-peroxidase technique (Vectastain Elite ABC kit; Vector Laboratories) with 3'3- diaminobenzidine (DAB; Vector Laboratories) as a substrate for peroxidase, was used to visualize the primary antibody. Finally, histological slices were washed, counterstained with hematoxylin and mounted on the permanent Vecta Mount medium (Vector Laboratories).

Immunohistochemical stains were made on five sections taken from each placenta and analyzed under Eclipse 600 (Nikon) microscope equipped with a Sony SSC-DC58AP camera. The specimens included a whole cross-section through the placenta containing all histological layers of the fetal and maternal part.

Results

Immunohistochemical data demonstrated the presence of P-gp, MDR3, and BCRP in the fetal and maternal part of the human placenta both, in healthy and diabetic women. MRP1 was identified mostly in the fetal part of the human placenta.

1. P-gp localization

Healthy placentas: P-gp was detected in all parts of the human placenta (Figure 1), namely, amniotic epithelium, syncytiotrophoblast apical plasma membrane, stem and tertiary

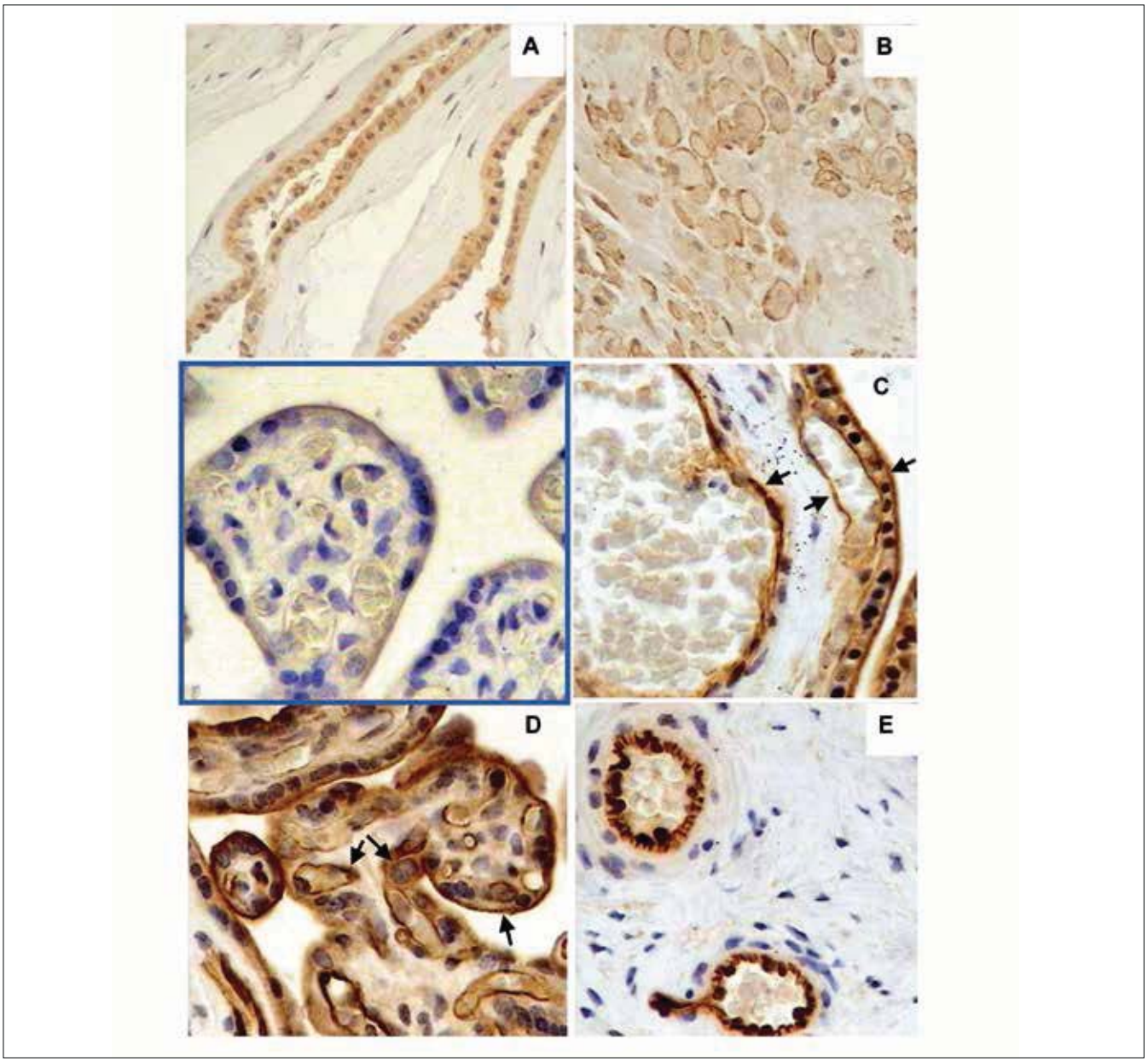

Figure 3. BCRP immunolocalization in normal and GDM human placentas at term.
BCRP protein detection in: A – amniotic epithelium (Control); B - decidual cells (Control). In these locations there were no visible differences when compared to GDM placentas. Strong immunoreactivity at apical syncytiotrophoblast plasma membrane and fetal blood endothelial cells was found in the villi (arrows) of control (C) and GDM (D) placentas, as well as in endothelial cells in the chorionic plate blood vessels, both in normal (not shown) and GDM placentas (E). Negative control is framed in blue. Magnification: A, B – 200 x; C, D, E and blue frame – 400x.

villi, and decidual cells. A higher expression of P-gp was observed in most villi, but in some villi its absence or very low positive reaction were found. P-gp was also localized in villous and extravillous cytotrophoblast, for example in the chorionic plate, as well as on the endothelial cells of fetal blood vessels, mostly in the chorionic plate and – to a lesser degree - in stem and tertiary villi.

GDM placentas: There were not distinct differences in P-gp localization in the syncytiotrophoblast between normal and diabetic placentas (Figure 1D). Nevertheless, P-gp expression in decidual (Figure 1E and F) and amnion epithelial cells was weaker comparing to controls.

2. MDR3 localization

Healthy placentas: MDR3 was found in all parts of the human placenta. MDR3 was expressed in amniotic epithelial cells, villous and extravillous cytotrophoblast, syncytiotrophoblast and decidual cells (Figure 2). The immunological reaction within the syncytiotrophoblast was very intense and showed diffuse distribution. The expression was high in the extravillous cytotrophoblast localized in the chorionic plate. On the other hand, there was very weak positive immunological reaction in the endothelial cells of the fetal blood vessels in the chorion and villi.

GDM placentas: We observed weaker MDR3 expression within the GDM placental syncytiotrophoblast when compared

