Soluble heparin proteoglycans released from stimulated mast cells induce uptake of low density lipoproteins by macrophages via scavenger receptor-mediated phagocytosis

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Abstract Stimulation of rat serosal mast cells in vitro triggers exocytosis of secretory granules from their cytoplasm. Thereupon, the granules lose their perigranular membranes, and about 40% of the heparin proteoglycans and all of the chondroitin sulfate proteoglycans that they initially contained are released into the incubation medium. At physiologic ionic strength and calcium ion concentration, the solubilized heparin proteoglycans, but not the chondroitin sulfate proteoglycans, form insoluble complexes with the low density lipoproteins (LDL) present. We calculated that the heparin proteoglycans could bind approximately seven times their own mass (M_r about 1×10^6) of LDL cholesterol. Using gold-labeled LDL, we observed massive phagocytosis of the heparin proteoglycan–LDL complexes by cultured mouse macrophages in vitro, which was inhibited by cytochalasin B. Uptake of LDL by mouse macrophages was 45-fold higher in the presence of solubilized heparin proteoglycans than in their absence, and continued unabated over a 72-h period, indicating that the uptake process was not under negative feedback control. Excess amounts of acetyl-LDL or polyinosinic acid inhibited the uptake of these insoluble heparin proteoglycan–LDL complexes, indicating that their phagocytosis was mediated by scavenger receptors of the acetyl-LDL receptor type. The experiments reveal the following pathophysiologic mechanism relevant to atherogenesis: stimulated mast cells secrete soluble heparin proteoglycans capable of forming insoluble complexes with LDL and thereby trigger uptake of LDL by macrophages through scavenger receptor-mediated phagocytosis.—**Lindstedt, K.A., J.O. Kokkonen, and P.T. Kovanen.** Soluble heparin proteoglycans released from stimulated mast cells induce uptake of low density lipoproteins by macrophages via scavenger receptor-mediated phagocytosis. *J. Lipid Res.* 1992. **33:** 65–75.

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The cytoplasm of rat serosal mast cells is filled with specific organelles, the secretory granules. These granules are composed of a proteoglycan matrix in which are embedded the other components, such as histamine and neutral proteases (1). The proteoglycans of rat serosal mast cells consist mostly (about 90%) of heparin proteoglycans, the remaining 10% being composed of highly sulfated chondroitin sulfate proteoglycans (2).

On stimulation, rat serosal mast cells exocytose a fraction of their granules in a process called degranulation (1). On reaching the extracellular medium, the granules lose those components that are weakly associated with the proteoglycan matrix (e.g., histamine). In contrast, the neutral proteases chymase and carboxypeptidase A remain tightly bound to the proteoglycan matrix, forming granule "remnants" suspended in the medium.

In previous studies we explored the role of the mast cell granule remnants in the metabolism of LDL (3). These extracellularly located remnants were shown to interact with LDL by binding the apolipoprotein B component of LDL to their surfaces (4). Binding of LDL to the heparin proteoglycans of the granule remnants was found to be a critical step in a sequence of events in which the granule remnants mediate the formation of foam cells (5, 6). The specific mechanism by which macrophages take up LDL involves initial stimulation of mast cells with ensuing extrusion of their secretory granules, binding of LDL by the exocytosed granule remnants, and, finally, phagocytosis of the LDL-laden granule remnants by macrophages.

In this report we present data showing that a considerable fraction of the granule heparin proteoglycans is solubilized from the exocytosed granules. These heparin proteoglycans are able to interact with

Abbreviations: LDL, low density lipoproteins; Ac-LDL, acetyl-LDL; PBS, Dulbecco's phosphate-buffered saline; EBME, Eagle's basal medium with Earle's salts and 20 mM HEPES; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PIA, polyinosinic acid.

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LDL, forming insoluble complexes that are taken up by mouse peritoneal macrophages via a specific mechanism of receptor-mediated phagocytosis.

MATERIALS AND METHODS

Materials and animals

Sodium [^{125}I]iodide (13–17 mCi/μg), [1,2-^{3}H]cholesteryl linoleate (69–77 mCi/mg), [U-^{14}C]sucrose (> 350 Ci/mmol), sodium [^{35}S]sulfate (> 5 mCi/μg), [^{3}H]dextran (334 mCi/g, M_r 70,000), and [1-^{14}C]oleic acid (50–60 mCi/mmol) were purchased from Amersham International. Compound 48/80, heparin chondroitin sulfate B, heparinase, chondroitinase ABC, bovine serum albumin, polyinosinic acid, and soybean trypsin inhibitor were obtained from Sigma. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Alcian Blue 8 GS was obtained from Fluka. Triton X-100 was purchased from BDH Chemicals. Human serum albumin was obtained from Kabi Diagnostica. Eagle's basal medium with Earle's salts and 20 mM HEPES (EBME) was purchased from Flow Laboratories. Dulbecco's phosphate-buffered saline (PBS) was obtained from Gibco. Male Wistar rats (200–500 g) and female NMRI mice (25–35 g) were purchased from Orion (Espoo, Finland).

Preparation, labeling, and modifications of LDL

Human LDL (d 1.019–1.050 g/ml) was isolated from plasma by sequential ultracentrifugation as described (7). LDL was iodinated by the iodine monochloride method (8, 9) and labeled with [^{14}C]sucrose as described (10).

[^{3}H]Cholesteryl linoleate-LDL ([^{3}H]CL-LDL) was prepared as described (11). Colloidal gold particles (15 nm) were prepared and conjugated with LDL as described by Robenek, Schmitz, and Assmann (12). Transmission electron microscopy, with negative staining, showed that the gold-LDL preparations contained 3–5 LDL molecules per gold particle. Moreover, negative staining demonstrated the presence of monodisperse preparations in which individual complexes were separated from one another. The concentration of LDL is expressed in terms of its protein concentration. For experiments, labeled LDL was diluted with unlabeled LDL to give the specific activities indicated in the figure and table legends. Acetyl-LDL (Ac-LDL) was prepared by treatment of LDL with acetic anhydride (13).

