ATP-Binding Cassette Transporter Expression in Human Placenta as a Function of Pregnancy Condition^S

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ABSTRACT:

Fetal drug exposure is determined by the type and concentration of placental transporters, and their regulation is central to the development of new treatments and delivery strategies for pregnant women and their fetuses. We tested the expression of several clinically important transporters in the human placenta associated with various pregnancy conditions (i.e., labor, preeclampsia, and preterm labor-inflammation). Placentas were obtained from five groups of women at the time of primary cesarean section: 1) term no labor; 2) term labor; 3) preterm no labor (delivered for severe preeclampsia); 4) preterm labor without inflammation (PTLNI); and 5) preterm labor with inflammation (PTLI). Samples were analyzed by Western blot and immunohistochemistry to identify changes in protein expression. Relative mRNA expression was determined by quantitative real-time polymerase chain reaction. A functional genomic approach was used to identify placental gene expression

and elucidate molecular events that underlie the given condition. Placental expression of ATP-binding cassette transporters from women in labor and women with preeclampsia was unaltered. Multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) and mRNA expression increased in placentas of women with preterm labor with inflammation. Molecular pathways of genes up-regulated in PTLI samples included cytokine-cytokine receptor interactions and inflammatory response compared with those in the PTLNI group. The mRNA expression of MDR1 and BCRP was correlated with that of interleukin-8, which also increased significantly in PTLI samples. These data suggest that the transfer of drugs across the placenta may be altered in preterm pregnancy conditions associated with inflammation through changes in MDR1 and BCRP.

Introduction

Drug treatment options during pregnancy and lactation are limited because few products have been tested for safety and efficacy in these two patient groups. The placenta is a partially protective barrier that limits fetal exposure to xenobiotics, which is attributed in part to the expression of transporter proteins on placental apical and basal membrane surfaces. Among the most abundant of the apically expressed

xenobiotic transporters on the maternal side of the placenta are multidrug resistance protein (MDR) 1 (P-glycoprotein; ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2), which handle the efflux of xenobiotics and metabolites out of the fetoplacental compartment (Jonker et al., 2000). The localization of MDR3 (ABCB4) and MRP1 (ABCC1) is less clear, but studies suggest that these transporters may be positioned on the basolateral membrane of the placenta where they transport substrates from mother to fetus (Nagashige et al., 2003; Evseenko et al., 2006). Additional transporters, including BCRP and MRP1, line the fetal capillaries, providing yet another barrier against xenobiotic entry (St-Pierre et al., 2000; Yeboah et al., 2006).

Expression of these clinically important transporters is dependent on gestational age. However, drug transporter expression and regulation in placenta of women with pregnancy pathology require further definition. Preterm labor is the leading cause of perinatal morbidity and mortality. Preeclampsia and inflammation, which are often secondary to uterine infection, are well recognized causes of preterm birth and, when diagnosed, frequently result in clinically indicated

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ABBREVIATIONS: MDR, multidrug resistance protein; ABC, ATP-binding cassette; MRP, multidrug resistance-associated protein; PCR, polymerase chain reaction; BCRP, breast cancer resistance protein; TNL, term no labor; TL, term labor; PTSPE, preterm no labor with indications for spontaneous preeclampsia; GA, gestational age; PTLNI, preterm labor without inflammation; PTLI, preterm labor with inflammation; ANOVA, analysis of variance; GO, Gene Ontology; FDR, false discovery rate; qRT, quantitative real-time; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; C_t, cycle threshold.

premature delivery. Treatment for the prevention of preterm birth has thus far been unsuccessful, and the rate of premature birth has increased over the years. We hypothesize that pregnancy conditions associated with preterm birth, such as spontaneous preterm labor, preeclampsia, and preterm labor-inflammation, alter the expression of drug transporters in human placenta. We applied immunohistochemical analysis, Western blot, and quantitative real-time PCR to determine the localization and protein and mRNA expression of transporters in a series of human placentas obtained from women with clinically diagnosed pregnancy conditions. In addition, we applied functional genomic profiling, an effective approach for obtaining mechanistic understanding of underlying disease through changes in gene expression (Mason et al., 2006), to gain insight into the processes associated with abnormal labor. We postulate that these processes may mediate the observed changes in transporter expression.

The results from these studies provide evidence for altered expression of MDR1 and BCRP during inflammation-associated spontaneous preterm labor. They also support the involvement of cytokine-mediated events as a means to explain the observed increase in MDR1 and BCRP expression. Overall, our data suggest that up-regulation of MDR1 and BCRP could alter drug transfer across the placenta. These results will help predict human fetal drug toxicity and drug delivery and offer new insights into the regulation of placental drug transporters and the impact of various pregnancy conditions on them.

