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High Glucose Alters Proteoglycan Expression and the Glycosaminoglycan Composition in Placentas of Women with Gestational Diabetes Mellitus and in Cultured Trophoblasts

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Abstract

Impaired glucose metabolism with diabetes may alter the expressions of proteoglycans (PGs), which may impair the biological functions of placenta. In this study, we investigated the expression of PGs and their conjugated glycosaminoglycan (GAG) composition in the placentas of mothers with gestational diabetes mellitus (GDM) and trophoblasts cultured in a high-glucose condition. The PGs by guanidine/HCl extraction and DEAE Sepharose fractionation followed by GAG degradation enzyme digestion analyses showed that the expression of chondroitin sulfate and/or dermatan sulfate (CS/DS) PGs was increased whereas the heparan sulfate (HS) PG was decreased in GDM placentas compared to controls. Western blot analyses demonstrated that the increased CS/DS PGs in GDM placentas were predominantly the small leucine-rich proteoglycans (SLRPs), decorin and biglycan. Increased mRNA expression level was consistently shown by quantitative real-time PCR. Immunohistochemistry indicated intensive staining of decorin and biglycan in the diabetic placenta with different localizations. Additionally, the basement membrane HSPG, perlecan was found to contain both CS/DS and HS in GDM placentas and plain HS in controls. Similar findings of PG alterations induced by hyperglycemia were observed in cultured trophoblast in a high-glucose condition. This study demonstrated that hyperglycemia induced not only the gene expressions of PGs but also alterations in the carried GAG type and composition.

Keywords: Biglycan; Decorin; Gestational diabetes mellitus; Glycosaminoglycan; Perlecan; Proteoglycan

1. Introduction

Diabetes mellitus during pregnancy may have adverse effects in the placenta including increased placental weight [1,2], oxidative stress [3], and nutrient transport and filtration problems [4]. Pathological studies revealed patchy syncytial necrosis, dilated rough endoplasmic reticulum, cytotrophoblastic hyperplasia, narrowing of the small vessels, focal thickening of the basement membranes, and related extracellular matrix alterations in the diabetic placenta [2–6].

Proteoglycan is a complex macromolecule composed of a core protein and one or more negatively charged polysaccharide chains called glycosaminoglycans (GAGs), which are covalently attached to the protein core [7,8]. The GAG is made up of repeating disaccharide units, classified into the four common types of heparin/heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS). In the placenta, proteoglycans are distributed in the endothelium, cell basement membranes, vessel walls, and villous stroma

Abbreviations: PG, proteoglycan; ECM, extracellular matrix; GDM, gestational diabetes mellitus; CS, chondroitin sulfate; DS, dermatan sulfate; SLRP, small leucine-rich proteoglycan; HS, heparan sulfate; GAG, glycosaminoglycan; KS, keratan sulfate; Hepase, heparintinase; Chase ABC, chondroitinase ABC; Chase B, chondroitinase B; CHAPS, 3-[(3)-cholamidopropyl-dimethyl-ammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; CPC, 1-hexadecyl pyridinium chloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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[9–13]. Detailed characterizations indicate the placental basement membrane tissue predominantly contains heparan sulfate proteoglycan (HSPG), whereas chondroitin sulfate and/or dermatan sulfate proteoglycans (CS/DS PGs) are mainly located in the intervillous space of the ECM [14]. Others and ourselves have demonstrated a placental HSPG, perlecan, to be present in the mesenchymal villi and cytotrophoblastic cell islands and cell columns, which consists in extravillous trophoblasts [9,15] and co-localizes with other basement membrane proteins, such as laminin and collagen type IV [9]. The HSPG, perlecan, composed of a 400–470 kDa core protein with three potential GAG attachment sites [16,17], is abundant in the extracellular basement membrane of vascularized tissues. It was implied that perlecan expression might be essential to placental vascularization during early gestation [15]. The other major PG in the placenta is CS/DS PG, which includes the small leucine-rich proteoglycans (SLRPs), decorin and biglycan. Decorin usually has one CS/DS GAG, whereas biglycan has two; they are the most-closely related molecules in the SLRP superfamily. It was shown that decorin and biglycan are associated with collagen type VI in placental stroma [10,18]. Immunocytochemistry and electron microscopic analyses suggested that SLRPs might be involved in the assembly of the ECM structure of the placenta through their regulation of collagen fibrillogenesis [19,20]. We showed that the expression of perlecan was significantly higher in placentas of mothers with GDM, and hyperglycemia induced the production of perlecan by trophoblasts [21]. Previous reports revealed that perlecan is involved in various diseases resulting from hyperglycemia-induced basement membrane alterations and vasculopathies [21–28]. It remains unclear whether hyperglycemia induces PG, especially decorin and biglycan expressions and changes in their conjugated glycosaminoglycan composition. Thus, in the present study, we investigated alterations in proteoglycans (PGs) and their conjugated glycosaminoglycan (GAG) composition in the GDM placenta. In addition, an in vitro culture system for trophoblasts exposed to high- and low-glucose conditions was also set up to demonstrate the hyperglycemic effect on the expressions of proteoglycans of interest and their GAG composition. These studies provide further information on alterations of proteoglycans induced by hyperglycemia that may help to understand the correlation of these molecules with complications in the diabetic placenta.

2. Materials and methods

2.1. Materials

Sixteen (eight normal as the controls and eight with GDM) third trimester placentas were obtained from pregnant women at gestational ages 36–40 weeks after a caesarean section or vaginal delivery. The diagnosis of GDM was based on a 100 g oral glucose load with two or more venous plasma glucose levels meeting the following criteria: fasting, $\geq$105 mg/L; 1 h, $\geq$190 mg/L; 2 h, $\geq$165 mg/L; or 3 h, $\geq$145 mg/L [29]. According to the clinical characteristics, women with GDM and poor glycemic control, including those clinically managed by diet alone ($n = 3$) or by diet and regular insulin ($n = 5$), were selected for the GDM group in this study. There were no significant differences in the clinical characteristics of patients with GDM and controls except for gravidity, 3.3 ± 1.8 vs. 1.3 ± 0.5; $p = 0.01$ and HbA1c, 5.8 ± 0.6 vs. 5.1 ± 0.3; $p = 0.013$ after undergoing dietary treatment or dietary with insulin treatment during pregnancy. The GDM group had a poor obstetric history with more instances of prior spontaneous abortions. The fetal birth weight (3421.3 ± 233.7 vs. 2952.4 ± 107.5 g) and placenta weight (672 ± 175.2 vs. 500 ± 35.4 g) in the GDM group were higher than those of controls but did not significantly differ ($p > 0.05$). Approval for this study was obtained from Mackay Memorial Hospital and informed consent was obtained from each participating subject.