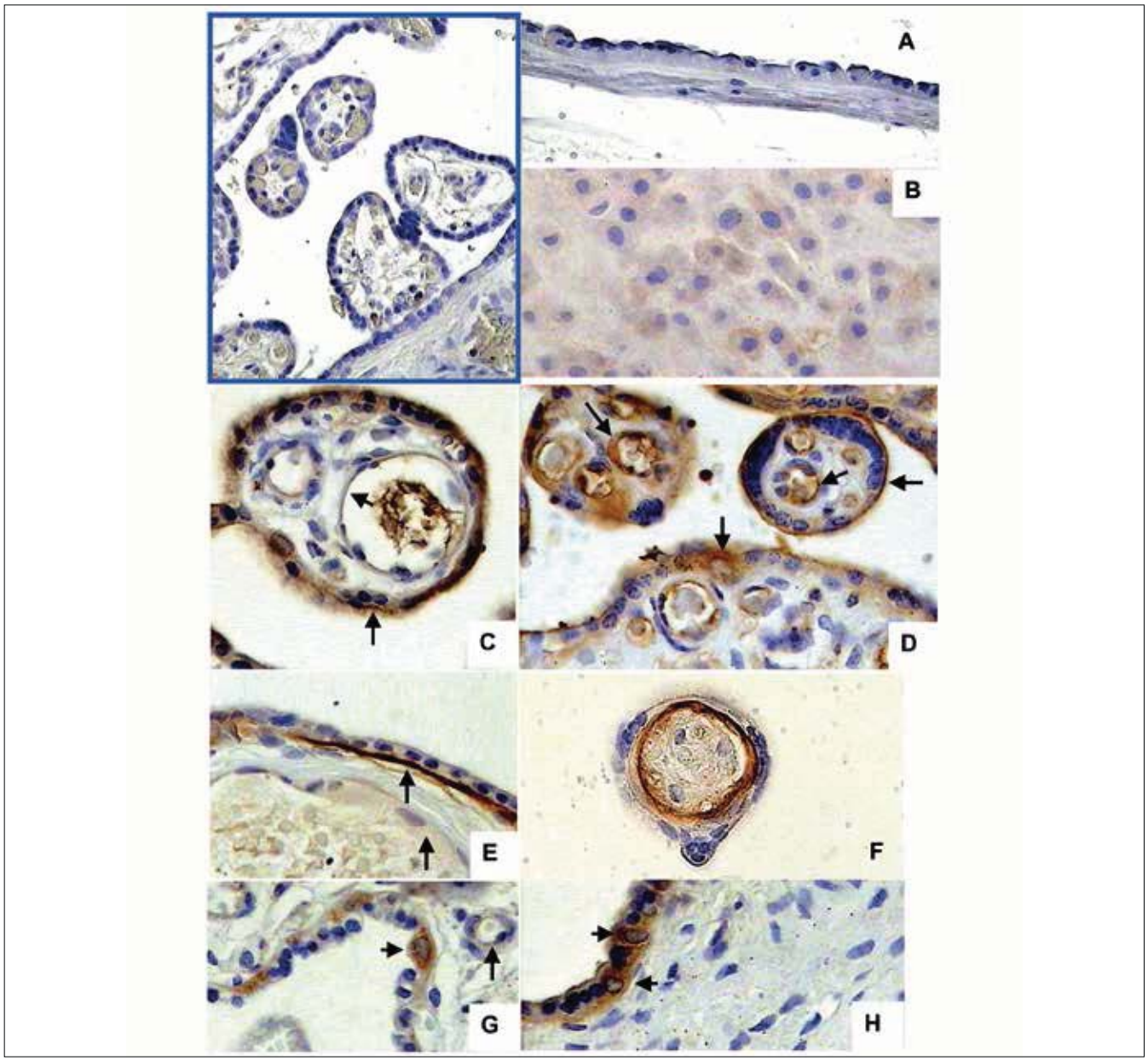

Figure 4. MRP1 immunolocalization in normal and GDM human placentas at term.
MRP1-negative reaction in the amniotic epithelium (A) and majority of decidual cells (B) was found in control placentas. There was no difference in GDM placentas when compared to controls. Distinct MRP1-positive reaction (arrows) was found in apical (C) and basal (E) syncytiotrophoblast plasma membranes, as well as the cytotrophoblast cells (G) located in tertiary villi of control placentas. Adequate locations are shown in GDM placentas at micrographs D, F and H, respectively. Note also (arrows) lack of positive reaction in controls (C, E, G) and poor positive reaction in a few GDM endothelial cells (D). Negative control is framed in blue. Magnification: A, B, and blue frame – 200x; C, D, E, F – 400x.

to normal ones. In few villi that expression appeared in the apical plasma membrane (Figure 2E). In most villi it was diffuse (Figure 2F). Finally, MDR3 immunoreactivity in amniotic epithelium was lower comparing to healthy placentas. There were no other differences in the MDR3 localization between normal and GDM placentas.