Materials and Methods

Study Design. Placenta were obtained with institutional review board approval and after written consent from five groups of women undergoing primary cesarean section at Yale University: 1) term no labor (TNL); 2) term labor (TL); 3) preterm no labor delivered for severe preeclampsia (PTSPE) [mean gestational age (GA), 30.3 weeks; range, 25.6–33.0 weeks]; 4) preterm labor unassociated with inflammation (PTLNI) (mean GA, 30.5 weeks; range, 25.3–36.6 weeks, histological chorioamnionitis, stage 0); and 5) preterm labor with inflammation (PTLI) (mean GA, 28.7 weeks; range, 28.0–33.3 weeks; histological chorioamnionitis, stage III). Labor was defined by the presence of regular uterine contractions accompanied by progressive cervical dilation. The diagnosis of intra-amniotic inflammation was based on an amniotic fluid mass restricted score of 3 or 4 plus >100 white blood cells/ μL^3 in the context of a positive amniotic fluid culture in a sample that was obtained by transabdominal amniocentesis (Buhimschi et al., 2005). These tests provide the most accurate tools currently available to maximize the likelihood of sample homogeneity. The mass restricted score provides qualitative information regarding the presence or absence of intra-amniotic inflammation. In brief, the score ranges from 0 to 4, depending on the presence (assigned a value of 1) or absence (assigned a value of 0) of each of four protein biomarkers (Buhimschi et al., 2005). A score of 3 to 4 indicates inflammation, whereas a score of 0 to 2 excludes it. This biomarker pattern is predictive of preterm birth, histological chorioamnionitis, and adverse neonatal outcome. A detailed description of the mass restricted method has been published previously (Buhimschi et al., 2005). Preeclampsia was defined according to established criteria from the American College of Obstetricians and Gynecologists as systolic blood pressure of 140 mm Hg or diastolic blood pressure of 90 mm Hg and proteinuria of at least +1 on dipstick testing, each on two occasions 4 to 6 h apart. In a 24-h urine collection, proteinuria was defined as ≥ 300 mg of protein. Indications for cesarean delivery in the PTLNI group were related to spontaneous preterm labor. The indication for cesarean delivery in the TNL and TL groups was related to breech presentation and an arrest of cervical dilation at ≥ 6 cm, respectively. Clinical data were retrieved from the medical records, and statistical analysis of patient demographics was performed using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test for multiple comparisons.

RNA Isolation and Microarray Preparation. Total RNA isolation and gene profiling of placenta were performed in triplicate for term and preterm samples using the Affymetrix GeneChip Human Genome U133 Plus 2.0

microarray (Affymetrix, Santa Clara, CA) as described previously (Mason et al., 2010).

Microarray Data Processing and Statistical Analysis. The quality of the microarray experiment was assessed as described by Chang et al. (2007) using bioconductor packages for statistical analysis of microarray data. Multidimensional scaling analysis was performed with the signal estimates to assess sample variability. The quality assessment and multidimensional scaling analyses identified and disqualified discordant sample chips. Signal data were obtained using the RMA algorithm. Differential gene expression between the individual pair-wise conditions was assessed by modified *t* tests as described previously (Kedziorek et al., 2010). The search for genes varying among the conditions was made by combining all the pair-wise comparisons above to construct an F test, which is equivalent to a one-way ANOVA for each gene except that the residual mean squares have been moderated between genes (Smyth, 2004). The *p* values for the tests provide a way to rank genes in terms of the evidence for differential gene expression to obtain the most likely differentially expressed genes between and among conditions. $p \leq 0.05$ and a 1.5-fold threshold were used as a cutoff for gene inclusion in our analysis.

Microarray Data Analysis. DAVID (Huang et al., 2009), an ontology-based Web tool, was used to evaluate statistical measures of knowledge-based groups of genes from publications and public resources. The biological functions of the genes in the placental groups were examined in DAVID on the basis of information from the Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes pathways, and gene descriptions from various public databases. We distinguished genes that were up-regulated and down-regulated (differentially expressed genes) and used DAVID to determine Gene Ontology categories that were overrepresented (enriched) with differentially expressed genes. The false discovery rate (FDR) filter identified categories (biological processes, pathways, or molecular functions) that were changed by random chance. The FDR was set at 10%, and GO categories with FDR $<10\%$ were considered significantly enriched.

Quantitative Real-Time PCR. Primer sequences for amplifications were chosen on the basis of previously published cDNA sequences (Supplemental Table 1). For normalization of the mRNA data, the endogenous reference gene 18s rRNA was used. All primer sets were tested to ensure efficiency of amplification over a wide range of template concentrations. SYBR Green (Bio-Rad Laboratories, Hercules, CA) was used for amplicon detection. A melt curve was used after amplification to ensure that all samples exhibited a single amplicon. Each sample was assayed in triplicate. The average C_t value (cycle threshold for target or endogenous reference gene amplification) was estimated using the software associated with the iCycler real-time PCR detection system (Bio-Rad Laboratories). Relative changes in mRNA expression of the target genes were analyzed using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001). In this method, the average ΔC_t was calculated by subtracting the average C_t value of the endogenous reference gene (18s rRNA) from the average C_t value of the target gene for the condition and control placental groups. Fold changes in mRNA expression of target genes in placenta from the condition groups (TL, PTSPE, PTLNI, and PTLI) were expressed relative to that of the TNL placental control group.

To validate (biological and statistical) the microarray results, we performed qRT-PCR on select genes that were differentially expressed and/or significantly different in comparisons of either PTLI versus PTLNI or PTLI versus TL.

Western Blot Analysis. Human placentas were processed according to methods described previously (Novotna et al., 2004). In brief, placentas were homogenized in buffer containing 250 mM sucrose, 10 mM Tris, 5 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) supplemented with phenylmethylsulfonyl fluoride (1 mM), pH 7.4. Crude membrane fractions were obtained through differential centrifugation. The homogenate was initially centrifuged at 10,000g for 10 min at 4°C. The resulting supernatant was then centrifuged at 36,000g for 70 min at 4°C. Protein concentration was determined using Bradford (Bio-Rad Laboratories) assays according to the manufacturer's protocol. Membrane protein (50–75 μg) was subjected to SDS-polyacrylamide gel electrophoresis on precast Tris-HCl gels (Bio-Rad Laboratories). The separated proteins were transferred to polyvinylidene difluoride membranes and blocked using 5% nonfat dry milk. Placental transporters were detected by incubating the membranes overnight at 4°C with a 1:500 dilution of the monoclonal primary antibody for