Heparintinase (Hepase) from *Flavobacterium heparinum* was purchased from Seikagaku, chondroitinase ABC (Chase ABC) from *Proteus vulgaris* and chondroitinase B (Chase B) from *F. heparinum* were purchased from Sigma. Monoclonal antibodies against human perlecan for immunoprecipitation were obtained from Chemicon (clone A7L6). Monoclonal antibodies against human perlecan for Western blot analysis were purchased from Zymed (clone 7B5). Antibodies against human decorin (LF136) and biglycan (LF51) were kindly provided by Dr. Larry W. Fisher from the National Institute of Health (Bethesda, MD, USA). All chemicals were purchased from Sigma (St. Louis, MO, USA) unless specifically stated otherwise.

2.2. Isolation of proteoglycans from the placenta

The protocol for extraction of PGs from the placenta followed that previously described by Yang et al. [9]. Placental tissues were minced and washed with phosphate-buffered saline (PBS) (0.15 M NaCl, pH 7.4, 1:20, w/v). Subsequently, the tissue slices were re-suspended in 4 M guanidine/HCl (15:20, w/v) containing 0.5% 3-[(3)-cholamidopropyl-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 2% β-mercaptoethanol, or 10 mM dithiothreitol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM diisopropyl fluorophosphates in propanol) and incubated end-over-end for at least 12 h at 4 °C, followed by centrifugation at $16{,}000 \times g$ for 60 min at 4 °C. The supernatant was then dialyzed to 7 M urea in 10 mM Tris–HCl (pH 8.3) at 4 °C with three buffer changes. The proteoglycan extract was stored at −20 °C for further analysis.

2.3. Fractionation of the placental proteoglycan extract

The placental PG extract in 7 M urea in 10 mM Tris–HCl (pH 8.0) was diluted with buffer A (10 mM Tris–HCl; pH 8.3) (1:1, v/v) and subjected to a DEAE Sepharose Fast Flow column (HR 5/10, Amersham Biosciences, Piscataway, NJ, USA), which had been pre-equilibrated with buffer A in a liquid chromatographic system (AKTA Basic 10, Amersham Biosciences, Piscataway, NJ, USA). After extensive washing with buffer A to remove any unbound material, the absorbed material was eluted by a salt gradient from 0 to 1 M NaCl in buffer A. Fractions comprising five and seven major peaks according to the positive absorbance at OD 280 and OD 214 were pooled for collection. The fractions in the individual peaks and their eluted salt concentrations were P1, fractions 1–5 (unbound material); P2, fractions 7–9 (0–0.08 M NaCl); P3, fractions 10–15 (0.08–0.2 M NaCl); P4, fractions 16–21 (0.3–0.35 M NaCl); P5, fractions 23–29 (0.35–0.48 M NaCl); P6, fractions 31–35 (0.6–0.7 M NaCl); P7, fractions 39–49 (0.8–1.0 M NaCl). The fractionated samples were stored at −20 °C until use. The protein concentration was determined by a protein assay kit (Bio-Rad, D*c* protein assay, Hercules, CA, USA).

2.4. Proteoglycan sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteoglycans (PGs) were separated by SDS-PAGE as described by Laemmli [30]. After fixation with 40% ethanol and 10% acetic acid, the gel was stained with alcian blue solution (0.5% alcian blue in 3% acetic acid) for the observation of intact PGs and subsequently stained with Coomassie blue G25 staining for the core protein.

2.5. Real-time PCR

Real-time PCR was performed as described previously by Fink et al. [31]. In brief, PCR amplification reactions were performed using an SYBR Green PCR Master Mix reagent kit (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) in an ABI PRISM 7700 sequence detector in a 25-μl reaction. 18S rRNA was used as an internal control. The primer sequences for gene amplification, designed using Primer Express (Applied Biosystems), were as follows: decorin, forward 5′CATCCGCATTGCTGATACCA3′ and reverse 5′AGTCCTTTCAGGCTAGCTGCATG3′; biglycan, forward 5′CATGAA CTGCATCGAGATGG3′ and reverse 5′GTCTCAGGGAGGTCTTTGG3′; and 18S rRNA, forward 5′CGAGCCGCCTGGATACC3′ and reverse 5′CCTCAGTTCCGAAAACCAACAA3′. The PCR was initiated after activation of the Amplitaq Gold enzyme (Applied Biosystems) in a reaction mixture by heating for 10 min at 95 °C. All genes were amplified by a first step of 15 s at 95 °C, followed by 1 min at 60 °C for 40 cycles. Cross-reactivity of the primers to genomic DNA was excluded by demonstration of a lack of amplification with human DNA. Additionally, target gene amplification was not detected in the absence of reverse transcriptase.

The relative amount of target gene expression was calculated by the comparative C_t method, which normalizes the copy number of the target genes to that of 18S rRNA. A validation experiment was performed to demonstrate that the efficiencies of amplification of gene of interest and the 18S rRNA reference gene were approximately equal. The absolute values of the slopes of the log input amount versus ΔC_t were 0.009 for decorin and 0.0033 for biglycan. Based on exponential amplification of the target and reference genes, the amount of amplified molecules at the threshold cycle could be normalized and compared [31]. The results are presented as the mean ± standard deviation, and the distributions were determined by the Kolmogorov–Smirnov test. The differences were assessed by the Mann–Whitney U test. A p value of <0.05 was considered significant.

2.6. Immunohistochemistry of placental decorin and biglycan

Placental tissue samples were collected and snap frozen in liquid nitrogen for cryosectioning. Indirect Immunofluorescence was performed on 5-μm thick cryosection. The sections were air-dried and fixed in ice-cold acetone for 10 min, then rehydrated with PBS for 5 min, blocked with protein block solution (Dako, High Wycombe, UK) for 20 min, and incubated with the rabbit antibody against human decorin (LF136; 1:400) or biglycan (LF51; 1:100 in PBS) for 1 h at room temperature. After three washes in PBS (5 min for each wash), the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (EnVision™ +/HRP Dual link Rabbit/Mouse, Dako) for 1 h at room temperature followed by 3,3′-diaminobenzidine tetrahydrochloride staining. The tissues were counterstained with Mayer's hematoxylin. All the immunostaining and image acquisition were carried out with identical settings for all tissues. The primary antibody was replaced by an appropriately diluted non-specific rabbit anti-human IgG (Dako) as a negative control.