Analysis of the material released from stimulated mast cells

Mast cells were isolated from rat peritoneal and pleural cavities as described (7). In the standard assay, 1–4 × 10^6 mast cells were incubated in 200 μl of buffer A (PBS containing 0.1 mg/ml of human serum albumin and 5.6 mM glucose). After preincubation at 37°C for 15 min, the cells were stimulated by addition of compound 48/80 (5 μg/ml), and incubation was continued for 15 min to allow completion of mast cell degranulation. The cells were then sedimented by centrifugation at 150 *g* for 10 min. The supernatant was removed and centrifuged at 12,000 *g* for 15 min to sediment the exocytosed mast cell granules (4). After centrifugation, the supernatant (12,000 *g*-supernatant, i.e., granule-free supernatant) was removed for analysis, and the 12,000 *g*-sediment (i.e., the granules) was washed once by resuspension in the above buffer and recentrifugation at 12,000 *g* for 5 min. The granule-free supernatant and the granules were then analyzed for their contents of Alcian Blue-reactive material (14), uronic acid (15), sulfate (16), and proteolytic enzymes, i.e., chymase and carboxypeptidase A (17). Before analysis the granules were dissolved by treatment with 2 M NaCl at room temperature for 30 min and dialyzed overnight against two changes of 5 l of distilled water. The granule-free supernatants were treated identically.

The relative concentrations of heparin and chondroitin sulfate glycosaminoglycans in the granule-free supernatant and in the granule fraction were determined by enzymatic digestions with heparinase and with chondroitinase ABC (18). The standard assay was conducted in 120 μl of buffer (50 mM Tris-chloride, pH 7.4, 1 mM calcium acetate, 100 μg/ml of trypsin inhibitor) containing the indicated amount of granule-free supernatant or granules and 17 U of heparinase or 0.05 U of chondroitinase ABC. Equal amounts (about 1 μg) of commercial heparin and chondroitin sulfate B were totally digested by heparinase and chondroitinase ABC, respectively, at these enzyme concentrations. After incubation at 37°C for 2 h, the material was dialyzed against distilled water and the content of Alcian Blue-reactive material was determined as described above. The concentration of heparin proteoglycans in the granule-free supernatant was determined by the Alcian Blue method, with commercial heparin as standard. The results were calculated by subtracting the proportion of chondroitin sulfate (30%) from the total amount of Alcian Blue-reactive material.

Analysis of the ^{35}S-labeled material released from stimulated mast cells

Mast cells were radiolabeled in vitro with sodium [^{35}S]sulfate as described (4), except that the cells were incubated in EBME containing 10 mg/ml of bovine serum albumin, 100 μg/ml of soybean trypsin inhibitor, and 100 international units/ml of penicillin (subsequently, this medium will be referred to as "cul-

ture medium") in a humidified incubator at 37°C for 5 h. After incubation, the cells were washed three times with PBS–glucose–albumin (buffer A). The radiolabeled mast cells were stimulated with compound 48/80 (5 μg/ml), and the granule-free supernatant was prepared as described above. NaCl was then added to give a final concentration of 2 M and incubation was continued at room temperature for 30 min. The granule-free supernatant was dialyzed against two changes of 5 l of distilled water, then against two changes of 5 l of 0.1 M Na_2SO_4, and finally against 5 l of distilled water to remove free [^{35}S] sulfate. After dialysis, the ^{35}S-labeled fractions were treated with 0.5 M NaOH (18) or with heparinase (4), and analyzed by gel filtration on a Sepharose 4B column. For this purpose, a 500-μl aliquot was applied to the column (1 × 60 cm) equilibrated with 2 M NaCl, and the column was eluted with 2 M NaCl at a flow rate of 3 ml/h at 4°C. Fractions of 1 ml were measured for ^{35}S radioactivity.

Analysis of the material released from membrane-covered granules upon treatment with Triton X-100

Granules with intact membranes were prepared essentially as described by Krüger, Lagunoff, and Wan (19). Purified mast cells ($5–10 \times 10^6$) were first radiolabeled in vitro with sodium [^{35}S]sulfate as described above, except that the incubation time was 18 h. After incubation the cells were washed three times with PBS containing 1.75 mg/ml of bovine serum albumin and 5.6 mM glucose, and resuspended in 500 μl of the above buffer. The cell suspension was sonicated in a Kerry water bath sonicator (Model Pul 125, Kerry Ultrasonics) at 4°C for 30 sec. Cellular debris was then sedimented by centrifugation at 150 *g* for 10 min at 4°C and the supernatant, containing the membrane-covered granules, was removed. Half of the supernatant was then added to 1 ml of the above buffer containing 0.1% (v/v) Triton X-100. The other half of the supernatant, which served as a control, was treated similarly, except that Triton X-100 was omitted. The samples were incubated at room temperature for 10 min, after which the granules were sedimented by centrifugation at 12,000 *g* for 15 min at room temperature. The supernatants were removed and the sedimented granules were resuspended in the above buffer to give the original volume. NaCl was then added to each sample to give a final concentration of 2 M. The amounts of ^{35}S-labeled material in the granule-free supernatant and in the granules were measured by gel filtration on a Sepharose 4B column, as described above. The material eluting in fractions 11–26 represented the ^{35}S-labeled heparin proteoglycans.

Sedimentation of LDL by soluble heparin proteoglycan

The standard sedimentation assay was conducted in 100 μl of culture medium or in 100 μl of buffer B (PBS supplemented with 10 mg/ml of bovine serum albumin and 5.6 mM glucose) containing the indicated concentrations of labeled or unlabeled LDL and of soluble heparin proteoglycans. After incubation at 37°C for 15 min, the amount of LDL sedimentable by heparin proteoglycans was determined by centrifugation at 12,000 *g* (4).

Incorporation of [^{14}C]oleate into cholesteryl esters and uptake of [^{14}C]sucrose-LDL by macrophages

Macrophages were harvested from unstimulated NMRI mice as described (6). Before experiments the dishes were washed twice with 2 ml of EBME and the medium was replaced with 1 ml of culture medium. In a standard assay, each dish received the indicated amounts of labeled or unlabeled LDL, soluble heparin proteoglycans, and [^{14}C]oleate-albumin to give a final oleate concentration of 200 μM. After incubation for the indicated times and lipid extraction in situ with hexane–isopropyl alcohol 3:2, the incorporation of [^{14}C]oleate into cholesteryl esters was determined by thin-layer chromatography (20). The uptake of [^{14}C]sucrose-LDL by macrophages was quantified as described previously (21).