3. BCRP localization

Healthy placentas: Very intense immunohistochemical reaction for BCRP was detected in placental tissues (Figure 3). Amniotic, decidual and cytotrophoblast cells showed diffuse distribution of BCRP in their cytoplasm. In addition, decidual cells exhibited marked BCRP expression in plasma membrane (Figure 3B). Strong positive BCRP reaction was found in apical syncytiotrophoblast plasma membrane, both in stem and tertiary villi, as well as in the endothelial cells of the fetal blood vessels in the chorionic plate and in all generations of villi.

GDM placentas: No visible differences in BCRP location between the controls and the GDM group were observed.

4. MRP1 localization

Healthy placentas: MRP1 expression was not observed in the amniotic epithelium and in the majority of decidual cells (Figures 4A and B), while in most endothelial cells of the blood vessels

it was low or undetectable (Figure 4C). MRP1 was localized predominantly on the basal surface of the syncytiotrophoblast plasma membrane. However, in some places the apical surface of the syncytiotrophoblast was also markedly stained (Figures 4C and E). It should be emphasized that both MRP1 localizations in the syncytiotrophoblast membrane were distinct but they were confined to some stem and tertiary villi. MRP1 expression was also observed in villous cytotrophoblast (Figure 4G).

GDM placentas: The main difference when comparing with the control group was slightly lower MRP1 expression in the syncytiotrophoblast of the GDM group (Figures 4D, F and H). Positive immunoreactivity in the amniotic epithelium cells was observed in one of the placentas from the GDM group.

Discussion

Localization of ABC transporters in the human placenta reflects their cell-specific expression. It means that increasing or decreasing range of their occurrence in the placental tissues may result from appearance or disappearance of protein expression in certain locations, what is dependent, among others, on gestation age. Most ABC transporters (P-gp, BCRP, MRP2, MRP4) are known to be localized on the apical surface of the syncytiotrophoblast. In turn, MRP1 and MRP3 are located both, in the basal and apical membranes; MRP5 and MDR3 in the basal plasma membrane of the syncytiotrophoblast [10, 12, 21, 22]. Furthermore, immunohistochemical studies showed that BCRP, MRP3 and MRP5 can also be found in the endothelium of fetal capillaries in the chorionic villi and BCRP can be expressed in the amniotic epithelium, chorion and decidua of placentas from the third trimester of pregnancy [13]. ABC transporter expression is most likely controlled by steroids, growth factors and cytokines secreted during pregnancy [10,13]. For instance, BCRP expression is negatively regulated by some cytokines and stimulated by estrogens, epidermal growth factor (EGF) and insulin-like growth factor II (IGF II). Although elevated cytokine concentrations play a role in pregnancy-induced insulin resistance, results of *in vitro* experiments on primary term trophoblast showed a decrease in BCRP mRNA expression after exposure to TNFα and Il-1β [12]. Recent studies indicate that TNF-α and IL-1β can inhibit most of ABC transporters located in the apical membrane of syncytiotrophoblast, while IL-6 stimulates or inhibits their expression, depending on the transporter. Basolateral syncytiotrophoblast transporters are relatively insensitive to cytokine stimulation. The fact that TNF-α and IL-1β reduce the expression of BCRP and MDR1 may have significant clinical implications in obstetric disorders linked with elevated cytokine concentrations, as it would result in increased fetal exposure to xenobiotics and trophoblast cell susceptibility to apoptosis [12, 23, 24, 25].

We found presence of P-gp in different parts of healthy term placentas, predominantly in the syncytiotrophoblast plasma membrane of stem and tertiary villi. The main functional significance of P-gp may result from its apical plasma membrane localization in the syncytiotrophoblast, where the transporter may be responsible for an efflux of harmful compounds from the fetal compartment, thus protecting the fetus against toxicity. It should be noted that similarly to other studies [26], we found no specific distribution pattern of the P-gp-positive reaction in different parts of the stem villi branches. We confirmed the presence of P-gp in the amniotic epithelial cells [5]. Also, we found a positive immunohistochemical reaction in the cytotrophoblast cells, in the endothelial cells of large fetal blood vessels in the chorionic plate and stem villi, and – to a lesser degree - of capillaries of tertiary villi. These localizations extend the barrier role of P-gp outside the apical surface of the syncytiotrophoblast and make it cytoprotective in terms of specific types of cells.

