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ORIGINAL ARTICLE

CHANGES OF PROTEOGLYCAN EXPRESSION AND GLYCOSAMINOGLYCAN CONSTITUENTS IN THE INTERVILLOUS SPACE OF THE PREGNANCY-INDUCED HYPERTENSION PLACENTA

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Abstract The changes in proteoglycan (PG) expression and glycosaminoglycan (GAG) constituents in the intervillous space of the pregnancy-induced hypertension (PIH) placenta were investigated. PGs and GAGs were purified from the extract of the placental intervillous space by the DEAE-Sephacel column and salt-concentration gradient method, and the GAG sugar chains were released by the actinase and cellulase treatments. The sugar chains from the placentas of normal pregnancy and PIH were compared by cellulose-acetate membrane electrophoresis. No difference was observed in the expressions of hyaluronic acid, heparan sulfate, and chondroitin sulfate, but a clear increase in the expression of dermatan sulfate (DS) in the placenta of the PIH was confirmed. An increase of the DS that specifically activates anticoagulants can be a body reaction to counteract the hemostatic condition observed in PIH.

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Key words: proteoglycans; glycosaminoglycans; pregnancy-induced hypertension; placenta; dermatan sulfate.

原　著

妊娠高血圧症候群胎盤の絨毛間腔におけるプロテオグリカン発現やグリコサミノグリカン構造の変化

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抄録　妊娠高血圧症候群（PIH）胎盤の絨毛間腔におけるプロテオグリカン（PG）発現やグリコサミノグリカン（GAG）糖鎖の構造について研究した．胎盤絨毛間腔から抽出したプロテオグリカンやグリコサミノグリカンを，塩濃度勾配を用いたDEAE-Sephacelカラムクロマトグラフィーで精製し，アクチナーゼやセルラーゼ酵素消化にてGAGを分離した．正常妊娠胎盤とPIH胎盤のGAG糖鎖についてセルロースアセテート膜電気泳動法を用いて比較した．ヒアルロン酸，ヘパラン硫酸，コンドロイチン硫酸には差違は認められなかったが，PIH胎盤におけるデルマタン硫酸（DS）の発現が増加していた．抗凝固因子を特異的に活性化するデルマタン硫酸の胎盤内での増加は、PIHで見られる凝固亢進に対抗するための生体内反応である可能性がある．

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キーワード：プロテオグリカン；グリコサミノグリカン；妊娠高血圧症候群；胎盤；デルマタン硫酸.

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Introduction

Pregnancy-Induced Hypertension (PIH) is a disease mainly characterized by high blood pressure, and is defined as "the development of hypertension after 20 weeks gestation, for 12 weeks after the delivery, or with albuminuria but not as an accidental complication in pregnancy." It occurs in 3 to 10% of pregnancies, and is a major cause of perinatal and maternal deaths[1]. Various studies have been conducted on the causes of this disease, yet a number of the problems remain to be solved.

Nevertheless, the recent studies revealed that the primary cause of PIH would be a poor placentation like a shallow invasion of the trophoblast and inadequate maternal spiral artery remodeling. The poor placentation will lead to endothelial perturbation of blood vessels by oxidative stress, leucocytes activation, a release of syncytiotrophoblast microvillous membrane fragment, lipid peroxides, cytokines, and etc. to bring about vasospasm, hypercoagulability, and vascular hyperpermeability. As a result, PIH has been shown to bring about multisystem disorder such as hepatonephrotic disorders including hypertension and albuminuria, thrombocytopenia, headache, upper abdominal pain, and spasm[2, 3].

The only fundamental cure available for PIH is delivery. A maternal condition can be cured or relieved with an improved prognosis by early delivery of the fetus and placenta so as to complete the gestation. As this disease is improved by the delivery of the fetus and placenta, the delivered parts, especially the placenta of PIH, have attracted a great deal of interest. A recent report discussed the cytokines that circulate in the placenta[3].

