Versican Interacts with Chemokines and Modulates Cellular Responses*

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We previously reported that versican, a large chondroitin sulfate proteoglycan, isolated from a renal adenocarcinoma cell line, ACHN, binds L-selectin. Here we report that versican also binds certain chemokines and regulates chemokine function. This binding was strongly inhibited by the chondroitinase digestion of versican or by the addition of soluble chondroitin sulfate (CS) B, CS E, or heparan sulfate. Furthermore, these glycosaminoglycans (GAGs) could bind directly to the chemokines that bind versican. Thus, versican appears to interact with chemokines via its GAGs. We next examined if versican or GAGs affect secondary lymphoid tissue chemokine (SLC)-induced integrin activation and Ca²⁺ mobilization in lymphoid cells expressing a receptor for SLC, CC chemokine receptor 7. Interestingly, whereas heparan sulfate supported both α₄β₁ integrin-dependent binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1)-IgG and Ca²⁺ mobilization induced by SLC, versican or CS B inhibited these cellular responses, and the extent of inhibition was dependent on the dose of versican or CS B added. These findings suggest that different proteoglycans have different functions in the regulation of chemokine activities and that versican may negatively regulate the function of SLC via its GAG chains.

Proteoglycans are proteins that carry glycosaminoglycans (GAGs).¹ The common GAGs are heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate, and hyaluronic acid. These GAGs have a large number of sulfate or carboxyl groups and, hence, have a strong negative charge, which makes it possible for proteoglycans to bind through their GAG chains various positively charged molecules such as certain growth factors, cytokines, and chemokines (1, 2). This interaction in the extracellular matrix or on the cell surface has been suggested to play important roles in vivo in the formation of immobilized gradients of these humoral factors, in their protection from proteolytic degradation, and in their presentation to specific cell-surface receptors (3–5). There is an increasing body of evidence indicating that HS proteoglycans are particularly important in promoting the oligomerization of chemokines and in facilitating their presentation to specific receptors (6–8). Sporadic reports indicate that CS proteoglycans may also have this function (9, 10). For instance, CS proteoglycans on human neutrophils specifically bind platelet factor 4 and are involved in the activation of neutrophils (9). An artificial proteoglycan modified with CS binds RANTES via its GAGs, which also induces the activation of T cells expressing this molecule on their surface (10). Thus, not only HS but also CS proteoglycans appear to bind chemokines and to play important roles in the regulation of chemokine functions.

Versican is a large CS proteoglycan (11, 12) expressed in various tissues (13). We have recently reported that versican derived from a renal adenocarcinoma cell line, ACHN, interacts through its CS chains with leukocyte adhesion molecules such as L-selectin and CD44 (14, 15), both of which have been implicated in leukocyte trafficking (16, 17). Since versican has abundant CS side chains and is secreted into the extracellular matrix, we speculated that it might also interact with certain chemokines to help form haptotactic gradients in the tissues where chemokines and versican are colocalized. In the present study we examined the ability of versican to bind a large panel of chemokines and the biological consequences of such binding. Our results indicate that versican can bind specific chemokines through its CS chains and that the binding tends to down-regulate the chemokine function. This raises the possibility that different proteoglycans have different functions in the regulation of chemokine activities and that certain proteoglycans, such as versican, may negatively regulate chemokine functions.