The literature lacks data on P-gp localization in human term placenta of GDM mothers. However, many disorders like preeclampsia, intrauterine growth restriction (IUGR) or GDM have been known to be connected with the activation of oxidative and inflammatory pathways. They may affect ABC transporter expression and localization [12,19]. Experiments on the primary cultures of the trophoblast, JAr and BeWo cell lines, showed downregulation of P-gp under inflammatory conditions [12]. In our study, we did not find any differences in P-gp location in GDM placentas comparing to controls, but P-gp expression in the amniotic epithelial and decidual cells was slightly weaker in diabetic placentas. One study demonstrated that P-gp mRNA, but not P-gp protein expression, was significantly elevated in GDM placentas [27], what supports our findings. Our results need further quantification, but they indicate that GDM diabetic environment can lead to a decrease in P-gp amniotic and decidual expression without changing the location in the placental tissues. In another study, the authors demonstrated that different subcellular locations of the transporter proteins were possible. They found changes in the P-gp localization from microvillous apical syncytiotrophoblast plasma membrane to its cytoplasmic compartment and basal membrane in retroviral transduced explants [28]. The significance of these observations arises from the fact that P-gp has been shown to take a part in active efflux of hydrophobic xenobiotics, their metabolites and steroid hormones. Cytostatics, HIV protease inhibitors, antibiotics, opioids, antimimetics, and diagnostic dye-rhodamine 123 are recognized P-gp substrates [2, 29]. P-gp plays an important clinical role is the limitation of fetal exposure to anthracyclines and taxanes that are recommended for pregnant women [29]. Materno-fetal transfer of doxorubicin was not shown even at high doses [17], decreasing the risk of fetus exposition. Furthermore, specific location of the observed changes in P-gp expression restricted to amniotic and decidual cells might indicate a modified transporter function in relation not only to exogenous, but also to endogenous substrates.

In our study, we detected MDR3 (P-gp homologue) mainly in the syncytiotrophoblast, where it showed diffuse distribution. MDR3 localization at the basal membrane of the syncytiotrophoblast seemed to be not obviously predominant, but other studies pointed to the basolateral position as the main place of MDR3 location [19, 30]. It could suggest an opposite direction of the substrate efflux, as compared to P-gp, i.e. towards the fetal circulation. MDR3 expression was also abundant in the amniotic epithelium, chorionic cytotrophoblast and decidual cell cytoplasm. On the other hand, some authors failed to detect MDR3 protein in the analyzed amnion membranes [5]. Strong expression of MDR3 protein in term placentas may suggest that it plays an important role in fetal development, however the physiological role of MDR3 in the human placenta remains unexplained. MDR3 is known to be involved in the efflux of phosphatidylcholine from cells, however some pharmaceutics (also P-gp substrates) are known to be its ligands [30].

There is no data available on MDR3 localization in GDM term placentas. In a few villi we found the expression of MDR3 to be located in the syncytiotrophoblast apical plasma membrane, but diffuse reaction within the syncytiotrophoblast was predominant. The change in the location of MDR3 from basal to apical surface of the syncytium might enhance xenobiotic efflux in GDM placentas, but that change covered only a small part of the chorionic villi. Moreover, MDR3 staining in the syncytiotrophoblast and amniotic epithelium of GDM placentas was weaker when compared to controls. It seems that changes in MDR3 expression may result in more important metabolic consequences than the observed changes in the transporter localization.