2.7. Cell culture of human trophoblasts

The trophoblast cell line, 3A-Sub-E (ATCC CRL-1584), was cultured in MEM (Gibco, Carlsbad, CA, USA) containing 1% FCS (Hyclone Laboratories, Logan, UT, USA) and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin) (Gibco, Carlsbad, CA, USA). Cells were plated at a density of 5×10^4 cells/cm^2 per 100-mm Petri dish and cultured for 72 h under a humidified 5% CO_2/95% air atmosphere at 37 °C in culture medium supplemented with 5.6 mmol/L D-glucose (the normal glycemic control), 30 mmol/L D-glucose (Merck, Whitehouse Station, NJ, USA; the hyperglycemic group), or 5.6 mmol/L D-glucose and 24.4 mmol/L mannitol as the osmotic control for 30 mM glucose, respectively. After 72 h, all cultures had achieved confluence, and cells were harvested for isolation of the proteoglycans as described in the next section.

2.8. Isolation of proteoglycans from cultured trophoblasts

To isolate the PGs from the cell lysate, 3×10^6 cells from each culture condition was pelleted and washed with PBS and then re-suspended in 3 ml PG extraction buffer (4 M guanidine/HCl in 50 mM sodium acetate (pH 5.8) containing 0.1% CHAPS and protease inhibitors) and incubated for overnight at 4 °C. After centrifugation, the supernatant was dialyzed to 7 M urea in 50 mM Tris (pH 6.8), and 150 mM NaCl including protease inhibitors and then stored at −20 °C for further analysis. To isolate the secreted PGs, the conditioned culture medium was collected, respectively, for the 1-hexadecyl pyridinium chloride (CPC) precipitation method [22]. Briefly, 10 ml of the medium was incubated with 1% CPC for 24 h at 25 °C for precipitation. The supernatant was discarded after centrifugation at $800 \times g$ for 1 h, and the precipitated PG–CPC complex was dissolved in 1 ml of 2 M NaCl in ethanol. Following the addition of 3 ml of absolute ethanol and incubation for 24 h at 25 °C, the PGs could be dissociated from the complex and precipitated. The precipitated PGs after centrifugation were then re-suspended in appropriate buffer for further analysis.

2.9. Perlecan immunoprecipitation

A two-cycle immunoprecipitation procedure was followed as described by Doolittle et al. [32]. The PG isolate (500 μg of the placental tissue extract or 150–270 μg of the cell lysate for each reaction) was pre-incubated with 50% protein G resin in 10 mM Tris, pH 8.0 (50 μl for the placental extract and 100 μl for the cell extract) for 1 h at 4 °C to remove any non-specific binding. Following centrifugation at $14{,}000 \times g$ for 20 s, the pre-cleared protein supernatant was transferred to a clean tube for immunoprecipitation by the addition of 0.5 μl of the monoclonal antibody against human perlecan (clone A7L6), and another new 50% protein G resin slurry in 10 mM Tris (pH 8.0) for end-over-end incubation overnight at 4 °C. The sample was then centrifuged for 20 s at $14{,}000 \times g$. Subsequently, the pellet was washed with 1 ml ice-cold 10 mM Tris (pH 8.0) three times, and the absorbed perlecan was then incubated with the desired buffer for SDS-PAGE or GAG identification analysis by enzyme digestion.

2.10. GAG identification of proteoglycans

The GAG carried by the PG of interest was determined by its susceptibility to different enzymes, i.e. chondroitinase ABC (Chase ABC) to digest both CS and DS types, chondroitinase B (Chase B) to DS, or heparintinase (Hepase) to digest HS. Followed by SDS-PAGE and Western blot analyses, the core protein could be revealed if the PG of interest was susceptible to the enzyme. The individual enzyme digestion protocol was based on the buffer and conditions suggested by the manufacturer. For Chase ABC (or Chase B) and Hepase double digestion, incubation with Hepase occurred prior to that with Chase ABC or Chase B for digestion.

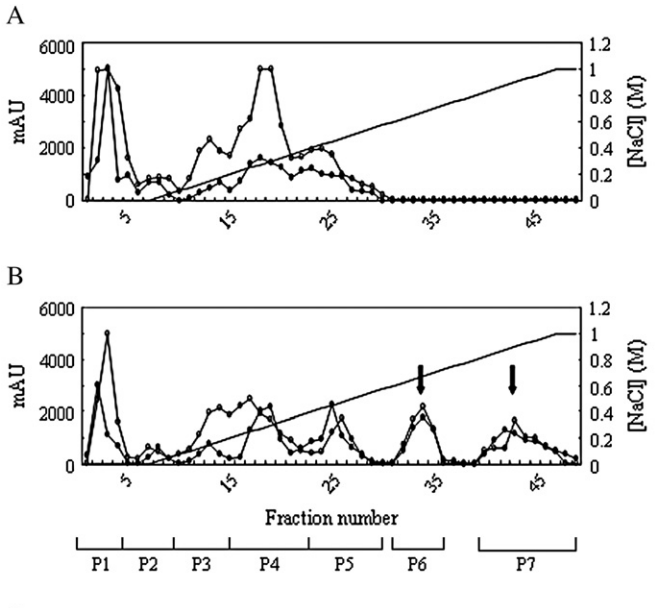
2.11. Western blots of perlecan, decorin, and biglycan

The sample was subjected to SDS-PAGE and then electro-blotted onto a PVDF membrane (Millipore, Bedford, MA, USA). Following blocking with 5% skimmed milk in Tris buffered saline (TBS, 50 mM Tris and 0.5 M NaCl; pH 8.0), the blot was then incubated with the monoclonal antibody against human perlecan (1:500, clone 7B5), decorin (1:500, LF136), or biglycan (1:1000, LF51) in TBS with 0.1% skimmed milk for at least 1 h at 4 °C. After three washes with TBST (TBS with 0.1% Tween-20), 5 min each time, the blot was soaked in a solution containing alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody at a dilution of 1:3000 in TBS with 0.1% skimmed milk for 1 h at room temperature. After an extensive washing with TBST, the blot was incubated with NBT/BCIP for colorimetric development.

3. Results

3.1. Expression of chondroitin/dermatan sulfate proteoglycan was increased in human placentas of patients with GDM

The PGs were isolated from the placenta with or without GDM by a guanidine/HCl extraction followed by DEAE Sepharose fractionation. The chromatogram indicated that most of the PGs from the term placentas and placentas of mothers with GDM were bound to the column and could be eluted at below 0.6 M NaCl (peaks 1–5, P1–P5) in the gradient (Fig. 1A, B). In addition, part of the PG extract from the placentas of mothers with GDM was eluted at a higher salt concentration of 0.6–1.0 M NaCl (peaks 6 and 7, P6 and P7) (Fig. 1B), indicating that the GDM placenta contains a portion of PGs with higher negatively charged density.



