Characterization of Proteoglycans of Human Placenta and Identification of Unique Chondroitin Sulfate Proteoglycans of the Intervillous Spaces That Mediate the Adherence of \textit{Plasmodium falciparum}-infected Erythrocytes to the Placenta*

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In pregnant women infected with \textit{Plasmodium falciparum}, the infected red blood cells (IRBCs) selectively accumulate in the intervillous spaces of placenta, leading to poor fetal outcome and severe health complications in the mother. Although chondroitin 4-sulfate is known to mediate IRBC adherence to placenta, the natural receptor has not been identified. In the present study, the chondroitin sulfate proteoglycans (CSPGs) of human placenta were purified and structurally characterized, and adherence of IRBCs to these CSPGs investigated. The data indicate that the placenta contains three distinct types of CSPGs: significant quantities of uniquely low sulfated, extracellular CSPGs localized in the intervillous spaces, minor amounts of two cell-associated CSPGs, and major amounts of dermatan sulfate-like CSPGs of the fibrous tissue. Of the various CSPGs isolated from the placenta, the low sulfated CSPGs of the intervillous spaces most efficiently bind IRBCs. Based on IRBC adherence capacities and localization patterns of various CSPGs, we conclude that the CSPGs of the intervillous spaces are the receptors for placental IRBC adherence. The identification and characterization of these CSPGs provide a valuable tool for understanding the precise molecular interactions involved in placental IRBC adherence and for the development of therapeutic strategies for maternal malaria. In the accompanying paper (Alkhalil, A., Achur, R. N., Valiyaveettil, M., Ockenhouse, C. F., and Gowda, D. C. (2000) \textit{J. Biol. Chem.} 275, 40357–40364), we report the structural requirements for the IRBC adherence.

Malaria during pregnancy is one of the major health problems among people living in endemic areas (1, 2). Adults in these regions generally have protective immunity against developing severe malaria (3). Despite this previously acquired immunity, women are susceptible to malaria pathogenesis when they become pregnant (1, 2). This is due to the selection of the placenta by an antigenically distinct phenotype of \textit{Plasmadium falciparum}, which is otherwise rarely present in infected individuals (4, 5). Multiplication of this clonal variant of the parasite leads to the accumulation of infected red blood cells (IRBCs)\(^1\) in high density in the placenta, causing severe clinical manifestations that include low birth weight, still birth, abortion, premature delivery, and maternal morbidity and mortality (1, 2, 6, 7).

\textit{P. falciparum} IRBCs do not generally remain in the peripheral circulation but instead adhere to the endothelial surfaces of the microvascular capillaries of specific organs of the host (8). This adherent property confers virulence to \textit{P. falciparum}, and it is believed to be the central event in malaria pathogenesis (9). Several studies have shown that cell adhesion molecules expressed on the surfaces of vascular endothelium, including CD36, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, platelet endothelial cell adhesion molecule-1/CD62, thrombospondin (10–20), and chondroitin 4-sulfate (C4S), are receptors for IRBC adherence (4, 5, 21–26). The adherence is mediated by the recognition of these receptors by proteins of the \textit{var} gene family, \textit{P. falciparum} erythrocyte membrane protein-1, expressed on the surfaces of infected erythrocytes (27–34). The parasite uses the adherence mechanism to avoid the clearance of IRBCs in the spleen, to overcome the host’s immune surveillance, and maintain up-regulation of cell adhesion molecules for efficient adherence and survival in the host. Thus, extensive accumulation of the parasites in vital organs and the consequent localized high concentration of parasite toxic metabolites and other factors that induce proinflammatory responses in the host lead to vascular damage and organ dysfunction (35–38). Characterization of the receptors that mediate the sequestration of IRBCs to the placenta is likely to provide tools for understanding the detailed molecular interactions between the receptors and parasite proteins on IRBCs and may offer strategies for the development of drugs and therapeutics for maternal malaria.

It has been shown that C4S mediates the adherence of \textit{P.}

\( ^1\) The abbreviations used are: IRBC, infected red blood cell; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; HSPG, heparan sulfate proteoglycan; PG, proteoglycan; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; GAG, glycosaminoglycan; DS, dermatan sulfate; HA, hyaluronic acid; PSS, bovine serum albumin; BD, blue dextran; Glc, glucose; HexN, hexosamine; GalNAc, N-acetylgalactosamine; GalN, galactosamine; GlcN, glucosamine; GlcA, glucuronic acid; GdnHCl, guanidine hydrochloride; NEM, N-ethylmaleimide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PBS, phosphate-buffered saline; TCS, phenylmethylsulfonyl fluoride; TLCK, N-o-tosyl-l-lysine chloromethyl ketone; TPCK, N-o-tosyl-l-phenylalanine chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; HS, heparan sulfate.
**P. falciparum Adherence to Human Placenta**

IRBCs to human placenta (4, 5, 39, 40). However, the CSPGs(s) involved in this process have not been identified, and thus there has not been a satisfactory explanation for the observed in vivo adherence pattern of the IRBCs in the placenta. It has been argued that thrombomodulin or another CSPG present on the syncytiotrophoblast cell surface that extends to the intervillous spaces is the receptor for IRBC adherence (25, 26, 41–43). However, this assumption does not agree with the in vivo IRBC adherence pattern, which is almost exclusively in the intervillous vascular spaces, and IRBCs are not directly attached to the syncytiotrophoblast cell surface (40, 44, 45).

In the placenta, maternal blood bathes the intervillous spaces where fetal villi lined with syncytiotrophoblast cells absorb nutrients, release waste, and exchange gases. Since maternal blood is not static but flows through the placenta at a rate of about 500 ml/min, the IRBCs cannot selectively accumulate in the intervillous spaces unless they adhere to a supporting immobilized extracellular matrix-like structure. An early study suggested that human placenta contains extracellular sialomucins, which can function as an electrochemical barrier in preventing maternal host lymphocytes from making direct contact with syncytiotrophoblasts (46). Consistent with this suggestion, the presence of an amorphous, electron-dense material in the fetal-maternal interface that could be stained with periodic acid-Schiff’s reagent and Hale colloidal iron has been described (47). However, neither the biochemical characterization of this specific material nor the existence of extracellular CSPGs in the intervillous spaces of the placenta has been reported. To explain the intriguing phenomenon of placental IRBC adherence, we reasoned that the placental intervillous space has a loose network of extracellular matrix containing CSPGs, and that these CSPGs support IRBC adherence.

Although several early studies reported the presence of a variety of GAGs including an abundance of HA in the placenta (48–55), a detailed, unequivocal biochemical characterization has been lacking, and almost nothing is known about the CSPGs of placental tissue. In this study, by differential extraction, we isolated CSPGs from different regions of the placenta, purified, and performed a detailed characterization. We demonstrate that the intervillous spaces of the placenta contain unique CSPGs with unusually low 4-sulfated CS chains, and these CSPGs can effectively bind IRBCs. The strategic location of the CSPGs in the intervillous spaces and their adherence characteristics indicate that these are the receptors for the opportunistic adherence of *P. falciparum* IRBCs to the placenta.