EXPERIMENTAL PROCEDURES

Reagents—Versican was isolated from the culture supernatant of a human renal adenocarcinoma cell line, ACHN, as described previously (14). Epithelial-derived neutrophil attractant 78 (ENA-78), growth-related gene (Gro-α), interleukin-8, γ interferon-inducible protein 10 (IP-10), platelet factor 4, lymphotactin, MCP-1, -2, -3, MIP-1α, MIP-1β, and RANTES were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). Recombinant human SDF-1α (18) was purchased from Genetics Institute. Recombinant human liver and activation-regulated chemokine (LARC), pulmonary and activation-regulated chemokine (PARC), and thymus and activation-regulated chemokine (TARC) were produced by using a baculovirus expression system and purified as described previously (19–21). EBI1-ligand chemokine was
produced in *Escherichia coli* and purified as described previously (22). SLC and goat anti-human SLC polyclonal antibodies were purchased from DAKO Japan Co. Ltd (Tokyo, Japan). Anti-versican monoclonal antibody (mAb) 2B1 (23), anti-CS mAb CS56 (24), chondroitin ABC (EC 4.2.7.4), chondroitin B (EC 4.2.7.2), keratanase (EC 3.2.1.103), chondroitin HS (pig skin cartilage), CS A (bird cartilage), CS D (shark cartilage), CS E (squid cartilage), and heparan sulfate (bovine kidney) were all purchased from Seikagaku Kogyo (Tokyo, Japan). Rat MadCAM-1 IgG (25) was kindly provided by Dr. Toshiko Iizuka (Tokyo Medical and Dental University).

**Cells**—The murine T cell line TK-1 (26) and murine pre B cell line L1.2 (27), kindly provided by Drs. Berhard Holzmann (Universitat Munich) and Eugene Butcher (Stanford University School of Medicine), respectively, were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-mercaptoethanol (RPMI-FCS). The human acute T cell leukemia Jurkat cells were also cultured in RPMI-FCS. The L1.2 cells stable-expressing transduced human CCR7 (L1.2/CCR7) was generated as described previously (21) and was cultured in RPMI-FCS containing 0.8 mg/ml Gentamicin (G418, Sigma).

**Dot Blot Analysis**—Various concentrations of chemokines were spotted onto a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To immobilize the chemokines, the membrane was incubated with 3% BSA, 0.1% NaN₃ in PBS at 4 °C. After blocking, the membrane was washed with PBS containing 1 mM CaCl₂, 1 mM MgCl₂ (PBS+) containing 0.05% Tween 20 and 0.1% BSA. Subsequently, the membrane was incubated with biotinylated versican (3 μg/ml) in the presence or absence of various GAGs (10 μg/ml) for 1 h at room temperature. After washing, the membrane was incubated with streptavidin-conjugated alkaline phosphatase (1:500, Promega, Madison, WI) for 1 h. To quantify the reaction, Blue Phos™ (Kirkegaard Perry Laboratories, Gaithersburg, MD) was added, and the optical density was read at 460 nm in a microtiter plate reader (InterMed, Tokyo, Japan). To determine the amount of unbound SLC in the wells, anti-versican core protein mAb 2B1 and anti-CS mAb CS56 were also spotted on the membrane to serve as positive controls for versican binding. As described previously, the binding of 125I-labeled SLC was counted in a gamma counter. All determinations were made in triplicate.

**RESULTS**

**Versican Binds Various Chemokines**—The binding of versican to 18 different chemokines was examined by dot blot analysis. Various chemokines were spotted onto a nitrocellulose membrane, biotinylated versican was added, and after washing, its binding was determined. Anti-versican core protein mAb 2B1 and anti-CS mAb CS56 were also spotted on the membrane to serve as positive controls for versican binding. As shown in Fig. 1A, biotinylated versican bound the CXC chemokines γ interferon-inducible protein 10 (IP-10), platelet factor 4, and SDF-1α, the C chemokine lymphotactin, and the CC chemokines MCP-1, MCP-2, RANTES, liver and activation-regulated chemokine (LARC), and SLC. In contrast, biotinylated versican failed to bind interleukin-8 or MIP-1α, which are known to bind HS (29–31).

We then performed a reverse assay, where the ability of immobilized versican to bind soluble chemokines was tested by ELISA. As shown in Fig. 1B, versican bound SLC and MCP-1 in a dose-dependent manner. Similarly, immobilized versican also bound SDF-1α and lymphotactin in a dose-dependent manner (data not shown).

