Widespread Expression of Chondroitin Sulfate-type Serglycins with CD44 Binding Ability in Hematopoietic Cells*

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Serglycin is a family of small proteoglycans with Ser-Gly dipeptide repeats and is modified with various types of glycosaminoglycan side chains. We previously demonstrated that chondroitin sulfate-modified serglycin is a novel ligand for CD44 involved in the adherence and activation of lymphoid cells. In this study, we investigated the production and distribution of CD44 binding serglycins in various hematopoietic cells and characterized their carbohydrate side chains. Immunoprecipitation analysis using CD44-IgG and polyclonal antibody against the serglycin core peptide demonstrated that various serglycin species capable of binding CD44 are produced by a variety of hematopoietic cells including lymphoid cells, myeloid cells, and a few tumor cell lines. Glycosaminoglycans on these serglycins, which are essential for CD44 binding, are composed of chondroitin 4-sulfate or a mixture of chondroitin 4-sulfate and chondroitin 6-sulfate, but no heparin or heparan sulfate side chain was detected. The serglycins are also secreted by normal splenocytes, lymph node lymphocytes, and bone marrow cells, whereas they are secreted in very small amounts by normal thymocytes. Secretion of serglycins is greatly enhanced by mitogenic stimulation with concanavalin A or lipopolysaccharide. Our results showed that serglycin, unlike hyaluronate, is produced and secreted in a functional (CD44 binding) form by many members of the hematopoietic system including various lymphocyte subsets. Our data suggest that serglycin may serve as a major ligand for CD44 in various events in the lymphohematopoietic system.

CD44, a cytoskeleton-associated cell-surface glycoprotein, acts as an adhesion molecule and signal transducer in the immune system (1, 2). Previous studies using anti-CD44 monoclonal antibodies showed that this molecule is involved in T cell activation (2–4), tumor metastasis (5, 6), hematopoiesis (7, 8), and lymphocyte homing (9). It has also been demonstrated that activation (2–4), tumor metastasis (5, 6), hematopoiesis (7, 8), and lymphocyte homing (9). It has also been demonstrated that activation (2–4), tumor metastasis (5, 6), hematopoiesis (7, 8), and lymphocyte homing (9). It has also been demonstrated that activation (2–4), tumor metastasis (5, 6), hematopoiesis (7, 8), and lymphocyte homing (9).

Recently, we reported that chondroitin sulfate-type serglycin is a novel ligand for CD44 (13). Serglycin is a secretory granule proteoglycan, and its mRNA is transcribed in hematopoietic lineage cells, yolk sac, and certain tumor cells (14). The transcription of the serglycin gene is regulated during tissue development and modulated by activation stimuli such as virus infection (15, 16). Although the function of serglycin remains to be fully determined, it has been suggested that it is involved in myeloid cell differentiation, as well as in cell-mediated cytotoxicity such that it helps packaging and stabilizing cytokines and proteases in secretory granules and transporting them to target sites when secreted extracellularly (14, 17–19). We have demonstrated that when the chondroitin sulfate-type serglycin, stored in secretory granules of a mouse T cell line, CTLL2, is secreted, it binds specifically to CD44 (13). We also showed that the addition of purified serglycin to CTL clones significantly enhances CD3-dependent granzyme release by these clones (13). Hyaluronate had no effect on the granzyme release, suggesting that the chondroitin sulfate-type serglycin has a unique function in CTL responses (13).

There are certain differences in post-translational carbohydrate modification and degradation of serglycin peptide core in different cell types (14). Serglycin carries mainly two types of carbohydrate chains, heparan sulfate or chondroitin sulfate. In human NK cells, the peptide core is thought to be cleaved in the secretory granule at its N and C termini, leaving a glycosaminoglycan attachment region consisting primarily of alternating serine and glycine residues (14). In rat LGL tumor cells, serglycin is metabolized by an endoglycosidase to individual chondroitin sulfate A in the secretory granule (14). In this regard, it is of note that neither the serglycin core peptide (as shown in this study) nor the modifying chondroitin sulfate alone can bind CD44 (13). Therefore, it is not clear whether all the serglycins synthesized by hematopoietic cells actually are able to bind CD44.