Electron microscopic examination of uptake of gold-LDL by macrophages

To each macrophage monolayer was added 1 ml of culture medium containing 100 μg/ml of gold-labeled LDL and 4 μg/ml of soluble heparin proteoglycans. After incubation at 37°C for 1 h, the dishes were washed twice with 2 ml of PBS and the cells were scraped off the dishes with a rubber policeman. After centrifugation at 150 *g* for 5 min at 4°C, the cell pellets were fixed with 2.5% (v/v) glutaraldehyde for 60 min, postfixed with 2% (v/v) osmium tetroxide for 60 min, dehydrated, and embedded in LX-112 embedding medium (Ladd Research Industries). Ultramicrotome sections were stained with uranyl acetate and lead citrate, and viewed with a JEOL 100 CX electron microscope.

Other assays

Protein was determined by the procedure of Lowry et al. (22), with bovine serum albumin as standard.

RESULTS

Sedimentation of ^{125}I-labeled LDL with material exocytosed from mast cells

Rat serosal mast cells were stimulated in vitro with compound 48/80 to induce degranulation. The mast cells were then removed by centrifugation at 150 *g*, and the exocytosed granules were separated from the supernatant by low speed centrifugation (see Materials and Methods). To verify the absence of contaminating granules in the granule-free supernatant, the activities of chymase and carboxypeptidase A, the two major enzymes of the granules, were determined. These enzymes are known to be tightly bound to the heparin proteoglycan matrix of the granules, and can thus be used as granule markers (1). As shown in **Table 1** (left columns), no enzyme activities were found in the granule-free supernatant, showing that this fraction was not contaminated with granules.

To assess the ability of the granule-free supernatant and the granules to sediment LDL, ^{125}I-labeled LDL was incubated with the two fractions. After incubation, the reaction mixtures were centrifuged at 12,000 *g* for 15 min. In accordance with previous findings (4) mast cell granules were able to sediment LDL. Unexpectedly, incubation of LDL with the granule-free supernatant also rendered LDL sedimentable at low centrifugal forces. Of the total capacity of the exocytosed material to sediment LDL, half could be attributed to the granules and half to the granule-free supernatant (Table 1, right column).

Characterization of the exocytosed granule-free material capable of sedimenting LDL

The sedimentation of LDL after incubation with the granule-free supernatant demonstrated the presence in this fraction of material capable of interacting with LDL. Inasmuch as LDL is known to interact with the glycosaminoglycans of serosal mast cells (4), we checked for the presence of these substances in the granule-free supernatant by three independent methods (**Table 2**). About 50% of the Alcian Blue-reactive material and about 40% of both the uronic acid and the sulfate released upon stimulation of mast cells were found in the granule-free supernatant, confirming the presence of glycosaminoglycans in this fraction.

For further characterization of the glycosaminoglycans capable of interacting with LDL, the ratio of the heparin to the chondroitin sulfate glycosaminoglycans in the granule-free supernatant was determined after enzymatic digestion (**Table 3**). Of the glycosaminoglycans in the granule-free supernatant, about two-thirds were susceptible to heparinase treatment and about one-third to chondroitinase ABC treatment, indicating that two-thirds of the glycosaminoglycans were of heparin type and one-third of chondroitin sulfate type. In contrast, most of the glycosaminoglycans in the granule fraction were degraded by heparinase and were resistant to digestion by chondroitinase ABC, indicating that the remaining "granule remnant" glycosaminoglycans were of the heparin type.

To assess the relative contributions of the solubilized heparin and chondroitin sulfate glycosaminoglycans to the sedimentation of the LDL, the granule-free supernatant was incubated with LDL after treatment with heparinase or with chondroitinase ABC (**Table 4**). Treatment of the supernatant with heparinase totally abolished its ability to render LDL sedimentable.[2] In contrast, chondroitinase ABC treatment had no effect on LDL sedimentation. Therefore, the ability of the granule-free supernatant to sediment LDL resided in its heparin glycosaminoglycan fraction.

The molecular weight of the heparin proteoglycans in the granule-free supernatant was determined using mast cells radiolabeled in vitro with sodium [^{35}S]sulfate. In accordance with the findings of Katz et al. (2), we could demonstrate that chondroitin sulfate glycosaminoglycans in rat serosal mast cells did not incorporate [^{35}S]sulfate. The ^{35}S-labeled material in the granule-free supernatant, when analyzed by gel filtration on a Sepharose 4B column, was found to elute as a single broad major peak with an estimated average M_r of 1×10^6 (**Fig. 1A**). To identify the released ^{35}S-labeled material present in the granule-free supernatant as heparin proteoglycans, we tested its susceptibility to treatments with mild alkali and with heparinase (18). After treatment with 0.5 M NaOH, the ^{35}S-labeled material was reduced in size to an approximate M_r of 1×10^5, as judged by the elution position of the [^{3}H]dextran (M_r 70,000) (Fig. 1B). Thus, the ^{35}S-labeled material in the granule-free supernatant appeared to be a heparin proteoglycan composed of about ten labeled heparin glycosaminoglycan chains. After heparinase treatment all the ^{35}S-labeled material was degraded to low-molecular-weight fragments (Fig. 1C).

[2]The approximate concentration of chondroitin sulfate proteoglycans in the experiment described in Table 4 was 2.5 μg/ml. We further tested the ability of the mast cell-derived chondroitin sulfate proteoglycans to sediment LDL using concentrations up to 10 μg/ml. No sedimentation of LDL was observed.