**EXPERIMENTAL PROCEDURES**

**Materials**—Term placentas were obtained from the Georgetown University Hospital. C4S (sturgeon notochord), super special grade CS (shark cartilage), *Proteus vulgaris* chondroitinase ABC, protease-free *P. vulgaris* chondroitinase ABC (120 units/mg), *Arthroboth rauraecus* chondroitinase AC II (87 units/mg), *Flavobacterium heparinum* chondroitinase B (25 units/mg), *F. heparinum* heparitinase (113 units/mg), and *Streptomyces hyalurolyticus* hyaluronidase (2000 turbidity reducing units/mg) were purchased from Seikagaku America (Falmouth, MA). C4Ss (bovine trachea and whale cartilage), HA (human umbilical cord), gelatin (300 bloom), PMSF, NEM, benzamidine, protein molecular weight standards for gel filtration, and bovine pancreas RNase (74 units/mg) were from Sigma. Pharmaceutical grade HA (Rooster comb) was from Anika Therapeutics Inc. (Woburn, MA). L-9-Dimethylmethylene blue was from Aldrich. TLCK, TPCK, and DNase I (grade II, 2000 units/ml) were from E. coli (strain B). C4Ss (bovine trachea and whale cartilage), gelatin (300 bloom), PMSF, NEM, benzamidine, and 0.1 mM NEM with gentle scraping. The combined extract was centrifuged, and the clear supernatant was divided into two equal portions; each was loaded onto a DEAE-Sepharose column (2.5 × 22 cm). The columns were washed successively with 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, and 20 mM NaOAc, 150 mM NaCl, pH 5.5. The effluents from both columns were combined and passed through another DEAE-Sepharose column (2.5 × 20 cm) and washed as above. The bound materials in each column were eluted with 20 mM NaOAc, 1.2 mM NaCl, pH 5.5. The eluate from each column was collected in 10-ml fractions, and aliquots were analyzed for uronic acid content by the carbazole method (56). Uronic acid-containing fractions were pooled, and dialyzed (molecular weight cut-off 8,500) against the same buffer. The column effluent was applied to a DEAE-Sephacel column (2.5 × 17 cm) equilibrated with the same buffer. The columns were washed until absorption of effluent was 0.280 nm at 280 nm was at background levels and eluted with a linear gradient of 0.1–0.9 mM NaCl in 50 mM NaOAc, 4 mM urea, pH 5.5. Fractions of 10 ml were collected, absorption at 280 and 280 nm was measured, and aliquots analyzed for uronic acid content (56).

The placenta tissue after the above extraction was cut into small pieces and combined with the above pellet and extracted 2 times with 600 ml of 25 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.0, containing 0.5% Triton X-100, 0.1 mM PMSF, 0.1 mM TLCK, 0.25 mM TPCK, 1 mM benzamidine, and 0.1 mM NEM with stirring for 6–8 h. The combined extract was centrifuged, and the clear supernatant was passed through a DEAE-Sepharose column (4.8 × 14 cm) equilibrated with the same buffer. The effluent was passed through another DEAE-Sepharose column (2.5 × 16 cm). The columns were washed first with 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, and then with 20 mM NaOAc, 150 mM NaCl, pH 5.5, until the absorption at 280 nm was at background levels. The bound materials were eluted with 20 mM NaOAc, 150 mM NaCl, pH 5.5 containing 0.1% CHAPS, and the elution was monitored by measuring absorption at 280 and 260 nm. The eluate was collected in 10-mI fractions, and those containing uronic acid were pooled, dialyzed against water, and lyophilized. This preparation contained a high level of nucleic acids; therefore, it was dissolved (10 mg/ml) in 50 mM Tris-HCl, 10 mM MgCl2, and 0.1 mM dithiothreitol, pH 7.8, and digested with a mixture of DNase (300 units/ml) and RNase (100 units/ml) in the presence of 0.1 mM PMSF, 0.1 mM TLCK, 0.25 mM TPCK, and 0.1 mM NEM at 37°C for 3 h. The incubation mixture was dialyzed against distilled water, lyophilized, dissolved in 50 mM NaOAc, 100 mM NaCl, pH 5.5, containing 4 mM urea, and chromatographed on a DEAE-Sepharose column (2.5 × 17 cm) using a linear gradient of 0.1–0.9 mM NaCl. The eluate was screened for uronic acid (280 nm), and neutral sugar (260 nm). The PG-containing fractions were pooled, dialyzed, and lyophilized.

The fibrous tissue of the placenta, which remained after extraction with detergent-containing buffer, was suspended in 600 ml of 50 mM Tris-HCl, 100 mM NaCl, pH 8.0, containing 10 mM EDTA, 0.5% Triton X-100, 6 mM urea, 0.1 mM PMSF, 0.1 mM TLCK, 0.25 mM TPCK, 1 mM benzamidine, and 0.1 mM NEM. The tissue was homogenized for 30 min using a Polytron homogenizer (Brinkmann, Switzerland), stirred at 4°C for 4 h, and centrifuged. The pellet was extracted two more times as above, and the combined supernatant divided into two portions, and each passed through separate DEAE-Sepharose columns (2.5 × 22 cm). The columns were washed first with 50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 6 mM urea, 10 mM EDTA and then with 50 mM NaOAc, 150 mM NaCl, 6 mM urea. The bound proteoglycans were eluted with 20 mM NaOAc, 1.2 mM NaCl, pH 5.5, 6 mM urea, and dialyzed. The dialysate was adjusted to 50 mM NaOAc, 100 mM NaCl, pH 5.5, 4 mM urea and chromatographed on a DEAE-Sepharose column (2.5 × 17 cm) using a linear gradient of 0.1–0.9 mM NaCl. The PG-containing fractions were pooled, dialyzed, and lyophilized.

**Cesium Bromide Density Gradient Centrifugation of GAGs/PGs**—The GAGs/PGs isolated by DEAE-Sepharose chromatography were dis...
solved (2 mg/ml) in 25 mM sodium phosphate, pH 7.2, containing 50 mM NaCl, 0.02% NaN₃, 4 mM GdnHCl and 42% (w/v) CsBr. The solutions were centrifuged in a Beckman 50 Ti rotor at 44,000 rpm for 65 h at 14 °C (57). Gradients were collected from the bottom of the tubes into 15 equal fractions, absorption at 260 and 280 nm was measured, and uronic acid contents determined (56). Fractions were pooled, dialyzed, lyophilized.

**Gel Filtration of GAGs/PGs**—The GAGs/PGs were sequentially chromatographed on columns of Sepharose CL-6B (2 × 65 cm) in 50 mM NaOAc, 150 mM NaCl, pH 6.0, containing 4 mM GdnHCl and Sepharose CL-4B (1.5 × 83 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, 4 mM GdnHCl. Fractions were collected and monitored for proteins and uronic acid content (56).

**Treatment with S. hyalurolyticus Hyaluronidase**—BCSPG-1/HA fraction (a mixture of CSPGs and HA, see Fig. IA) purified by CsBr density gradient centrifugation and Sepharose CL-6B chromatography was dissolved in 0.5 ml of 20 mM NaOAc, 150 mM NaCl, pH 6.0. The solution was incubated with S. hyalurolyticus hyaluronidase (50 units/ml) for 2 h at 0–4 °C (58). The digest was chromatographed on a Sepharose CL-6B column (1 × 49 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, containing 4 mM GdnHCl.

**Purification of the Cell-associated CSPGs**—The mixture of DCSPGs/DSHS PGs (Fig. 1B) purified by CsBr density gradient centrifugation (Fig. 2B) and Sepharose CL-6B chromatography (Fig. 3B) was treated with heparitinase (25 milliunits/mg PG) in 0.5 ml of 50 mM Tris-HCl, 1 mM calcium acetate, pH 7.0, containing 0.1 mM PMSF, 0.1 mM TLCK, 0.25 mM TPCK, and 0.1 mM NEM at 60 °C for 3 h (59). The digest was chromatographed on a Sepharose CL-6B column (1 × 49 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, containing 4 mM GdnHCl.

**Release of the GAG Chains from the Purified CSPGs**—The PGs were treated with 0.1 M NaOH, 1 mM NaBH₄ at 45 °C for 18 h under nitrogen (60). The solution was cooled in an ice bath, neutralized with 1 M HAc, and dried in a rotary evaporator. Boric acid was removed by repeated evaporation with 0.1% acetic acid in methanol. The residue was dried and chromatographed on a Sepharose CL-6B column (1 × 49 cm) in 0.2 M NaCl. Fractions were collected and aliquots assayed for uronic acid content (56). Positive fractions were combined, dialyzed (molecular weight cut-off 3,500) against distilled water, and lyophilized.