The aim of the present study was to examine the extent of the expression of CD44-binding serglycins in hematopoietic cells and to characterize their chondroitin sulfate side chains. Our results showed that the chondroitin sulfate-type serglycin capable of binding CD44 is secreted by a wide range of hematopoietic cells, including malignant cell lines and normal cells, and that mitogenic stimulation greatly enhances its secretion. We found also that the glycosaminoglycan side chains of the CD44 binding serglycins consist of mainly conventional chondroitin sulfate such as chondroitin 4-sulfate, chondroitin 6-sulfate, or a mixture of both. The results that a variety of blood cells produce the CD44 binding serglycin implies that serglycin may serve as a major ligand for CD44 in the hematopoietic system.
Expression of CD44 Ligand Serglycin in Hematopoietic Cells

MATERIALS AND METHODS

Animals—Female C57BL/6 (6–8 weeks of age) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Female Japanese White rabbits (1.5–2.0 kg body weight) were purchased from the Shiraishi Laboratory Farm (Tokyo, Japan). All experiments were performed according to the Guidelines for Animal Use and Experimentation as set by our institutions.

Antibodies and Reagents—Mouse cytotoxic T cell clones were kindly provided by Dr. S. Aizawa (Department of Physiology and Pathology, The National Institute of Radiological Science). The culture medium was RPMI 1640 containing 10% fetal calf serum (lot PK89, Mitsubishi Kasei Co., Tokyo, Japan), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μg/ml of streptomycin, 1% (v/v) 100% non-essential amino acids (Flow Laboratories, Irvine, CA), 100 units/ml penicillin, and 100 μg/ml of streptomycin. Preparation of CD44-IgG was performed as described previously (10). Control human IgG was purchased from Sigma. Chondroitinase ABC (from Proteus vulgaris, protease-free) and hyaluronidase (from Streptomyces hyalurolyticus) were purchased from Seikagaku Kogyo (Tokyo).

Preparation of Polyclonal Antibera Specific for Serglycin Core Peptide—Female Japanese White rabbits were immunized with 125 μg of the synthetic peptide (see Fig. 2A) suspended in Freund’s complete adjuvant and boosted at intervals of 2 weeks with the same amount of the peptide in Freund’s incomplete adjuvant. The peptide was derived from a region that lies adjacent to the Ser-Gly repeats and has no homology to any known molecules according to the homology search using EMBL protein data base, release 27.0. The peptide was synthesized using Peptide Synthesizer model 433A and Fmoc 9-fluorenylmethyl ester, as described previously (22). Expression of CD44 Ligand Serglycin in Hematopoietic Cells

Enzyme-linked Immunosorbent Assay—Purified serglycin was obtained as described previously (13) and coated on 96-well microtiter plates overnight at 4 °C. Immobilized serglycin was digested with chondroitinase ABC (0.2 units/ml) for 60 min at 37 °C, and then nonspecific sites were blocked with 1% bovine serum albumin in PBS for 2 h at room temperature. Binding of CD44-IgG (10 μg/ml) or polyclonal anti-serglycin core protein antibody, pSG (30 μg/ml), to each well was examined as described previously (13).

Radioactive Labeling and Immunoprecipitation—Metabolic labeling with [35S]methionine and [35S]sodium sulfate was performed as described previously (20). Immunoprecipitation analysis was performed using protein G-Sepharose (10-μl beads) conjugated with 10 μg of either CD44-IgG, pSG, or 50 μl of preimmune serum. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (4–20% gradient gel). Autoradiography was analyzed using a FUJIX BAS2000 image analyzer (Fuji Photo Film Co., Kanagawa, Japan).

Reverse Transcription and PCR Amplification of RNA—The total cellular RNA was isolated using ISOGEN (Wako Pure Chemical Industries, Osaka). First strand synthesis was carried out using SuperScript™ RNaseH− reverse transcriptase (Life Technologies, Inc., Tokyo) with oligo(dT) primer. The cDNA products were then used as template for PCR in which the following primers, 5’CTCAAAAGATTTCATCTCCAAATG and 5’GGTGAATATATACATGGTCT3’, were used. The 3’ primer was located 152 base pairs downstream of the 5’ primer. After amplification, the PCR products were run on agarose gel.

Disaccharide Analysis of 35S-Labeled Chondroitin Sulfates—Cells were incubated overnight in the presence of [35S]sodium sulfate, and 35S-labeled serglycin in culture supernatants were immunoprecipitated with CD44-IgG. After treatment with chondroitinase ABC, the immunoprecipitates were subjected to disaccharide analysis by high performance liquid chromatography using Partisil-10 SAX (Whatman), as reported previously (21).