MDR1 (F4; Sigma-Aldrich, St. Louis, MO) and BCRP (clone BXP-21; Millipore Bioscience Research Reagents, Temecula, CA), a 1:500 dilution for MDR3 (clone P3 II-26; Millipore Bioscience Research Reagents), a 1:400 dilution for MRP1 (MRPr1; Enzo Life Sciences, Inc., Plymouth Meeting, PA), and a 1:50 dilution for MRP2 (M2 III-6; Millipore Bioscience Research Reagents). The membranes were immunoblotted using peroxidase-conjugated secondary antibody and detected using the ECL detection system (GE Healthcare Biosciences, Pittsburgh, PA). Equivalence of protein loading was confirmed by secondary immunoblotting with anti- β -actin antibody.

Immunohistochemical Analysis. Immunohistochemical detection of MDR1 and BCRP was performed on frozen sections of placenta from each of the five groups of women ($n = 3$ /group). Placental sections were blocked and incubated overnight at 4°C with the MDR1 and BCRP monoclonal antibodies and dilutions used for Western blots. Biotin-labeled secondary antibodies were visualized using peroxidase-conjugated streptavidin (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine (Sigma-Aldrich) as the substrate. Slides were then counterstained with hematoxylin followed by dehydration in a graded series of ethanol dilutions, cleared by xylene substitute, and mounted with DPX mountant (Sigma-Aldrich). Control incubations did not include primary antibody.

Statistical Analysis. Statistical analysis was done with GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA). Quantitative real-time PCR results were reported as fold change in mRNA expression of target genes (mean $\pm$ S.E.M.) for each placental group relative to the mRNA expression found in the TNL control placental group. Mean fold changes in mRNA expression in all the groups were compared by ANOVA followed by the post hoc Student-Newman-Keuls multiple comparison test. Pearson coefficient analysis was used to determine the correlation between the fold changes in mRNA expression of the target genes. Statistical significance was set at $p < 0.05$.

Results

Clinical Characteristics of Placental Samples. There were no pathological changes in placentas from TNL, TL, and PTLNI groups on the basis of histological evaluation. The samples from the PTLI group were associated with histological stage III chorioamnionitis (full-thickness inflammation of both chorion and amnion). This was complemented by inflammation of the amnion (range, grades 1–3; mode, grade 3), inflammation of the chorion-decidua (range, grades 3–4; mode, grade 3), and funisitis (range, grade 1–4; mode, grade 3). Histological grading was based on the four-grade system devised by Salafia et al. (1989). Pathological abnormalities associated with placentas from the PTSPE group consisted of placental infarcts less than 3 cm, fibrin deposition, decidual vascular thrombosis, decidual hemorrhage, necrosis, and hyperplastic arteriosclerosis. Furthermore, there were no statistically significant differences in gestational age or fetal birth weight among those women who delivered term (no labor versus labor) or preterm (preeclampsia, inflammation, labor, and no labor) or in maternal age (data not shown). Thus, the observed differences in the ABC transporters among the placental groups are probably the result of the pregnancy condition rather than any variation in maternal age or variation in gestational age within term or preterm groups (i.e., PTLNI versus PTLI).

Expression and Localization of Placental Drug Transporters. Levels of MDR1, MDR3, MRP1, MRP2, and BCRP were determined from immunoblot analyses. Greater expression of MDR1 and BCRP was observed in placentas of women with preterm labor (Fig. 1, A and B) than in placentas of those with term labor. Furthermore, protein expression was higher in the placentas of women in the PTLI group than in those of women in the PTLNI group (Fig. 1, A and B). Other laboratories have shown that MDR1 expression is not dependent on the region of the sample or on cesarean versus vaginal delivery (Camus et al., 2006; Sun et al., 2006). MRP1, MRP2, and MDR3 were present in all samples, but their expression was

Fig. 1. Immunoblot analysis of protein expression of MDR1 (A), BCRP (B), MRP2 (C), MRP1 (D), and MDR3 (E) in human placentas from women after primary cesarean section during TNL, TL, PTSPE, PTLNI, and PTLI.

variable and did not appear to be dramatically affected by pregnancy condition (Fig. 1, C–E).

The proper cellular localization is essential for transporters to perform their transport function. Immunohistochemical analysis verified that the observed changes were due to MDR1 and BCRP (Fig. 2) expression at the membrane of the syncytiotrophoblast cells. BCRP was also localized to fetal blood vessel endothelial cells (Fig. 2).

In many instances, the regulation of these transporters occurs at transcription. Given the range of gestational ages in each placental group (condition), we increased the number of placental samples ($n = 6–10$), after Western blot analysis and immunohistochemical analysis, used for semiquantitative real-time PCR. We found significant increases in MDR1 and BCRP gene expression in the PTLI samples (Fig. 3), which corresponds with their observed protein levels in this condition. There were no changes in MRP1 and MRP2 gene expression among the given conditions. MDR3 mRNA levels were significantly increased in the PTLI group, and there were higher levels in the PTSPE group than in the TL and TNL groups.