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Placenta	Total wet weight (g)	Total extracted PG (%)	HSPG (%)	CS/DS PG (%)
Control (n= 8)	500 ± 35.4	15.90 ± 0.6661	34.73 ± 0.0017	17.83 ± 0.0068
GDM (n= 8)	672 ± 175.2	16.67 ± 0.9348	25.37 ± 0.0301*	49.85 ± 0.0362*

Fig. 1. DEAE Sepharose chromatography of proteoglycans (PGs) extracted from control placentas (A) and placentas with gestational diabetes mellitus (GDM) (B). The placental PG extract (500 μg) was applied to the DEAE column and eluted with a NaCl gradient from 0 to 1 M (solid line). The absorbance at 280 nm (solid dotted line) and at 214 nm (open dotted line) was measured in mAU units. It resolved five (P1–P5) and seven (P1–P7) peaks for the PGs extracted from control placentas and the GDM placentas, respectively. A portion of the PGs from the placenta with GDM was eluted at higher salt concentration (P6 and P7) (arrow). (C) Distribution of the HSPG and CSPG in control and GDM placentas. The yield of PGs extracted from control and GDM placentas and distribution of the type of placental PG determined by GAG degradation enzyme digestion are shown. The value is presented as mean ± SD, $*p < 0.05$ compared to the control.

To identify the type of GAG on the PGs in placenta, enzymatic analyses were carried out by incubation of the PG with the GAG degradation enzyme, heparintinase (Hepase) or chondroitinase ABC (Chase ABC). Chase ABC could digest both CS and DS whereas Hepase could digest HS of PG containing GAG type. This was followed by SDS-PAGE for which the susceptibility of the PG to the enzyme was observed by comparing the band intensity of the PG and the revealed core protein before and after digestion. The revealed protein bands and the band intensity depend on GAG degradation enzyme digestion. Results of the PG content and the distribution of the HSPG and CSPG in placenta and the placenta with GDM are summarized in Fig. 1C. It was shown that there were no significant differences in the total wet weight and in the yield of the total PG extract between the placentas of mothers with GDM and the controls (Fig. 1C). Significantly increased expressions of CS/DS PGs and decreased expressions of HSPGs were found in GDM placentas compared to controls (Fig. 1C). It was also revealed that the highly negatively charged PG extract from P6 and P7 in GDM placentas were susceptible to Chase ABC (Fig. 2B) rather than Hepase (Fig. 2A), suggesting they were CS/DS PGs. In addition, after Chase ABC digestion, the protein bands of the CS/DS PGs in P6 and P7 at 64, 40, and 36 kDa were revealed (Fig. 2B), implying that they might belong to the small leucine-rich proteoglycans (SLRPs), biglycan and decorin.

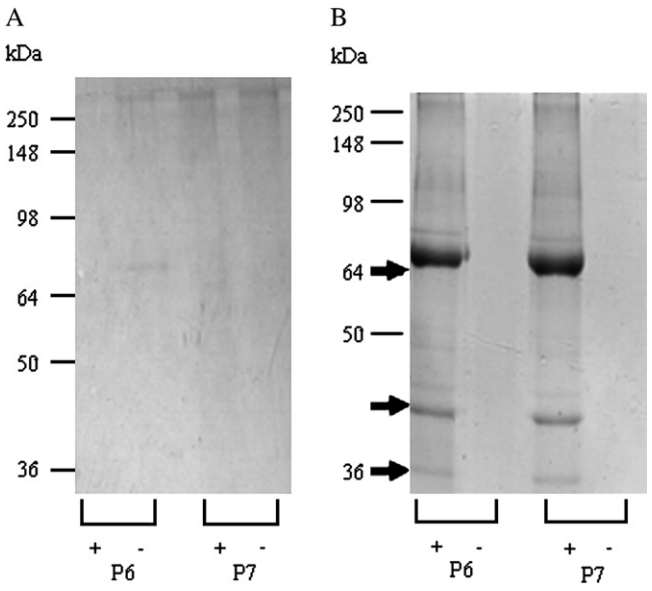

Fig. 2. Characterization of the DEAE Sepharose fractionated proteoglycans (PGs) in P6 and P7 from the GDM placentas. Ten micrograms of the PG extract from P6 and P7 were incubated with (+) or without (−) heparintinase (Hepase) (A) and chondroitinase ABC (Chase ABC) (B) for digestion. The results were revealed by 10% SDS-PAGE followed by alcian blue and Coomassie blue staining. The PGs in P6 and P7 were susceptible to Chase ABC and resistant to Hepase, suggesting they are CS/DS PGs. Significant protein bands of 60, 40, and 36 kDa (arrow) appeared after Chase ABC digestion. The protein molecular weight maker in kDa is shown in the left.

3.2. The increased expressions of CSPG in placentas of mothers with GDM include the SLRPs, biglycan and decorin

To confirm whether the increased CS/DS PGs from P6 and P7 in the placenta with GDM were biglycan and decorin, Western blot analyses using specific antibody against the core protein of decorin or biglycan were carried out following both Chase ABC and Chase B digestions. Results showed that the 60 kDa protein band in normal placenta and both 64 and 60 kDa proteins in the placenta with GDM were indeed biglycan (Fig. 3A) and the 36 kDa protein was decorin (Fig. 3B). The determination of GAG substitution by examining the susceptibility of the placental PG to various GAG degradation enzyme analyses indicated that biglycan, which has two GAG chains, in the placentas of mothers with GDM might be substituted by both CS and DS GAGs (Fig. 3A, right panel) as compared to biglycan being predominantly composed of DS GAGs in the controls (Fig. 3A, left panel). In addition, the anti-biglycan antibody detected 64 kDa protein while the PG extract was digested with Chase B and the 60 kDa band following Chase ABC digestion in placentas of mothers with GDM, suggesting that the 60 kDa biglycan core protein was substituted with both DS and CS and the increased CS content in biglycan was approximately 4 kDa (Fig. 3A, right panel). Decorin, which has one GAG, in GDM placentas, on the other hand, might predominantly be substituted with DS, as compared to that with CS in control placentas (Fig. 3B). A higher molecular weight band around 40 kDa might be due to incomplete digestion.

