Macrophage Receptors for Lumican
A Corneal Keratan Sulfate Proteoglycan

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**Purpose.** Keratan sulfate proteoglycans (KSPGs) of the cornea exhibit a characteristic change in glycosylation resulting from stromal inflammation and scarring. To examine potential roles for these molecules in the pathobiology of the cornea, the authors investigated interaction of inflammatory macrophages with KSPGs in vitro.

**Methods.** Attachment and spreading of mouse peritoneal macrophages were examined on surfaces coated with corneal proteoglycans, intact or with modified glycosylation. Solution-phase interactions were demonstrated using soluble proteoglycans labeled with $^{125}$-Iodine or with fluorescein. The affinity and specificity of these interactions were determined by competitive inhibition with unlabeled proteoglycans.

**Results.** Macrophages did not adhere to intact corneal KSPGs but did attach and spread rapidly on the lumican core protein after the removal of keratan sulfate chains. Arterial lumican, a nonsulfated form of this proteoglycan, also stimulated macrophage attachment. Labeled arterial lumican specifically bound to macrophages with high affinity. Flow cytometry demonstrated a high proportion of macrophages binding lumican. Lumican binding was inhibited by divalent cation-chelators and by polyanions. Inhibition and kinetics of lumican binding were distinct from interaction of macrophages with maleated bovine serum albumin, collagen, laminin, and fibronectin.

**Conclusions.** The highly sulfated KSPGs of cornea do not promote macrophage adhesion; however, the low-sulfate lumican present in pathologic corneas may act to localize macrophages in regions of inflammation. The lumican receptor differs from macrophage scavenger receptors and from receptors for several other extracellular matrix molecules. Invest Ophthalmol Vis Sci. 1997;38:1159-1167.

Corneal transparency is a direct result of the unique connective tissue of the corneal stroma. Molecular components of the stroma that distinguish it from other fibrous connective tissues can be identified in the collagen types that form the stromal collagen fibrils and the unusual proteoglycan composition of the matrix surrounding the collagen fibrils.1,2 Both of these components may be necessary for maintenance of the ultrastructure critical for transparency.3,4 Stromal proteoglycans are of particular interest in that they contain a unique class of molecules present only in the cornea. These compounds, known as the corneal keratan sulfate proteoglycans (KSPGs), constitute the majority of stromal proteoglycans. The KSPGs consist of three related proteins, each covalently linked to one to three keratan sulfate chains.3,5 Each of these proteins is found in a nonsulfated form in tissues other than the cornea, but only in the cornea do these proteins assume the character of proteoglycans.3,6-8 Along with dermatan sulfate, the keratan sulfate chains of the cornea provide the primary water-binding component of the stroma, against which the corneal endothelium works to maintain the hydration level required for corneal transparency.9 Alterations of the glycosaminoglycan composition of the cornea, such as occur...
in some heritable diseases, often result in loss of corneal transparency, thereby linking the water-binding function of keratan sulfate to transparency.\textsuperscript{10,11}

Three different proteins have been identified attached to keratan sulfate in corneal stroma. The cDNA sequences and primary structures of all three have been elucidated. Two of these proteins, lumican and keratocan, are closely related 37-kDa glycoproteins and share approximately 40% sequence identity at the amino acid level.\textsuperscript{7} The third KSPG protein recently was shown to be the product of a gene named osteoglycin, originally cloned from an osteogenic cell line. This 25-kDa protein shares structural features with lumican and keratocan but has less sequence identity.\textsuperscript{8} Features common among the KSPG proteins include six highly conserved cysteine residues and a series of leucine-rich repeats. These repeats are found in an increasing number of proteins and have been associated with a beta-pleated sheet secondary structure as well as a propensity toward protein-protein and protein-cell interactions.\textsuperscript{12-14} The high abundance of the KSPG proteins in the corneal stroma and the interactive character of their common structural elements suggest that, in addition to providing sites for keratan sulfate attachment, these proteins may contribute to the stromal function by interactions with other proteins or with cells. In fact, interaction of lumican protein with laminin and with corneal collagen has been observed previously.\textsuperscript{15,16} It seems likely that these proteins may also interact with cells in normal or pathologic corneal stroma.