**Isolation and Analysis of the Core Proteins**—The PGs (5 mg) were treated with protease- and BSA-free chondroitinase ABC (1 unit, protease inhibitors added as a precaution) in 0.5 ml of 100 mM Tris-HCl, 50 mM NaOAc, pH 8.0, at 37°C for 6 h (61). The solution was adjusted to 4 mM GdnHCl, reduced with 100 mM dithiothreitol at 42 °C under nitrogen for 2 h, and alkylated with 150 mM iodoacetamide for 3 h at room temperature in the dark. The solution was chromatographed on a Sepharose CL-6B column (1 × 49 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, containing 4 mM GdnHCl, and the elution was monitored by measuring the absorption at 280 nm. The protein-containing fractions were pooled and dialyzed against 100 mM NH₄HCO₃. The amino acid analyses were carried out in a Beckman 7300 analyzer using an ion exchange column with post-column ninhydrin detection by the Biotechnology Resource Laboratory (Yale University, New Haven, CT).

**Enzymatic Digestions of the PGs and GAG Chains**—The purified PGs or GAGs were digested with 3.4 M GdnHCl. Fractions were collected and monitored for proteins and the supernatant was dialyzed against 100 mM NH₄HCO₃. The amino acid contents were determined using standard sugar solutions.

**Carbohydrate Composition Analysis**—For hexosamine analysis, the PGs or GAG chains were hydrolyzed with 3.4 M HCl at 100 °C for 6 h. For neutral sugar analysis, PGs or core proteins were hydrolyzed with 2.5 M trifluoroacetic acid at 100 °C for 5 h. The hydrolysates were dried in a Speed-Vac and analyzed on a CarboPac PA1 high pH anion-exchange column (4 × 250 mm) using a Dionex BioLC HPLC system coupled to a pulsed amperometric detector (64). The elution was performed with 20 mM sodium hydroxide, and the response factors for the monosaccharides were determined using standard sugar solutions.

**Determination of the Sulfate Contents of PGs**—Determination of the sulfate contents of PGs or GAGs was determined according to the procedure of Farnedale et al. (65). Briefly, the PG or GAG (5 μg) in 100 μl of water was mixed with 2.5 ml of 1.9-dimethylmethylene blue (16 μg/ml) in 40 mM glycine-HCl, 42 mM NaCl, pH 3.0, in polystyrene tubes. Immediately after mixing at room temperature, the color was measured at 525 nm. A fully 4-sulfated CS (0.5 to 10 μg) from sturgeon notochord was used as the standard.

**Other Analytical Procedures**—The uronic acid contents were determined using standard sugar solutions. **Distribution of the Sulfate Contents of PGs**—The sulfate contents of PGs or GAGs were determined according to the procedure of Farnedale et al. (65).
RESULTS

Isolation of Proteoglycans from Human Placenta—The placenta was extracted with a large volume of an isotonic buffer by gentle scraping to solubilize uronic acid-containing macromolecules. After centrifugation, the combined pellet and tissue were extracted with a detergent-containing buffer to obtain the cell-associated PGs. Finally, the fibrous tissue was extracted with a detergent-containing buffer to obtain the PGs of the tissue matrix. These procedures resulted in the complete extraction of the uronic acid-containing macromolecules in the placenta. The GAGs/PGs in each of these extracts were isolated by adsorption onto DEAE-Sephacel and elution with a linear NaCl gradient. After centrifugation, the combined pellet and tissue were extracted with a detergent-containing buffer to obtain the cell-associated PGs. Finally, the fibrous tissue was extracted with a buffer containing detergent and 6 M urea to solubilize PGs of the tissue matrix. These procedures resulted in the complete extraction of the uronic acid-containing macromolecules in the placenta. The GAGs/PGs in each of these extracts were isolated by adsorption onto DEAE-Sephacel and elution with 1.2 M NaCl. The GAGs/PGs were then purified by chromatography on DEAE-Sepharose using a salt gradient, CsBr density gradient centrifugation, and size-exclusion chromatography on Sepharose CL-6B and Sepharose CL-4B. Scheme 1

FIG. 1. Purification of PGs of human placenta by DEAE-Sepha-
cel chromatography. The GAGs/PGs, obtained by the initial DEAE-
Sephacel chromatography of various extracts of the placenta, were rechromatographed on DEAE-Sephacel columns (2.5 ¥ 17 cm) equili-
brated with 50 mM sodium acetate, 100 mM NaCl, 4 M urea, pH 5.5. The columns were washed with the same buffer and eluted with a linear gradient of 0.1–0.9 M NaCl. Ten-ml fractions were collected, and ab-
sorption at 280 and 260 nm was measured. Aliquots (50–125 µl) of fractions were assayed for uronic acid by the carbazole method. A–C, PGs from buffer extract (PGs of the inter villous spaces), buffer/deter-
genent extract (cell-associated PGs), and buffer/detergent/6 M urea extract (PGs of the fibrous tissue), respectively. GAG/PG-containing fractions were pooled as indicated by horizontal bars. The NaCl gradient pattern in panels B and C is as shown in panel A.

FIG. 2. Purification of PGs of human placenta by CsBr density gradient centrifugation. The placental PGs isolated by DEAE-
Sephacel chromatography (Fig. 1) were fractionated by CsBr density gradient centrifugation in a Beckman 50 Ti rotor as described under “Experimental Procedures.” Gradients were aspirated from the bottom of the tubes using a peristaltic pump by carefully inserting glass cap-
illaries (1 mm outer diameter); 0.67-ml fractions were collected, screened for protein (280 nm), and aliquots assayed for uronic acid (530 nm). The density (g/ml) of fractions was determined by weighing. A–C, BCSPG-2 (PGs of the inter villous spaces that eluted at 0.35 M NaCl in Fig. 1A), cell-associated PGs eluted at 0.5 M NaCl (Fig. 1B), and PGs of the fibrous tissue eluted at 0.7 M NaCl (Fig. 1C), respectively. The PG-containing fractions were pooled as indicated. CsBr densities of fractions in B and C were similar to that shown in A. The BCSPG-1/HA mixture (Fig. 1A) was similarly purified; its density gradient centrifugation fractionation pattern was very similar to that shown in panel A for BCSPG-2.

TABLE I
Yield and composition of GAGs/PGs of human placenta during different stages of isolation and purification

| Extraction type (location of GAGs/PGs in the placenta) | Yielda | Uronic acid contentb | Protein contentc |
|--------------------------------------------------------|--------|---------------------|-----------------|
| GAGs/PGs                                               | A      | B       | C       | A      | B       | C       | A      | B       | C       |
|--------------------------------------------------------|--------|-------------------|-----------------|
| PBS, pH 7.2 (intervillous spaces)                       |        |        |        |        |        |        |        |        |        |
| BCSPG-1/HA                                             | 12.0   | 2.8    | 1.15   | 0.8    | 0.6    | 0.30   | 6.8    | 0.9    | 0.43   |
| BCSPG-2                                               | 140.5  | 40.1   | 14.0   | 5.8    | 4.7    | 4.2    | 105.5  | 14.2   | 4.4    |
| Buffer/detergent (cell-associated)                     |        |        |        |        |        |        |        |        |        |
| DCSPGs and DHSPGs                                      | 74.8   | 48.3   | 3.4d   | 4.5f   | 1.9    | 0.9    | 35.2   | 19.4   | 1.2    |
| Buffer/detergent/urea (fibrous tissue)                 | 134.6  | 102.7  | 47.1f  | 10.7e  | 9.2e   | 7.1e   | 74.4   | 37.6   | 17.1   |

a Average yield based on five different preparations from 5 placentas.

b By the carbazole method.

c By the BCA method.

d The combined yield of DCSPG-1, DCSPG-2 and DHSPGs.