Immunohistochemical studies showed that BCRP is localized in the syncytiotrophoblast and additionally in the endothelium of fetal capillaries, amniotic epithelium, chorion and maternal decidua [12, 13]. Our results confirmed these findings. We detected intense positive staining of BCRP localized at the maternal-facing side of the syncytiotrophoblast, mostly in the stem and tertiary villi. Strong positive BCRP reaction was found in the endothelial cells of the blood vessels of fetal circulation. Such location seems to be strictly connected with the role of BCRP as the component of placental barrier reducing fetus exposure to xenobiotics. BCRP is involved in the transport of many drugs, including anticancer drugs like mitoxantrone, captothecin derivatives (topotecan, irinotecan), epirubicin, etoposide, methotrexate, daunorubicin, and nucleoside analogues - zidovudine and lamivudine. Prazosin, dipyridamole, phytoestrogens and drugs that are widely used in the therapy of pregnant patients (nitrofurantoin, cimetidine or glyburide), are among other substrates of BCRP. Many of those are also P-gp substrates [13]. Amniotic epithelial, cytotrophoblast and some decidual cells (including both cytoplasm and plasma membrane) also showed BCRP expression. Such different locations of BCRP in the placenta can reflect its protective function against the effects of hypoxia and oxidative stress and maintenance of stem cell regeneration potential [24, 31].

We did not notice any significant differences in BCRP localization between control and GDM placentas, what may result in unchanged materno-fetal disposition of therapeutics, including oral hypoglycemic agents. In addition, it was shown in the studies of Anger et al., that average placental BCRP mRNA expression in insulin-managed diabetes did not significantly differ from controls [27].

MRP transporters have higher substrate specificity than P-gp. Numerous clinically important anionic drugs are preferred substrates for MPR. MRP proteins are involved in the transport of methotrexate, etoposide, vinblastine, vincristine, HIV protease inhibitors and some fluoroquinolone antibiotics [2, 16]. In our studies MRP1 was localized both on basal and apical surface of the syncytiotrophoblast, but the basal membrane was the predominant place of its location. Interestingly, MRP1 expression in the endothelial cells was very low and it was not observed in the majority of maternal decidual cells and the amniotic epithelium. Such localization in human placenta may give evidence to its different endogenous function in delivering nutrients to rapidly growing fetus, as compared to BCRP and P-gp. That role of MRP1 was suggested to be more important than providing barrier protection to limit the entry of foreign compounds into the fetal circulation [19]. Similar results were obtained by other authors [32], with the exception of Aye et al. [5], who successfully localized MRP1 in the amniotic epithelium. It should be noted the single cases of observed MRP1 expression in normal amniotic epithelium and described in the literature, as well as found by us in GDM placentas, indicate that there might be significant differences in the MRP1 expression in the amniotic epithelium between individuals. The only difference we found in GDM diabetic placentas was a lower MRP1 immunoreactivity in the syncytiotrophoblast. Results of the experiments where the primary term trophoblast was treated with cytokines, showed increased MRP1 mRNA expression, but not altered protein levels [12]. If our observations were to be confirmed in quantitative studies, it might indicate that the GDM-dependent mechanism of decreasing MRP1 expression was not related to cytokines.

Our study was performed on placentas delivered from diet-managed GDM women. Oral hypoglycemic agents are used in pregnancy very carefully because of the risk of hypoglycemia in the newborns. Oral antihyperglycemic agent glyburide (sulfonylurea derivative), can easily bind with proteins and is actively displaced by P-gp, BCRP, MRP3 and MRP1 from placenta to the maternal circulation [17, 33]. MRP1 was shown to be mainly associated with the transport of glyburide, while BCRP and P-gp of metformin. Different antidiabetic agents used in the combination therapy (glyburide, metformin, rosiglitazone) and transferred by the same transporter can compete with one another. Such competition results in an increase of drug concentrations in fetal circulation [33].

Conclusion

In conclusion, changes in ABC transport protein localization in the human placenta, related to GDM, were indistinct. The changes in transporter protein expression, namely weaker MRP1 and MDR3 positive reaction in syncytiotrophoblast, slightly lower expression of P-gp in decidual and amniotic epithelial cells, and MDR3 in the amniotic epithelium, were much more distinct. These changes should be confirmed by protein quantification. Basal syncytiotrophoblast transporters: MRP1 and MDR3 seem to be more sensitive to GDM-specific environment than apical proteins, what may result in biodisposition of both, endogenous substrates and drugs.