TABLE 1. Sedimentation of ^{125}I-labeled LDL by the material released from stimulated mast cells

Fraction	Enzyme Activities		^{125}I-Labeled LDL Sedimented
	Chymase	Carboxypeptidase A	
	$\Delta A_{256} \times 10^2$/min/$10^6$ mast cells	$\Delta A_{254} \times 10^3$/min/$10^6$ mast cells	$\mu g/10^6$ mast cells
150 *g* Supernatant	13.7 (100)	21.8 (100)	3.89 (100)
Granule-free supernatant	< 0.1 (< 1)	< 0.2 (< 1)	1.81 (48)
Granules	14.5 (106)	22.8 (105)	1.79 (46)

Purified mast cells (4×10^6) were preincubated in 200 µl buffer A at 37°C for 15 min. The cells were stimulated with compound 48/80 (5 µg/ml), and incubation was continued for 15 min to allow complete mast cell degranulation. The cells were then sedimented by centrifugation at 150 *g* for 10 min to yield the 150 *g* supernatant. From the 150 *g* supernatant, a granule-free supernatant and granules were prepared as described in Materials and Methods. The activities of chymase and carboxypeptidase A in each fraction were assayed as described in Materials and Methods. Each LDL sedimentation assay contained 100 µg/ml of ^{125}I-labeled LDL (40 cpm/ng) and a 10-µl aliquot of one or the other of the above fractions in 100 µl buffer B. After incubation at 37°C for 15 min, the amounts of ^{125}I-labeled LDL sedimented were determined. Each value is a mean of duplicate incubations. Values in parentheses represent percentages of control values.

Origin of the heparin proteoglycans in the granule-free supernatant capable of sedimenting LDL

The elution profile found for the released ^{35}S-labeled heparin proteoglycans is in good agreement with the results previously obtained for the heparin proteoglycans (average M_r 1×10^6) found in intact granules of rat serosal mast cells by Yurt and coworkers (18). We could demonstrate that the increase in the amount of proteoglycans in the granule-free supernatant closely paralleled the degree of mast cell degranulation, measured as histamine release, which ranged from 7% to 50%. Also this finding suggests that the soluble proteoglycans were derived from the exocytosed mast cell granules. To demonstrate unequivocally that the heparin proteoglycans in the granule-free medium were derived from the granules, we prepared ^{35}S-labeled granules with intact perigranular membranes (see Materials and Methods). The process of exocytosis was mimicked by treating the membrane-covered granules with Triton X-100, which selectively strips off their membranes (19). After this treatment, the amount of ^{35}S-labeled heparin proteoglycans present in the granule-free supernatant was determined by gel filtration on a Sepharose 4B column as described above (see Fig. 1). In preparations of granules with intact membranes, 92% (104,000 dpm) of the total labeled heparin proteoglycan was found in the granules and 8% (9200 dpm) in the granule-free supernatant (**Table 5**). Triton X-100 treatment of the granules resulted in complete loss of the perigranular membranes, as shown by the complete release of histamine from the granules (data not shown). Loss of the perigranular membrane led to a fivefold increase in the amount of heparin proteoglycans in the granule-free supernatant, the proportion of the ^{35}S-labeled heparin proteoglycans in the granule-free supernatant being 43% (49,000 dpm). Repeated treatment of the granules with Triton X-100 did not result in any further loss of heparin proteoglycans. Moreover, no chymase activity was released from the granules upon treatment with Triton X-100 (Table 5), indicating that treatment of granules with the detergent did not lead to solubilization of the residual matrix of the formed granule remnants. Thus, intact rat serosal mast cell granules contain a distinct fraction (about 40%) of the heparin proteoglycans that dissolves from the granules upon loss of the perigranular membrane. This fraction of heparin proteoglycans will be referred to as the "soluble heparin proteoglycans."

LDL binding characteristics of the soluble heparin proteoglycans

To explore the LDL binding characteristics of the soluble heparin proteoglycans, ^{125}I-labeled LDL was incubated in the presence of increasing concentrations of the granule-free supernatant. As in previous experiments, the incubation conditions were kept physiologic, i.e., under physiologic ionic strength and concentration of $CaCl_2$. After incubation at 37°C for 15 min, the reaction mixtures were centrifuged at 12,000 *g* and the amounts of ^{125}I-labeled LDL were then measured (**Fig. 2A**). At this low-speed centrifugation no sedimentation of ^{125}I-labeled LDL occurred in the presence of the granule-free supernatant unless the heparin concentration was more than 2 µg/ml. Above this critical concentration the amount of sedimentable ^{125}I-labeled LDL increased linearly, indicating progressive formation of insoluble complexes between LDL and the soluble heparin proteoglycans.

To study the stoichiometry of the sedimentation of heparin proteoglycans with LDL, the experiment was repeated using [^{35}S]sulfate-labeled soluble heparin proteoglycans and an LDL preparation labeled with [^{3}H]cholesteryl linoleate (11). It can be seen from Fig. 2B that LDL and heparin proteoglycans sedi-

TABLE 2. Release of Alcian Blue-reactive material, uronic acid, and sulfate from stimulated mast cells

Fraction	Alcian Blue-Reactive Material	Uronic Acid	Sulfate
	$A_{678}/10^6$ mast cells	*μg/10^6 mast cells*	
150 *g* Supernatant	0.90 (100)	2.96 (100)	3.72 (100)
Granule-free supernatant	0.43 (48)	1.26 (43)	1.56 (42)
Granules	0.59 (66)	1.84 (62)	2.50 (67)

Mast cells were stimulated and the material released was fractionated as described in the legend to Table 1. The contents of Alcian Blue-reactive material, uronic acid, and sulfate in each fraction were determined as described in Materials and Methods. Each value is a mean of duplicate incubations. Values in parentheses represent percentages of control values.

mented in parallel. From the specific activities of [^{3}H]cholesteryl linoleate-labeled LDL and [^{35}S]sulfate-labeled heparin proteoglycans, it could be calculated that heparin proteoglycans were able to bind approximately seven times their own mass of LDL cholesterol.[3]