Functional Characteristics of Genes Overexpressed in PTLI. *PTLI compared with PTLNI.* We identified 127 genes that were overexpressed (≥ 1.5 ; $p \leq 0.05$) in PTLI compared with PTLNI. The enrichment of these genes was categorized by GO (pathways, biological processes, and molecular functions). Pathway analysis using the Kyoto Encyclopedia of Genes and Genomes database revealed only one significantly enriched pathway, cytokine-cytokine receptor interaction (FDR 7.7). Significantly up-regulated genes were further categorized by biological processes and molecular functions. We identified seven biological processes that were significantly enriched: 1) response to wounding (FDR 0.6); 2) inflammatory response (FDR 0.8); 3) regulation of cell motion (FDR 3.9); 4) regulation of cell

FIG. 2. Immunohistochemical localization of MDR1 and BCRP in human placenta. Results show MDR1 and BCRP localization to the membrane of the syncytiotrophoblast cells (arrows) in all tissue conditions ($n = 3/\text{tissue group}$). BCRP was also localized to the fetal blood vessel endothelial cells (arrowheads). The MDR1 and BCRP controls are indicative of immunostaining without primary antibody. Original magnification, $100\times$. Scale bars (in control), $120\ \mu\text{m}$.

proliferation (FDR 5.7); 5) defense response (FDR 6.4); 6) positive regulation of signal transduction (FDR 7.7); and 7) positive regulation of cell motion (FDR 8.0). Three molecular functions were significantly enriched: 1) growth factor binding (FDR 0.1); 2) cytokine binding (1.3); and 3) cytokine receptor activity (FDR 8.4).

PTLI compared with TL. We identified 137 genes that were over-expressed (≥ 1.5 ; $p \leq 0.05$) in PTLI compared with TL. We found only the focal adhesion pathway (FDR 9.9) to be significantly enriched. Nine biological processes enriched: 1) female pregnancy (FDR 0.4); 2) tube development (FDR 3.3); 3) ossification (FDR 3.8); 4) positive regulation of kinase activity (FDR 4.3); 5) bone development (FDR 5.1); 6) positive regulation of transferase activity (FDR 5.2); 7) wound healing (FDR 7.08); 8) regulation of locomotion (FDR 7.3); and 9) anion transport (FDR 9.4); two molecular functions were enriched: 1) growth factor binding (FDR 1.95); and 2) actin binding (FDR 5.7).

Functional Characteristics of Genes Underexpressed in PTLI.

PTLI compared with PTLNI. We identified 216 genes that were underexpressed (≥ 1.5 ; $p \leq 0.05$) in PTLI compared with PTLNI. There was only one significantly enriched pathway: extracellular matrix-receptor interaction (FDR 1.7). Ten biological processes were found to be significantly enriched: 1) unsaturated fatty acid metabolic process (FDR 0.6); 2) fatty acid metabolic process (FDR 1.1); 3) branching morphogenesis of a tube (FDR 1.8); 4) morphogenesis of a branching structure (FDR 3.2); 5) eicosanoid metabolic process (FDR 3.9); 6) tube morphogenesis (FDR 6.8); 7) negative regulation of binding (FDR 8.7); 8) lipid biosynthesis process (FDR 8.9); 9) positive regulation of cell adhesion (FDR 9.2); and 10) eicosanoid biosynthesis process (FDR 9.4). Six molecular functions were significantly enriched: 1) lipid binding (FDR 0.4); 2) coenzyme binding (FDR 1.8); 3) cofactor binding (FDR 3.6); 4) actin binding (FDR 6.7); 5) peroxidase activity (FDR 8.1); and 6) oxidoreductase activity, acting on peroxide as acceptor (FDR 8.1).

PTLI compared with TL. There were 140 genes underexpressed (≥ 1.5 ; $p \leq 0.05$) in PTLI compared with TL. No pathways or biological processes were significantly enriched (FDR $< 10\%$). Lipid binding (FDR 1.8) was the only molecular function that was significantly enriched.

Biological Validation of Microarray Gene Expression. To verify the microarray results, highly differentially expressed genes including β_1 adrenergic receptor (*ADRB1*), eosinophil major basic protein, also referred to as proteoglycan 2 (*MBP* or *PRG2*), stanniocalcin 1 (*STC1*), and hydroxysteroid (11- β) dehydrogenase 2 (*HSD11 β 2*) were selected and analyzed by qRT-PCR. We confirmed changes in expression (direction and magnitude) of these genes between PTLI and PTLNI (Supplemental Table 2) and PTLI and TL (Supplemental Table 3). Overall, the direction of change in gene expression by qRT-PCR was consistent with the microarray analysis of these four genes. Additional genes encoding human chorionic gonadotropin β polypeptide (*βhCG*), retinoid X receptor α (*RXR α*), and GATA binding protein 2 (*GATA2*) were used to confirm statistical significance of microarray genes.

Changes in the mRNA Expression of Proinflammatory Cytokines in Various Placental Conditions. Previous reports have indicated inverse correlations between MDR1 and proinflammatory cytokines. However, neither TNF- α nor IL-6 mRNA expression was altered, and fold changes in IL-8 mRNA expression were significantly increased (12.1-fold, $p < 0.001$) in PTLI compared with TNL (Fig. 4). IL-8 mRNA expression in PTLI was greater than that in other conditions including PTSPE, in which the fold change in IL-8 mRNA expression (6.3-fold, $p < 0.05$) was greater than that of TL but not that of PTL with and without inflammation (Fig. 4). The fold changes in

FIG. 3. Relative changes in the gene expression (A, MDR1; B, BCRP; C, MRP2; D, MRP1; and E, MDR3) of ABC transporters was determined by real-time PCR in human placenta ($n = 6-10$) from women with various pregnancy conditions. The mean fold change in ABC transporter genes, normalized to the endogenous reference gene, 18S rRNA and relative to the expression of the TNL control, was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. Differences between all possible pairs of group means were determined by one-way ANOVA followed by a Student-Newman-Keuls multiple comparison post hoc test. Data are presented as mean $\pm$ S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, no significant difference. There were no differences in the mean fold changes of MRP1 and MRP2. Relative mRNA expression in PTLI was significantly higher for MDR1 ($p < 0.01$), BCRP ($p < 0.001$), and MDR3 ($p < 0.05$), by 6.4-, 3.7-, and 12.7-fold, respectively, than that in the TNL placental group.

mRNA expression of IL-8 were correlated with that of MDR1 (Pearson $r = 0.50$, $p < 0.05$, respectively) and that of BCRP (Pearson $r = 0.65$, $p < 0.00$) among the placental groups.