Acknowledgments

This work was supported by the institutional grants from the Medical University of Silesia: no. KNW-1-072/D/1/0 and KNW-1-007/D/2/0.

Oświadczenie autorów

1. Danuta Kozłowska-Rup – współautor: koncepcji, założeń, metod, manuskryptu, protokołu, analizy i interpretacji danych, korekta i aktualizacja literatury, zebranie materiału, wykonanie badań immunohistochemicznych, korekta i akceptacja ostatecznego kształtu manuskryptu, współfinansowanie pracy.

2. Piotr Czekaj - współautor: koncepcji, założeń, metod, manuskryptu, protokołu, analizy i interpretacji danych, korekta i aktualizacja literatury, korekta i akceptacja ostatecznego kształtu manuskryptu, współfinansowanie pracy – autor zgłaszający i odpowiedzialny za manuskrypt.
3. Danuta Plewka –współautor metod oraz analizy danych, korekta i aktualizacja literatury, wykonanie badań immunohistochemicznych.
4. Jerzy Sikora – współautor założeń i protokołu, zebranie materiału, konsultacja danych klinicznych, ostateczna weryfikacja i akceptacja manuskryptu

Źródło finansowania:

Praca była finansowana ze środków na finansowanie badań uczestników studiów doktoranckich; umowa numer: KNW-1-072/D/1/0 i KNW-1-007/D/2/0.

Konflikt interesów:

Autorzy nie zgłaszają konfliktu interesów oraz nie otrzymali żadnego wynagrodzenia związanego z powstawaniem pracy.