Ability of soluble heparin proteoglycans to promote uptake of LDL by macrophages

The significance of the interaction between LDL and soluble mast cell-derived heparin proteoglycans, in terms of cellular metabolism of LDL, was tested by measuring the uptake of LDL by cultured mouse macrophages in vitro. For this purpose, increasing amounts of the granule-free supernatant were added to macrophage monolayers in an incubation medium containing LDL. As an index of LDL uptake we measured the incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate in the macrophages, an intracellular process known to correlate with the amount of cholesterol entering the cell (23). The results showed that the synthesis of cholesteryl esters in the macrophages closely followed the formation of insoluble complexes of LDL and heparin proteoglycans (**Fig. 3** with inset). Thus, at low heparin proteoglycan concentrations, neither complex formation nor stimulation of cholesteryl ester synthesis was observed, and more than 2 μg/ml of soluble heparin proteoglycans was required for each process to occur. At the highest heparin proteoglycan concentration used (12 μg/ml), the rate of LDL uptake by macrophages was 45-fold that observed in the absence of the proteoglycans. These observations indicate that the soluble heparin proteoglycans promote uptake of LDL by cultured mouse macrophages, provided insoluble complexes are formed between them and LDL.

TABLE 3. Content of heparin and chondroitin sulfate glycosaminoglycans in the material released from stimulated mast cells

Treatment	Alcian Blue-Reactive Material	
	Granule-Free Supernatant	Granules
	A_{678}/fraction	
None	0.12 (100)	0.15 (100)
Heparinase	0.04 (35)	0.01 (6)
Chondroitinase ABC	0.09 (79)	0.16 (106)

Mast cells were stimulated and granule-free supernatant and granules were prepared as described in the legend to Table 1. Aliquots of each fraction corresponding to material released from 0.3 × 10^6 mast cells were treated with heparinase or with chondroitinase ABC, after which the contents of Alcian Blue-reactive material in each fraction were determined as described in Materials and Methods. Each value is a mean of duplicate incubations. Values in parentheses represent percentages of control values.

[3]In LDL, 1 ng cholesteryl linoleate corresponds to approximately 2.4 ng total cholesterol.

Mechanism of uptake of LDL-heparin proteoglycan complexes by cultured mouse macrophages

Uptake of LDL-heparin proteoglycan complexes by macrophages was visualized by using gold-labeled LDL. This was done by incubating gold-labeled LDL with macrophage monolayers in the presence of the granule-free supernatant for 60 min and then preparing the dishes for transmission electron microscopy.

TABLE 4. Sedimentation of ^{125}I-labeled LDL by heparin and chondroitin sulfate glycosaminoglycans in the granule-free supernatant

Treatment	^{125}I-Labeled LDL Sedimented
	ng/assay
None	234
Heparinase	< 1
Chondroitinase ABC	212

Mast cells were stimulated and the granule-free supernatant was prepared as described in the legend to Table 1. Aliquots of the granule-free supernatant corresponding to material released from 0.3 × 10^6 mast cells were treated with heparinase or chondroitinase ABC, as described in Materials and Methods. Each LDL sedimentation assay contained 200 μg/ml of ^{125}I-labeled LDL (34 cpm/ng) and the enzyme-treated granule-free supernatant in 100 μl buffer B. After incubation at 37°C for 15 min, the amounts of ^{125}I-labeled LDL sedimented were determined. Each value is a mean of duplicate incubations.

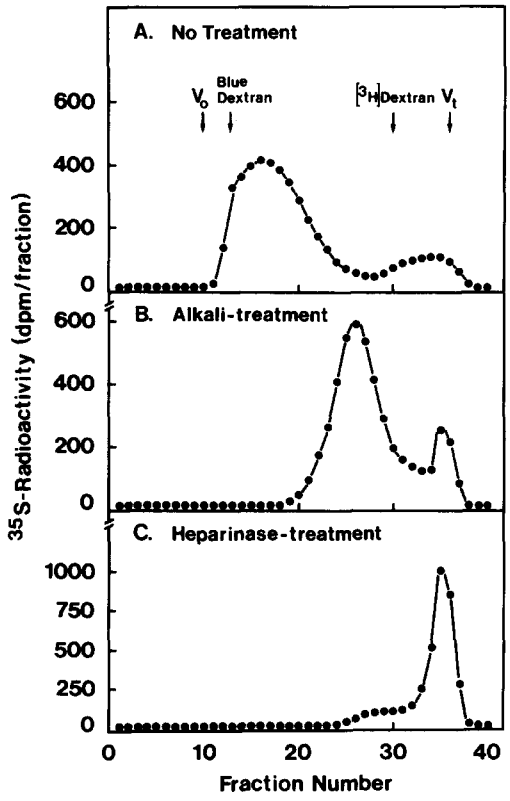

Fig. 1. Sepharose 4B analysis of the ^{35}S-labeled material released into the granule-free supernatant from stimulated mast cells. Analysis of the material without treatment (A) and after treatment with alkali (B) or heparinase (C). Mast cells were radiolabeled with sodium [^{35}S]sulfate and stimulated with compound 48/80. The ^{35}S-labeled material in the granule-free supernatant was treated with 0.5 M NaOH or with heparinase, and a 500-μl aliquot (4800 dpm) was applied to a Sepharose 4B column (1 × 60 cm) equilibrated with 2 M NaCl. The column was eluted with 2 M NaCl at a flow rate of 3 ml/h at 4°C. Fractions of 1 ml were measured for their ^{35}S radioactivity. The void volume of the column (V_o), the total volume of the column (V_t), and the elution positions of Blue Dextran (M_r 2×10^6) and [^{3}H]dextran (M_r 70,000) are indicated by arrows.

Fig. 4 shows a macrophage after massive phagocytosis of gold-labeled LDL. No phagocytosis or aggregation of gold-labeled LDL could be seen in control experiments performed without the granule-free supernatant (not shown). Moreover, it was found that cytochalasin B (10 μg/ml), a known inhibitor of phagocytosis (24), totally abolished the heparin proteoglycan-mediated uptake of LDL by macrophages (data not shown).