Proteoglycan (PG), a constituent of the extracellular matrix, is a sugar protein with the sugar chain glycosaminoglycan (GAG) bound to a core protein, and is responsible for a variety of functions such as the maintenance of tissue construction, and cellular adhesion/movement[4]. Recently its anti-inflammatory effect has gained wide attention. We investigated the anti-inflammatory effect of PG, reporting on the suppressed productions of IL-1β, IL-6, and IL-8 that are important for the onset of chorioamnionitis and cervical maturation[5].

Research on PG and GAG is conducted regarding the embryonic adnexa as well; in a comparative study on the umbilical cords from normal pregnancy and PIH, an increased sulfated GAG of Wharton's jelly in the PIH group was found[6]. In PIH, substances such as lipid peroxides or cytokines increase[7], suggesting that a difference in composition of PGs and GAGs in the placenta would be involved in pathophysiology of PIH. In this study, we investigated the changes associated with the proteoglycan and glycosaminoglycan in the intervillous space of the PIH placenta.

Materials and Methods

The placental tissues

This study was approved by the Ethical Committee of the Institutional Review Board of our institution. The placental tissues were obtained in 14 cases of single-fetus deliveries performed at the Hirosaki University Hospital or its affiliated hospitals with the informed consent of the pregnant women. The blood and hematomas were removed from the placental tissues immediately following the deliveries, and the placental tissues were kept at −20 to −80℃.

There were 7 placentas from normal pregnancy as the control with the mean (± standard deviation) maternal age of 29.4 ± 4.1, and the blood pressures were in the normal range (102−135 mmHg for systole, and 54−79 mmHg for diastole). Only 1 case was positive for albuminuria. The mean of the gestation weeks was 37.1 ± 1.8. The mean weight of the delivered neonates was 2738.6 ± 393.3 g with

Table 1. -Characteristics of the women included in this study

	Control (n = 7)	PIH (n = 7)
Maternal age (y)	29.4 ± 4.1	33.9 ± 7.2
Gravidity	1.4 ± 1.6	0.6 ± 0.7
Parity	0.7 ± 0.7	0.2 ± 0.4
Week of delivery	37.1 ± 1.7	36.8 ± 3.7
Cesarean section (%)	57.1	71.1
Placenta weigth (g)	553.9 ± 115.6	392.1 ± 124.7[a]
Fetal birth weight (g)	2738.6 ± 393.3	2179.9 ± 731.8

[a]p = 0.002: PIH, pregnancy-induced hypertension.

a placental mass of 553.9 ± 115.6 g (Table 1). On the other hand, there were 7 placentas from pregnant women diagnosed as PIH with the mean age of 33.9 ± 7.2; the ranges of the blood pressures were 138−213 mmHg for systole, and 74−117 mmHg for diastole. Four cases were strongly positive for albuminuria (57.1%). The mean of the gestation weeks was 36.8 ± 3.7. The mean weight of the delivered neonates was 2179.9 ± 731.8 g with a placental mass of 392.1 ± 124.7 g, which is significantly less compared to the placentas in normal pregnancy (Table 1).

Isolation of the PGs from the intervillous space of placenta

The placentas that had been kept frozen were thawed on ice. The umbilical cords and the fetal membrans were removed from the placentas, and 100−150 g each of the placental tissues were used; the placental tissues were cut so as to transverse the intervillous space. The extract was obtained by rubbing the tissues gently with the fingers in the ice-cooled phosphate buffer saline (pH 7.2) of 600 mL containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 10 mM N-ethylmaleimide. Then, it was centrifuged at 4,500 rpm for 10 minutes. The supernatant was combined with the DEAE-Sephacel column (5.0 × 5.0 cm). This column was washed with 25 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 10 mM EDTA, and 4 M urea until the UV absorbance <0.02 at 280 nm; then, it was equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 0.15 M NaCl and 4 M urea. The bound GAGs and PGs were eluted in 0.15 M sodium acetate buffer (pH 5.5) containing 4 M urea with a linear concentration gradient of 0.15−0.8 M NaCl. An aliquot of 5 mL each was taken, and the UV absorbance was measured at 280 nm; the uronic acid content in a constant volume was measured using the carbazole-sulfuric acid method (530 nm). The fractions positive to the uronic acid were desalted by dialysis and concentrated by the extracorporeal ultrafiltration method.