Cell Aggregation Assay—Mouse thymoma cell line BW5147 cells were harvested, washed with PBS, and adjusted to 2.5 × 106 cells/ml in Ca2+- and Mg2+-free PBS. In the next step, 500 μl of the nonaggregated single cell suspension was placed into each well of a 4-well Lab-Tek chamber slide (Nunc Inc.) into which purified serglycin (100 μg/ml) was added. Then the cell suspension was rotated on a shaker at 80 rpm for 15 min at room temperature. Aggregation was assessed by phase contrast microscopy. In the antibody blocking experiments, 50 μg/ml of a function-blocking anti-CD44 antibody KM201 (7) was added to the cell suspension prior to the aggregation assay.

Cell Adhesion Assay—Flat-bottom wells of 96-well microtiter plate were coated with anti-human IgG (50 μg/ml) at 4 °C overnight. After washing with PBS, purified CD44-IgG or control human IgG was added to the wells, incubated for 60 min, washed, followed by the addition of various concentrations of purified serglycin. After washing, we examined cell adhesion to these wells using cells labeled with a fluorescent dye 2’,7’-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester, as described previously (22).

RESULTS

Secretion of Serglycin by Various Hematopoietic Cell Lines—The secretion of serglycin was examined by immunoprecipitation analysis using recombinant soluble CD44 (CD44-Ig) on culture supernatants from various hematopoietic cell lines. The cell lines included BW5147 (thymoma), MLV-induced YAC-1 (T lymphoma), EL-4 (T lymphoma), X63 (myeloma), BAF/B03 (pro-B), WEHI3 (myelomonocyte), P815 (mastocytoma), J774 (macrophage), and a number of CTL clones. This technique has enabled us in the past to identify chondroitin sulfate-modified serglycin as a novel ligand for CD44 in a mouse T cell line CTLL-2 (20). As shown in Fig. 1, sulfated macromolecules similar to those detected in CTLL-2 were precipitated with CD44-IgG from culture supernatants of EL-4, X63, BAF/B03, WEHI3, J774, P815, and some CTL clones but not from YAC-1 or BW5147. These radiolabeled molecules were not precipitated with control human IgG or other Ig fusion proteins such as LEC-IgG (Fig. 1, Ref. 20). The sulfated molecules migrated as a broad band that disappeared by chondroitinase ABC digestion (data not shown), suggesting that the sulfated macromolecules are modified with chondroitin sulfate that can bind CD44. Chondroitinase-resistant sulfated molecules were not detected, suggesting that chondroitin sulfate-type proteoglycans represent the major CD44 binding material in these cell lines.

To determine whether these CD44 binding molecules are identical to chondroitin sulfate-type serglycin, we prepared a polyclonal antibody against a stretch of the peptide representing part of the mouse serglycin peptide core (Fig. 2A). The specificity of the polyclonal anti-serglycin antibody (pSG) was verified by enzyme-linked immunosorbent assay. Interestingly, affinity purified pSG did not recognize intact mouse serglycin purified from mouse T cell line CTLL-2 (Fig. 2B, left panel) to which binding of CD44-Ig was readily recognized (Fig. 2B, right panel). Treatment with chondroitinase ABC, however, resulted in a dose-dependent binding of pSG to serglycin (Fig. 2B, left panel) but abolished CD44-Ig binding (Fig. 2B, right panel).
These findings suggest that pSG recognizes a protein epitope hidden by glycosaminoglycan chains, whereas CD44-Ig recognizes a glycosaminoglycan epitope on serglycin. In support of this conclusion, pSG immunoprecipitated successfully the glycosaminoglycan-deprived serglycin core protein derived from CTLL-2, whereas preimmune serum did not (Fig. 2C).

Using pSG, we then investigated whether the core protein obtained from various hematopoietic cell lines represented that of serglycin. To this end, [35S]methionine-labeled serglycin precipitated with CD44-IgG was treated with chondroitinase ABC, and the obtained peptide core was precipitated with pSG (lane a) or control preimmune serum (lane b). Lane c represents chondroitinase-treated serglycin immunoprecipitated with CD44-IgG.

