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Proteomic analysis of perfusate from *in situ* rat placenta perfusion

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Abstract

Background: The placenta is the most important organ for the development of the embryo and fetus because of its transport functions. Over the past several decades, a small number of proteins were tested that can cross the placenta, however, there have been no high throughput and comprehensive studies of protein crossing of the placenta.

Methods: To evaluate the *in situ* perfused placenta model and applicate it to investigate the protein transfer functions of the placenta, the rat placenta was perfused *in situ* and the proteome of the perfusate was analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in the present study.

Results: To eliminate the effects of remnants of fetal plasma in the perfusate, a two-tailed t-test of relative quantitation based on spectral counts was performed between the perfusate and fetal plasma. Twenty two proteins in the perfusate were significantly enriched ($p<0.05$) in comparison to the fetal plasma. Most enriched proteins were located in the cytoplasma, membrane, or matrix. There were few plasma proteins that were obviously enriched in the perfusate when we examined alpha fetal protein as the indicator of fetal plasma remnants.

Conclusions: We generated no definite evidence that plasma proteins cross the placenta. The placenta acts as a protein barrier in perfused condition. However, some of the proteins identified in the perfusate may be useful as biomarkers of the integrity of placenta barrier and the degree of placental injury.

Keywords: Placenta perfusion, proteome, placenta barrier, placenta transport function

Introduction

The placenta is an important organ for the optimal growth and development of the embryo and fetus during pregnancy because of its major functions in the absorption of nutrients, excretion of waste products, and gas transfer [1,2]. Over the past several decades, placenta transport functions have been studied in the rat [3-6], pig [7-11], sheep [12], rabbit [13], and human [14,15]. Most of these studies focused on transfer of ions, nutrients, gas, and drugs [2,16,17], whereas protein transfer across the placenta has been rarely studied. Using an isotope labelling technique and enzyme-linked immunosorbent assay, proteins such as albumin [18-20], globulins [18-20], transferrin [18], antibodies [21,22], thyroid hormones [23,24], C-reactive protein [25], granulocyte-macrophage colony-stimulating factor [26], and protease inhibitors [27,28] have been reported to cross the placenta barrier. The number of proteins that were tested is very small compared to the thousands of plasma proteins as there have been no high throughput and comprehensive studies of protein crossing of the placenta.

Artificial perfusion of the fetal umbilical circulation of the placenta has proved very useful in understanding of the mechanisms regulating solute exchange in sheep [12], guinea pigs [7-10,29], rats [6,30,31], and humans [21]. Perfusion of the placenta *in situ* has been used for solute transfer studies in the rat and mouse [30,32]. In the rat, *in situ* placental perfusion has proven particularly useful in the investigation of the mechanisms that control placental ion transport [33]. We hypothesized that this *in situ* placental perfusion model may be useful for proteins transfer studies in rat.

Proteomic technology can identify a large number of proteins in one analysis. This is the first study to comprehensively investigate proteins transfer from maternal circulation to fetal circulation across the placenta using an *in situ* placental perfusion model combined with proteomic technology.

Materials and methods

Experiment design

The artificial perfusion of the fetal umbilical circulation of the placenta was performed and the perfusate was analyzed by proteomic technology. In our study, three placenta perfusate samples and three umbilical cord plasma samples as contral were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). After protein identifications using Mascot search algorithms, the relative protein abundance was estimated based on spectral counts and a two-tailed t-test was used to analyze significant differences in identified proteins

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between two different specimens ($p<0.05$).

Sample preparation

This study was approved by the Institute of Basic Medical Sciences Animal Ethics Committee at the Peking Union Medical College (Animal Welfare Assurance Number: # A5518-01). All rats were housed and handled under ethical conditions, according to the international rules of animal care as specified in the International Animal Welfare Recommendations. Pregnant Sprague-Dawley rats weighing 350 g were purchased from the Vital River Company (Beijing, China). These pregnant rats were anesthetized by pentobarbitone injection of 30 mg/kg sodium thiobutabarbital on d 21 of gestation (term=23 d). Each rat was then immobilized on its back in a thermostated (37°C) bath of isotonic saline. After laparotomy and hysterotomy, each fetus was delivered and its umbilical artery and vein were canulated as previously described [30]. The placenta was perfused (0.5 ml/min) with a Krebs-Ringer solution containing the following in mmol/L:118NaCl, 4.7KCl, 24.9$NaHCO_3$, 1.4$CaCl_2$, and 1.18KH_2PO_4. This solution also contained 0.2% D-glucose, 6% Dextran (40 000 mol wt), and essential amino acids, and it was pH 7.4 adjusted by continuous gassing with 95% O_2 and 5% CO_2. The solution was warmed to 37°C using a heat exchanger prior to perfusion. The perfusion pressure was monitored via a side arm in the arterial catheter. At the beginning of the perfusion, the first 0.5 ml of perfusate, which contained mostly umbilical cord plasma, was collected. After 10 min perfusion, no erythrocytes were apparent after centrifuging the perfusate from the fetal circulation. Subsequently, 20 ml of perfusate was used in a 40 min circulatory perfusion. If there was leakage in the experiment, that experiment was discarded. The cells were removed by centrifugation at 1000 g for 15 min. The resulting perfusate or serum was then centrifuged at 12000 g for 15 min at 4°C to remove any remaining cellular debris. The supernatant was collected and frozen at -80°C [34].