Releasing of the GAG sugar chains from the purified placental PGs

The concentrated PGs/GAGs solution was digested by the actinase E in 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ at 50℃ for 12 hours, resulting in a peptide-GAGs form. The peptide-GAGs were precipitated with the 4-fold volume of the salt-saturated ethanol. Then, the GAG chain was digested by the enzyme cellulase that can free the GAG chain without damage and possess end-β-xylosidase activity in 0.1 M sodium acetate buffer (pH 5.0) at 37℃ for 12 hours. The enzyme was heat inactivated for 5 minutes at 100℃. After centrifugation, the supernatant was recovered and precipitated with 4-fold volume of the salt-saturated ethanol to obtain the GAG fractions.

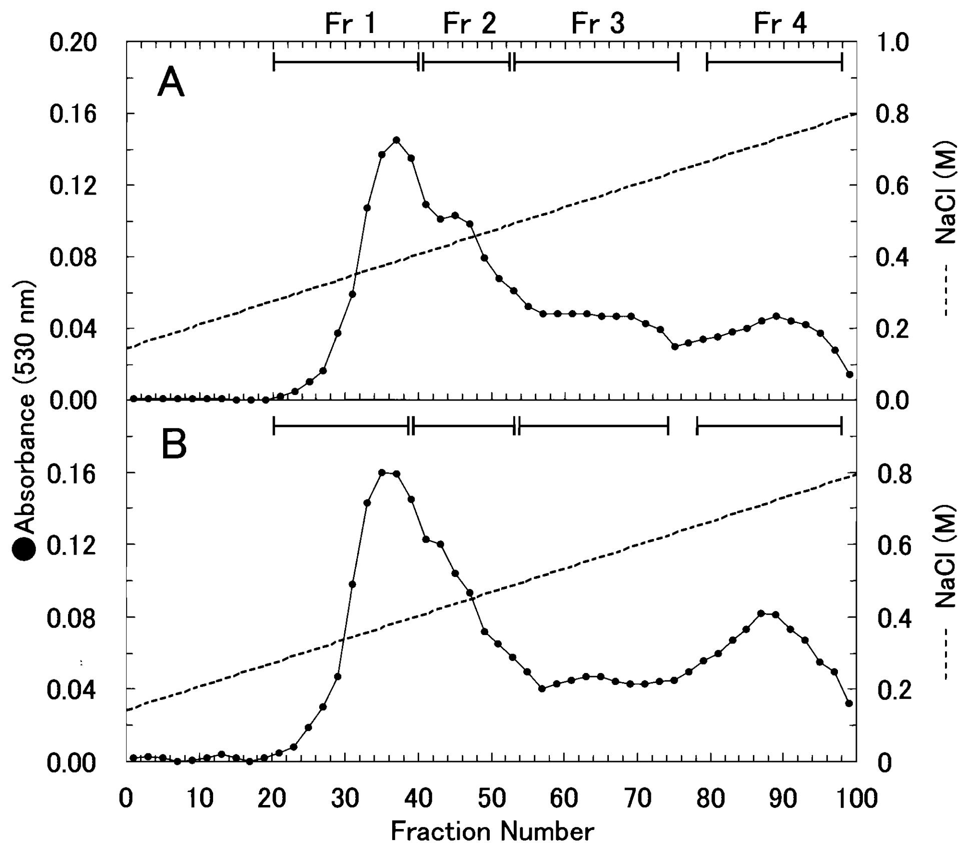

Figure 1 Isolation of the proteoglycans and glycosaminoglycans from the intervillous space of placentas. The experimental method was described in the Materials and Methods. A and B represent the peaks from placentas of the normal pregnancy and pregnancy-induced hypertension using DEAE-Sephacel column chromatography, respectively. Fr, fraction. Isolation pattern shown is one representative of seven experiments.

