Proteoglycans Predominantly Synthesized by Human Brain Microvascular Endothelial Cells in Culture are Perlecan and Biglycan

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It has been suggested that the properties of vascular endothelial cells differ depending on the type of blood vessel. Proteoglycans are macromolecules that contribute to vascular properties through regulation of endothelial cell functions. In this study, we characterized proteoglycans synthesized by cultured human brain microvascular endothelial cells by biochemical techniques. The experiments indicated that the cells synthesize and secrete two types of proteoglycans: large heparan sulfate proteoglycans bearing heparan sulfate chains of approximately M_r~68000 and chondroitin/dermatan sulfate proteoglycans bearing chondroitin/dermatan sulfate chains of approximately M_r~48000. The heparan and chondroitin/dermatan sulfate proteoglycans were identified as perlecan and biglycan, respectively, by Western blot analysis. A part of perlecan was associated with the cell layer, while most of biglycan was secreted into the medium. These results suggest that the characteristics of proteoglycans synthesized by brain microvascular endothelial cells are similar to the characteristics of those synthesized by previously reported arterial endothelial cells. Regulation of endothelial cell functions by proteoglycans may be independent of blood vessel types.

Key words ——— endothelial cell, proteoglycan, vascular, perlecan, biglycan

INTRODUCTION

Regulation of vascular endothelial cell functions often differs depending on the type of blood vessel; for example, vascular endothelial growth factor is highly expressed in the cells from microvascular tissue,[1)] but not in those from arterial tissue.[2)] In addition, the secretion of tissue plasminogen activator, one of the fibrinolytic proteins expressed in vascular endothelial cells,[3,4)] is suppressed by the basic fibroblast growth factor (FGF-2) in human umbilical vein endothelial cells but enhanced in human aortic endothelial cells *in vitro*.[5)]

Arterial endothelial cells synthesize proteoglycans, including a large heparan sulfate proteoglycan perlecan,[6)] members of the syndecan family of transmembrane proteoglycans,[7,8)] the cell surface-associated proteoglycan glypican-1,[7)] and a small leucine-rich dermatan sulfate proteoglycan biglycan,[9,10)] as an extracellular matrix component. However, the predominant proteoglycans synthesized by arterial endothelial cells are perlecan and biglycan.[11)]

Proteoglycans are involved in the regulation of vascular endothelial cell functions that are mediated by growth factors and cytokines, such as transforming growth factor-β and FGF-2, to which some proteoglycans bind. On the other hand, the types of proteoglycans that are synthesized by brain microvascular endothelial cells have not been completely determined. It is possible that the characteristics of endothelial cell proteoglycans are different in the brain and artery; this difference may influence the regulation of endothelial cell functions in different manners in these two tissues.

Since vascular endothelial cells cover the luminal surface of blood vessels in a monolayer, the endothelial cell layer can be the first target of toxic substances. Although proteoglycans are one of the important components of the extracellular matrix, the proteoglycans synthesized by human brain microvascular endothelial cells have not yet been clari-

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fied. In this study, we characterized proteoglycans synthesized by human brain microvascular endothelial cells *in vitro*.