Macrophages are inflammatory cells not normally present in the corneal stroma. In response to corneal damage and infection, macrophages accumulate rapidly in affected areas, occasionally initiating loss of corneal transparency and angiogenesis. Because of these severe potential consequences of stromal macrophage accumulation, we investigated the ability of corneal KSPGs to stimulate macrophage attachment. Our results show that the KSPG core protein, lumican, is recognized by receptors on macrophages and that keratan sulfate chains on the lumican protein interfere with interaction between lumican and this receptor.

METHODS

Materials

Bovine corneal KSPG, arterial lumican, and endo-\beta-galactosidase were prepared as described previously.\textsuperscript{6,17,18} Corneal lumican was obtained from corneal KSPG by treatment with endo-\beta-galactosidase followed by ion-exchange chromatography as described previously.\textsuperscript{5} Lumican was deglycosylated using trifluoromethanesulfonic acid as described previously\textsuperscript{6} or was treated with 0.4 U/ml of N-glycanase (Genzyme, Boston, MA) in 3 M urea, 0.1 M Tris–phosphate, pH 6.8, overnight at 37°C. Acid soluble bovine skin collagen was obtained from Sigma Chemical (St. Louis, MO). Mouse Engelbreth–Holm–Swarm tumor laminin and human plasma fibronectin were purchased from Becton–Dickinson (Bedford, MA). Maleated bovine serum albumin (mal-BSA) was prepared by direct treatment of BSA with excess maleic anhydride in 0.1 M sodium bicarbonate, pH 8.5.\textsuperscript{19}

Macrophages

The C3HeB/FeJ mice were obtained from Jackson Laboratory (Bar Harbor, ME) or were bred in the animal breeding facilities in the Division of Biology. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Macrophages were elicited in peritoneal exudate by intraperitoneal injection of 1.5 ml of thioglycolate broth and harvested 5 days later by washing the peritoneal cavity with 10 to 20 ml of ice cold phosphate-buffered saline. The cells were centrifuged, washed, and counted using a hemocytometer. Previous use of this procedure found that >90% of cells prepared in this manner were macrophages.\textsuperscript{20}

Cell Attachment Assay

Proteins were allowed to adsorb to the surface (0.16 cm\textsuperscript{2}) of Costar 96-well culture dishes at a concentration of 10 \mu g/ml in 50 \mu l of CMF (0.12 M sodium chloride, 10 mM potassium chloride, 10 mM sodium phosphate, 10 mM glucose, pH 7.2) overnight at 4°C. The surface then was rinsed three times with CMF and, when noted, the wells were treated additionally with 100 \mu g/ml collagen in CMF for 2 hours at room temperature to block nonspecific macrophage adsorption to the plastic.\textsuperscript{21} Macrophages were suspended in prewarmed serum-free Dulbecco’s modified Eagle’s nutrient mixture F-12 Ham (DME/F12) at a concentration of 5 \times 10\textsuperscript{5} cells/ml and 0.1 ml was transferred to each coated culture well. Attachment was carried out at 37°C in a humidified incubator with 5% carbon dioxide for the length of time noted for each experiment. In some experiments, phorbol-12-myristate-13-acetate (PMA) (Sigma) was included in the culture medium at a concentration of 50 ng/ml. The culture wells were washed three times with warmed medium using a multichannel pipette, and then the cells were fixed and stained for 2 hours at room temperature in 50 \mu l CMF containing 1% formaldehyde and 25 \mu g/ml of the fluorescent dye Dil (1,1’-diododecyl 3,3',3'-tetramethylindocarbocyanine; Molecular Probes, Eugene, OR).\textsuperscript{22} The fixed cells were covered with CMF containing 30% sucrose and stored at 4°C until analysis. The attached cells were photographed using a Zeiss fluorescence microscope equipped with a rhoda-
mine filter set. Each photographic field was centered on a culture well and included 80% of the surface area. Cells in each field were counted manually or the digitized photographs were analyzed using NIH Image software to determine the fraction of the total image covered by attached cells. Measurements from three to six fields were averaged for each determination. Differences between means were compared with the single-sided Student’s t-test using $P < 0.02$ (98% confidence) as a criterion for significance.