Enzymatic Digestions of GAG chains

The released GAG chains (25 nmol) were treated with chondroitinase ABC or chondroitinase AC-II. They were incubated in 120 μl of chondroitinase ABC (5 milliunits) dissolved in 0.1 M Tris-HCl buffer (pH 8.0) including 0.01% bovine serum albumin (BSA) and 30 mM sodium acetate at 37℃ for 18 hours or incubated 120 μl of chondroitinase AC-II (5 milliunits) dissolved in 0.1 M sodium acetate buffer (pH 6.0) including 0.01% BSA at 37℃ for 18 hours.

Cellulose-acetate membrane electrophoresis

Cellulose-acetate membrane electrophoresis was conducted on each digested GAG fraction using the Acid Mucopolysaccharide Kit. Using a cellulose-acetate membrane (6 cm width), the electrophoresis was conducted for 35 minutes at 1 mA/cm with the buffer of 0.47 M formic acid/0.1 M pyridine (pH 3.0). For the staining, 0.1% Alcian blue/0.1% acetic acid was used while 0.1% acetic acid was used as the decolorizing solution.

Results

Figure 1A and 1B show elution pattern of intravillous space GAGs/PGs of normal and PIH placentas, respectively, separated by DEAE-Sephacel column chromatography using the salt-concentration gradient. The intervillous space GAGs/PGs were represented by the 4 peaks for the elution at the salt concentrations 0.40, 0.45, 0.55, and 0.75 M in the control and PIH groups. Although elution profiles of the intervillous

Table 2. Difference in relative amount of glycosaminoglycans between Fractions 1 and 4 in the placentas of control and pregnacy-induced hypertension (PIH)

	Uronic acid	
	Control (n = 7)	PIH (n = 7)
Fraction 1	1.00	1.00
Fraction 4	0.39 ± 0.19	0.70 ± 0.32[a]

[a]$p < 0.05$

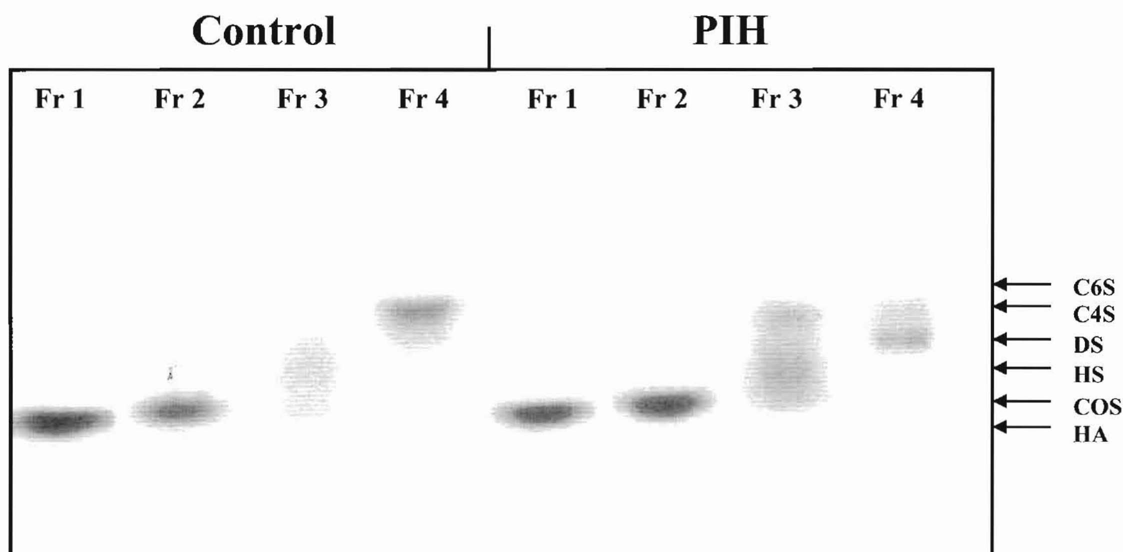

Figure 2 Expression patterns of glycosaminoglycans extracted from placentas of normal pregnancy and pregnancy-induced hypertension (PIH). C6S, 6-chondroitin sulfate; C4S, 4-chondroitin sulfate; DS, dermatan sulfate; HS, heparan sufate; COS, chondroitin; HA, hyaluronic acid; Fr, fraction. Expression pattern shown is one representative of seven experiments.