To clarify whether the expressions of CS/DS PGs biglycan and decorin in placentas with GDM were also increased at mRNA level, quantitative real-time PCR was carried out in the study. It demonstrated that the expressions of biglycan (Fig. 4A, left panel) and decorin (Fig. 4A, right panel) at the mRNA level in human placentas with GDM were, respectively, up-regulated to 2- and 2.5-fold compared to the control. Immunohistochemistry consistently showed that the expressions of biglycan and decorin were more prominent in GDM placentas (Fig. 4B, panels B and E), as compared to the controls (Fig. 4B, panels A and D). However, the distribution of biglycan differed from that of decorin. Biglycan was found in endothelial cells and smooth muscle cells of vessel walls, and the cell surface of trophoblasts (Fig. 4B, panels A and B), whereas decorin was mainly localized to vessel walls and ECM stroma of the chorionic villi, and no immunoreactivity was observed in either villous or extravillous trophoblasts (Fig. 4B, panels D and E). These suggest that hyperglycemia might not only induce gene expressions of the PGs but also alterations of the GAG type and composition in GDM placentas.

3.3. Hyperglycemia induced increased chondroitin sulfate substitution of perlecan in GDM placentas

Since we found that hyperglycemia might result in alterations of the GAG type and composition. Our prior study showed that the expression of basement membrane PG perlecan in GDM placentas was increased and its core protein was substituted by both HS and CS/DS [9], the type of GAG substitution on perlecan was also investigated. Perlecan from the PG extract of the placenta was isolated by immunoprecipitation. The core protein of perlecan (~400 kDa) could be revealed by Western blot using the antibody against the perlecan only if its substituted GAG chains were removed by appropriate GAG degradation enzyme digestion. The results demonstrated that perlecan isolated from control placentas predominantly carried HS (Fig. 5A), since perlecan was not susceptible to

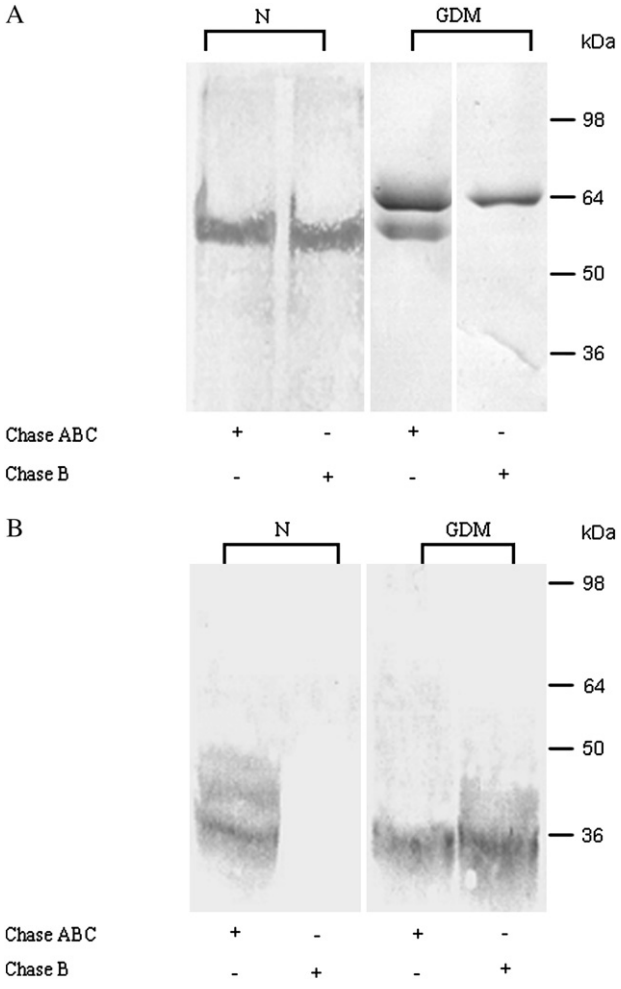

Fig. 3. Identification of decorin and biglycan in human placentas with and without gestational diabetes mellitus (GDM). Equal amounts were used of the proteoglycan extract from the control placenta (N) and P6 of the placenta with GDM (GDM) with (+) and without (−) Chase ABC or Chase B digestion followed by Western blot analyses using anti-biglycan (A) or anti-decorin (B) antibodies to detect the core protein. (A) The anti-biglycan antibody recognized the 60 kDa protein band in the control placenta after either Chase B or Chase ABC digestion. A 64 kDa band after Chase B digestion and the 60 kDa band following Chase ABC digestion were revealed in the PGs extracted from the placenta with GDM. (B) The 36 kDa protein was recognized by the anti-decorin antibody. The decorin core protein in the control placenta was revealed only after Chase ABC digestion, indicating it carried CS. Biglycan and decorin from the placenta with GDM contained both CS and DS, since they were susceptible to both Chase ABC and Chase B. The protein molecular weight maker (kDa) is shown in the right.