Protein preparation

The perfusate and umbilical cord plasma were dialyzed using a 3 kD dialysis bag, then ethanolamine and hydrochloric acid were added to a final concentration of 20mM, which brought the solution to a final pH of 10. The samples were loaded onto a Q-HP anion exchange column (GE company) at a flow rate of 1ml/min, then eluted with 6M guanidine hydrochloride buffer. The eluent was replaced by water with 0.1% formic acid and concentrated with a centrifugal filter device (Centricon® Plus-20, cut-off 3 000 molecular weight; Millipore, Bedford, MA, USA).

Protein digestion in gels

To remove the Dextran, the protein was digested in gels. The extracted proteins (20 μg) were dissolved by mixing the samples with loading buffer, boiled for 5 min, and loaded onto 12% SDS-PAGE gels. Then, all of the proteins in each sample were digested and extracted according to a previously described method [35].

Mass spectrometry (MS) analysis

In our study, three analyses of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) were performed on the placenta perfusate and umbilical cord plasma samples. For these LC-MS/MS analyses, the peptides were sequentially loaded onto a trap column (Waters, Millford, MA, USA) at a flow rate of 20 μL/min with mobile phase (0.1% formic acid, 99.9% water). The trap column effluent was then transferred to a reversed-phase microcapillary column (0.1×150 mm, packed with Magic C18, 5 μm, 200 Å; Michrom Bioresources, Inc, FOB Auburn, CA, USA) in an ACQUITY UPLC system (Waters, Millford, MA, USA). Separation of the peptides was performed at a flow rate of 500 nL/min and coupled to an online analysis by tandem MS using LTQ Orbitrap Velos (Thermo Fisher Scientific, San Jose, USA). The elution gradient for the reversed-phase column was changed from 95% solvent A (0.1% formic acid, 99.9% water) to 40% solvent B (0.1% formic acid, 99.9% acetonitrile). The run time was 60 min. The MS was programmed to acquire data in a data-dependent mode [36]. All survey scans were acquired in the Orbitrap mass analyzer and the lock mass option was enabled for the 445.120025 ion [37]. The MS survey scan was obtained for the m/z range 300–2000 amu with a resolution of 60000, followed by data-dependent MS/MS scans (isolation width of 3 m/z, dynamic exclusion for 0.5 min), and the twenty most intense ions were fragmented in the ion trap by collision-induced dissociation with a normalized collision energy of 35%, activation q value 0.25, and activation time of 10 ms.

Protein identification

Tandem mass spectra were extracted from raw files using the Mascot search engine (Matrix Science, MASCOT Daemon ver. 2.2.2) with a mass range from 600–5000 Da. The processed spectral data were then searched against the IPI rat version 3.81 protein database (39 602 sequences and 21 027 041 residues) using Mascot search algorithms (ver. 2.2.0). In each case, a reversed database was used to determine the false discovery rates (FDR). The search criteria was as follows: trypsin as a protease was allowed with a maximum of two missed cleavages, carbamidomethylation at cysteine sites was set as a fixed modification, whereas acetylation at the protein N-terminus and oxidation at methionine sites were set as variable modifications. Mass tolerances of ±10 ppm for precursor ions and 0.6 Da for fragments detected using the LTQ and the Orbitrap were used in the database search. Under these conditions, the FDR was less than 3% and the significance threshold was $p<0.05$.

Quantification

The relative protein abundance was estimated based on spectral counts (SCs) of each given protein [38]. To reduce the bias of the peptide amount loaded in each experiment, the SCs were normalized for each protein by dividing the SC by the total SC identified in each run [39]. A two-tailed t-test was

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used to analyze significant differences in identified proteins between two different specimens ($p<0.05$) [40].