The high value was because of strong nonspecific color development due to contaminants.

The combined yield of DS/CSPGs.

The values were significantly low because of lower extinction coefficient of IdoUA (29%) compared with GlcA (100%). For corrected uronic acid contents of the purified PG fractions, see Table II.
summarizes the protocols used for the isolation and purification of PGs, and Table I shows the yield and composition of PGs at various stages of purification.

**Purification and Characterization of Proteoglycans from Intervillous Spaces of Human Placenta—** Chromatography on DEAE-Sephacel using a NaCl gradient resolved the GAGs/PGs of the intervillous spaces into three distinct peaks (Fig. 1A). The minor and major peaks that eluted at 0.25 and 0.35 M NaCl were designated as BCSPG-1/HA and BCSPG-2, respectively. Compositional analysis revealed that the minor peak eluting at 0.5 M NaCl corresponds to the PGs extracted with the detergent-containing buffer (see below); this material was combined with the respective extract (see Fig. 1B). Both BCSPG-1/HA and BCSPG-2 contained high levels of proteins and low levels of nucleic acids (Table I). CsBr density gradient centrifugation separated the major portions of proteins and some nucleic acids (fractions 11–15 and fraction 1 in Fig. 2A) from BCSPG-1/HA and BCSPG-2 (fractions 2–10 in Fig. 2A); the PGs remained in the moderate buoyant density (average p = 1.430 g/ml) regions. BCSPG-2 and BCSPG-1/HA were purified further by chromatography on Sepharose CL-6B (Fig. 3A and data not shown); both CSPGs eluted as single unsymmetrical peaks separated from the low molecular weight contaminants (fractions 48–59). BCSPG-1/HA and BCSPG-2 each appear to contain two distinct CSPG populations; a high molecular weight peak (Kav = 0.05, Mr = 1 × 10⁶) that is partially resolved from a relatively low molecular weight PG (Kav = 0.15, Mr = 570,000). BCSPG-1/HA contains a higher proportion of the high molecular weight PG (data not shown) compared with BCSPG-2, also evident from the elution patterns of BCSPG-1 and BCSPG-2 on Sepharose CL-4B (data not shown).

Hexosamine compositional analysis revealed that the purified BCSPG-1/HA contains 84% GalN and 16% GlcN (Table II). This fraction was treated with *S. hyalurolyticus* hyaluronidase, an enzyme that specifically degrades HA, and chromatographed on Sepharose CL-6B (data not shown). Approximately 15% of the GAG chains of the BCSPG-1/HA fraction were degraded by the enzyme to low molecular weight materials that eluted at the totally included volume, and the remainder of the GAG/PG fraction eluted at the same elution volume as that of the untreated fraction (data not shown). Bio-Gel P-6 chromatography of the enzyme-degraded, uronic acid-containing material revealed that it was a mixture of tetra- and hexasaccharides formed by the enzymatic degradation of HA (data not shown). Consistent with this conclusion, hexosamine compositional analysis showed the exclusive presence of GlcN in these oligosaccharides, and chondroitinase ABC degraded them into disaccharides (data not shown). The portion of BCSPG-1/HA that was resistant to *S. hyalurolyticus* hyaluronidase contained 96% GalN and 4% GlcN (Table II), and it was degraded quantitatively by chondroitinase ABC or chondroitinase AC II into disaccharides (see Table III) but completely resistant to heparitinase (data not shown). These results and the presence of a significant amount of protein indicated that BCSPG-1/HA was a mixture of a low sulfated CSPG (85–90%) and HA (10–15%). The PG obtained after the removal of HA from BCSPG-1/HA fraction was designated as BCSPG-1.

Hexosamine analysis revealed that BCSPG-2 contains 97% GalN and 3% GlcN (Table II). The GAG chains of BCSPG-2 were resistant to *S. hyalurolyticus* hyaluronidase, or heparitinase, but they were quantitatively converted to disaccharides by chondroitinase ABC or chondroitinase AC II. These results demonstrated that the BCSPG-2 fraction does not contain HA or HS.

BCSPG-1 and BCSPG-2 were purified further by gel filtration on Sepharose CL-4B, which completely separated the minor high molecular weight protein contaminants from the uronic acid-containing materials (data not shown). The above results demonstrate that the placental intervillous spaces contains a relatively high level of unusually low sulfated, extracellular CSPGs and a very low level of HA.

**Purification and Characterization of Cell-associated Proteoglycans of the Placenta—** The DEAE-Sephacel chromatography using NaCl gradient fractionated the cell-associated PGs obtained from the detergent-buffer extract into four distinct peaks: three minor peaks eluting at 0.25, 0.35, and 0.7 M NaCl, and a major peak at 0.5 M NaCl (Fig. 1B). Hexosamine analysis revealed that the 0.25 M NaCl-eluted fraction contained ~75% GalN and ~25% GlcN. Treatment with *P. falciparum* hyaluronidase converted about ~20% of the GlcN-containing material into a mixture of hexa- and tetrascarachides; the remainder of GlcN appears to represent glycoprotein oligosaccharides. These results suggested that the GAGs/PGs fraction that eluted at 0.25 M NaCl was, as in the case of BCSPG-1/HA, a mixture of ~20% HA and ~80% low sulfated CSPG (see above).

Hexosamine composition and analysis of the disaccharides released by the chondroitinase ABC or chondroitinase AC II digestion revealed that the material eluted at 0.35 M NaCl from the DEAE-Sephacel column was similar to BCSPG-2 (see Fig. 1A). Thus, the fractions eluted at 0.25 and 0.35 M NaCl from the DEAE-Sephacel column represent the residual BCSPG-1/HA and BCSPG-2 that were not solubilized under gentle extraction procedure using buffer alone. These fractions were combined with BCSPG-1/HA and BCSPG-2, respectively. The composition of the minor fraction that eluted at 0.7 M NaCl from the DEAE-Sephacel column was similar to that of the PG extracted with urea-containing buffer, and it was pooled with the respective material (see Fig. 1C).

The cell-associated PG fraction that eluted at 0.5 M NaCl contained high levels of nucleic acid contaminants. Upon CsBr density gradient centrifugation both PGs and nucleic acids...
ent disaccharides have similar molar extinction coefficients. "Experimental Procedures." ABC, and the digests were analyzed by HPLC as described under placental PGs or intact purified PGs were digested with chondroitinase settled to high buoyant density regions (Fig. 2B). The uronic acid-containing material (fractions I–9 in Fig. 2B) was recovered, treated again with nucleases, and rechromatographed on Sepharose CL-6B; the PGs were eluted as two partially resolved peaks, fraction I and II (Fig. 3B). Fractions I and II contained ~65% GlcN and ~35% GaIN, and ~60% of the GAGs in these fractions were degraded by heparitinase, suggesting that each PG fraction was a mixture of CSPG and HSPG. Therefore, fractions I and II (Fig. 3B) were combined together, treated with heparitinase, and chromatographed again on Sepharose CL-6B (Fig. 4). The heparitinase-digested, uronic acid-containing material was eluted in the totally included volume (fractions 48–64), whereas the enzyme-resistant PG fractions eluted as two partially separated peaks (Fig. 4). These were separately pooled and analyzed for hexosamine, uronic acid, and protein content (Table II). The material eluted in fractions 20–25 contained predominantly protein with little or no uronic acid. Fractions 26–34 (designated as DCSPG-1) and fractions 35–46 (DCSPG-2) had similar hexosamine compositions, ~90% GaIN and ~10% GlcN (Table II). DCSPG-1 and DCSPG-2 have significantly lower molecular mass, ~250 kDa ($K_v \approx 0.30$) and ~55 kDa ($K_v \approx 0.58$), respectively, compared with BCSPG-1 or BCSPG-2 (compare Fig. 4 with Fig. 3A). The GAG chains of these PGs were quantitatively digested to disaccharides by either chondroitinase ABC or chondroitinase AC II, indicating that these are CSPGs.