References

1. Marin JJ, Briz O, Serrano MA. A Review on the molecular mechanisms involved in the placental barrier for drugs. Curr Drug Deliv. 2004, 1 (3), 275-289.
2. Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. Clin Pharmacokinet. 2004, 43 (8), 485-514.
3. Van der Aa EM, Peereboom-Stegeman JH, Noordhoek J, [et al.]. Mechanisms of drug transfer across the human placenta. Pharm World Sci. 1998, 20 (4), 139-148.
4. Ganapathy V, Prasad PD, Ganapathy ME, Leibach FH. Placental transporters relevant to drug distribution across the maternal-fetal interface. J Pharmacol Exp Ther. 2000, 294 (2), 413-420.
5. Aye IL, Paxton JW, Evseenko DA, Keelan JA. Expression, localisation and activity of atp binding cassette (abc) family of drug transporters in human amnion membranes. Placenta. 2007, 28 (8-9), 868-877.
6. Mason CW, Buhimschi IA, Buhimschi CS, [et al.]. ATP-binding cassette transporter expression in human placenta as a function of pregnancy condition. Drug Metab Dispos. 2011, 39 (6), 1000-1007.
7. Ni Z, Mao Q. ATP-binding cassette efflux transporters in human placenta. Curr Pharm Biotechnol. 2011, 12 (4), 674-685.
8. Meyer zu Schwabedissen HE, Jedlitschky G, Gratz M, [et al.]. Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation. Drug Metab Dispos. 2005, 33 (7),896-904.
9. Hahnova-Cygalova L, Ceckova M, Staud F. Fetoprotective activity of breast cancer resistance protein (BCRP, ABCG2): expression and function throughout pregnancy. Drug Metab Rev. 2011, 43 (1), 53-68.
10. Myllynen P, Immonen E, Kummu M, Vähäkangas K. Developmental expression of drug metabolizing enzymes and transporter proteins in human placenta and fetal tissues. Expert Opin Drug Metab Toxicol. 2009, 5 (12), 1483-1499.
11. Vähäkangas K, Myllynen P. Drug transporters in the human blood-placental barrier. Br J Pharmacol. 2009, 158 (3), 665-678.
12. Evseenko DA, Paxton JW, Keelan JA. Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors. Drug Metab Dispos. 2007, 35 (4), 595– 601.
13. Mao Q. BCRP/ABCG2 in the placenta: expression, function and regulation. Pharm Res. 2008, 25 (6), 1244–1255.
14. Maeda K, Sugiyama Y. Impact of genetic polymorphisms of transporters on the pharmacokinetic, pharmacodynamic and toxicological properties of anionic drugs. Drug Metab Pharmacokinet. 2008, 23 (4), 223-235.
15. Rice EG, Illanes SE, Mitchell MD. Gestational diabetes mellitus: a positive predictor of type 2 diabetes? Int J Endocrinol. 2012, ID 721653.
16. Weier N, He SM, Li XT, [et al.]. Placental drug disposition and its clinical implications. Curr Drug Metab. 2008, 9 (2), 106-121.
17. Gedeon C, Behravan J, Koren G, Piquette-Miller M. Transport of glyburide by placental ABC transporters: implications in fetal drug exposure. Placenta. 2006, 27 (11-12), 1096-1102.
18. Kozłowska-Rup D, Czekaj P. Barrier role of ABC family of proteins in human placenta. Ginekol Pol. 2011, 82 (1), 56-63.Polish.
19. Aye IL, Keelan JA. Placental ABC transporters, cellular toxicity and stress in pregnancy. Chem Biol Interact. 2013, 203 (2), 456-466.
20. Polish Gynecological Society standards of medical care in management of women with diabetes. Ginekol Pol. 2011, 82 (6), 474-479. Polish.
21. Ganapathy V, Prasad PD. Role of transporters in placental transfer of drugs. Toxicol Appl Pharmacol. 2005, 207 (2), 381-387.
22. St-Pierre MV, Ugele B, Gambling L, Shiverick KT. Mechanisms of drug transfer across the human placenta - a workshop report. Placenta. 2002, 23, 159–164.
23. Kirwan JP, Hauguel-De Mouzon S, Lepercq J, [et al.].TNF- Is a predictor of insulin resistance in human pregnancy. Diabetes. 2002, 51 (7), 2207-2213.
24. Evseenko DA, Murthi P, Paxton JW,[et al.]. The ABC transporter BCRP/ABCG2 is a placental survival factor, and its expression is reduced in idiopathic human fetal growth restriction. FASEB J. 2007, 21 (13), 3592-3605.
25. Petrovic V, Teng S, Piquette-Miller M. Regulation of drug transporters during infection and inflammation. Mol Interv. 2007, 7 (2), 99-111.
26. Sun M, Kingdom J, Baczyk D, [et al.]. Expression of the multidrug resistance p-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. Placenta. 2006, 27 (6-7), 602-609.
27. Anger GJ, Cressman AM, Piquette-Miller M. Expression of ABC Efflux transporters in placenta from women with insulin-managed diabetes. PLoS One. 2012, 7 (4), e35027.
28. Atkinson DE, Sibley CP, Fairbairn LJ, Greenwood SL. MDR1 P-gp expression and activity in intact human placental tissue; upregulation by retroviral transduction. Placenta. 2006, 27 (6-7), 707-714.
29. Ceckova-Novotna M, Pavek P, Staud F. P-glycoprotein in the placenta: Expression, localization, regulation and function. Reprod Toxicol. 2006, 22 (3), 400-410.
30. Evseenko DA, Paxton JW,Keelan JA. ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast. Am J Physiol Regul Integr Comp Physiol. 2006, 290 (5), 1357-1365.
31. Jonker, JW, Smit JW, Brinkhuis M, [et al.]. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst. 2000, 92 (20), 1651–1656.
32. Nagashige M, Ushigome F, Koyabu N, [et al.]. Basal membrane localization of MRP1 in human placental trophoblast. Placenta. 2003, 24 (10), 951–958.
33. Hemauer SJ, Patrikeeva SL, Nanovskaya TN, [et al.]. Role of human placental apical membrane transporters in the efflux of glyburide, rosiglitazone, and metformin. Am J Obstet Gynecol. 2010, 202 (4), 383.