MATERIALS AND METHODS

Materials —— Human brain microvascular endothelial cells and Western blotting blocking reagent were purchased from Dainihon Pharmaceutical (Osaka, Japan). HuMedia EG-2, which is a growth medium for human vascular endothelial cells, was purchased from Kurabo (Osaka, Japan). Dulbecco's modified Eagle's medium and collagen-coated tissue culture dishes and plates were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Iwaki (Chiba, Japan), respectively. [^{35}S]Na_2SO_4 (carrier-free) was purchased from MP Biomedicals (Irvine, CA, U.S.A.). DEAE-Sephacel, benzamidine, Tris base, dextran blue, phenylmethanesulfonyl fluoride, and papain (1.7 units/mg solid) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Chondroitinase ABC (EC 4.2.2.4 derived from *Proteus vulgaris*), heparinase II [derived from *Flavobacterium heparinum* (*F. heparinum*)], and heparinase III (EC 4.2.2.8 derived from *F. heparinum*) were purchased from Seikagaku (Tokyo, Japan). ECL Western blotting detection reagents, horseradish peroxidase-linked protein A, nitrocellulose membranes (Hybond ECL), Hyperfilm ECL, Sepharose CL-2B, Sepharose CL-6B, and PD-10 columns (disposable Sephadex G-25M) were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Urea, phenol red, and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Rabbit antiserum against perlecan core protein (EY-9) was kindly provided by Dr. John Hassell (Shriners Hospital for Children, Tampa, FL, U.S.A.); rabbit antisera against bovine biglycan (LF-96) and decorin (LF-94) were kindly provided by Dr. Larry Fisher (National Institute of Dental Research, Bore Research Branch, Bethesda, MD, U.S.A.). Cetylpyridinium chloride and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Incorporation of [^{35}S]Sulfate into Glycosaminoglycans —— Human brain microvascular endothelial cells were cultured in HuMedia EG-2 in 100-mm dishes in a humid atmosphere of 5% CO_2 in air until confluent. They were transferred into 24-well culture plates at a density of 1×10^4 cells/cm^2 and cultured until confluent. After washing the cells twice with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, the medium was replaced by a fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and the cells were subsequently incubated at 37°C for 6, 12, 24, or 48 hr in the presence of [^{35}S]sulfate (2 MBq/ml). After incubation, the conditioned medium was harvested, and solid urea was added up to a concentration of 8 M. The cell layer was washed twice with ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and harvested after extraction with 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM *N*-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min. The medium and cell extracts were used to determine the incorporation of [^{35}S]sulfate into glycosaminoglycans by the cetylpyridinium chloride precipitation method[12] as follows: portions of the extracts were spotted on filter papers and washed five times for 1 hr in 1% cetylpyridinium chloride with 0.05 M NaCl. The radioactivity of the proteoglycans precipitated on the dried filter paper was measured by liquid scintillation counting.

Characterization of Proteoglycans —— Human brain microvascular endothelial cells in 100-mm dishes were incubated at 37°C for 48 hr in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in the presence of [^{35}S]sulfate (2 MBq/ml). After incubation, the cell extracts were prepared as described above and chromatographed on PD-10 columns equilibrated in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base to obtain high molecular mass (> 3 kDa) macromolecules. For separation of proteoglycans on the basis of their hydrodynamic size, the macromolecules were then chromatographed on a Sepharose CL-2B column (0.9×80 cm) in 8 M urea buffer containing 0.25 M NaCl. Proteoglycan-containing peaks were eluted at K_{av}s of 0.3–0.6 and 0.6–0.8 (the high M_r subclass and the low M_r subclass, respectively). The subclasses were pooled and applied to DEAE-Sephacel (5 ml of resin) in 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.1 M NaCl, 0.5% Triton X-100, and 50 mM Tris base. Unbound radioactivity was removed from the column by washing with 30 ml of the buffer. Bound radioactivity was eluted from the column with a linear gradient of 0.25–0.7 M NaCl in urea buffer (total volume of 50 ml). Peaks containing proteoglycans, which were characteris-

tic for each subclass, were pooled and concentrated by application of the diluted samples to 0.3-ml DEAE-Sephacel minicolumns and eluting bound radioactivity with sequential washes of 8 M urea buffer containing 3 M NaCl. The proteoglycans were precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol and 80 μg/ml carrier chondroitin sulfate for 2 hr at –20°C; the precipitation was repeated three times. The precipitated proteoglycans were digested with 30 μg of papain in 0.1 M acetate buffer (pH 7.0) containing 5 mM EDTA and 5 mM cysteine at 65°C for 4 hr or with both 13.8 U/ml heparinase II and 27.6 U/ml heparinase III in 0.2 M Tris–HCl buffer (pH 7.0) containing 10 mM calcium acetate or with 1.7 units/ml chondroitinase ABC in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 mg/ml bovine serum albumin (BSA) and 3 mM sodium acetate at 37°C for 4 hr. The digested samples were chromatographed on a Sepharose CL-6B column (0.9 × 80 cm) in 0.2 M Tris–HCl buffer (pH 7.0) with 0.2 M NaCl. The void volume and the total volume were estimated by the elution position of dextran blue and phenol red, respectively. Estimates of the glycosaminoglycan chain size were made by comparison of the experimentally determined Sepharose CL-6B elution K_{av} with a previously published curve of log M_r versus K_{av} on Sepharose CL-6B for chondroitin sulfate chains of various known M_r.[13]