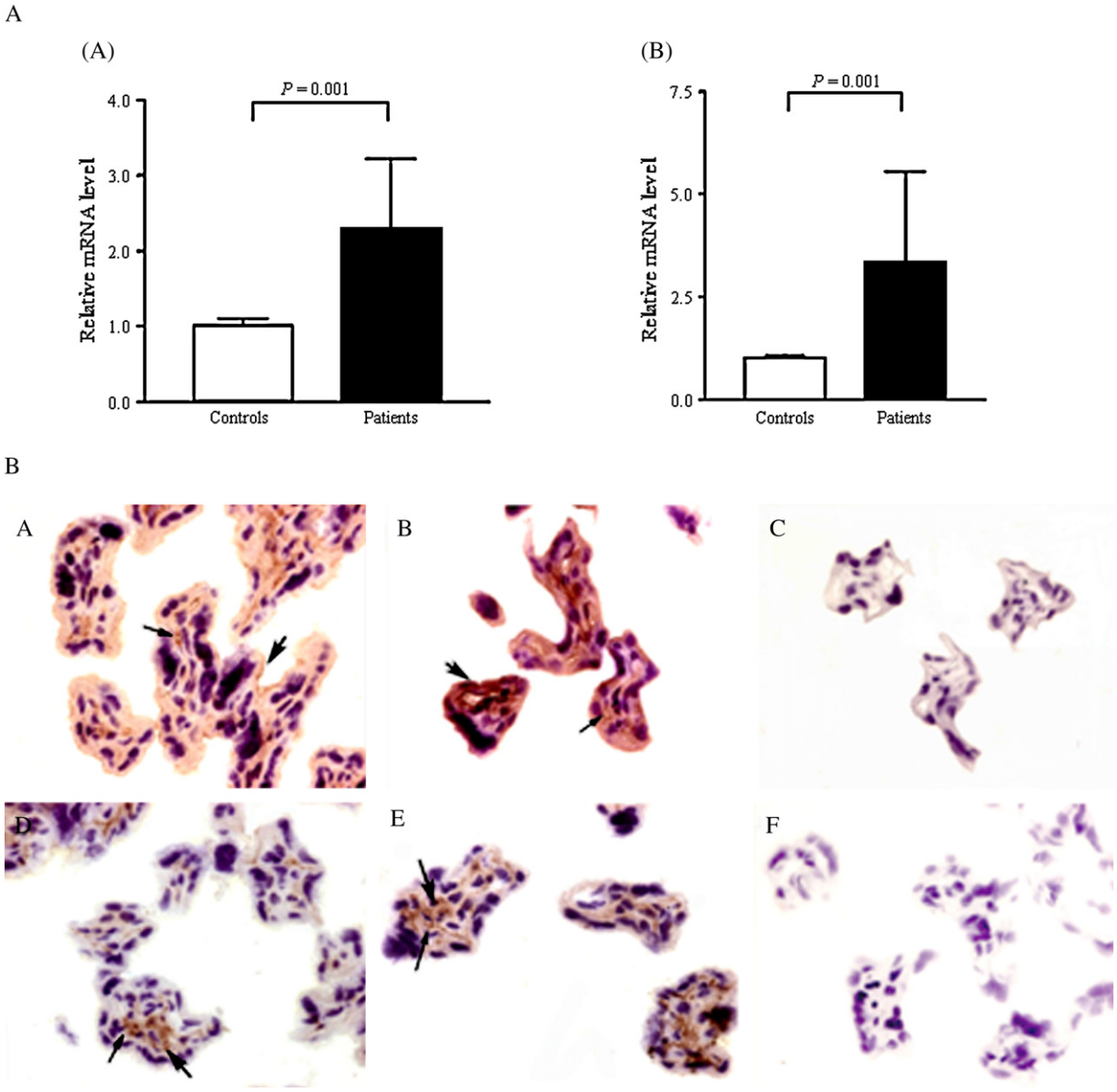

Fig. 4. (A) The mRNA levels of biglycan and decorin in control placenta and the gestational diabetes mellitus (GDM) placenta were quantitated by real-time PCR. Expressions of biglycan (A) and decorin (B) in placentas from women with GDM (patients, $n = 8$) and normal gestational age-matched controls (control, $n = 8$) were determined by quantitative real-time PCR. The relative amount of biglycan and decorin mRNA in each group was normalized against the control group ($p = 0.001$ compared to the control; error bar, SD). (B) Immunohistochemistry of biglycan and decorin in control placentas (A, D) and the placentas with GDM (B, C, E, F). Immunoreactivity to biglycan (A and B) and decorin (D and E) was relatively stronger in GDM placenta (B and E) than those of control (A and D). The biglycan (A and B) was mainly found in the endothelial cells and smooth muscle cells (small arrow) of vessel wall, and the surface of syncytiotrophoblast layer (large arrow), whereas decorin (D and E) was mainly localized to the extracellular matrix stroma (large arrow) and vessel wall (small arrow) of the chorionic villi and no immunoreactivity was observed in either villous or extravillous trophoblasts. The GDM placentas were stained with non-specific rabbit anti-human IgG as negative controls (C and F; magnification ×400).

either Chase ABC or Chase B alone, and the core protein appeared after Hepase digestion (Fig. 5A). In contrast, the perlecan core protein in GDM placentas was revealed following either Hepase or Chase ABC digestion, but not to be shown after Chase B digestion (Fig. 5B). It indicated that the placental perlecan contains both HS and CS/DS GAGs and perlecan from GDM placentas contained an increased amount of CS. Therefore, hyperglycemia indeed altered the GAG type and composition of the PGs in GDM placentas.

3.4. High glucose induced increased expression of chondroitin sulfate/dermatan sulfate substitution on biglycan, decorin, and perlecan in cultured trophoblasts

To clarify the previously observed alterations of proteoglycans in GDM placentas induced by hyperglycemia, human trophoblasts cultured in high-glucose (30 mM) medium to mimic the hyperglycemic condition were generated, and the

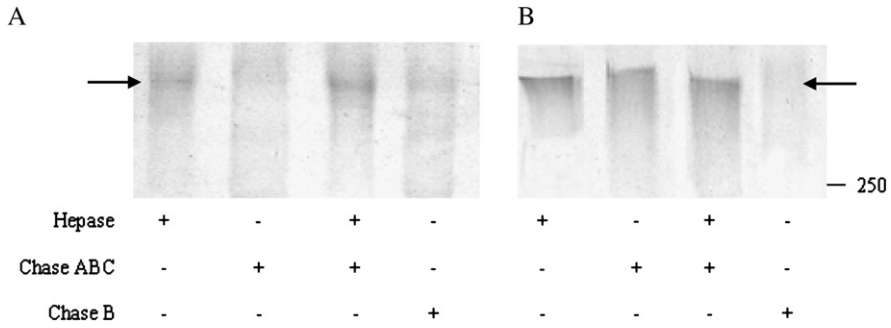

Fig. 5. Characterizations of the glycation on perlecan in control placentas and gestational diabetes mellitus (GDM) placentas. Perlecan was isolated from the equal amount of the proteoglycan (PG) extracted from control placenta (A) and the placenta with GDM (B) by immunoprecipitation. Followed by glycosaminoglycan (GAG) degradation enzyme digestion with (+) or without (−) heparintinase (Hepase), chondroitinase ABC (Chase ABC), or chondroitinase B (Chase B) as indicated, the perlecan core protein (∼400 kDa, arrow) was revealed by Western blot analyses using another anti-perlecan antibody (clone 7B5). (A) Perlecan in control placenta was sensitive to Hepase but not to Chase ABC or Chase B, and the core protein band appeared after digestion. (B) The perlecan from the placenta with GDM was sensitive to both Hepase and Chase ABC but not to Chase B, suggesting that the GAG of perlecan contained heparan sulfate (HS) and chondroitin sulfate (CS). A 250 kDa standard protein marker is shown in the right.

expression of previously studied PGs was investigated. The observations in a high-glucose condition were also compared to that in the osmotic controls (30 mM mannitol) and in regular medium containing a low-glucose (5.6 mM) condition.

The conditioned medium and cell lysate were respectively collected for the analyses. Both biglycan and decorin were found to be secreted predominantly into the culture medium. The expression levels of biglycan in the high-glucose conditioned medium and in the osmotic control were significantly increased compared to those in the low-glucose medium (Fig. 6). The GAG type analysis indicated that biglycan in the high-glucose condition may carry the GAGs containing both DS and CS, since the 60 kDa band appearing as the core protein of biglycan was shown after both DS and CS GAGs were removed by Chase ABC digestion (Fig. 6A). Biglycan expressed in the low-glucose condition (5.6 mM) predominantly contained CS, since a prominent amount of a 60 kDa core protein of biglycan was detected only after Chase ABC digestion (Fig. 6A). Increased expression of decorin and predominantly DS substitution on the decorin core protein were also found in cultured trophoblasts in the high-glucose condition and in the osmotic control (Fig. 6B). In addition, the osmotic effect was greater than high-glucose condition on the expression of decorin (Fig. 6B).