Annotation

Proteins showing significant changes were noted in the Uniprot database (http://www.uniprot.org/uniprot/) and Wikipedia (http://en.wikipedia.org/wiki/).

Results and discussion

Identifications of proteins in placental perfusate and umbilical cord plasma

Proteins from three placental perfusates and umbilical cord plasma samples were identified using one dimensional LC-MS/MS. In total, 367 proteins were identified in all six MS runs (**Supplement Tables S1** and **S2**).

Comparison of protein patterns between placental perfusate and umbilical cord plasma

Using a two-tailed t-test, compared to umbilical cord plasma, 22 proteins were found to have significantly higher levels of expression and 10 were found to have significantly lower levels of expression in placental perfusate ($p<0.05$) (**Table 1**) (details in **Supplement Tables S1** and **S2**).

Some injury-related proteins in placenta without placental barrier damaged

Tissue specific and subcellular locations of all proteins showing significant changes ($p<0.05$) were analyzed. Proteins such as annexin A2, tropomyosin, nidogen-1, nidogen 2, col6a3 uncharacterized protein, and Transglutaminase 2 were enriched in the perfusate. These same proteins show high levels of genetic content in the placenta of the mouse [41]. Many of these significantly enriched proteins were identified as being located in the cytoplasm, basement membranes, and the cell surface or matrix in the Uniprot database or other databases, the details of which were shown in **Table 1**. Based on these findings, we suggest that the placenta may be partly damaged when perfused *in situ*. However, physiological perfusion of the placenta *in situ* does not disrupt placental morphology and remains useful in the investigation of the mechanisms regulating placental transport [30,32]. These proteins may be biomarkers of early injury to the placenta while the barrier functions of the placenta remain intact.

Placenta barrier for plasma proteins

Alpha fetal protein (AFP) is synthesised and released from fetal liver, so it may be a good indicator of the remnants of fetal plasma. In our experiment, AFP levels were decreased significantly in the placental perfusate, which indicates that fetal plasma contamination was significantly reduced. Also, serotransferrin, heparin cofactor 2, hemoglobin alpha 2 chain, and other secreted plasma proteins were significantly decreased, which suggests that most of the fetal plasma was washed away. If a protein was enriched and noted as a plasma protein, it probably can cross the placenta barrier from the mother's side. Hemopexin, transthyretin, and platelet factor 4 were enriched. Hemopexin is synthesized in the liver and secreted into the blood, and it has been reported that the hemopexin receptor is present in the human placenta [42], which may help hemopexin cross the placenta. The reasons for the selective enrichment of transthyretin and platelet factor 4 are not clear, however these two proteins play important physiological functions.

Some proteins can cross the placenta [19,22,24] and some mechanistic and characterization studies of proteins that cross the placenta have been performed. The effects of molecular charge and molecular size on macromolecular permeability of the fetal endothelium has been studied in the guinea pig placenta [11,43]. However, in our study, few plasma proteins were found to pass the placenta, which may be a result of our quantitative methods and the low efficiency for protein passage of the placenta. In previous studies, a small percentage of proteins passed the placental barrier over a time period of longer than 3 hours [18,19]. Our quantification method was based on spectral counts, which is a semi-quantitative method, and this approach may not reflect the small changes in protein levels across the placenta.

Antibodies have been shown to cross the placenta in studies using isotopically labelled serum proteins [22] and using an *in vitro* perfused human placenta model [21]. It has been determined that IgG Fc receptors are expressed by cells of the placenta, and suggested that they have a possible role in the materno fetal transmission of passive immunity [44]. That there was no obvious change in antibody levels in our study may be related to the limitted perfusion time. However, the placenta would have undergone more damage if we had used a longer perfusion time. With the further development of this perfusion technique, there will be less damage made to the placenta, which will allow for longer perfusion time and possibly the identification of proteins that pass the placenta.

Stable isotope labeling with amino acids (SILAC) *in vivo* may be the best available isotope labeling technology because of its high labeling efficiency, high-throughput, simple operation, accurate quantitation, and wide range of applications from cell line extension to understaning organization at the whole animal level [45]. It can label a large number of proteins in one study, and these labeled proteins can be used for quantitative proteomic analysis. Furthermore, the placenta remains intact during such a study. This would be an excellent method to use in future studies of placental protein transfer.