**Purification and Characterization of Proteoglycans of the Fibrous Tissue of the Placenta—**On DEAE-Sepharacel chromatography using NaCl gradient, the crude PGs isolated from the urea-containing buffer extract, eluted as two peaks, a minor at 0.5 M NaCl, and a major at 0.7 M NaCl (Fig. 1C). The hexosamine compositional analysis and digestion with heparitinase and chondroitinase ABC revealed that the 0.5 M NaCl-eluted peak was a mixture of residual cell-associated HSPGs and CSPGs that were not extracted with detergent-containing buffer (see Fig. 1B). Therefore, this was combined with PGs extracted with detergent-containing buffer for further purification (see above). The PGs that eluted at 0.7 M NaCl had significant level of nucleic acid contamination. Upon CsBr density gradient centrifugation, both PGs and nucleic acids fractionated to the high density region (Fig. 2C). The PG-containing fractions (fractions 1–8) were combined and purified by gel filtration on Sepharose CL-6B in the presence of 4 M GdnHCl (Fig. 3C). This procedure separated the nucleic acid contaminants, and the PGs eluted as two unresolved peaks, fraction I (DS/CSPG-1, 20–25%) and fraction II (DS/CSPG-2, 75–80%), with $K_v$ of 0.09 ($M_r \approx 800,000$) and 0.38 ($M_r \approx 150,000$), respectively. The GAG chains of both DS/CSPG-1 and DS/CSPG-2 were completely susceptible to chondroitinase ABC, but only partially susceptible to chondroitinase AC II or chondroitinase B (see characterization of GAG chains below), indicating that the GAG chains are CS/DS polysaccharides.

**Characterization of GAG Chains of the Purified Placental PGs—**Aliquots of the PGs were subjected to β-elimination with

### Table II

**Yield and composition of the CSPGs purified from human placenta**

| PGs          | Yield (mg/placenta) | Protein | Uronic acid | HexN | Sulfate | Man-Gal | HexN composition (mol %) |
|--------------|---------------------|---------|-------------|------|---------|--------|-------------------------|
| BCSPG-1HA    | 1.1 (1.0)           | 37 (34) | 25 (29)     | 31 (34)| <0.5    | 6 (3)  | 84 (96) | 16 (4)  |
| BCSPG-2      | 14                  | 30      | 32          | 35   | ~1      | 2      | 97        | 3       |
| DCSPG-1      | 0.4                 | 27      | 28          | 34   | 6       | 5      | 92        | 8       |
| DCSPG-2      | 0.8                 | 31      | 26          | 33   | 5       | 5      | 90        | 10      |
| DS/CSPG-1    | 9                   | 36      | 24          | 27   | 11      | 2      | 91        | 9       |
| DS/CSPG-2    | 35                  | 35      | 25          | 27   | 11      | 2      | 94        | 6       |

*By the BCA method.

*By the carbazole method.

*Estimated according to the procedure of Farndale et al. (68).

*By the high pH anion-exchange HPLC of 2.5 mM trifluoroacetic acid hydrolysates of the purified PGs or their core proteins.

*The numbers in parentheses indicate the values for BCSPG-1 obtained after the digestion of HA with S. hyalurolyticus hyaluronidase.

The high protein content in this PG fraction is because of color interference by impurities.

### Table III

**Disaccharide compositions of GAGs of the purified placental CSPGs**

The GAGs released by the alkaline β-elimination of the purified placental PGs or intact purified PGs were digested with chondroitinase ABC, and the digests were analyzed by HPLC as described under "Experimental Procedures."

| PGs         | Disaccharides* |
|-------------|---------------|
|             | Δdi-0S | Δdi-4S | Δdi-6S |
| BCSPG-1     | 98     | 2      | 0      |
| BCSPG-2     | 92     | 8      | 0      |
| DCSPG-1     | 52     | 33     | 15     |
| DCSPG-2     | 60     | 27     | 13     |
| DS/CSPG-1   | 8      | 52     | 40     |
| DS/CSPG-2   | 2      | 78     | 20     |

* Calculated from the areas of the peaks by assuming that the different disaccharides have similar molar extinction coefficients.

Fig. 4. Purification of cell-associated CSPGs. DCSPGs/DHSPGs fraction (Fig. 3B, 13 mg from 4 placentas) was treated with heparitinase in the presence of protease inhibitors, and the solution was chromatographed on Sepharose CL-6B (1 × 49 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, containing 4 M GdnHCl. Fractions (0.67 ml) were collected, screened for protein (280 nm), and aliquots assayed for uronic acid (530 nm). CSPG-containing fractions were pooled as indicated. The elution positions of BD, BSA, and Glc are indicated.
Analysis of GAGs of the placental CSPGs. The GAGs (200–400 μg) released by NaOH/NaBH₄ β-elimination of the purified placental CSPGs were chromatographed on Sepharose CL-6B columns (1 × 49 cm) in 0.2 mM NaCl, 0.67 ml fractions were collected, and aliquots assayed for uronic acid (530 nm). A, CS chains from BCSPG-2; B, CS chains from DCSPG-1 (●) and DCSPG-2 (○); C, DS/CS chains from DS/CSPG-2. The GAG-containing fractions were pooled as indicated. The elution positions of BD, Glc, and GAG standards (SCS, shark cartilage CS, M₉ 60,000; FCS, fish (sturgeon notochord) CS, M₉ 37,000; BCS, bovine tracheal CS, M₉ 25,000) are indicated. The GAG chains of BCSPG-1, and DS/CSPG-1 were also analyzed and their elution patterns were similar to those of the GAGs of BCSPG-2 and DS/CSPG-2, respectively.

NaOH/NaBH₄, and the sizes of the released GAGs were assessed by gel filtration on a calibrated Sepharose CL-6B column calibrated with GAGs of known molecular weights (Fig. 5). The CS chains of BCSPG-2 and DCSPG-1 eluted as single broad peaks indistinguishable from one another with an estimated average M₉ 60,000 (Fig. 5A, and data not shown). HPLC analysis of the disaccharides formed by the chondroitinase ABC digestion revealed that the GAG chains of BCSPG-1 and BCSPG-2 contained 98% and 92% nonsulfated and 2% and 8% of 4-sulfated disaccharides, respectively (Table III), indicating that the CS chains of BCSPG-1 and BCSPG-2 are unusually low sulfated. These results agree with the low charge densities of these CSPG fractions (see Fig. 1A).

Upon gel filtration on a calibrated Sepharose CL-6B column, the GAGs of DCSPG-1 eluted as a major broad peak (average M₉ 46,000) and a small proportion of lower molecular weight GAGs not resolved from the major peak (closed circles in Fig. 5B). The GAG chains of DCSPG-2 eluted as two partially separated peaks, a minor peak with the same elution volume as that of the major GAGs of DCSPG-1, and a major heterogeneous peak of low molecular weight GAGs (M₉ 20,000, Fig. 5B). The GAG chains of both DCSPG-1 and DCSPG-2 were completely digested into disaccharides by chondroitinase ABC or chondroitinase AC II; 52–60% 4-sulfated, 27–33% 4-sulfated, and 13–15% 6-sulfated disaccharides (Table III). These results agree with the moderate charge densities of DCSPG-1 and DCSPG-2 (see Fig. 1B).