Analysis of Proteoglycan Core Proteins —— Confluent cultures of human brain microvascular endothelial cells in a 100-mm dish were incubated at 37°C for 48 hr in 6 ml of fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After incubation, proteoglycans were extracted from the cell layer and the conditioned medium under a dissociative condition in the presence of 8 M urea. The extract was concentrated on 0.3-ml DEAE-Sephacel minicolumns and precipitated with 1.3% potassium acetate in 95% ethanol. The precipitated proteoglycans were digested with or without heparinase II/III or chondroitinase ABC, and SDS-polyacrylamide gel electrophoresis was performed on a 4–12% acrylamide gradient slab gel with a 3% stacking gel according to Laemmli's procedure.[14] The SDS-polyacrylamide gel was equilibrated in 25 mM Tris transfer buffer (pH 9.5) with 20% methanol and transferred to a nitrocellulose membrane for 90 min with a semidry transfer apparatus (Atoo, AE-6677). The membrane was blocked and exposed overnight to primary antibodies against perlecan, biglycan, and decorin (each dilution 1 : 1000) at 4°C. After incubation of the blot with horseradish peroxidase-linked protein A, bands that bound to the primary antibodies were visualized by an enzyme-linked chemiluminescence procedure on Hyperfilm ECL.

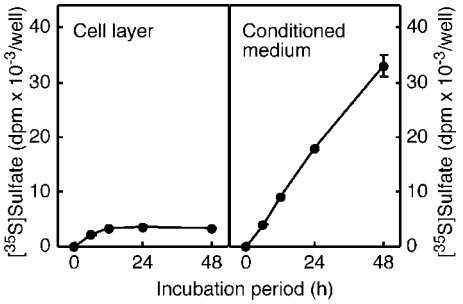

Fig. 1. Accumulation of [^{35}S]Sulfate-Labeled Glycosaminoglycans in the Cell Layer and the Conditioned Medium of Cultured Human Brain Microvascular Endothelial Cells

Confluent cultures of the cells were incubated at 37°C for 6, 12, 24, and 48 hr in the presence of [^{35}S]sulfate. Values are means ± S.E. of six samples.

RESULTS

Figure 1 shows the accumulation of glycosaminoglycans in the cell layer and the conditioned medium of human brain microvascular endothelial cells. The accumulation plateaued by 12 hr in the cell layer but increased steadily in the conditioned medium during a 72-hr labeling period, suggesting that a part of the proteoglycans synthesized by the cells is associated with the cell layer and the remainder is secreted into the medium. It is postulated that human brain microvascular endothelial cells produce extracellular matrix type proteoglycans, such as perlecan, versican, biglycan, and decorin, rather than the transmembrane or cell surface-associated type proteoglycans such as those of the syndecan family and glypican.

[^{35}S]Sulfate-labeled proteoglycans obtained from the cell layer and the conditioned medium of human brain microvascular endothelial cells were separated by Sepharose CL-2B chromatography on the basis of their hydrodynamic size. As shown in Fig. 2, the proteoglycans were eluted as a single peak at a K_{av} of 0.3–0.6 in the cell layer. In the conditioned medium, the proteoglycans eluted at a K_{av} of 0.6–0.8 accompanied with a shoulder that corresponds to the proteoglycan peak in the cell layer. These results indicate that the cells synthesize a high M_r subclass

of proteoglycans that can be associated with the cell layer and a low M_r subclass of proteoglycans that is secreted into the medium.