Discussion

Expression patterns of placental ABC transporters vary with gestational age and medical condition during pregnancy. The general consensus is that MDR1 and BCRP expression decline (Gil et al., 2005; Mathias et al., 2005; Sun et al., 2006; Meyer zu Schwabedissen et al., 2006), whereas MRP2 and MDR3 levels increase with gestational age toward term (Patel et al., 2003; Meyer zu Schwabedissen et al., 2005). These changes may reflect a physiological adaptation to the changing requirements for fetal protection, especially in the preterm period. However, several discrepancies have been observed, particularly in humans. For example, Mathias et al. (2005) reported that BCRP expression in human placenta does not change significantly with gestational age, whereas Yeboah et al. (2006) showed that placental BCRP levels increased toward term, whereas mRNA expression remained unchanged. The placental samples used here encompass two very distinct gestational time points: a preterm pregnancy period (28–31 weeks) and a term pregnancy period (38–41 weeks). Because this is not a continuous time course analysis, we cannot infer gestational regulation of the transporters inspected. However, we do observe relatively higher expression of MDR1 and BCRP in placental samples from preterm women compared with those from term women (Fig. 1, A and B).

There were no apparent changes in expression levels of the ABC transporters in response to labor (term or preterm), which is consistent with prior reports in which expression levels of BCRP in human reproductive tissues (fetal membranes and attached deciduas) (Yeboah et al., 2008) and MDR1 in human placenta (Sun et al., 2006) were not altered by labor at term. Our data further support the fact that MDR1 and BCRP expression does not change with preterm labor. Changes in MRP1, MRP2, and MDR3 expression were less apparent in crude membrane fractions of placental tissue. These preparations differ from isolated syncytiotrophoblasts in purity and may explain potential differences with other results, specifically in the extent of BCRP and MRP1 expression, which is also localized to the fetal capillary endothelial cells. However, immunohistochemical analysis revealed that the cellular localization of MDR1 and BCRP was not altered in the placental groups. Furthermore, mRNA expression appears to parallel that of protein expression. We suspect that the observed differences in protein expression are due to the specific pregnancy condition rather than to variation in experimental design.

Preterm birth is the leading cause of perinatal morbidity and mortality. A large proportion of preterm births are associated with pre-eclampsia and inflammation, often secondary to infection. It is increasingly clear that inflammation (outside of that associated with pregnancy) affects the expression of drug transporters (Petrovic et al., 2007). We found that both MDR1 and BCRP (protein and mRNA expression) are highest in placentas from women with inflammation (Figs. 1, A and B, and 3, A and B). Given their high white blood cell

FIG. 4. Relative changes in mRNA expression of proinflammatory cytokines. A, mRNA levels of IL-6, TNF- α , and IL-8 were analyzed by quantitative real-time PCR. The mean fold change in ABC transporter genes, normalized to the endogenous reference gene, 18s rRNA, and relative to the expression of the TNL control, was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. Differences between all possible pairs of group means were determined by one-way ANOVA followed by a Student-Newman-Keuls multiple comparison post hoc test. Data shown are the mean $\pm$ S.E.M. from 6 to 10 independent placentas from each of the five groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, no significant difference. There were no differences in the mean fold changes of TNF- α and IL-6. Relative mRNA expression in PTSPE and PTLI was significantly higher for IL-8 by 6.3-fold ($p < 0.05$) and 12.1-fold ($p < 0.001$), respectively, than that in the TNL placental group. B, fold changes in mRNA expression of IL-8 transcripts were correlated with those for MDR1 and BCRP in the human placentas of woman with pregnancy conditions. Correlation analysis was performed using Pearson correlation.

counts, it is probable that inflammation (i.e., stage III chorioamnionitis) is a response to uterine infection. These data represent the first evidence of direct infection-mediated transporter regulation.

Our findings differ from prior literature reports noting transporter down-regulation during inflammation caused by inflammatory cytokines such as TNF- α , IL-6, and endotoxin (i.e., LPS) in rats (Sukhai et al., 2001; Chen et al., 2005; Wang et al., 2005) and human primary placental cells (Evseenko et al., 2007). We offer several possible explanations for these differences: the impact of LPS-induced inflammation on drug transporters has yet to be evaluated at different gestational stages, and previous reports have indicated that preterm placentas respond differently to LPS than those at term, specifically in their patterns of cytokine release (Holcberg et al., 2007). More importantly, common clinical infections of the reproductive compartments are associated with microorganisms that lack LPS, such as *Ureaplasma* species, *Mycoplasma hominis*, and group B *Streptococcus*. It is evident that different pathogens or pathogen components elicit diverse patterns of gene expression and cytokine release (Flad et al., 1993; Ueyama et al., 2005). For example, IL-8 was significantly elevated in amniotic fluid and umbilical cord blood in cases of intrauterine *Ureaplasma* infection, which was not observed with other pathogens (Witt et al., 2005). Taken together, these results indicate that stimulation of alternative cytokines or inflammatory mediators could have contrasting effects on ABC transporters. Thus, observed differences in transporter regulation among various experimental models are not surprising. This is evident in cases of patients with inflammation from rheumatoid arthritis in whom an increase, rather than a decrease, in MDR1 expression is observed (Llorente et al., 2000). It is clear that the impact of inflammation on drug transporters in the human placenta is still in a nascent stage. The development of

models that more closely mimic the human pathological pregnancy condition will expound differences in transporter regulation, including the need to evaluate various inflammatory pathogens and or stimuli during pregnancy.