**Receptor Assay**

Arterial lumican in CMF was $^{125}$I-labeled to a specific activity of $3 \times 10^8$ cpm/μg using the insoluble catalyst IODOGEN (Pierce Chemical, Rockville IL) as described previously. Approximately 4.4 ng of labeled lumican in the presence of variable amounts of unlabeled lumican was incubated with $10^5$ macrophages in 0.3 ml of CMF containing 1 mg/ml BSA on ice for 2 hours. The cells were washed by centrifugation in the same buffer three times, and the radioactivity in cell pellets and supernatants was determined. The bound:free ratio was determined for each concentration point in triplicate and the arithmetic mean used for further calculations. In some assays, washing of macrophages was carried out in the presence of 2 mM ethylenediaminetetraacetic acid (EDTA), and incubation was carried out in the presence of EDTA and 5 mM calcium chloride. The results of these assays were similar to those carried out without chelators.

**Flow Cytometry**

Carbohydrate moieties on arterial lumican (250 μg) were oxidized with 10 mM periodic acid for 1 hour at room temperature in 100 μl of 0.1 M sodium acetate, pH 6, and 1 mM EDTA, followed by precipitation of the protein in 80% ethanol. The oxidized lumican then was dissolved in 50 μl CMF and reacted with 5 μl of fluorescein semithiocarbazide (Sigma) 100 mg/ml in dimethylsulfoxide at room temperature in the dark for 2 hours. The unreacted dye was removed by passage over a 1-ml column of Sephadex G50 in CMF, and the labeled lumican was diluted to 1 μg/μl in CMF. The fluorescein-lumican was incubated at a concentration of 60 nM (4 μg/ml) with $5 \times 10^5$ macrophages in 0.5 ml CMF containing 1 mg/ml BSA on ice for 3 hours. Unbound lumican was removed by washing the cells three times centrifugally, and then the cells were fixed in cold CMF with 3.7% formaldehyde. Fluorescence of 10⁴ individual cells was determined using a Becton-Dickinson (San Jose, CA) FACScan flow cytometer using fluorescein filters as described previously.

**RESULTS**

Preliminary experiments investigating the ability of corneal proteoglycans to promote cell attachment found that corneal KSPG exhibited antiadhesive properties for several cell types, but that a nonsulfated form of the KSPG protein lumican purified from artery supported rapid attachment and spreading of mouse peritoneal macrophages. As shown in Figure 1, after 30 minutes in serum-free medium, more macrophages adhered to surfaces coated with the nonsulfated lumican than those coated with collagen, fibronectin, or laminin. Sulfated (corneal) KSPG appeared to inhibit macrophage attachment. Microscopic examination of the cells attached under these conditions (Fig. 2) showed that macrophages attached to arterial lumican had spread on this surface (Fig. 2A), whereas the majority of cells attached to corneal KSPG remained rounded (Fig. 2B).

A time course of macrophage attachment, shown in Figure 3, revealed that macrophages eventually attach to surfaces coated with collagen, laminin, and corneal KSPG in numbers similar to their attachment to nonsulfated lumican. The primary difference in the cellular response to these substrata was the initial rate of attachment and spreading. Attachment to nonsulfated lumican was extremely rapid and reached a maximum value within 30 minutes (Fig. 3A), whereas maximal attachment to the other extracellular matrix components required 3 hours in the case of laminin (Fig. 3C) or 15 hours in the case of collagen or sulfated...
glycosylation might play in mediating macrophage attachment, we examined the effects of partial and complete deglycosylation of the lumican protein. Arterial lumican, a glycoprotein that contains essentially oligomeric nonsulfated keratan sulfate, was treated with endo-β-galactosidase to remove these oligomers or with N-glycanase to remove all glycosylation. As listed in Table 1, these treatments had no significant effect on the ability of this protein to induce macrophage attachment. Lumican, purified from corneal KSPG after removal of its keratan sulfate chains with endo-

FIGURE 2. Spreading of macrophages attached to lumican. Macrophages were allowed to attach and spread on arterial lumican (A) or corneal keratan sulfate proteoglycans (B), then fixed and stained with the fluorescent dye Dil, as described in Methods. Bar = 50 μm.