Conclusion

Based on the proteomic analysis of perfusate from *in situ* rat placenta perfusion in our present study, few plasma proteins were obviously enriched in the perfusate when we examined alpha fetal protein as the indicator of fetal plasma remnants. We generated no definite evidence that plasma proteins cross the placenta and the placenta acts as a protein barrier in

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Table 1. Significant changes in protein levels between placental perfusate and umbilical cord plasma as assessed using two-tailed t-tests.

IPI	Description	T-value	Down/Up	Tissue (gene expression in mouse)	Subcellular location
IPI00325146	Isoform Short of Annexin A2	17.2	Up	Placenta highly expressed	Basement membrane, Extracellular matrix,Secreted
IPI00214905	Tropomyosin alpha-4 chain	13.4	Up	Placenta highly expressed	Cytoplasm
IPI00231929	Isoform M1 of Pyruvate kinase isozymes M1/M2	13.2	Up		Cytoplasm
IPI00563457	RGD1563545 similar to nidogen 2	8.7	Up		Basement membrane,cell surface
IPI00366081	Desmoplakin isoform 2	7.9	Up		Basement membrane,cell surface
IPI00421429	Junction plakoglobin	7.4	Up		Cell junction
IPI00209416	Ephrin-B1	6.8	Up	Placenta lowly expressed	Membrane
IPI00231136	Nidogen-1	6.7	Up	Placenta highly expressed	Basal lamina(GO)
IPI00197711	L-lactate dehydrogenase A chain	5.8	Up	Placenta lowly expressed	Cytoplasm
IPI00372786	Isoform 1 of Nidogen-2	5.5	Up	Placenta highly expressed	Extracellular matrix(GO)
IPI00421832		4.8	Up		
IPI00372705	Gsdma Uncharacterized protein	4.7	Up		
IPI00195372	Elongation factor 1-alpha 1	4.3	Up	Placenta lowly expressed	Cytoplasm
IPI00565677	Col6a3 Uncharacterized protein	4.0	Up	Placenta highly expressed	Extracellular matrix(GO)
IPI00959248	LOC683720 rCG50520-like	3.5	Up		
IPI00195516	Hemopexin	3.5	Up	Liver highly expressed	Secretedin plasma
IPI00205135	Transglutaminase 2	3.4	Up	Placenta highly expressed	Tissue transglutaminase
IPI00208644	COP9 signalosome complex subunit 1	3.4	Up	Placenta lowly expressed	Cytoplasm
IPI00199973	Leucine-rich repeat-containing protein 51	3.2	Up		Cytoplasm
IPI00464815	Alpha-enolase	3.2	Up	Placenta lowly expressed	Cytoplasm, membrane
IPI00324380	Transthyretin	3.2	Up	Epithelium and liver	Secreted
IPI00206634	Platelet factor 4	2.9	Up		Secreted.
IPI00205036	Hemoglobin alpha 2 chain	-2.8	Down		
IPI00886485	Rat T-kininogen	-2.9	Down	Placenta lowly expressed	Secreted.in plasma
IPI00782621		-3.4	Down		
IPI00363974	Isoform 1 of Gelsolin	-3.4	Down	Placenta lowly expressed	Cytoplasm, Secreted
IPI00564924	Gem (nuclear organelle) associated protein 5	-3.4	Down	Placenta lowly expressed	
IPI00191740	Isoform 1 of Alpha-fetoprotein	-3.8	Down	Liver highly expressed	Secreted in plasma
IPI00210947	Serpind1 Heparin cofactor 2	-4.3	Down	Liver highly expressed	Secretedin plasma
IPI00396889	Protein convertasesubtilisin/kexin type 9	-4.3	Down		Cytoplasm, Secreted
IPI00231282	Kng1;Kng2 Isoform LMW of Kininogen-1	-5.1	Down	Placenta lowly expressed	Secretedin plasma
IPI00679202	Tf Isoform 1 of Serotransferrin	-8.0	Down	Liver highly expressed	Secretedin plasma

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perfused condition. However, some of the proteins identified in the perfusate may be useful as biomarkers of the integrity of placenta barrier and the degree of placental injury.

Additional files

Supplement Table S1
Supplement Table S2

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	LW	LJ	RX	ML	SM	YG
Research concept and design	✓	--	--	--	--	✓
Collection and/or assembly of data	✓	✓	✓	✓	✓	--
Data analysis and interpretation	✓	--	--	--	--	--
Writing the article	✓	--	--	--	--	--
Critical revision of the article	--	--	--	--	--	✓
Final approval of article	--	--	--	--	--	✓
Statistical analysis	✓	--	--	--	--	--

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