Disaccharide Analysis of CD44 Binding Serglycin—To characterize glycosaminoglycans on serglycin involved in CD44 recognition, disaccharides were obtained from the serglycin preparations by chondroitinase ABC digestion and subjected to high performance liquid chromatography on a Partisil-10 SAX column with stepwise elution using increasing concentrations of KH2PO4 (Fig. 4). The composition of the disaccharide obviously differed from one cell to another, but the majority of disaccharides on serglycin were of chondroitin 4-sulfate-type and/or chondroitin 6-sulfate-type. Serglycin synthesized by BAF/BO3 and P815 was modified exclusively with chondroitin 4-sulfate, whereas serglycin from X63 was modified mainly by chondroitin 4-sulfate and chondroitin 6-sulfate. Serglycin from EL-4 was modified with several types of chondroitin sulfates with chondroitin 6-sulfate being the dominant type. Products corresponding to heparin, heparan sulfate, hyaluronate, or any unique disaccharides were not observed. These results strongly suggest that the CD44-binding serglycins are predominantly modified by conventional chondroitin sulfates and that the binding of CD44 to serglycin is apparently independent of the composition of the modifying chondroitin sulfate disaccharides.

Secretion of Serglycin by Normal Lymphoid Cells—In the next step, we examined whether normal hematopoietic cells...
produce serglycins capable of binding CD44. For this purpose, suspensions of lymph node cells, splenocytes, thymocytes, and bone marrow cells were incubated in the presence of sodium [35S]sulfate, and immunoprecipitation analysis was performed on their culture supernatants using CD44-IgG. As shown in Fig. 5A, low levels of sulfated macromolecules were detected in the culture supernatants of splenocytes and lymph node cells, whereas a higher level was detected in bone marrow cells. Stimulation of lymphoid cells with concanavalin A or lipopolysaccharide markedly increased the secretion of sulfated macromolecules (Fig. 5B). CD44-IgG-immunoprecipitable serglycin showed approximately 2–10-fold increase in [35S]incorporation.

**DISCUSSION**

We reported previously that chondroitin sulfate-type serglycin is a novel ligand for CD44 and that the chondroitin sulfate side chain plays an important role in recognition by CD44 (13). The serglycin gene is transcribed in most leukocytes including neutrophils, monocytes, granulocytes, and lymphocytes (15). However, glycosaminoglycans modifying the serglycin peptide core are heterogeneous in their disaccharide composition, length, extent of sulfation, or position of sulfation (14, 23). Hence, it is not clear whether these cells actually produce serglycins that can bind CD44. In the present study, we demonstrated, for the first time, that a variety of hematopoietic cells produce chondroitin sulfate-type serglycin capable of binding CD44. Disaccharide analysis showed that the glycosaminoglycans modifying the CD44 binding serglycin differ from one cell type to another and consist of mainly conventional chondroitin sulfates, such as chondroitin 4-sulfate, chondroitin 6-sulfate, or a mixture of both. This indicates that the binding circumstances (Fig. 6A). The aggregation was completely inhibited by anti-CD44 monoclonal antibody, KM201 (Fig. 6C), suggesting that serglycin-CD44 interaction is directly responsible for the aggregation and that serglycin acts as a bridge between CD44 on the surface of different cells. The homophilic cell clustering was observed even in the absence of Ca2+ and Mg2+ cations, indicating that neither integrins nor selectins are involved in this process. To explore further the mechanism of serglycin-induced cell aggregation, we performed the following cell adhesion assay using recombinant CD44 and purified serglycin. When serglycin was added to wells where CD44-Ig had been immobilized, BW5147 cells bound avidly (Fig. 6D). The process was specifically inhibited by anti-CD44 (not shown). In contrast, BW5147 cells failed to bind when CD44-Ig or human IgG alone was added to wells in the absence of anti-human IgG. This indicates that serglycin acted as a linker between cell-surface CD44 and CD44-Ig immobilized to wells, confirming the above speculation that serglycin is involved in cell-cell interaction by bridging CD44 molecules.

**FIG. 6. Aggregation of BW5147 cells through CD44-serglycin interaction.** A, BW5147; B, BW5147 in the presence of 100 μg/ml purified serglycin; C, as in B but with 50 μg/ml purified anti-CD44 (KM201); D, adhesion of BW5147 to serglycin captured by immobilized CD44-IgG. Cell adhesion assay was performed as described under "Materials and Methods."

**FIG. 5. Production of chondroitin sulfate-type serglycin by normal hematopoietic cells.** A, secretion of serglycin-like molecules by normal hematopoietic cells derived from various lymphoid organs. B, enhanced secretion of serglycin-like molecules by mitogen-stimulated lymphoid cells. In both A and B, the cells were labeled with [35S]sulfate, and immunoprecipitation analysis was performed as described under "Materials and Methods." Immunological probes used were as follows: a, CD44-IgG; b, human-IgG. C, detection of transcription of serglycin gene in normal lymphoid cells by reverse transcription-PCR.
ability of serglycin to CD44 is independent of its chondroitin sulfate composition. Although neither heparin nor heparan sulfate nor any unique disaccharide was detected from the CD44 binding serglycins, it remains to be formally tested if heparin-type serglycins, such as those produced by connective tissue mast cells (14), can actually bind CD44.