When chromatographed on a calibrated Sepharose CL-6B column, the GAGs of both DS/CSPG-1 and DS/CSPG-2 eluted as a single symmetrical peak corresponding to an average M₉ 55,000 (Fig. 5C). HPLC analysis of the disaccharides formed by the digestion of GAG chains with chondroitinase ABC showed 52% and 78% 4-sulfated, 40% and 20% 6-sulfated, and 8% and 2% nonsulfated disaccharides for DS/CSPG-1 and DS/CSPG-2, respectively (Table III). Chondroitinase AC II converted 40% of the GAG chains of DS/CSPG-2 to disaccharides and 60% of the GAGs to higher molecular weight fragments (data not shown). With chondroitinase B, ~65% of GAG chains were digested to di- and tetrasaccharides and the remainder was present as higher molecular weight fragments (data not shown). From these results, it was estimated that DS/CSPG-2 contains 40% CS and 60% DS structural features. Similarly, chondroitinase AC II or chondroitinase B digestion of the GAG chains of DS/CSPG-1 showed 60–70% CS and 30–40% DS content (data not shown).

Analysis of Core Proteins of the Purified Placental PGs—The core proteins of BCSPG-1 and BCSPG-2 were released by treatment with chondroitinase ABC in the presence of protease inhibitors, disulfide bonds reduced, and S-alkylated, and then chromatographed on Sepharose CL-6B in the presence of 4 M GdnHCl (Fig. 6). Both PGs released a high and a low molecular mass core protein (fractions I and II, Kᵅ 0.12 and 0.57, respectively); each eluted as a single symmetrical peak corresponding to an average M₉ 33% (by data not shown). A major portion of the high molecular mass core proteins (fraction I in Fig. 6) of BCSPG-1 and BCSPG-2 barely entered the gel. However, the low molecular mass core proteins (fraction II in Fig. 6) electro-
phoresed as broad bands with mobility corresponding to 55 kDa (Fig. 7A, and data not shown). The core proteins of both PGs were stained with neither Coomassie Blue nor Alcian Blue but with ammoniacal silver. Both high and low molecular mass core proteins contained low levels (5%) of glycoprotein type carbohydrate, suggesting that the lack of staining with Coomassie Blue is not because of high carbohydrate content (Table II); instead, it appears to be due to their high acidic nature (see below).

Amino acid analysis indicated that the high molecular mass core proteins (fraction I in Fig. 6) of BCSPG-1 and BCSPG-2 were very similar, except for relatively higher proportions of Ser and Gly in the core protein of BCSPG-1 (Table IV). These core proteins contain high levels of acidic amino acids and low levels of basic amino acids; Glu, Ser, Gly, Thr, Ala, and Asp accounted for ~80% of the composition (Table IV). The low molecular mass core proteins of BCSPG-1 and BCSPG-2 (fraction II in Fig. 6) also showed similarity in their amino acid compositions, except for the presence of significantly higher proportions of Ser and Gly and lower proportions of Thr, Pro, and Arg in BCSPG-1. The amino acid compositions of the high and low molecular mass core proteins of both BCSPG-1 and BCSPG-2 show significant resemblance to the previously characterized rat and human cartilage aggrecans (see Table IV for comparison) (71, 72).

The core proteins of cell-associated CSPG fractions (DC-
SPG-1 and DCSPG-2) were released by treatment with chondroitinase ABC and analyzed by SDS-PAGE (Fig. 7B). DCSPG-1 showed clusters of 50-, 54-, 58-, and 62-kDa core proteins and two high molecular mass bands at 235 and 290 kDa; the latter may be due to contaminant proteins since they also present in untreated PG. DCSPG-2, consistent with the predicted low molecular weight by gel filtration on Sepharose CL-6B (see Fig. 4), showed a single 18-kDa band. It is known that human placental syncytiotrophoblasts contain a low level of the CSPG form of thrombomodulin (73). It is widely believed that this PG mediates the placental IRBC adherence (25, 26, 41–43). Therefore, we analyzed the core proteins of cell-associated CSPGs of the placenta for thrombomodulin by Western blot using anti-thrombomodulin polyclonal antibody. A faint band observed upon SDS-PAGE (see Fig. 7B). Thus, it appears that the CSPG form of thrombomodulin is a minor component of the cell-associated PGs of human placenta.

SDS-PAGE analysis of core proteins released from the PGs of various tissues (Table V) (75–78). Therefore, we analyzed the core proteins of previously reported DSPGs (number of residues/1000 amino acids)

| Amino acid | DS/CSPG-2 | Human fetal membrane PG | Bone PG | Fibroblast PG |
|------------|-----------|-------------------------|---------|---------------|
| Asp        | 130       | 108                     | 137     | 136           |
| Thr        | 44        | 60                      | 34      | 48            |
| Ser        | 79        | 77                      | 67      | 81            |
| Glu        | 98        | 101                     | 79      | 84            |
| Pro        | 68        | 72                      | 85      | 68            |
| Gly        | 84        | 103                     | 69      | 70            |
| Ala        | 49        | 62                      | 37      | 44            |
| Val        | 64        | 66                      | 61      | 68            |
| Met        | 8         | 14                      | 14      | 9             |
| Ile        | 51        | 48                      | 54      | 59            |
| Leu        | 121       | 99                      | 131     | 122           |
| Tyr        | 26        | 17                      | 33      | 21            |
| Phe        | 34        | 34                      | 36      | 27            |
| His        | 33        | 34                      | 33      | 27            |
| Lys        | 75        | 57                      | 57      | 81            |
| Arg        | 33        | 34                      | 48      | 34            |
| Trp        | ND        | ND                      | 3       | 3             |
| Cys        | ND        | 11                      | 22      | 18            |

The composition obtained from the deduced amino acid sequence of the core protein of DSPG from the human fetal membrane (74).

The composition obtained from the deduced amino acid sequence of the human bone PG I core protein (75).

The composition obtained from the deduced amino acid sequence of the human fibroblast PG 40 core protein (77).

ND, not determined.

**TABLE V**

Amino acid composition of the core protein of human placental DS/CSPG-2 and core proteins of previously reported DSPGs (number of residues/1000 amino acids)

The core proteins were hydrolyzed under vacuum with 100 μl of 6 M HCl, 0.2% phenol for 16 h at 115 °C.

| Amino acid | DS/CSPG-2 | Human fetal membrane PG | Bone PG | Fibroblast PG |
|------------|-----------|-------------------------|---------|---------------|
| Asp        | 130       | 108                     | 137     | 136           |
| Thr        | 44        | 60                      | 34      | 48            |
| Ser        | 79        | 77                      | 67      | 81            |
| Glu        | 98        | 101                     | 79      | 84            |
| Pro        | 68        | 72                      | 85      | 68            |
| Gly        | 84        | 103                     | 69      | 70            |
| Ala        | 49        | 62                      | 37      | 44            |
| Val        | 64        | 66                      | 61      | 68            |
| Met        | 8         | 14                      | 14      | 9             |
| Ile        | 51        | 48                      | 54      | 59            |
| Leu        | 121       | 99                      | 131     | 122           |
| Tyr        | 26        | 17                      | 33      | 21            |
| Phe        | 34        | 34                      | 36      | 27            |
| His        | 33        | 34                      | 33      | 27            |
| Lys        | 75        | 57                      | 57      | 81            |
| Arg        | 33        | 34                      | 48      | 34            |
| Trp        | ND        | ND                      | 3       | 3             |
| Cys        | ND        | 11                      | 22      | 18            |

Adherence of *P. falciparum* IRBCs to Purified PGs of Human Placenta—The purified placental CSPGs were assessed for their ability to adhere IRBCs in a cytoadherence assay. The various CSPGs of human placenta significantly differ in their IRBC binding capacity, which is related to the differences in the structure of their GAG chains. Both BCSPG-1 and BCSPG-2 very efficiently bound IRBCs in a concentration-dependent manner and exhibited similar binding capacities (Fig. 8 and Table VI). These CSPGs supported significant levels of IRBC adherence at coating concentrations as low as 12 ng/ml, and at 50 ng/ml both PGs bound IRBCs at high densities. At coating concentrations of 100–200 ng/ml, both BCSPG-1 and BCSPG-2 exhibited saturating levels of IRBC adhesion (Table VI). The IRBC adhesion was completely abolished upon incubation of the CSPG-coated plates with chondroitinase ABC or heparanidase, whereas treatments with *S. hyalurolyticus* hyaluronidase or heparitinase had no effect. The adhesion of IRBC was inhibited by C4S but not by C6S, HA, HS, chondroitin, heparin, dextran sulfate, or pentosan polysulfate (see accompanying paper (Ref. 70)), indicating that the adhesion is C4S-specific.