**Versican Binds Chemokines in a GAG-dependent Manner**—To examine whether the chemokine binding is mediated by the GAG chains on versican, the effects of GAG-degrading enzymes on the binding of SLC to versican were determined. As shown in Fig. 2, *top panel*, both chondroitinase ABC and chondroitinase B inhibited the binding of versican to SLC, whereas neither of these enzymes affected the reactivity of versican with anti-versican core protein mAb 2B1 (Fig. 2, *middle panel*). In addition, chondroitinase ABC as well as chondroitinase B almost completely abrogated the reactivity of versican with the
antibody CS mAb CS56, whereas keratanase failed to alter the reactivity of versican with mAb CS56 (Fig. 2, bottom panel). These results suggest that the GAGs on versican play an important role in chemokine binding. However, the possibility remains that the core protein of versican is also involved in chemokine binding, since treatment with chondroitinases did not completely inhibit the binding of SLC to versican.

To further investigate the involvement of GAGs in the chemokine binding, we examined whether exogenously added soluble GAGs could inhibit the binding of chemokines to versican. Interestingly, CS B, CS E, or HS almost completely abolished the binding of most of the chemokines examined, whereas CS C and CS D inhibited the binding of a restricted set of chemokines, and chondroitin and CS A were only slightly inhibitory (Fig. 3A). Given that CS B, CS E, and HS were extremely potent inhibitors of the chemokine binding to versican, we speculated that these GAGs might have particularly high chemokine binding abilities compared with other types of GAGs.

To further examine this issue, various GAGs were immobilized on plastic wells, and the binding of SLC was determined by ELISA. As shown in Fig. 3B, SLC bound strongly to CS B, CS E, and HS in a dose-dependent manner but bound other GAGs only weakly or not at all, consistent with the results shown in Fig. 3A. SLC did not bind to a nonsulfated type of GAG, chondroitin. Collectively, these results indicate that the GAG chains of versican are instrumental in chemokine binding and that the sulfation of GAG chains may affect the binding.

Versican Down-regulates the αβ7-Integrin-mediated Cell Adhesion Induced by SLC—We next investigated the possible functional consequences of the versican-chemokine interaction. First, we examined whether versican could influence the SLC-induced activation of integrins using αβ7-integrin- and CCR7-expressing TK-1 mouse T cells. SLC, which we found to bind versican strongly, can facilitate the adhesion of αβ7 integrin-positive cells to their ligand, MAdCAM-1 (32). To examine the effect of versican on this chemokine action, SLC was coimmobilized in wells with MAdCAM-1-IgG alone or with MAdCAM-1-IgG and versican. To evaluate nonspecific binding, human IgG was used instead of MAdCAM-1-IgG. As shown in Fig. 4A, left panel, SLC enhanced the binding of TK-1 cells to MAdCAM-1-IgG, showing a bell-shaped dose-response pattern in the absence of versican. This up-regulated adhesion was apparently mediated by the interaction between αβ7 integrin and MAdCAM-1, since the effect was completely abrogated by anti-αβ7 mAb and anti-MAdCAM-1 mAb (data not shown). By contrast, SLC immobilized on versican up-regulated the cell adhesion only marginally, if at all (Fig. 4A, right panel). The lack of strong up-regulation of TK-1 adhesion by SLC complexed with versican was not simply due to the reduced availability of SLC to TK-1, because the actual amount of SLC...
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**Fig. 3. Certain GAGs are involved in chemokine binding to versican.** A, binding of versican to chemokines is inhibited by certain GAGs. Chemokines were spotted onto nitrocellulose membranes (50 ng/spot). After blocking, biotinylated versican was applied to each chemokine spot in the presence or absence of 10 μg/ml GAGs. In the case of SLC, GAGs were added at a concentration of 30 μg/ml. The amount of bound versican was quantified using a densitometer. Values are expressed as the percentage of specific binding compared with the control without GAGs. B, SLC directly binds certain GAGs. Chondroitin (CH), CS A, CS B, CS C, CS D, CS E, or HS (50 μg/ml) was coated onto the wells of an ELISA plate. After blocking, SLC (0.01, 0.03, 0.1, or 0.3 μg/ml) was added to the wells and incubated for 1 h. The binding of SLC to GAGs was assessed by ELISA. The values shown have been corrected by subtracting the background. Representative results from three separate experiments are shown. IP-10, γ interferon-inducible protein 10; PF-4, platelet factor 4; LARC, liver and activation-regulated chemokine.