A high level of production of CD44 binding serglycin was observed in bone marrow cells. In this context, it is noteworthy that the expression of the serglycin gene occurs very early in hematopoiesis (15) and that CD44 is involved in bone marrow hematopoiesis (7, 8). Because the abrogation of CD44-hyaluronate interaction leads to disruption of early lymphohematopoiesis in vitro (7), it is thought that the interaction with hyaluronate is important for CD44 in the regulation of hematopoiesis. However, given that serglycin is ubiquitously expressed by hematopoietic cells and that it binds to the hyaluronate binding site or its close vicinity on the CD44 core protein (13), it would be interesting to determine in future studies of the CD44-serglycin interaction contributes at all to the generation of hematopoietic cells in the bone marrow. In addition, other than the possible interaction with CD44, exocytosed serglycin may also be important for the binding of hematopoietic growth factors possibly by increasing their local concentrations and make them accessible to immature hematopoietic cells. Several studies have already indicated the importance of adequate concentrations of diffusible cytokines/growth factors by immobilized proteoglycans in the regulation of cell proliferation and adhesion (19, 24–26).

Our results also showed that normal lymph node cells and splenocytes also produce the CD44 binding serglycin, which is markedly enhanced by stimulation with concanavalin A or lipopolysaccharide. This confirms and further extends previous observations that mitogen-stimulated T lymphocytes produce chondroitin sulfate proteoglycans that are secreted rapidly into the extracellular space (27, 28). Our observation that thyocytes do not produce the CD44 binding serglycin but transcribe the serglycin gene whereas spleen and lymph node cells actually produce the CD44 binding serglycin indicates that the production of serglycin with a CD44 binding ability may correlate with T cell differentiation. Although the exact function of serglycin in the T cell lineage remains to be fully explored, the results of our previous study indicate that serglycin acts on killer T cells and augments the release of their granzyme (13).

With regard to the receptor expression, most T lymphocytes express CD44 but do not bind hyaluronate unless they are activated (29, 30). This is also the case with binding to serglycin (13). This indicates that, although hyaluronate or serglycin may be found frequently in the hematopoietic milieu, ligation with CD44 occurs only when appropriate activation stimulus is present, which may provide specificity for what would otherwise be an uncontrolled interaction between a ubiquitously expressed cell-surface receptor and common components of the lymphohematopoietic system. Furthermore, the restricted expression of serglycin in the hematopoietic cell lineage, which is quite different from the ubiquitous expression of hitherto described ligands for CD44 such as hyaluronate, fibronectin, and collagen, may also help ensure the specificity of CD44 function.

Cell aggregation and binding experiments indicated that serglycin can mediate strong homophilic as well as heterophilic cell aggregation by linking CD44 molecules on opposing cells. Although it has been shown previously that stimulation of CD44 induces cell adhesion mediated by non-CD44 molecules under certain experimental conditions (22, 31), homophilic cell aggregation induced by the addition of serglycin is apparently not mediated by other adhesion molecules such as integrins or selectins, since aggregation was not affected by chelators of divalent cations such as EDTA. Our recent findings that the binding is seen even in the presence of function-blocking antibodies against β1 or β2 integrin also excludes the involvement of integrin. 2

Finally, it is of interest whether high endothelial cells in mucosal lymph nodes produce serglycin, since CD44 was initially identified as lymphocyte homing receptor of mucosal lymphoid tissues (9). Suggestion has also been made that a counter-receptor for CD44 on high endothelial cells is not hyaluronate but an as yet unidentified type (32). However, preliminary experiments in our laboratory using polyclonal anti-serglycin core peptide antibody indicate that serglycin is not present in high endothelial venules in mouse mesenteric lymph nodes at least at readily detectable levels. Therefore, it is still not clear at present that serglycin plays any role in lymphocyte homing.

The in vitro significance and function of the serglycin-CD44 interaction in various cell types remains to be determined. However, our results describing the production of serglycin serve as an important basis for future elucidation of the biological function of serglycin and CD44. Collectively, given that the CD44-binding serglycin is widely expressed in hematopoietic cells and that its expression is restricted to the hematopoietic tissue under normal circumstances (14), it is tempting to suggest that serglycin may serve as a major ligand for CD44 in the hematopoietic system.

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