Hence, we adopted a functional genomic approach to identify potential mechanisms driving changes in gene expression during PTLI. We hypothesized that underlying inflammatory events may account for the observed MDR1 and BCRP regulation. Because the PTLI group is defined, in part, by labor, it was logical to compare this placental group with those also associated with labor, specifically PTLNI and TL. In general, functional pathways and biological processes associated with pregnancy and development were found to be enriched (overrepresented) with genes overexpressed in PTLI compared with TL. Of interest, these events appeared to be similar in comparisons of genes up-regulated in PTLNI compared with TL (supplemental data). When we compared PTLI with PTLNI, we found that genes were up-regulated in processes associated with inflammation and cellular regulation, in particular, the cytokine-cytokine receptor interaction pathway, as were molecular functions related to cytokine activity. These results provide biological relevance for the given PTLI condition and further suggest that proinflammatory cytokines may be involved in the pathways regulating MDR1 and BCRP. Thus, we evaluated the correlation between expression of well recognized proinflammatory cytokines, IL-6, IL-8, and TNF- α and MDR1 and BCRP.

IL-8 is a potent chemotactic agent and activates neutrophils, potentiating the host defense mechanism against inflammation. It is thought to be constitutively produced by the human placenta (Shimoya et al., 1992) independent of preterm versus term delivery (Keelan et al., 1999). IL-8 is increased in placental tissue during

chorioamnionitis (Lockwood et al., 2006) as well as in amniotic fluid and cord blood from women with intrauterine infection. We found a significant fold increase in IL-8 mRNA expression in placentas in PTLI, whereas there were no differences in placentas in preterm versus term pregnancy as demonstrated in comparisons between PTLNI and TL and TNL (Fig. 4). These results are consistent with the literature. Fold changes in IL-8 mRNA expression were correlated with that of MDR1 and that of BCRP. On the basis of the aforementioned association between IL-8 and inflammation-infection, these data support altered expression of MDR1 and BCRP in placentas of women with preterm labor and inflammation. Of interest, there were no changes in mRNA expression of other proinflammatory cytokines, TNF- α and IL-6. However, changes in these cytokines may be more apparent in the amniotic fluid or the maternal or fetal serum.

We observed elevated placental mRNA expression of IL-8 in women with PTSPE compared with women with term labor (TNL and TL). These results are consistent with reports of increased IL-8 production in trophoblasts (Bowen et al., 2005) and elevated IL-8 levels in maternal and umbilical cord serum as well as amniotic fluid of preeclamptic women (Nakabayashi et al., 1998; Laskowska et al., 2007). In contrast, Wang et al. (1999) found a decrease in placental IL-8 production in preeclampsia. Additional experiments may be required to determine the association of preeclampsia and cytokine-specific production.

In this study, we did not detect significant changes in protein or mRNA expression of the multidrug-associated proteins, MRP1 and MRP2. LPS and proinflammatory cytokines have been shown to down-regulate MRP2 expression in the liver of rodents (Teng and Piquette-Miller, 2008); however, there are currently no data to support inflammatory-induced changes in MRP2 and MRP1 expression in humans and in placental tissue. Although MDR3 has generally been considered a liver-specific transporter, MDR3 expression in human term and preterm placentas has been described previously (Patel et al., 2003); however, its physiologic function in syncytiotrophoblasts remains speculative. We observed that MDR3 levels were not altered to the same extent as its mRNA expression. Others have also indicated discrepancies in MDR3 and its mRNA expression in trophoblasts, which may be attributed, in part, to translational regulation (Evseenko et al., 2006).

In the present study, we found that MDR1 and BCRP are significantly regulated in human placenta. Prior studies have shown that MDR1 and BCRP are coregulated in various tissue barriers to enhance tissue protection from xenobiotics. For example, de Vries et al. (2007) showed that these two transporters act in concert to limit the penetration of topotecan at the blood-brain barrier. Like the blood-brain barrier, the placenta protects against harmful toxic substances and restricts the entry of therapeutic agents. Therefore, changes in placental expression of these transporters could have a profound impact on drug efficacy or toxicity. We further demonstrated that both MDR1 and BCRP expression increase in association with underlying inflammation. Up-regulation of MDR1 and BCRP in placenta during preterm inflammation and/or labor could significantly impair therapeutic intervention. For example, MDR1 and BCRP transport a variety of drugs including drugs necessary for fetal therapy. BCRP/Bcrp1 significantly limits the fetal level of nitrofurantoin, an antibiotic commonly used to treat urinary tract infections during pregnancy (Zhang et al., 2007), whereas MDR1/Mdr1a/b transports antibiotics such as azithromycin, erythromycin, clarithromycin, levofloxacin, and rifampin (Thurauf and Fromm, 2006), agents currently used to prevent maternofetal infections. MDR1 may also limit the transplacental transfer of protease inhibitors such as nelfinavir, ritonavir, saquinavir, and lopinavir, which are used in human immunodeficiency virus-

infected women to prevent transmission to the fetus. At the present time, perinatal drug therapy in an inflamed and/or infected maternal-fetal milieu is secondary to clinical premature fetal delivery. Further studies will be needed to demonstrate that placental MDR1 and BCRP expression during preterm inflammatory conditions directly correlates with drug exposure and outcome. A variety of placental transporters localize to the maternal interface of the placenta, the fetal membrane surface, or both. Additional studies should be focused on other important placental transporter proteins and their regulation under various pregnancy conditions.