| A | Binding (%) |
|---|-------------|
| CH | CS A | CS B | CS C | CS D | CS E | HS |
| 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 500 | 500 | 500 | 500 | 500 | 500 | 500 |

**Discussion**

It is generally accepted that proteoglycans interact with small molecular weight humoral mediators such as chemokines that are produced in the same tissue. These interactions protect the small molecules from degradation, help them form concentration gradients in situ, and help to present them to specific receptors on the cell surface (1–5). However, it is not known whether proteoglycans always regulate chemokine function positively, let alone the exact identity of the proteoglycan involved. We next examined another effect of versican on chemokine functions by examining the intracellular Ca2+ response elicited by SLC and SDF-1β in L1.2/CCR7 (22) and Jurkat cells (33), respectively. As shown in Fig. 5A, leukotriene D4, SLC, or SDF-1β induced a rapid and transient intracellular Ca2+ response. However, versican inhibited the chemokine-induced intracellular Ca2+ response in a dose-dependent manner. Versican did not affect the leukotriene D4-induced intracellular Ca2+ response (Fig. 5A). When GAGs were used instead of versican, CS B was again inhibitory on SLC-induced intracellular Ca2+ response, whereas CS A and HS were not (Fig. 5B). These results further suggest that versican inhibits the ability of chemokines to elicit intracellular signals, and it does so, at least in part, via its GAG chains.

**How Does Versican Inhibit the Effects of SLC?** To investigate the mechanism by which versican inhibits chemokine functions, we examined whether versican affects the binding of SLC to CCR7-expressing cells. As shown in Fig. 6A, the binding of 10 ng/ml 125I-SLC to L1.2/CCR7 cells was inhibited by a 200-fold excess amount of unlabeled SLC, which reduced the binding to the background level. In contrast, SLC binding was not significantly inhibited by even 10 μg/ml versican, even though the intracellular Ca2+ response induced by 1 μg/ml SLC in L1.2/CCR7 cells was inhibited by 0.4 μg/ml versican (Fig. 5). Taking the molecular size of SLC (15 kDa) and versican (1,600 kDa) into account, this means that the intracellular Ca2+ response was inhibited at a molar ratio of 1:0.0037 (SLC:versican), whereas the binding of SLC to its receptor was uninhibited, even at a molar ratio of 1:9.37 (SLC:versican). Thus, the down-regulation of chemokine function by versican is unlikely to be due to the inhibition of SLC binding to its receptor. We next examined if GAGs affect the binding of SLC to L1.2/CCR7 cells. The binding of 10 ng/ml 125I-SLC to L1.2/CCR7 cells was inhibited by 50 μg/ml but not by lower doses of CS B or HS (5 and 0.5 μg/ml), whereas, as shown in Fig. 5, the intracellular Ca2+ response induced by 1 μg/ml SLC was inhibited by 50 μg/ml of CS B. Taking the molecular size of CS B (11–25 kDa) into consideration, this result implies that the SLC-induced intracellular Ca2+ response was inhibited by CS B at a molar ratio of 1.300–68.2 (SLC-CS B), whereas the binding of 125I-SLC to L1.2/CCR7 cells was inhibited only at much higher molar ratios (1:3,000–6,820).