space GAGs/PGs were similar between normal and PIH groups, the peak for the Fr 4 of the control group was relatively small compared to the larger corresponding peak for the PIH group. The relative amount of GAGs of Fr 4 to that of Fr 1 was 0.70 ± 0.32 in PIH group and was significantly lower than that of control group (0.39 ± 0.19, $P<0.05$) (Table 2). In order to characterize GAG composition of 4 fracions, cellulose-acetate membrane electrophoresis was performed for each fraction (Figure 2). The bands were observed at the following positions: Fr 1 at the chondroitin/hyaluronic acid, Fr 2 at the chondroitin, Fr 3 at the chondroitin sulfate/dermatan sulfate and heparan sulfate, and Fr 4 at the chondroitin sulfate/dermatan sulfate. Almost the same patterns were observed in the electrophoresis for the Fr 1–3 of the control and PIH groups. The Fr 4 consists of chondroitin sulfate and dermatan sulfate, but the PIH group showed an increased expression of the dermatan sulfate than that of chondroitin sulfate (Figure 2). Figure 3 shows cellulose-acetate membrane electrophoresis of Fr 4 following enzymatic digestion by chondroitinase ABC and chondroitinase AC-II. Chondroitinase ABC is a digestive enzyme for 6-chondroitin sulfate, 4-chondroitin sulfate, and dermatan sulfate while chondroitinase AC-II digests the 6-chondroitin sulfate, 4-chondroitin sulfate, and hyaluronic acid. As shown in Figure 3, chondroitinase ABC treatment digested all

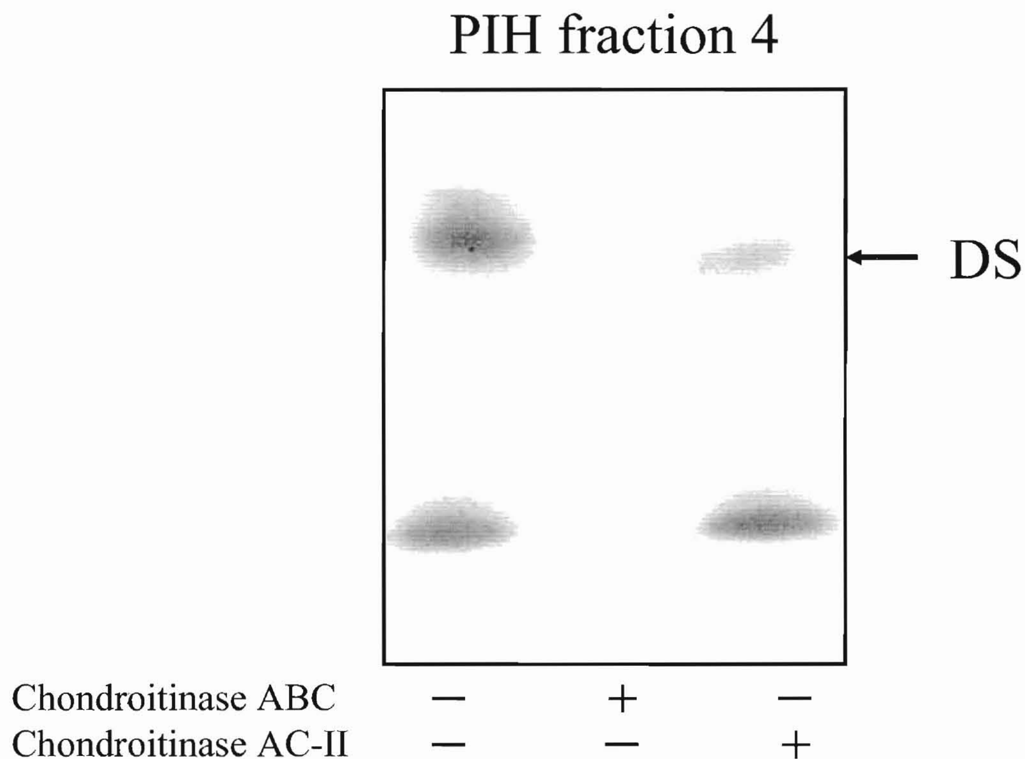

Figure 3 Enzymatic Digestions of GAG chains. The fraction 4 extracted from pregnancy-induced hypertension placenta was digested chondroitinase ABC and chondroitinase AC-II.

bands, while chondroitinase AC-II treatment did not, indicating the Fr 4 is the one corresponding to the dermatan sulfate.