The high and low M_r subclasses were further purified by DEAE-Sephacel ion-exchange chromatography on the basis of their charge density differences (Fig. 3). A major part of the radioactivity of the high M_r subclass from either the cell layer or the conditioned medium was eluted by NaCl at 0.4 M. On the other hand, the low M_r subclass from the conditioned medium was eluted by NaCl at 0.4 and 0.55 M. However, it is postulated that the former peak is due to contamination of the high M_r subclass and the latter peak is representative of the low M_r subclass.

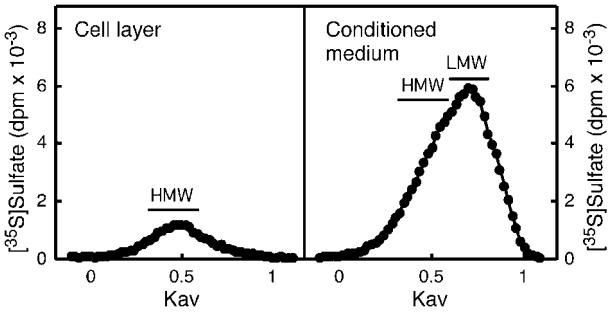

Fig. 2. Sepharose CL-2B Molecular Sieve Chromatography of [^{35}S]Sulfate-Labeled Proteoglycans Extracted from the Cell Layer and the Conditioned Medium of Human Brain Microvascular Endothelial Cells

Confluent cultures of the cells were incubated at 37°C for 48 hr in the presence of [^{35}S]sulfate. *Horizontal bars* indicate the fractions that were pooled and chromatographed on a DEAE-Sephacel ion-exchange column (see Fig. 3). HMW, the high M_r subclass; LMW, the low M_r subclass.

The glycosaminoglycan composition of the high and low M_r subclasses obtained from the conditioned medium purified by DEAE-Sephacel chromatography was analyzed by Sepharose CL-6B chromatography in order to characterize proteoglycans bound to the subclasses (Fig. 4). The high M_r subclass was sensitive to digestion with either papain or heparinase, suggesting that the subclass is composed of heparan sulfate proteoglycans. On the other hand, it was suggested that the low M_r subclass consisted of chondroitin/dermatan sulfate proteoglycans because this subclass was sensitive to either papain or chondroitinase ABC. The length of heparan sulfate chains in the high M_r subclass and chondroitin/dermatan sulfate chains in the low M_r subclass were M_r~68000 and ~41000, respectively. The high M_r subclass from the cell layer could not be analyzed because of the low [^{35}S]sulfate radioactivity.

It was shown that the major proteoglycans synthesized by human brain microvascular endothelial cells are large heparan sulfate proteoglycans and small chondroitin/dermatan sulfate proteoglycans. Arterial endothelial cells synthesize and secrete perlecan and biglycan as a large heparan sulfate proteoglycan and a small chondroitin/dermatan sulfate proteoglycan, respectively.[11] In addition, the expression of decorin, another small chondroitin/dermatan sulfate proteoglycan, is induced during the formation of neovessels both *in vitro*[15,16] and *in vivo*.[17] Based on these results, Western blot analysis for perlecan, biglycan, and decorin was performed to identify the proteoglycan core proteins synthesized by human brain microvascular endothelial cells (Fig. 5). The expression of perlecan and biglycan