Authorship Contributions

Participated in research design: Mason, Weiner, and Swaan.

Conducted experiments: Mason and Dong.

Contributed new reagents or analytic tools: I.A. Buhimschi and C.S. Buhimschi.

Performed data analysis: Mason and Swaan.

Wrote or contributed to the writing of the manuscript: Mason, Weiner, and Swaan.

References

- Bowen RS, Gu Y, Zhang Y, Lewis DF, and Wang Y (2005) Hypoxia promotes interleukin-6 and -8 but reduces interleukin-10 production by placental trophoblast cells from preeclamptic pregnancies. *J Soc Gynecol Invest* **12**:428–432.
- Buhimschi IA, Christner R, and Buhimschi CS (2005) Proteomic biomarker analysis of amniotic fluid for identification of intra-amniotic inflammation. *BJOG* **112**:173–181.
- Camus M, Deloménie C, Didier N, Faye A, Gil S, Dauge MC, Mabondzo A, and Farinotti R (2006) Increased expression of MDR1 mRNAs and P-glycoprotein in placentas from HIV-1 infected women. *Placenta* **27**:699–706.
- Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, et al. (2007) Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* **26**:745–752.
- Chen YH, Wang JP, Wang H, Sun MF, Wei LZ, Wei W, and Xu DX (2005) Lipopolysaccharide treatment downregulates the expression of the *pregnane X receptor*, *cyp3a11* and *mdr1a* genes in mouse placenta. *Toxicology* **211**:242–252.
- de Vries NA, Zhao J, Kroon E, Buckle T, Beijnen JH, and van Tellingen O (2007) P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clin Cancer Res* **13**:6440–6449.
- Evseenko DA, Paxton JW, and Keelan JA (2006) ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast. *Am J Physiol Regul Integr Comp Physiol* **290**:R1357–R1365.
- Evseenko DA, Paxton JW, and Keelan JA (2007) Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors. *Drug Metab Dispos* **35**:595–601.
- Flad HD, Loppnow H, Rietschel ET, and Ulmer AJ (1993) Agonists and antagonists for lipopolysaccharide-induced cytokines. *Immunobiology* **187**:303–316.
- Gil S, Saura R, Forestier F, and Farinotti R (2005) P-glycoprotein expression of the human placenta during pregnancy. *Placenta* **26**:268–270.
- Holcberg G, Amash A, Sapir O, Sheiner E, Levy S, and Huleihel M (2007) Perfusion with lipopolysaccharide differently affects the secretion of tumor necrosis factor- α and interleukin-6 by term and preterm human placenta. *J Reprod Immunol* **74**:15–23.
- Huang da W, Sherman BT, and Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**:44–57.
- Jonker JW, Smit JW, Brinkhuis RF, Maliapaard M, Beijnen JH, Schellens JH, and Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst* **92**:1651–1656.
- Kedzior DA, Muja N, Walczak P, Ruiz-Cabello J, Gilad AA, Jie CC, and Bulte JW (2010) Gene expression profiling reveals early cellular responses to intracellular magnetic labeling with superparamagnetic iron oxide nanoparticles. *Magn Reson Med* **63**:1031–1043.
- Keelan JA, Marvin KW, Sato TA, Coleman M, McCowan LM, and Mitchell MD (1999) Cytokine abundance in placental tissues: evidence of inflammatory activation in gestational membranes with term and preterm parturition. *Am J Obstet Gynecol* **181**:1530–1536.
- Laskowska M, Laskowska K, Leszczynska-Gorzela B, and Oleszczuk J (2007) Comparative analysis of the maternal and umbilical interleukin-8 levels in normal pregnancies and in pregnancies complicated by preeclampsia with intrauterine normal growth and intrauterine growth retardation. *J Matern Fetal Neonatal Med* **20**:527–532.
- Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{- $\Delta\Delta C_T$} method. *Methods* **25**:402–408.
- Llorente L, Richard-Patin Y, Díaz-Borjón A, Alvarado de la Barrera C, Jazek-Ocampo J, de la Fuente H, Gonzalez-Amaro R, and Diaz-Jouanen E (2000) Multidrug resistance-1 (MDR-1) in rheumatic autoimmune disorders. Part I: Increased P-glycoprotein activity in lymphocytes from rheumatoid arthritis patients might influence disease outcome. *Joint Bone Spine* **67**:30–39.
- Lockwood CJ, Arcuri F, Toti P, Felice CD, Krikun G, Guller S, Buchwalder LF, and Schatz F (2006) Tumor necrosis factor- α and interleukin-1 β regulate interleukin-8 expression in third trimester decidua cells: implications for the genesis of chorioamnionitis. *Am J Pathol* **169**:1294–1302.
- Mason CW, Hassan HE, Kim KP, Cao J, Eddington ND, Newman AH, and Voulalas PJ (2010) Characterization of the transport, metabolism, and pharmacokinetics of the dopamine D3 receptor-selective fluorenyl- and 2-pyridylphenyl amides developed for treatment of psychostimulant abuse. *J Pharmacol Exp Ther* **333**:854–864.