KSPG (Figs. 3B and 3C). Activation of the macrophages with the phorbol ester PMA (solid circles) had no significant effect on the attachment of the cells to nonsulfated lumican at 15 minutes, but showed a moderate stimulation (20% to 30%) beginning at 30 minutes. Macrophage attachment to collagen and corneal KSPG also was moderately stimulated by PMA activation at times >30 minutes incubation. As reported previously, macrophage attachment to laminin was strongly stimulated by PMA activation. 25

In addition to their attachment to lumican, macrophages also attach rapidly to uncoated tissue culture plastic (Fig. 1). We took advantage of the low initial rate of macrophage attachment to collagen to use this protein in short-term assays to block nonspecific cell attachment to plastic. Figure 4 presents the results of an assay in which plastic surfaces were coated with a range of concentrations of nonsulfated lumican followed by collagen blocking. Under these conditions, >90% of cell attachment was lumican dependent. Macrophages bound and spread only in wells treated with >3 μg/ml arterial lumican.

Both sulfated and nonsulfated forms of lumican are highly glycosylated. To address the role that this glycosylation might play in mediating macrophage attachment, we examined the effects of partial and complete deglycosylation of the lumican protein. Arterial lumican, a glycoprotein that contains essentially oligomeric nonsulfated keratan sulfate, was treated with endo-β-galactosidase to remove these oligomers or with N-glycanase to remove all glycosylation. As listed in Table 1, these treatments had no significant effect on the ability of this protein to induce macrophage attachment. Lumican, purified from corneal KSPG after removal of its keratan sulfate chains with endo-

FIGURE 3. Time course of macrophage attachment. Macrophages were incubated in culture wells coated with arterial lumican (A), corneal keratan sulfate proteoglycan (B), collagen (C), and laminin (D) in the presence of phorbol-12-myristate-13-acetate (solid circles) or with no additions (open circles) under the conditions similar to those described for Figure 1. Attached cells were fixed and counted as described in Methods. Tick marks on the ordinate represent 500 cells/mm², and error bars show the standard deviation of triplicates.

FIGURE 3. Time course of macrophage attachment. Macrophages were incubated in culture wells coated with arterial lumican (A), corneal keratan sulfate proteoglycan (B), collagen (C), and laminin (D) in the presence of phorbol-12-myristate-13-acetate (solid circles) or with no additions (open circles) under the conditions similar to those described for Figure 1. Attached cells were fixed and counted as described in Methods. Tick marks on the ordinate represent 500 cells/mm², and error bars show the standard deviation of triplicates.
FIGURE 4. Lumican-dependent macrophage attachment. Culture wells were coated overnight with arterial lumican at various concentrations and then blocked for 2 hours with collagen at 100 μg/ml. Macrophages were allowed to attach for 30 minutes and processed as described in Methods. The ordinate shows the percentage of the total area covered by attached cells. Error bars show the standard deviation of five measurements.

β-galactosidase, like arterial lumican, promoted macrophage attachment. Corneal lumican, fully deglycosylated using trifluoromethanesulfonic acid, also promoted cell attachment. These results show that macrophage surface receptors recognized the lumican protein both in glycosylated and deglycosylated forms but that presence of sulfated keratan sulfate chains blocked the interaction with the protein.