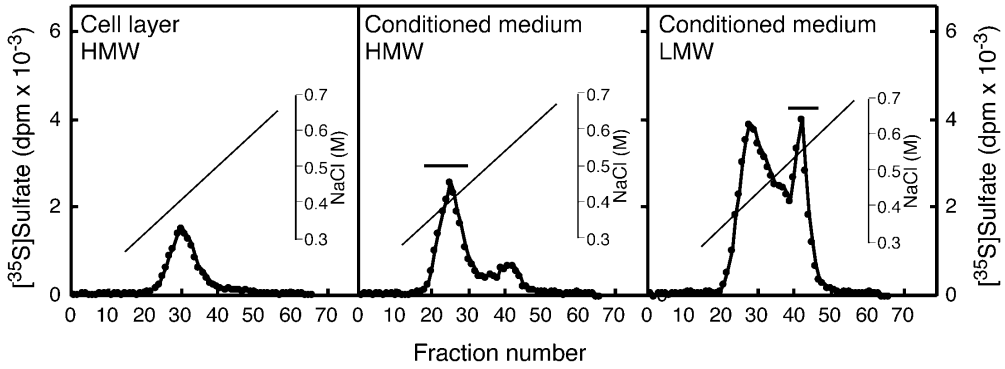

Fig. 3. DEAE-Sephacel Ion-Exchange Chromatography of the High and Low M_r Subclasses Obtained from the Cell layer and the Low M_r Subclass Obtained from Conditioned Medium of Human Brain Microvascular Endothelial Cells

The high and low M_r subclasses were chromatographed on a DEAE-Sephacel column with a linear gradient of 0.25–0.7 M NaCl in 8 M urea buffer. *Horizontal bars* indicate the fractions that were pooled and chromatographed on a Sepharose CL-6B column, before and after digestion with papain, heparinase II/III, or chondroitinase ABC (see Fig. 4).

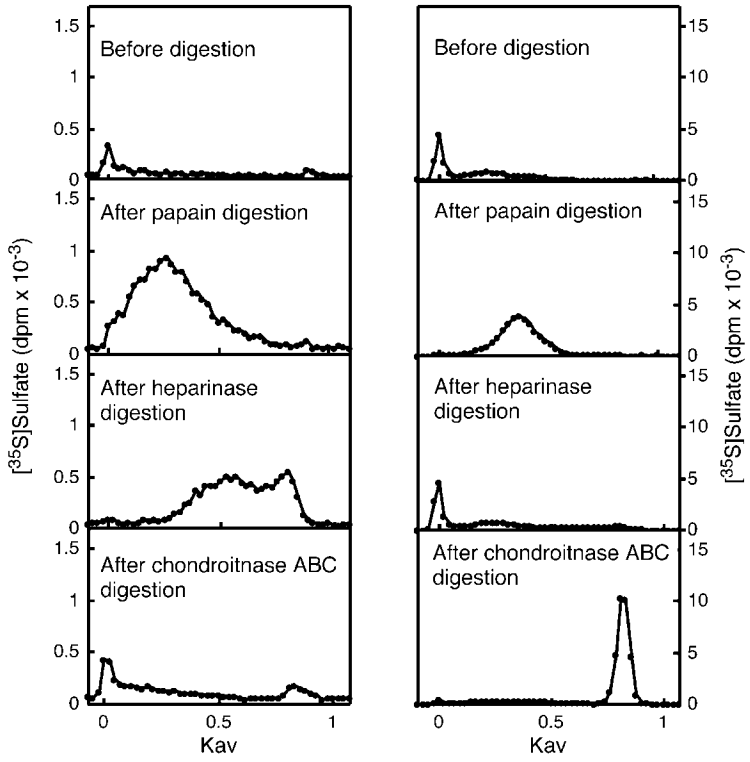

Fig. 4. Characterization of Glycosaminoglycan Chains Bound to the High (left panels) and Low (right panels) M_r Subclasses Purified by Sepharose CL-4B Chromatography and DEAE-Sephacel Chromatography

The high and low M_r subclasses were chromatographed on a Sepharose CL-6B column, before and after digestion with papain, heparinase II/III, or chondroitinase ABC. The chain size of glycosaminoglycans was estimated by comparison with a published chondroitin sulfate calibration.[13)]

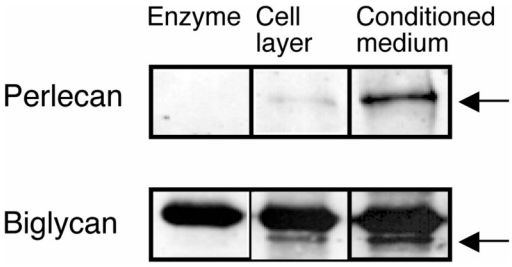

Fig. 5. Western Blot Analysis of Perlecan and Biglycan Core Proteins

Proteoglycans extracted from the cell layer and the conditioned medium of human brain microvascular endothelial cells and digested with heparinase II/III or chondroitinase ABC were run on a 4–12% gradient slab gel and probed with antibodies for perlecan and biglycan. Arrows indicate the bands of perlecan and biglycan core proteins.

was observed whereas that of decorin was not confirmed (not shown) in both the cell layer and the conditioned medium.