Finally, regulation of the heparin proteoglycan-mediated uptake of LDL by cultured mouse macrophages was investigated. As shown in **Fig. 5**, uptake of LDL-heparin proteoglycan complexes continued unabated over a period of 72 h, indicating failure of down-regulation, i.e., unregulated uptake of LDL. Since unregulated uptake of LDL by cultured mouse macrophages in vitro is known to be mediated by "scavenger receptors," we measured the ability of known inhibitors of this process to prevent the uptake of LDL-heparin proteoglycan complexes by macrophages. As shown in **Table 6**, a 10-fold excess of acetyl LDL, or a concentration of 0.1 mg/ml of polyinosinic acid, were both able to inhibit the uptake of LDL-heparin proteoglycan complexes, indicating that proteoglycan-induced uptake of LDL depends on scavenger receptors, and more specifically, of the acetyl-LDL receptor type (25, 26). A control experiment showed that neither inhibitor had any effect on the formation of insoluble complexes between LDL and soluble heparin proteoglycans.

DISCUSSION

The present studies reveal that the secretory granules of rat serosal mast cells contain two fractions of heparin proteoglycans, one of which (the soluble fraction) is released from the granules when the perigranular membrane is lost at exocytosis, while the other remains tightly bound to the granule remnants. These experiments did not reveal the exact mechanism by which the soluble fraction was released from the granules. However, neither chymase nor car-

TABLE 5. Release of soluble heparin proteoglycans and chymase from membrane-covered granules on treatment with Triton X-100

Treatment	^{35}S-Labeled Heparin Proteoglycans		Chymase	
	Granule-Free Supernatant	Granules	Granule-Free Supernatant	Granules
	dpm × 10^{-3}		Δ A_{256} × 10^3/*min*	
None	9.2	104	< 0.5	38.4
Triton X-100	49	64	< 0.5	40.8

Membrane-covered granules were prepared from ^{35}S-labeled mast cells (7×10^6) and the release of soluble [^{35}S]heparin proteoglycans from the ^{35}S-labeled granules on treatment with Triton X-100 was estimated by gel filtration on a Sepharose 4B column as described in Materials and Methods. The material eluting in fractions 11–26 represented the [^{35}S]heparin proteoglycans (see Fig. 1A). The amounts of chymase released from membrane-covered granules were determined as described in Materials and Methods.

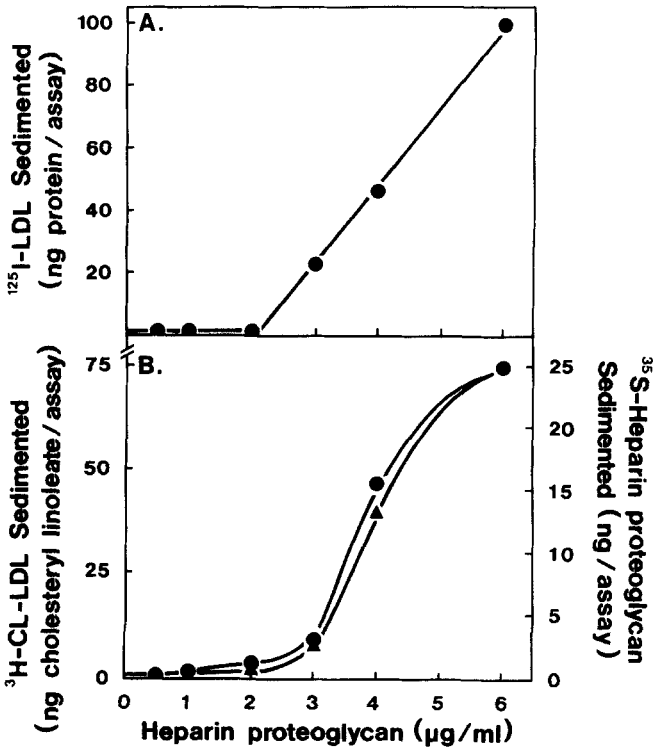

Fig. 2. Co-sedimentation of LDL and heparin proteoglycan as a function of the concentration of the soluble heparin proteoglycans. A. Each reaction mixture contained 200 μg/ml of ^{125}I-labeled LDL (16 cpm/ng) and the indicated concentration of soluble heparin proteoglycans in 100 μl of buffer B. After incubation at 37°C for 15 min, the amounts of ^{125}I-labeled LDL sedimented were determined. B. The experiment described above was repeated, using ^{35}S-labeled heparin proteoglycans and [^{3}H]CL-LDL ([^{3}H]cholesteryl linoleate-labeled LDL). Each reaction mixture contained 200 μg/ml of [^{3}H]CL-LDL as protein corresponding to 160 μg/ml of cholesteryl linoleate (22,500 dpm/μg of cholesteryl linoleate) and the indicated concentration of soluble heparin proteoglycans in 100 μl of buffer B. After incubation at 37°C for 15 min, the amounts of [^{35}S]heparin proteoglycans (▲) and [^{3}H]CL-LDL (●) sedimented were determined.

boxypeptidase A was released from the granules, which strongly suggests that the soluble fraction already exists as a separate pool within the intact membrane-covered granules.

The solubilized heparin proteoglycans were capable of interacting with LDL to form insoluble complexes, i.e., they rendered LDL sedimentable at low-speed centrifugation. Contrary to most studies on the formation of insoluble complexes between LDL and proteoglycans (27–34), the current studies were conducted in a medium having both physiologic ionic strength and physiologic concentrations of divalent cations. As in the case of the association of LDL with synthetic and biologic polyanions (35), ionic strength profoundly influenced the interaction between LDL and the mast cell-derived heparin proteoglycans. Thus, even a slight increase in ionic strength above physiologic level (170 mM NaCl) prevented the formation of insoluble complexes. Moreover, lowering of ionic strength (10 mM NaCl) increased the formation of insoluble complexes by 10-fold. Moreover, maximal formation of insoluble complexes between LDL and proteoglycans from bovine (27) or human (34) aorta has been shown to require a high concentration of calcium chloride (30 mM). Thus, at physiologic ionic strength or normal calcium chloride levels, the formation of insoluble complexes between LDL and various types of proteoglycans is far from maximal. We suggest that formation of insoluble complexes between LDL and the mast cell-derived soluble heparin proteoglycans, as shown to occur under physiologic conditions in vitro, could conceivably occur in vivo when mast cells are stimulated to secrete soluble heparin proteoglycans into their extracellular microenvironment. Further studies are needed, however, since we do not know whether the proteins present in the extracellular fluid, which also have affinity for heparin, would inhibit the formation of these insoluble complexes.