A large number of macrophage cell surface receptors have been identified, several of which can be grouped by biologic function. One such group potentially involved in lumican binding is that of the integrin proteins, a family of receptors that recognizes extracellular matrix molecules. Integrin–ligand interactions require divalent cations for activity and are inhibited by chelators such as EDTA. Scavenger receptors represent a family of divalent cation-independent macrophage cell surface proteins that interact with a broad range of ligands, including denatured proteins, dextran sulfate, and some proteoglycans.

In Figure 5, we examined conditions of macrophage attachment that could implicate participation of scavenger or integrin receptors in lumican binding. The ability of macrophages to bind arterial lumican was examined in the presence of EDTA or dextran sulfate. As seen in Figure 5, both of these agents completely blocked attachment of macrophages to lumican-coated surfaces. Attachment to fibronectin, a serum protein recognized by several integrin receptors, was

TABLE 1. Effect of Glycosylation on Macrophage Attachment to Lumican

<table>
<thead>
<tr>
<th>Protein</th>
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<th>Treatment</th>
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KSPG = keratan sulfate proteoglycans.
* Culture wells were coated with 10 μg/ml protein and blocked with collagen as in Figure 4. Macrophage attachment was assayed.
† Enzymatic deglycosylation was carried out with N-glycanase and chemical deglycosylation with trifluoromethanesulfonic acid.
completely blocked by EDTA but only moderately inhibited by dextran sulfate. Attachment to mal-BSA, a ligand for the macrophage scavenger receptor, was not reduced significantly in the presence of EDTA but was almost eliminated by dextran sulfate. The sensitivity to chelation of divalent cations clearly distinguishes the lumican receptor activity from that of the macrophage scavenger receptor. The sensitivity of lumican binding to dextran sulfate also suggests that different receptors are involved in binding fibronectin and lumican.

In Figure 6, we examined the affinity of soluble lumican in direct binding to macrophages. In the saturation analysis shown in Figure 6A, binding of $^{125}$I-labeled lumican to the cells was competed effectively with unlabeled lumican at concentrations greater than 2 nM (open circles). In the presence of EDTA (Fig. 6A, solid circles), little specific (competable) binding of $^{125}$I-Lumican could be shown. Analysis of these data using Scatchard plotting (Fig. 6B) shows a nonlinear curve, suggesting binding sites of both high and low affinities present on the macrophage surface. The initial slope of the plot extrapolates to a value of 1 to 2 nM (dotted line, Fig. 6B), suggesting a high-affinity binding constant in the low nanomolar range.

The cell attachment and lumican binding studies presented do not directly elucidate the proportion of the macrophage population that interacts with lumican. To examine the distribution of lumican receptor activity on individual cells within this population, macrophages were incubated with fluorescein-labeled lumican in the presence or absence of EDTA or with unlabeled lumican. The intensity of fluorescence of individual cells, determined using flow cytometry, is shown in Figure 7. In the presence of labeled lumican (Fig. 7A), an increase was observed in the fluorescence of a large proportion of the cells, indicating that many of the macrophages bound the fluorescent lumican. This binding was significantly reduced by the addition of unlabeled lumican (Fig. 7B) or by EDTA (Fig. 7C), showing that the interaction between fluorescein-labeled lumican and macrophages has a specificity of binding and a requirement for divalent cations similar to that observed in the previous interaction experiments.

**DISCUSSION**

The data presented here provide evidence that mouse peritoneal macrophages bind to lumican, a core protein of corneal KSPG. Nonsulfated forms of lumican adsorbed to plastic surfaces acted to stimulate rapid cell attachment and spreading. Additionally, in solution, lumican labeled with either radioactive iodine or with fluorescein, bound with high affinity to macrophages in a dose-dependent manner that could be competed with unlabeled lumican. These results indicate the presence of a lumican receptor on the cell surface of peritoneal macrophages.