The IRBCs adhered to purified cell-associated DCSPG-1 in a concentration-dependent manner, but the binding efficiency of DCSPG-2 was significantly low (Table VI). In the case of DCSPG-1, the coating concentration required for levels of IRBC
adhesion comparable to those of BCSPG-1 or BCSPG-2 was severalfold higher, and saturating levels of adhesion was observed at >0.8 μg/ml concentration. A lower binding capacity of DCSPG-2 compared with DCSPG-1 may occur at least in part because of the presence of a significant amount of protein contamination (Fig. 4), which could not be eliminated during various purification steps employed. Furthermore, the adherence capacities of IRBCs to the cell-associated CSPGs were significantly weaker compared with those of BCSPG-1 or BCSPG-2, since, in the case of former, IRBCs bound weakly and they tend to detach under washing conditions used for the latter.

Both DS/CSPG-1 and DS/CSPG-2 adhered IRBCs very poorly; high coating concentrations (>0.8 μg/ml) were required to obtain appreciable level of IRBC binding (Table VI). The binding of IRBCs by DS/CSPG-1 and DS/CSPG-2 was significantly weaker compared with BCSPG-1 or BCSPG-2; when the adhesion assay was performed in parallel, most IRBCs initially bound to DS/CSPG-1 and DS/CSPG-2 were detached under washing conditions normally employed for BCSPG-1 or BCSPG-2. When the IRBC adherence capacity of DS/CSPG-1 and DS/CSPG-2 were compared, the former shows relatively higher binding compared with the latter. This agrees with the higher level of CS structural features in DS/CSPG-1 compared with that of DS/CSPG-2.

DISCUSSION

The results presented in this paper provide a complete picture of the nature of PGs present in human placenta and their localization in the tissue. The data also indicate a detailed structural characterization of various CSPGs present in human placenta and the identification of CSPGs that mediate the adherence of P. falciparum IRBCs to the placenta. The major findings are as follows. 1) human placenta contains three distinct types of CSPGs: extracellular CSPGs localized in the intervillous spaces, cell-associated CSPGs, and DS/CSPGs of the tissue matrix. 2) The extracellular CSPGs of the intervillous spaces contain unusually low sulfated CS chains (only 2–8% of the disaccharide repeats are 4-sulfated), and these CSPGs efficiently bind IRBCs. 3) Based on the IRBC binding characteristics and tissue localization pattern of various PGs, BCSPG-1 and BCSPG-2 were identified as the receptors for the IRBC adherence in the placenta.

BCSPG-1 and BCSPG-2 together account for ~24% of the total PGs in the placenta. As indicated by the elution pattern on DEAE-Sephacel columns, both BCSPG-1 and BCSPG-2 have unusually low overall charge densities. The occurrence of such a low sulfated chondroitin chains is very unusual. In CSPGs of various cells and tissues so far studied, usually >70% of the disaccharide repeats of the GAG chains are sulfated. However, an earlier study reported the presence of very low sulfated (~2.1% by total weight) chondroitin chains in bovine cornea (79). The reported sulfate content corresponds to the presence of 11% sulfated and 89% non-sulfated disaccharide repeats in the chondroitin chains of cornea, and it is comparable to the sulfate content in the GAG chains of BCSPG-2, the major CSPG in the intervillous spaces of human placenta. It is likely that the unusually low sulfated chondroitin chains of human placental CSPGs and those of bovine cornea (presumably present in the form of CSPG) have specific biological functions.

BCSPG-1 and BCSPG-2 resemble one another closely with respect to their core proteins, GAG chains, overall hydrodynamic sizes, and buoyant densities but they differ significantly in overall charge densities. Both CSPGs contain a high and a low molecular mass core proteins; although the proportions of these core proteins are different in these CSPGs, they resemble one another in their molecular sizes and amino acid compositions. The GAG chains of both BCSPG-1 and BCSPG-2 are also very similar in their molecular size (~60,000) but they differ in sulfate content (~2% and ~8%, respectively). Based on the types of core proteins present in BCSPG-1 and BCSPG-2, each must be a mixture of two distinct PGs, a high molecular mass and a low molecular mass CSPG. Since the intact high and low molecular weight populations of BCSPG-1 and BCSPG-2 in each case corresponds to ~1 × 10^6 and ~570,000, and the sizes of their core proteins are ~670,000 and ~56,000, respectively, these PGs should contain about 6–8 GAG chains. Thus, the CSPGs of the placental intervillous spaces contain fewer CS chains compared with high molecular mass cartilage proteoglycans (80). The moderate buoyant densities of BCSPG-1 and BCSPG-2 also agree with the presence of fewer GAG chains and low sulfate contents in these PGs.

The low sulfated GAG chains of the CSPGs of the human placental intervillous spaces present intriguing possibilities regarding their occurrence. 1) The GAG chains are the products of specific cell types in which the levels of the Golgi sulfotransferase activity may be down-regulated, or the core proteins may carry specific information for low levels of sulfation of GAG chains. 2) The GAGs might have been produced by the removal of sulfate groups from normally sulfated CS4 chains by sulfatases in the intervillous spaces of the placenta. However, the latter is unlikely because the existence of such an extracellular sulfatase has not been demonstrated so far in any normal eukaryotic cell or tissue. Therefore, it appears that the former possibility is most likely.

The high molecular mass core proteins of BCSPG-1 and BCSPG-2 contain high proportions of Ser and Gly, suggesting the possibility of Ser/Gly repeats and thus potential for the attachment of multiple CS chains. However, our data indicate the presence of only 6–8 CS chains in the CSPGs, suggesting the possibility that all potential sites are not utilized for the attachment of GAG chains. The amino acid composition of the low molecular mass core proteins of BCSPG-1 and BCSPG-2 shows significant similarity to the high molecular mass core protein (Table IV), although there are some characteristic differences, particularly, in the proportions of Thr, Ser, Pro, Arg, and Gly. Considering overall similarities, it appears that both high and low molecular mass core proteins of BCSPG-1 and BCSPG-2 are related to the core protein of cartilage aggregicans (71, 72). The data also suggest the possibility for the existence of a family of genes related to these core proteins in the placenta. Although the amino acid composition of the core proteins of BCSPG-1 and BCSPG-2 closely resembles that of the cartilage aggregicans, they differ significantly from the latter with respect to core protein and GAG chain sizes. Whereas the cartilage aggregicans contain ~200-kDa core protein and ~20-kDa CS chains, the CSPGs of the placental intervillous spaces have >600-kDa core protein and ~60-kDa CS chains. In this respect, the larger placental CSPGs are similar to the large aggregating CSPGs of chick embryonic limb bud cartilage mesenchyme, which have 500–550-kDa core proteins and ~60-kDa GAG chains (81). The sizes of the GAG chains of placental CSPGs are comparable to 70-kDa CS chains of embryonic skeletal muscle mesenchyme large nonaggregating CSPGs (82). Based on these similarities, it appears likely that the high molecular weight BCSPG-1 and BCSPG-2 population represents the embryonic status of the fetus, and the PGs are likely to be produced by syncytiotrophoblasts, the cells of fetal origin.