A large amount of perlecan was found in the cell lysate of trophoblasts after 24 h of exposure to a high-glucose condition. Compared to the osmotic control and low-glucose condition, the expression of perlecan in trophoblasts was significantly increased in the high-glucose condition (Fig. 7A). In addition, the GAG composition of perlecan was also altered as observed in GDM placentas. The perlecan expressed in trophoblasts cultured in the low-glucose medium predominantly contained HS (Fig. 7B), whereas the perlecan induced by the high-glucose condition carried more CS/DS than HS since it was more sensitive to Chase ABC (Fig. 7C) than to Hepase (Fig. 7B) in the GAG enzyme digestion analyses. Although the perlecan expressed by trophoblasts was also susceptible to Chase B, the resulting band was higher than that after Hepase or Chase ABC digestion (Fig. 7D), indicating that DS was present but not the only constituent in the GAGs of perlecan. Double digestion with both Hepase and Chase ABC (Fig. 7E) or Hepase and Chase B (Fig. 7F) revealed the core protein band of perlecan to be 400 kDa. The increased expression levels of PGs in trophoblasts cultured in the high-glucose condition were not due to the increased growth rate, since the proliferation rate showed no significant changes in short-term cultured (1–3 days) trophoblasts under different glucose concentrations in our study (data not shown).

The GAG composition determined by the GAG degradation enzyme digestion analysis in both placentas and trophoblasts at high- and low-glucose conditions is summarized in Table 1. Observations of altered GAG compositions in biglycan, decorin, and perlecan corresponding to the high- and low-glucose conditions in placentas were similar to those in trophoblasts.

4. Discussion

Our present studies show that hyperglycemia increases the expression of PGs, including decorin, biglycan, and perlecan, which carry predominantly CS/DS types of GAG in human GDM placentas and in trophoblasts cultured in a high-glucose condition. Hyperglycemia induced increased expressions of CS/DS PGs were significant and it might be associated with the complications of GDM placentas of diabetic patients with poor glucose control. We have also identified the increased CS/DS PGs including the SLRPs, decorin and biglycan in the ECM and perlecan in the basement membrane of placenta. Similar observations were also reported by the studies in other diabetic tissues and cells. In the high-glucose condition, the mRNA level of decorin in the diabetic kidney as well as in cultured glomerular mesangial cells and in tubular epithelial cells [33] and biglycan in bovine myocardial endothelial cells were found to be up-regulated [27]. Another study suggested that promoters of the decorin gene may respond to the high-glucose condition in cultured human mesangial cells

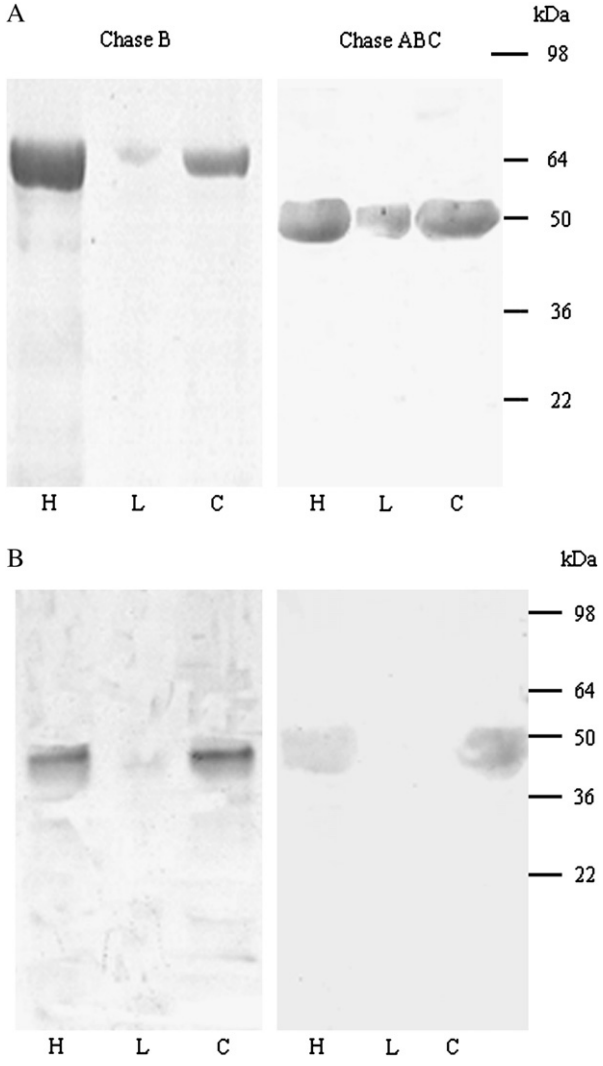

Fig. 6. Characterization of biglycan and decorin in trophoblasts cultured in high-glucose (H), low-glucose (L), and osmotically controlled (M) conditions. An equal amount of conditioned medium was collected for proteoglycan (PG) isolation by CPC-ethanol precipitation as described in Section 2. Following incubation with the indicated glycosaminoglycan (GAG) degradation enzyme and the subsequent Western blot analyses, the GAG compositions of biglycan and decorin were determined. (A) A 64 kDa protein after Chase B digestion and the 60 kDa core protein after Chase ABC digestion were revealed, suggesting that the biglycan in trophoblasts carries both dermatan sulfate (DS) and chondroitin sulfate (CS) GAGs. The expression level of biglycan in the trophoblasts cultured in the high-glucose condition was greatly increased compared to those in the osmotic control and in the low-glucose conditions. (B) A significantly increased amount of decorin containing predominantly DS was expressed by trophoblasts exposed to high glucose and the osmotic control compared to the low-glucose condition. The migration positions of the known molecular weight (kDa) standard markers are shown in the right.