**DISCUSSION**

It is generally accepted that proteoglycans interact with small molecular weight humoral mediators such as chemokines that are produced in the same tissue. These interactions protect the small molecules from degradation, help them form concentration gradients in situ, and help to present them to specific receptors on the cell surface (1–5). However, it is not known whether proteoglycans always regulate chemokine function positively, let alone the exact identity of the proteoglycan involved. We next examined another effect of versican on chemokine functions by examining the intracellular Ca2+ response elicited by SLC and SDF-1β in L1.2/CCR7 (22) and Jurkat cells (33), respectively. As shown in Fig. 5A, leukotriene D4, SLC, or SDF-1β induced a rapid and transient intracellular Ca2+ response. However, versican inhibited the chemokine-induced intracellular Ca2+ response in a dose-dependent manner. Versican did not affect the leukotriene D4-induced intracellular Ca2+ response (Fig. 5A). When GAGs were used instead of versican, CS B was again inhibitory on SLC-induced intracellular Ca2+ response, whereas CS A and HS were not (Fig. 5B). These results further suggest that versican inhibits the ability of chemokines to elicit intracellular signals, and it does so, at least in part, via its GAG chains.

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Furthermore, we found that versican or certain types of CS can inhibit the function of SLC; i.e. SLC that was complexed with versican or CS B was significantly less efficient than SLC alone in inducing \( \alpha_2 \beta_1 \) integrin activation as well as the intracellular \( \text{Ca}^{2+} \) response, and the extent of inhibition by versican or CS B was dose-dependent. In contrast, SLC that was complexed with HS was as efficient as SLC alone in its chemokine activities. These findings suggest that the interaction between proteoglycans and chemokines results in different biological consequences, depending on the proteoglycans involved, and that certain proteoglycans or their GAGs alone may negatively regulate the function of chemokines in certain situations.

Although CS proteoglycans bear negatively charged GAG chains that can interact with positively charged residues of chemokines, a nonspecific electrostatic interaction may not be the sole factor determining the interaction between CS proteoglycans and chemokines. The results shown in Fig. 3 that nonsulfated chondroitin failed to interact with SLC indicates that sulfation of the GAG chains is important in the interaction. However, CS E but not CS D could interact with SLC even though both are composed of disulfated disaccharides, indicating that sulfation at a specific position is critical. In addition, CS B and CS E, but not CS A, interacted with SLC even though all of these GAGs have 4-sulfation on N-acetylgalactosamine residues, suggesting that 4-sulfation on N-acetylgalactosamine residues is not sufficient but that sulfation of another position(s) and/or the structure of uronic acid residues is critical for chemokine binding.

Based on the observations that binding of the CC chemokine SLC to versican was inhibited by chondroitinase B, which specifically cleaves the CS B chain, and that CS B itself bound SLC directly, it is likely that CS B or a GAG containing a CS B-type structure on versican is actually involved in the chemokine binding. This hypothesis is supported by our GAG composition analysis using FACE (fluorophore-assisted carbohydrate electrophoresis) and immunological analysis using anti-GAG mAbs, which indicated that versican is modified with at least CS B and CS C (15). However, we do not know currently whether versican is modified with CS E or a similar moiety, and further structural analysis will be required to understand the exact carbohydrate structure responsible for SLC binding.

The mechanism by which versican or a specific GAG chain (versican/GAG) inhibits SLC function remains unclear, but several possibilities can be considered: (i) versican/GAG inhibits SLC binding to specific chemokine receptors; (ii) versican/GAG competes with endogenously expressed cell-surface proteoglycans that may help present SLC to its receptors; (iii) versican/GAG transduces a negative signal by binding to molecules different from chemokine receptors; (iv) versican/GAG allows SLC to bind its receptor even after forming a complex with SLC but inhibits an appropriate signal to be transduced.