Discussion

The results of the present study clearly demonstrated that the placenta of women who had normal full term delivery contains unique PGs and GAGs using salt-concentration gradient DEAE-Sephacel chromatography. Achur et al characterized PGs and GAGs in the placenta and found that chondroitin sulfate chains in the normal placenta are (i) the PG in the intervillous space at low-sulfated chondroitin sulfate/hyaluronic acid, (ii) the PG containing low-sulfated chondroitin sulfate, (iii) the cellular PG containing chondroitin sulfate and heparan sulfate, and (iv) the PG in the connective tissue of placenta containing chondroitin sulfate/ dermatan sulfate[9]. When the results of their study are compared with those of the present reports, it is shown that the Fr 1 corresponds to (i), Fr 2 to (ii), Fr 3 to (iii), and Fr 4 to (iv), respectively. As shown in Figure 1B, our results have confirmed those of Achur et al[9] and shown for the first time that the expression of dermatan sulfate was increased in the PIH placenta as compared to the normal placenta.

Dermatan sulfate, also known as chondroitin sulfate B (CS-B), is composed of linear polysaccharides assembled as disaccharide units containing a hexosamine, N-acetyl galactosamine (GalNAc) or glucuronic acid (GlcA) joined by β 1,4 or 1,3 linkages respectively[10]. Dermatan sulfate is expressed in many mammalian tissues and is implicated in many pathological conditions such as ischemic cardiovascular lesion, tumorigenesis, infection, wound repair, and fibrosis[10]. In addition, one of the putative activities of dermatan sulfate is to activate the

expression of Heparin cofactor II (HCII) [11]. Giri et al have clearly demonstrated that dermatan sulfate isolated from placentas from women with uncomplicated pregnancies after vagina delivery stimulates HCII activity[11]. HCII is a GAG-dependent thrombin inhibitor found in both the maternal and fetal blood[12], and an about 50% reduction in HCII activities has been shown in severe PIH. A correlation between decreased HCII activity and inadequate functioning of the placenta has been also suggested[13]. Growing evidence has indicated that an antithrombin deficiency during pregnancy is a predominant risk factor for venous thrombosis and embolism, and indeed it is shown that the fetal mortality increases by 1.4 times in thrombophilic women[14]. Based upon these observations, it is presumed that reduced HCII activity seen in PIH pregnancies would be attributable to production failure of dermatan sulfate in the placenta. However, as shown in this study, we could not find a decrease in dermatan sulfate in placentas from women with PIH. Against our prediction, results of the present study showed a significant increase of fraction 4. Although as shown in Figure2, Fraction 4 consists of chondroitin sulfate, dermatan sulfate and unidentified components, the increase in this fraction was attributed to an increase in the component of dermatan sulfate. Thus far, we did not have any data that can account for this discrepancy, but it is possible that an increase in dermatan sulfate was the results of body reaction toward many damages caused PIH at the placental tissues. It has been shown that dermatan sulfate is involved in wound healing of the tissues, by activating endothelial leukocyte adhesion through stimulation of ICAM-1 or by promoting fibroblast growth factor-2 (FGF-2) activity. Both ICAM-1 and FGF-2 are shown to be present at the placenta and increase in PIH.