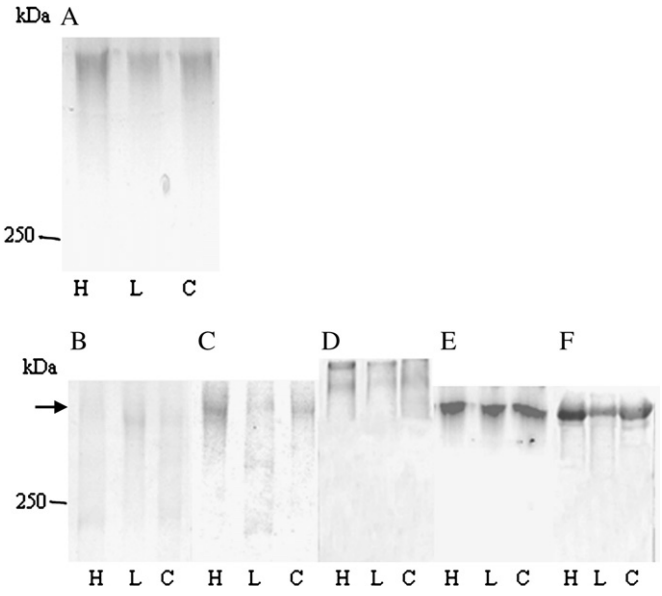

Fig. 7. Characterization of the glycosaminoglycan (GAG) composition for perlecan expressed by trophoblasts cultured in high glucose (H), low glucose (L), and the osmotically controlled (M) media. (A) Isolation of perlecan by immunoprecipitation. An equal amount of the proteoglycan extract from the cell lysate was immunoprecipitated with anti-perlecan antibody and then subjected to a 6% SDS-PAGE with alcian blue and Coomassie blue staining. The typical smear band of intact proteoglycans was revealed. The high-glucose condition induced the largest amount of perlecan expression compared to the osmotic control and the low-glucose condition. (B–F) GAG degradation enzyme digestion followed by Western blot analyses of the immunoprecipitated perlecan in cultured trophoblasts. The isolated perlecan was incubated with (B) Hepase, (C) Chase ABC, (D) Chase B, (E) Hepase and Chase ABC, and (F) Hepase and Chase B, respectively. Subsequently, the perlecan core protein (arrow) was revealed by Western blot. Perlecan expressed in the low-glucose condition contained more heparan sulfate (HS) than chondroitin sulfate/dermatan sulfate (CS/DS), whereas the perlecan in the high-glucose condition carried more CS/DS than HS. The perlecan contained no plain DS type of GAG since Chase B digestion resulted in a higher molecular weight band than the perlecan core protein as shown in panel D. A 250 kDa standard protein marker is shown in the left.

[34]. However, a study reported that a hyperglycemic condition (30 mM) increased the expressions of biglycan and perlecan but not those of decorin in rat vascular smooth muscle cells [35] and bovine myocardial endothelial cells [27]. It suggested that the expression of PGs responding to the hyperglycemic condition may be variable in different cell types and various PGs may be differentially regulated by glucose. Our studies in the cultured trophoblast at high- and low-glucose conditions demonstrated that the hyperglycemic effect on the altered expressions of proteoglycans might, at least in part, be contributed by osmotic stress. The osmotic stress might be synergistic with the metabolic effect of glucose on the increased expressions of biglycan and perlecan, whereas the osmotic stress has even greater effect than high-glucose condition on decorin expression. The altered osmolarity induced PG synthesis was also found in articular chondrocytes and in intervertebral disk cells [36–38]. Although the mechanism for gene regulation of the PG in response to osmolarity remains unclear, it is suggested that it might rely on the localization, structure, or embryological origins of the cells [37].

The basement membrane PG perlecan, which was found to, at least in part, carry CS/DS rather than HS in GDM placentas was unusual. It suggested that high glucose may regulate the glycation of PGs at the post-translational modification level. Increases in DS content in the CS/DS PGs, decorin and biglycan were also identified in our studies. In other studies on mesangial cells of diabetic rats, a hyperglycemic condition produced much more DS in the cultured medium and a high

Table 1
Summary of the glycosaminoglycan (GAG) composition in biglycan, decorin, and perlecan in high- and low-glucose conditions in the placentas and in trophoblasts determined by GAG degradation enzyme digestion and Western blot analyses

	Placentas[a]						Trophoblasts					
	L[b]			H[c]			L			H		
	CS	DS	HS	CS	DS	HS	CS	DS	HS	CS	DS	HS
Biglycan	±	+	−	+	+	−	+	+	−	+	+	−
Decorin	+	−	−	+	+	−	−	−	−	+	+	−
Perlecan	−	±	+	+	±	+	±	+	+	+	+	+

L, low-glucose condition; H, high-glucose condition; +, presence of expression; ±, possible presence of expression; −, absence of expression.
[a] Sixteen third trimester placentas from pregnant women at gestational ages of 36–40 weeks.
[b] L, $n = 8$ from normal placentas.
[c] H, $n = 8$ from GDM placentas.

degree of cell-associated HS compared to that from normal rats was also reported [39]. A decrease in the number of HS GAG chains on the perlecan core protein was found in human aortic endothelial cells cultured in a high-glucose condition [22]. The regulatory mechanism of glucose in the biosynthesis of GAG was little known. A hyperglycemic effect on reduction of *N*-deacetylase, which is a key enzyme for heparan sulfate biosynthesis in diabetic rat was reported [40]. The other studies on the expressions of *N*-deacetylase/*N*-sulphotransferase 1 and 2 in patients with type II diabetes showed no significant differences compared to that in non-diabetic patients [41]. Marano et al. [42] suggested that hyperglycemic effect that altered the glycation of PGs might be mediated by regulation of the catabolism of the GAG-linked protein molecules.

Hyperglycemia-induced alterations of PG expression and its GAG composition may impair the biological functions of the placenta. Perlecan with decreased HS substitution in a hyperglycemic condition may contribute to the nutrient transport and filtration changes found in the diabetic placenta [4]. In other vascularized organs, decreased basement membrane HSPG has been shown to be associated with diabetic nephropathy and altered permeability of glomeruli [43,44], or atherosclerosis promotion by impaired lipoprotein clearance in the liver [26] and in artery walls [22]. In addition, it has been suggested that the DS content in decorin and biglycan might affect blood coagulation in term placenta [45,46]. Whether the hyperglycemia-induced GAG alterations and PG expression in placenta as the observations in our study were correlated to any of the problems or defects described above require further studies.

Hyperglycemia-induced altered expressions of PGs in placenta may be mediated by cytokines, such as transforming growth factor-beta (TGF-β). In diabetic kidney, high glucose induced TGF-β expression, which stimulated HSPG expression in mesangial cells [47]. Decorin was found to antagonize the TGF-β activity and attenuated the increased production of ECM [48]. It suggested that decorin may counter-regulate TGF-β activity on ECM deposition induced by hyperglycemia [49].

This study demonstrates that the increased expressions of CS/DS PGs in GDM placenta are indeed induced by hyperglycemia. The high-glucose condition also affects the post-translational glycosylation for GAG substitution of PGs. The hyperglycemia-induced alterations in PG may influence matrix remodeling, which in turn correlates with complications of diabetic placenta.

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