The CSPGs of placental intervillous spaces differ from the previously described cartilage aggregating CSPGs in having negligible amounts of glycoprotein type carbohydrate (80, 83). High molecular weight cartilage aggregating CSPGs contain as
many as 100 CS chains and a number of keratan sulfate and O-linked oligosaccharide chains (80). In this respect, the placental CSPGs appear to resemble small nonaggregating cartilage CSPGs, which contain 2 or 3 CS chains without appreciable amount of other carbohydrates (84).

The intervillous spaces of human placenta contain, besides BCSPG-1 and BCSPG-2, a small amount of HA, which accounts for 1–2% of the total GAGs in this location. This finding agrees with the results of a previous study that reported the presence of a small proportion of HA in human placenta compared with CS and DS (52). However, in contrast to these results, several other studies have reported the presence of high levels of HA on the syncytiotrophoblast cell lining of the placental villi (48–55). The previously reported high content of HA was based on experimental approaches that did not discriminate HA from other PGs in placenta. Size exclusion chromatography on Sepharose 40354 indicated that this PG fraction is a mixture of 20–25% DS/CSPG-2 and CCl-6B indicated that this PG fraction is a mixture of 20–25% DS/CSPG-2 and 55,000) and overall sulfate content of the PGs. The average values of at least three independent assays (each in duplicate); standard deviations were generally within ±5%.

### Table VI

| Coating concentration (ng/ml) | BCSPG-1 | BCSPG-2 | DCSPG-1 | DCSPG-2 | DS/CSPG-1 | DS/CSPG-2 |
|-----------------------------|--------|--------|---------|---------|-----------|-----------|
| 3200                        | 5125   | 5114   | 5113    | 2545    | 1815      | 993       |
| 1600                        | 5110   | 5145   | 5086    | 1522    | 1316      | 781       |
| 800                         | 5170   | 5096   | 4786    | 1210    | 1154      | 613       |
| 400                         | 5100   | 5175   | 4358    | 523     | 811       | 325       |
| 200                         | 5055   | 5123   | 2947    | 116     | 404       | 202       |
| 100                         | 4112   | 4523   | 1645    | 105     | 219       | 106       |
| 50                          | 3225   | 3348   | 1045    | 73      | 144       | 66        |
| 25                          | 1445   | 1874   | 423     | 56      | 73        | 48        |
| 12                          | 965    | 924    | 105     | 42      | 37        | 34        |
| 6                           | 253    | 379    | 58      | 28      | 19        | 17        |
| 3                           | 174    | 215    | 62      | 16      | 14        | 13        |
| 2                           | 152    | 179    | 51      | 11      | 12        | 10        |

*The average values of at least three independent assays (each in duplicate); standard deviations were generally within ±5%.

The adherence of IRBCs to BCSPG-1 and BCSPG-2 is mediated by their GAG chains because the binding was completely abolished upon prior treatment of the CSPG-coated plates with chondroitinase ABC or testicular hyaluronidase but not with enzymes that specifically degrade HA or HS (70). These data, when considered with the location of BCSPG-1 and BCSPG-2 in the intervillous spaces, where IRBCs accumulate in high density in the P. falciparum-infected pregnant women (40, 44, 45),
establish that these CSPGs are the natural receptors for adhesion of IRBCs to the placenta.

Although the cell-associated CSPGs, DCSPG-1 and DCSPG-2, of the placenta can also support the IRBC adherence, the binding by these CSPGs was significantly lower when compared with binding by BCSPG-1 or BCSPG-2 (see Table VI). The overall proportion of cell-associated CSPGs is significantly lower compared with the levels of CSPGs of the intervillous spaces: 2% and 24%, respectively. Moreover, in *P. falciparum*-infected placenta, IRBCs rarely adhere directly to the surfaces of syncytiotrophoblasts, the cell type that is in direct contact with maternal blood in the placenta (40, 45). Thus, the cell-associated CSPGs cannot be the major receptors for placental IRBC adherence.

Although the DS/CSPGs of placenta, particularly DS/CSPG-1, bind IRBCs, the adherence is significantly weak, and high density binding is apparent only under mild washing conditions. Moreover, DS/CSPG-1 and DS/CSPG-2 are most likely present in the collagen network of the fibrous tissue; therefore, they are not accessible for IRBC adherence in the placenta.

It is widely believed that thrombomodulin, a 105–120-kDa transmembrane thrombin-binding protein modified with one C4S chain on the syncytiotrophoblast surfaces, is the receptor for the *in vivo* IRBC adherence (25, 26, 41–43). However, this does not agree with our data, or with those previously published (40, 45). First, thrombomodulin is expressed throughout the vascular endothelium (arteries, veins, capillaries, and lymphatics) of almost all organs (~100,000 molecules/cell) except in the brain and syncytiotrophoblasts of human placenta, where it functions as a cell surface anticoagulant (85). Second, the *in vivo* IRBC adherence pattern (40, 45) differs from the expression of thrombomodulin on the surfaces of syncytiotrophoblasts (85). Third, only 10–20% of the total thrombomodulin occurs in the form of CSPG, and the remainder lacks C4S chains (86); thus, the amount of CSPG form of thrombomodulin available is very low. The reported yield of thrombomodulin in a previous study was 0.88 mg of protein from 5 kg of placenta (73); this corresponds to ~14 μg of CSPG form/placenta (~400 g). Consistent with these data, thrombomodulin was barely detectable by Western blotting of the cell-associated CSPG pools. Together, these observations strongly suggest that, although the PG form of thrombomodulin or another CSPG on trophoblastic cell surfaces can support the adherence of IRBC in adhesion assays, they are not the major receptors for IRBCs.

Very recently, HA has been reported as an additional receptor for placental IRBC adherence (87). However, our study indicates that HA may not be a receptor for IRBC adherence since the level of HA in the intervillous spaces of placenta, where IRBCs accumulate in pregnant women, is very low.2 Besides, the adherence of IRBCs to human umbilical cord HA could not be abolished by treatment of the coated plates with *S. hyalurolyticus* hyaluronidase, and highly purified HA neither supports IRBC adherence nor was able to inhibit IRBC adherence to umbilical cord HA even at 200 μg/ml concentration. Moreover, treatment of human umbilical cord HA with *S. hyalurolyticus* hyaluronidase yielded a CSPG, which supports IRBC adhesion.2 Thus, it appears that the reported binding of IRBC to umbilical cord HA was due to the presence of CSPG contaminants in the HA preparations used (87).

BCSPG-1 and BCSPG-2 of the placenta must be associated with a loose network of matrix in the intervillous spaces. The effective extraction of the CSPGs by gentle washing of the placental intervillous spaces with an isotonic buffer is consistent with this conclusion. It is possible that these CSPGs may correspond to or may be the components of the previously described, amorphous, electron-dense material present in the fetal-maternal interface (47), and they may function as chemical barriers in masking fetal antigens from the maternal immune system. Additionally, as in cartilage and aorta, aggregates of CSPGs may play a key role in maintaining shape of the intervillous spaces to facilitate maternal blood flow, and in sustaining and dissipating the pressure load developed by continual filling of intervillous spaces with blood. Furthermore, in other PGs, the GAG chains of the CSPGs may be involved in adsorbing essential components including metal ions, growth factors, and nutrients from maternal blood and mobilizing them in the intervillous spaces for effective uptake by fetal villi. The low sulfation of the GAG chains may be better suited for this function since the low charge density of GAG chains may facilitate the effective transfer of the adsorbed materials by a relatively weak interaction compared with the expected stronger interactions if the GAGs were to be highly sulfated. In any event, the accumulation of CSPGs with low sulfated GAG chains in the intervillous spaces suggest that these molecules play an important role in the function of the placenta, and *P. falciparum* exploits this opportunity for better colonization and thus its survival.

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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

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