Another hypothesis that can account for discrepancy between an increased dermatan sulfate content and decreased anticoagulant activities usually seen in PIH subjects is that the composition of GAGs is altered. Chen et al demonstrated that hyperglycemia induces gene expressions of PGs and that it also alters carried GAG type and composition both in vivo and in vitro[15]. The risk of having PIH increases by 3 times in gestational diabetes mellitus (GDM) as a result of the placenta being exposed to hyperglycemia[15]. Interestingly, the GAG/PG was investigated for the GDM placenta, and an increased dermatan sulfate was reported[15]. Although GDM and PIH cannot be discussed on the same line, it is notable to emphasize that the alteration in composition of GAG/PGs in the placenta is involved in pathophysiology of these abnormalities.

Further studies are anticipated to relate the roles of the proteoglycans and the PIH with the function of the placenta.

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References

1) Xiong X, Buekens P, Pridjian G, Fraser WD. Pregnancy-induced hypertension and perinatal mortality. J Reprod Med 2007;52:402-6.

2) Zhou JF, Wang XY, Shangguan XJ, Gao ZM, Zhang SM, Xiao WO, Chen CG. Increased oxidative stress in women with pregnancy-induced hypertension. Biomed Environ Sci 2005;18:419-26.

3) Dowling O, Rochelson B, Way K, Al-Abed Y, Metz CN. Nicotine inhibits cytokine production by placenta cells via NFkappaB: Potential role in pregnancy-induced hypertension. Mol Med 2007;13:576-83.

4) Higuchi T, Tamura S, Tanaka K, Takagaki K, Saito Y, Endo M. Effects of ATP on regulation of galactosyltransferase-I activity responsible for synthesis of the linkage region between the core protein and glycosaminoglycan chains of

proteoglycans. Biochem Cell Biol 2001;79:159-64.

5) Tanaka K, Taniguchi R, Higuchi T, Ozaki T, Mizunuma H, Takagaki K. 4-Methylumbelliferone inhibits hyaluronate synthesis in human uterine cervical fibroblasts. J Obstet Gynaecol Res 2007;33:772-6.

6) Romanowicz L, Bankowski E, Jaworski S. Stimulation of glycosaminoglycan biosynthesis by umbilical cord serum of newborns delivered by mothers with EPH gestosis (preeclampsia). Pathobiology 2000;68:264-9.

7) Cester N, Straffolani R, Rabini RA, Magnanelli R, Salvolini E, Gaiassi R, Mazzanti L, Romanini C. Pregnancy induced hypertension: a role for peroxidation in microvillus plasma membranes. Mol Cell Biochem 1994;131:151-5.

8) Farach-Carson MC, Carson DD: Perlecan: a multifunctional extracellular proteoglycan scaffold. Glycobiology 2007;17:897-905.

9) Achur RN, Valiyaveettil M, Alkhalil A, Ockenhouse CF, Gowda DC. Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. J Biol Chem 2000;275:40344-56.

10) Trowbridge JM, Gallo RL. Dermatan sulfate: new functions from old glycosaminoglycans. Glycobiology 2002;12:117-25.

11) Giri TK, Tollefsen DM. Placental dermatan sulfate: isolation, anticoagulant activity, and association with heparin cofactor II. Blood 2006;107:2753-8.

12) Reverdiau-Moalic P, Delahousse B, Body G, Bardos P, Leroy J, Gruel Y. Evolution of blood coagulation activators and inhibitors in the healthy human fetus. Blood 1996;88:900-6.

13) Bellart J, Gilabert R, Cabero L, Fontcubetra J, Monasterio J, Miralles RM. Heparin cofactor II: a new marker for pre-eclampsia. Blood Coagul Fibrinolysis 1998;9:205-8.

14) Vossen CY, Preston FE, Conard J, Fontcuberta J, Makris M, van der Meer FJ, Pabinger I, Palareti G, Scharrer I, Souto JC, Svensson P, Walker ID, Rosendaal FR. Hereditary thrombophilia and fetal loss: a prospective follow-up study. J Thromb Haemost 2004;2:592-6.

15) Chen CP, Chang SC, Vivian Yang WC. High glucose alters proteoglycan expression and the glycosaminoglycan composition in placentas of women with gestational diabetes mellitus and in cultured trophoblasts. Placenta 2007;28:97-106.