The first possibility, that versican inhibits the binding of SLC to CCR7, appears unlikely since versican inhibited the SLC-induced intracellular \( \text{Ca}^{2+} \) responses (Fig. 5) at a dose that did not affect the binding of SLC to its receptor (Fig. 6A). The observations that the SLC-induced intracellular \( \text{Ca}^{2+} \) response was inhibited by CS B at a molar ratio of 1:30–68 (Fig. 5) and that the binding of \( ^{125} \text{I}-\text{SLC} \) to CCR7-expressing cells
Fig. 5. Versican and CS B inhibit Ca\(^{2+}\) mobilization induced by chemokines. L1.2 cells stably transfected with CCR7 (L1.2/CCR7) and Jurkat cells were loaded with Fura-2-AM and stimulated with stimulators that had been preincubated for 10 min with the indicated concentrations of versican (A) or 50 \(\mu\)g/ml GAGs (B). The arrowhead indicates the time of application of the stimulators. The intracellular calcium concentration was monitored by measuring the fluorescence ratio (F340/F380). Representative results from three separate experiments are shown. The scale is shown as a bar on the right.

was not inhibited at this dose range (Fig. 6B) suggest that CS B may also exert its inhibitory function without inhibiting the binding of SLC to its receptor. Concerning the second possibility, that versican/GAG competes with endogenous proteoglycans that may help present SLC to its specific receptor CCR7 and, thus, inhibit SLC signaling, our experiments showed that SLC specifically bound to a single class of high affinity receptors expressed on L1.2/CCR7 cells with a \(K_d\) of 2.26 nM (data not shown), indicating that endogenous cell-surface proteoglycans are not involved in SLC binding in this cell line. In addition, our preliminary experiments showed that the binding of SLC to L1.2/CCR7 cells was not altered by treating the cells with chondroitinase or heparitinase (data not shown). In line with this notion, it was recently reported that cell-surface GAG chain expression is not necessary for chemokines such as MIP-1\(a\), MIP-1\(b\), and RANTES to exert their biological effects (34). Collectively, the available data suggest that versican/GAG does not inhibit SLC signaling through inhibiting SLC binding to CCR7. Rather, the inhibition of SLC signaling is probably at least partly due to inhibition of the chemokine signal after SLC binds its receptor. Still other possible mechanisms remain to be completely excluded.

Versican binds certain chemokines but not all of them. It is notable that versican binds SDF-1\(\beta\) that is expressed in normal human skin as well as in inflammatory infiltrates of autoimmune skin diseases (37). In the inflamed skin, a large number of mononuclear cells expressing the receptor for SDF-1\(\beta\), CXCR4, selec-
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tively accumulates where SDF-1β is localized (37), suggesting the possibility that SDF-1β is involved in the infiltration of mononuclear cells into the inflamed skin. To inhibit excessive infiltration of mononuclear cells, however, an excess amount of SDF-1β produced in inflammatory infiltrates may have to be eliminated locally. In this sense, our result showing that versican-SDF-1 interaction results in down-regulation of SDF-1β activity (Fig. 3A) is interesting, pointing to the possibility that versican expressed in the inflamed skin interacts with SDF-1β to modulate the infiltration of mononuclear cells by down-regulating SDF-1β activity, although this has yet to be evidenced experimentally. In addition, several CS proteoglycans, i.e. L-selectin-reactive CS proteoglycans of 150 and >200 kDa (38) and endoglycan (39), have been reported to be comnicated in lymph node high endothelial venules where SLC is produced (36). It would be interesting to examine whether these CS proteoglycans can interact with SLC to regulate the function of SLC in situ.

Given that only specific kinds of GAGs can bind chemokines (Fig. 3), it is likely that only versican species modified with specific GAGs can bind chemokines. We previously showed that versican derived from ACHN binds via its GAG chains, a leukocyte adhesion molecule, L-selectin (14), and that certain GAGs such as CS B, CS E, and HS bind L-selectin directly (15). This specificity of binding is quite similar to what we observed for chemokine binding (Fig. 3B); i.e. those GAGs that bind L-selectin also bind certain chemokines, and those GAGs that do not bind L-selectin fail to bind the chemokines. Thus, we speculate that versican species modified with L-selectin-reactive GAGs also bind chemokines, which may be of particular importance in certain in vivo situations such as in inflammatory responses, where L-selectin and chemokines are comcomitantly involved. The biological significance of the interaction between versican and chemokines is an important subject for further investigation.

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