DISCUSSION

Proteoglycans play important roles in the regulation of vascular endothelial cell functions including proliferation,[18)] migration,[19)] angiogenesis,[18)] and anticoagulant properties.[7)] In the present study, it was found that human brain microvascular endothelial cells in culture predominantly synthesize and secrete perlecan with heparan sulfate chains of M_r~68000 and biglycan with chondroitin/dermatan sulfate chains of M_r~41000. It has been shown that cultured bovine aortic endothelial cells also synthesize and secrete perlecan and biglycan with glycosaminoglycan chains of M_r~74000 and ~39000, respectively.[11)] This suggested that the characteristics of proteoglycans in brain microvascular endothelial cells are almost similar to those in arterial endothelial cells, at least with regard to the type of proteoglycan core proteins and the length of glycosaminoglycan chains. However, the fine structure of glycosaminoglycan chains in both arterial and brain microvascular endothelial proteoglycans remains to be elucidated.

Microvessels are composed of endothelial cells and pericytes that wrap around and along endothelial cells.[20)] It has been suggested that endothelial cells and pericytes regulate each others functions in

small vessels. For example, pericytes inhibit the proliferation and movement of endothelial cells.[21,22] On the other hand, endothelial cells promote the growth of pericytes by secretion of endothelin-1.[23] Since the inhibition of endothelial cell growth by pericytes is achieved by close apposition of these two cell types, the extracellular matrix may be involved in modulating endothelial cell behavior. Recently, we have shown that bovine retinal pericytes predominantly synthesize and secrete chondroitin/dermatan sulfate proteoglycans, such as versican, biglycan, and decorin, with a small amount of heparan sulfate proteoglycans.[24] It is possible that proteoglycans synthesized by bovine retinal pericytes differ from those synthesized by human brain microvascular pericytes. However, it is likely that the differential expression of proteoglycans in endothelial cells and pericytes may influence the interaction between these two cell types with regard to the manner in which they regulate each others behavior.

Vascular endothelial cells can be the first target of toxic substances, such as heavy metals and biologically active chemicals in various tissues, because the monolayer of cells is a barrier between blood and the underlying tissue. Nolan and Shaikh[25] suggested that the initial effect of acute cadmium administration is on the integrity and permeability of the vascular endothelium and other necrotic changes in the underlying tissue occur secondarily. We have shown that cadmium,[26,27] lead,[27–29] arsenite,[30] and the biologically active sulfated polysaccharide sodium spirulan[31–33] influence the functions, including proteoglycan metabolism, of vascular endothelial cells. Proteoglycans are involved in the regulation of vascular endothelial cell functions; however, excluding a few exceptions such as the inhibition of vascular endothelial repair by lead, the toxicity or biological activity that mediate altered proteoglycan metabolism has not been completely understood.[27,28] The present data will be useful for similar studies by providing basic information on brain microvascular endothelial cell proteoglycans.

The present data demonstrated that human brain microvascular endothelial cells predominantly synthesize and secrete perlecan and biglycan as proteoglycans. It has been shown that arterial endothelial proteoglycan synthesis is regulated by growth factors/cytokines such as connective tissue growth factor[34] and transforming growth factor-β[11] and influenced by the heavy metal lead.[27,35,36] Based on the previous and present results, further investigation should be performed to clarify the mechanisms responsible for the toxic effects of injurious chemicals, heavy metals, and pathological factors on the brain tissue.

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