The mode of uptake of the complexes of LDL and heparin proteoglycans was found to be phagocytosis. This conclusion is based on the findings that uptake was inhibited by cytochalasin B and, when viewed by

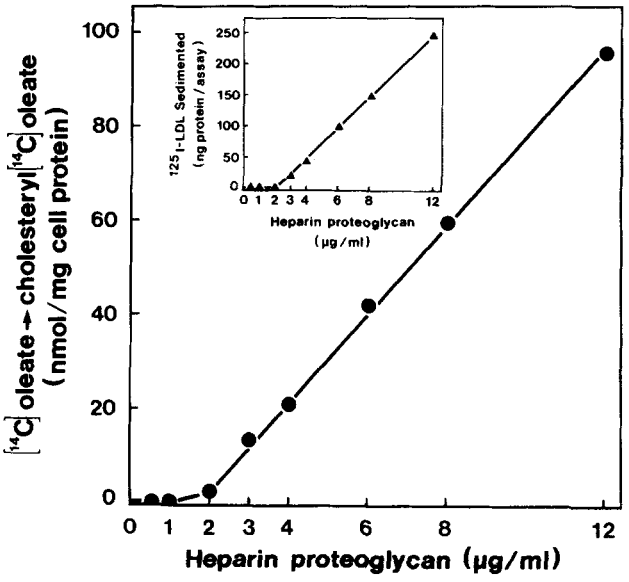

Fig. 3. Formation of cholesteryl esters in macrophages incubated with soluble heparin proteoglycans and LDL. To each macrophage monolayer was added 1 ml of culture medium containing 100 μg/ml of LDL, 200 μM [^{14}C]oleate-albumin (9700 dpm/nmol), and the indicated concentrations of soluble heparin proteoglycans. After incubation at 37°C for 18 h, the cellular content of cholesteryl [^{14}C]oleate was determined. A value of [^{14}C]oleate incorporated into cholesteryl esters (2.2 nmol/mg protein) in macrophage monolayers incubated with LDL alone has been subtracted from the values obtained with LDL and heparin proteoglycans. The inset shows the results of a sedimentation experiment conducted as described in the legend to Fig. 2A, except that the assay contained 100 μg/ml of ^{125}I-labeled LDL (87 cpm/ng) in culture medium.

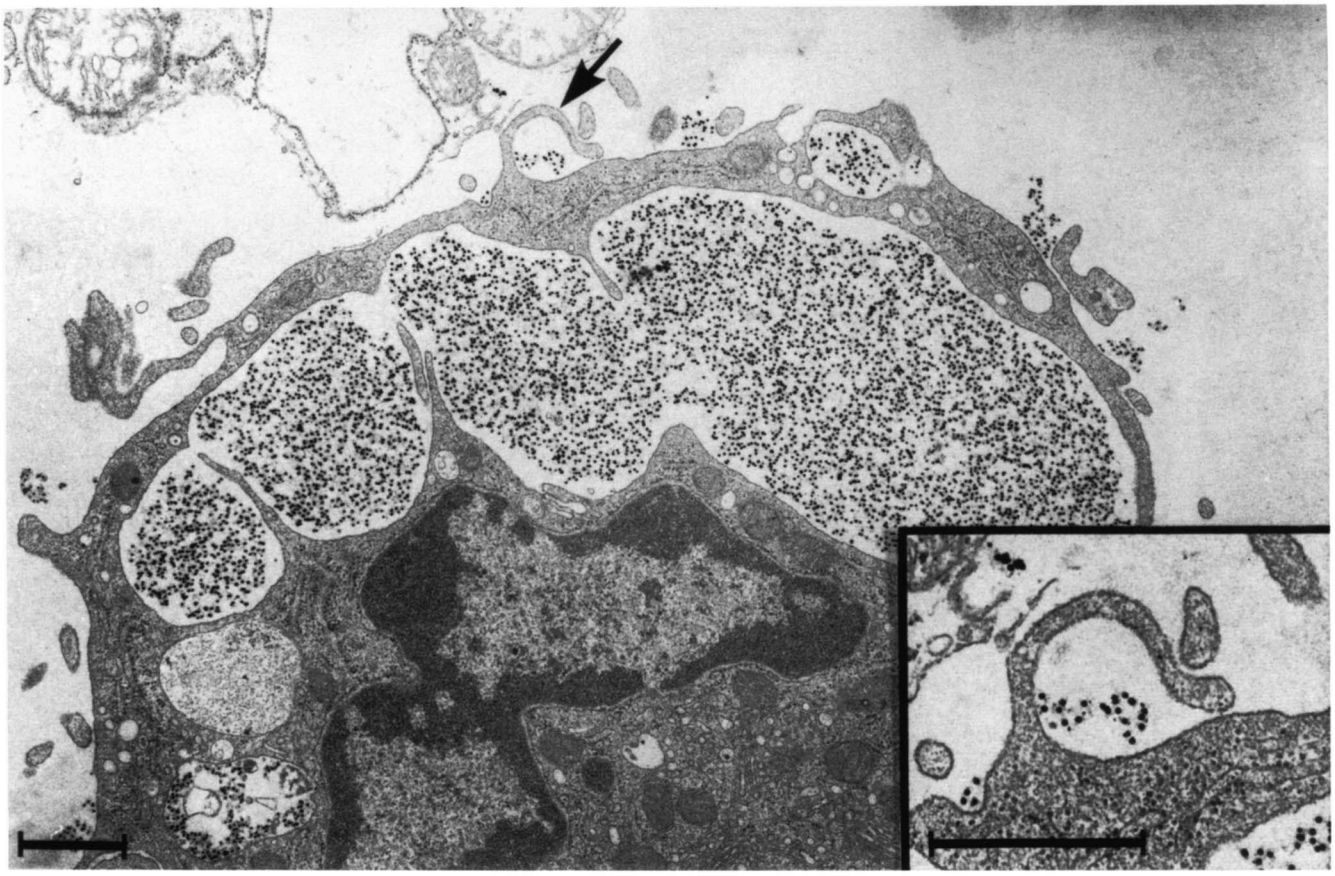

Fig. 4. Phagocytosis by a macrophage of the gold-labeled LDL-heparin proteoglycan complexes. To each macrophage monolayer was added 1 ml of culture medium containing 4 μg/ml of soluble heparin proteoglycans and 100 μg/ml of gold-labeled LDL. After incubation at 37°C for 1 h, the cells were prepared for transmission electron microscopy. The arrow points to the phagocytosis of a small gold-labeled LDL-heparin proteoglycan aggregate (see inset); bar = 1 μm.