- Mason CW, Swaan PW, and Weiner CP (2006) Identification of interactive gene networks: a novel approach in gene array profiling of myometrial events during guinea pig pregnancy. *Am J Obstet Gynecol* **194**:1513–1523.
- Mathias AA, Hitti J, and Unadkat JD (2005) P-glycoprotein and breast cancer resistance protein expression in human placenta of various gestational ages. *Am J Physiol Regul Integr Comp Physiol* **289**:R963–R969.
- Meyer zu Schwabedissen HE, Grube M, Dreisbach A, Jedlitschky G, Meissner K, Linnemann K, Fusch C, Ritter CA, Völker U, and Kroemer HK (2006) Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP). *Drug Metab Dispos* **34**:524–533.
- Meyer zu Schwabedissen HE, Jedlitschky G, Gratz M, Haenisch S, Linnemann K, Fusch C, Cascorbi I, and Kroemer HK (2005) Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation. *Drug Metab Dispos* **33**:896–904.
- Nagashige M, Ushigome F, Koyabu N, Hirata K, Kawabuchi M, Hirakawa T, Satoh S, Tsukimori K, Nakano H, Uchiumi T, et al. (2003) Basal membrane localization of MRP1 in human placental trophoblast. *Placenta* **24**:951–958.
- Nakabayashi M, Sakura M, Takeda Y, and Sato K (1998) Elevated IL-6 in midtrimester amniotic fluid is involved with the onset of preeclampsia. *Am J Reprod Immunol* **39**:329–334.
- Novotna M, Libra A, Kopecky M, Pavek P, Fendrich Z, Semecky V, and Staud F (2004) P-glycoprotein expression and distribution in the rat placenta during pregnancy. *Reprod Toxicol* **18**:785–792.
- Patel P, Weerasekera N, Hitchins M, Boyd CA, Johnston DG, and Williamson C (2003) Semi quantitative expression analysis of MDR3, FIC1, BSEP, OATP-A, OATP-C, OATP-D, OATP-E and NTCP gene transcripts in 1st and 3rd trimester human placenta. *Placenta* **24**:39–44.
- Petrovic V, Teng S, and Piquette-Miller M (2007) Regulation of drug transporters during infection and inflammation. *Mol Interv* **7**:99–111.
- Salafia CM, Weigl C, and Silberman L (1989) The prevalence and distribution of acute placental inflammation in uncomplicated term pregnancies. *Obstet Gynecol* **73**:383–389.
- Shimoya K, Matsuzaki N, Taniguchi T, Kameda T, Koyama M, Neki R, Saji F, and Tanizawa O (1992) Human placenta constitutively produces interleukin-8 during pregnancy and enhances its production in intrauterine infection. *Biol Reprod* **47**:220–226.
- Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**:Article3.
- St-Pierre MV, Serrano MA, Macias RI, Dubs U, Hoehli M, Lauper U, Meier PJ, and Marin JJ (2000) Expression of members of the multidrug resistance protein family in human term placenta. *Am J Physiol Regul Integr Comp Physiol* **279**:R1495–R1503.
- Sukhai M, Yong A, Pak A, and Piquette-Miller M (2001) Decreased expression of P-glycoprotein in interleukin-1 β and interleukin-6 treated rat hepatocytes. *Inflamm Res* **50**:362–370.
- Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG, and Gibb W (2006) Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. *Placenta* **27**:602–609.
- Teng S and Piquette-Miller M (2008) Regulation of transporters by nuclear hormone receptors: implications during inflammation. *Mol Pharm* **5**:67–76.
- Thuermer N and Fromm MF (2006) The role of the transporter P-glycoprotein for disposition and effects of centrally acting drugs and for the pathogenesis of CNS diseases. *Eur Arch Psychiatry Clin Neurosci* **256**:281–286.
- Ueyama J, Nadai M, Kanazawa H, Iwase M, Nakayama H, Hashimoto K, Yokoi T, Baba K, Takagi K, Takagi K, et al. (2005) Endotoxin from various gram-negative bacteria has differential effects on function of hepatic cytochrome P450 and drug transporters. *Eur J Pharmacol* **510**:127–134.
- Wang JH, Scollard DA, Teng S, Reilly RM, and Piquette-Miller M (2005) Detection of P-glycoprotein activity in endotoxemic rats by 99mTc-sestamibi imaging. *J Nucl Med* **46**:1537–1545.
- Wang Y, Baier J, Adair CD, Lewis DF, Krueger S, Kruger T, Gurski M, and Brown E (1999) Interleukin-8 stimulates placental prostacyclin production in preeclampsia. *Am J Reprod Immunol* **42**:375–380.
- Witt A, Berger A, Gruber CJ, Petricevic L, Apfalter P, and Husslein P (2005) IL-8 concentrations in maternal serum, amniotic fluid and cord blood in relation to different pathogens within the amniotic cavity. *J Perinat Med* **33**:22–26.
- Yeboah D, Kalabis GM, Sun M, Ou RC, Matthews SG, and Gibb W (2008) Expression and localisation of breast cancer resistance protein (BCRP) in human fetal membranes and decidua and the influence of labour at term. *Reprod Fertil Dev* **20**:328–334.
- Yeboah D, Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG, and Gibb W (2006) Expression of breast cancer resistance protein (BCRP/ABCG2) in human placenta throughout gestation and at term before and after labor. *Can J Physiol Pharmacol* **84**:1251–1258.
- Zhang Y, Wang H, Unadkat JD, and Mao Q (2007) Breast cancer resistance protein 1 limits fetal distribution of nitrofurantoin in the pregnant mouse. *Drug Metab Dispos* **35**:2154–2158.

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