The current results provide several pieces of information characterizing the specificity of the lumican receptor. The macrophage–lumican interaction was
More than two dozen cell surface receptors have been identified on macrophages. Properties of the lumican–macrophage interaction distinguish the lumican receptor from some of these previously characterized molecules. The requirement for divalent cations shows that the lumican receptor is distinct from the widely studied macrophage scavenger receptor. The scavenger receptor is a constitutive macrophage protein that binds a wide range of ligands, including denatured proteins, low-density lipoproteins, and some proteoglycans in a divalent ion-independent manner. We observed occasional low-affinity divalent cation-independent interactions with lumican that might have been the result of interactions with the scavenger receptor; however, it was clear that the high-affinity interactions with lumican were not related to the scavenger function of these cells.

Divalent cations are required by a number of known macrophage receptors, including the integrins, a widespread family of receptors that recognizes extracellular matrix molecules. Integrins are involved in cell interactions with laminin, collagen, and fibronectin. The data presented here suggest that macrophage interactions with each of these molecules differ significantly from interactions with lumican. Lumican binding was much more rapid than that to acid solvable, type I collagen (Fig. 3). This characteristic, in fact, was useful in using collagen as a blocking agent to examine properties of the specific binding of macrophages to lumican-coated plastic (Figs. 4 and 5, Table 1). Macrophage binding to laminin has been reported to be dependent on cellular activation, a finding confirmed in Figure 3. This property distinguishes the laminin–macrophage interaction from that with lumican, which is largely independent of cell activation. Binding of macrophages to fibronectin showed similar kinetics to lumican binding (data not shown); however, binding to lumican was strongly inhibited by polyanions such as dextran sulfate (Fig. 5), whereas binding to fibronectin showed only modest inhibition by dextran sulfate (Fig. 5). These results exclude the participation of several common integrin molecules as lumican receptors, but numerous other divalent ion-dependent receptors have been identified on macrophage surfaces, and it is likely others remain to be identified. More studies will be required for identification of the molecules that comprise the lumican receptor.

The mouse peritoneal macrophages used in this study display cell surface receptors characteristic of inflammatory macrophages found in other tissues, including corneal macrophages. Therefore, it seems likely that lumican receptor activity may be expressed in vivo in tissues in which lumican is located. In cornea, the accessibility of lumican to cells bearing this receptor may be modulated by the stromal kerato-
cytes. The receptor appears to interact with moieties in the lumican protein, as evidenced by the ability of fully deglycosylated lumican to stimulate macrophage attachment (Table 1). Nonsulfated glycosylation of this protein, such as is found in arterial lumican, did not appear to alter the lumican–macrophage interaction. Sulfated corneal KSPG, of which lumican represents a major protein component, however, was not bound by macrophages. This finding suggests that the presence of the keratan sulfate chains on the lumican protein blocks its interaction with the macrophage cell surface receptors. This idea is consistent with the inhibition of the macrophage–lumican interaction by other sulfated polymers such as dextran sulfate (Fig. 5) as well as the reported antiadhesive character of keratan sulfate. On the other hand, there is good reason to predict that the lumican protein participates in such an interaction. The leucine-rich repeats present in this and the other two KSPG proteins are known to be active in protein–protein interactions and have been found in both cell surface receptors and their ligands.

Lumican, as well as the other corneal KSPG proteins (keratocan and osteoglycin), are unique in terms of the variable nature of their state of glycosylation. In noncorneal tissues, these proteins exist as nonsulfated glycoproteins, whereas in the cornea, they are linked to long, highly sulfated keratan sulfate chains. Thus, in normal corneal tissue, the current data would predict that little macrophage–lumican interaction should occur. In pathologic conditions of the cornea, such as bacterial keratitis, bullous keratopathy, and in healing stromal wounds, expression of the highly sulfated keratan sulfate chains is reduced or eliminated, and lumican occurs in its nonsulfated glycoprotein form. In these conditions, our results would predict that corneal tissue would show an increased adhesiveness for macrophages based on interaction with the nonsulfated lumican in the extracellular matrix of these regions. The alteration in KSPG sulfation that occurs in pathologic corneas may therefore serve as a mechanism of localizing inflammatory cells in regions of the cornea experiencing pathologic changes.

Key Words
cornea, lumican, macrophage, proteoglycan, receptor

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References


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