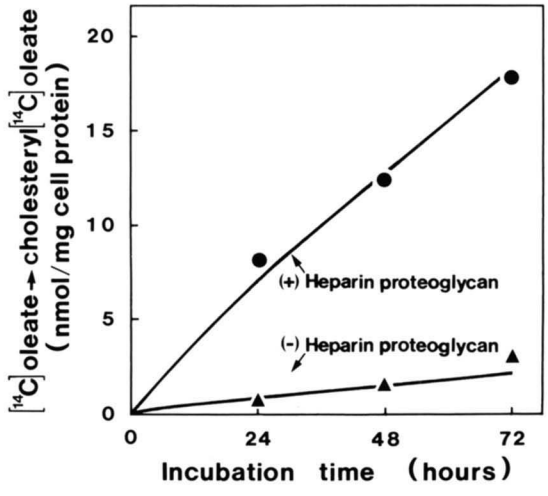

Fig. 5. Unregulated LDL uptake by macrophages induced by soluble heparin proteoglycans. To each macrophage monolayer was added 1 ml of culture medium containing 100 μg/ml of LDL, 200 μM [^{14}C]oleate-albumin (15,100 dpm/nmol), and 3 μg/ml of soluble heparin proteoglycans (●). Every 24 h each monolayer received additional LDL (100 μg/ml) and heparin proteoglycans (3 μg/ml). In the control experiment no heparin proteoglycans were added (▲). After incubation at 37°C for the indicated times, the cellular content of cholesteryl [^{14}C]oleate was determined.

electron microscopy, the ingested material appeared in typical phagosomes (see Fig. 4). These findings agree with results obtained with LDL complexed with arterial proteoglycans (33). Furthermore, LDL aggregated by vortexing (36) or by treatment with phospholipase C (37) or with 4-hydroxynonenal (38) are ingested by macrophages via phagocytosis.

The scavenger receptors on cultured mouse macrophages appear to mediate phagocytosis of the complexes between LDL and soluble mast cell heparin proteoglycans. This is shown, for example, by the observation that the uptake process of the LDL-heparin proteoglycan complexes continued at a constant rate over a period of 72 h, indicating failure of negative feedback regulation, a finding typical for uptake processes mediated by scavenger receptors (39). Recently, the scavenger receptor-mediated uptake of molecules by macrophages has been shown to be heterogeneous (40). In addition to the classical acetyl-LDL receptor (23), the presence of other types of scavenger receptors has been demonstrated (25, 26). In the present study, the binding site on the macrophage surface responsible for the recognition of the

TABLE 6. Scavenger receptor-mediated uptake by macrophages of the LDL-heparin proteoglycan complexes

Addition	[^{14}C]Sucrose-LDL Uptake by Macrophages
	μg/mg cell protein
LDL + heparin proteoglycan	15.8 (100)
LDL + heparin proteoglycan + Ac-LDL	0.2 (1)
LDL + heparin proteoglycan + PIA	3.4 (22)

Each macrophage monolayer received 1 ml of culture medium containing 100 μg/ml of [^{14}C]sucrose-LDL (3800 dpm/μg), 4 μg/ml of soluble heparin proteoglycans, and 1 mg/ml of Ac-LDL or 100 μg/ml of polyinosinic acid (PIA). After incubation at 37°C for 18 h, the cellular content of [^{14}C]sucrose-LDL was determined as described in Materials and Methods. A value of [^{14}C]sucrose-LDL taken up (6.4 μg/mg protein) by macrophage monolayers incubated with LDL alone has been subtracted from the values obtained with LDL and heparin proteoglycan. Each value is a mean of duplicate incubations. Values in parentheses represent percentages of control values.

LDL-heparin proteoglycan complexes appeared to be the acetyl-LDL binding site. During uptake by macrophages, acetyl-LDL competed fully with the LDL-heparin proteoglycan complexes for binding to the cell surface. Moreover, polyinosinic acid, a compound reported to inhibit the interaction of acetyl-LDL with its macrophage binding site (41), was found to inhibit uptake of the LDL-heparin proteoglycan complexes. These findings contrast with those obtained with LDL complexed with arterial proteoglycans. Such complexes appear to be recognized partly by scavenger receptors other than the classical acetyl-LDL receptor (30, 42).

Using radioactively labeled heparin proteoglycans, we found that the rates of uptake and degradation of the proteoglycans by cultured mouse macrophages were not influenced by the formation of complexes with LDL (data not shown). Thus, it appears that the uptake process is not specific for the complexes, but that the LDL in these complexes is ingested by the already operative process of proteoglycan uptake, which is mediated by the acetyl-LDL receptors.

We have previously shown that the exocytosed granule remnants induce uptake of LDL by cultured mouse macrophages (5). They do this by carrying LDL into the macrophages on being themselves phagocytosed. Thus, it appears that after mast cell stimulation, two heparin proteoglycan fractions are formed, one remaining in the insoluble granule remnants and the other being released from the granules, both of which are capable of carrying LDL into the macrophages. We calculate from the in vitro data that, of the total capacity of rat serosal mast cells to carry LDL into macrophages, about half is accounted for by the granule remnants and half by the soluble heparin proteoglycans. Therefore, it is conceivable that the newly described soluble heparin proteoglycan fraction makes a substantial contribution to the capacity of stimulated mast cells to induce uptake of LDL by macrophages, a cellular process associated with the development of atherosclerosis.

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