Heparin Proteoglycans Released From Rat Serosal Mast Cells Inhibit Proliferation of Rat Aortic Smooth Muscle Cells in Culture

Yenfeng Wang, Petri T. Kovanen

Abstract—Mast cells are present in the human arterial intima. To study whether mast-cell degranulation influences the rate of proliferation of smooth muscle cells, we cocultured sensitized (IgE-bearing) rat serosal mast cells and rat aortic smooth muscle cells (SMCs). When sensitized mast cells were stimulated to degranulate with antigen, the rate of proliferation of the cocultured SMCs decreased sharply. This inhibitory effect was found to be due mainly to the very high molecular weight (\(M_r\)) heparin proteoglycans (average \(M_r\) 750 000) released from the stimulated mast cells. When the heparin proteoglycans were purified from mast-cell granule remnants and added to the SMC culture, they were found to block the cell cycle at the \(G_0\)\(\rightarrow\)S transition and the exit from the \(G_2\)/M phase, their inhibitory effect resembling that of commercial heparin. However, in contrast to the reported dependence of the inhibitory effect of commercial heparin on the release of transforming growth factor-\(\beta\) from serum, the inhibitory effect of the mast cell–derived heparin proteoglycans in the presence of serum was not transforming growth factor-\(\beta\) dependent. Moreover, the effect of the mast cell–derived heparin proteoglycans was more efficient than that of commercial heparins of high (average \(M_r\) 15 000) and low (average \(M_r\) 5000) molecular weight. We also purified heparin glycosaminoglycans (average \(M_r\) 75 000) from the mast cell–derived heparin proteoglycans and found that they also inhibited SMC growth efficiently, although less strongly than their parent heparin proteoglycans. These results reveal, for the first time, that mast cells are able to regulate SMC growth. Thus, activated mast cells, by releasing heparin proteoglycans, possibly participate in the regulation of SMC growth in the human arterial intima, the site of atherogenesis. (Circ Res. 1999;84:74-83.)

Key Words: atherosclerosis ● heparin proteoglycan ● mast cell ● proliferation ● smooth muscle cell

Accumulation of smooth muscle cells (SMCs) in the human arterial intima has been recognized as an important feature of atherosclerosis.1,2 Especially during the late stages of atherogenesis, the SMCs, which originate either in the intima or in the underlying media, may undergo abnormally regulated proliferation.3–5 However, compared with the SMCs in restenotic lesions, the rate of proliferation of the SMCs in atherosclerotic lesions is low. Accordingly, as reported recently, the fraction of the SMCs that bear markers of ongoing cell proliferation in advanced human atherosclerotic lesions is 3.6% (versus 15.2% in restenotic lesions), when detected with an antibody against the proliferating cell nuclear antigen, or 7.2% (versus 20% in restenotic lesions), when the mRNA of proliferating cell nuclear antigen is detected with an antisense probe.5 Such a relatively low rate of growth, together with the observation that a fraction of the SMCs in the lesions undergo apoptotic cell death,6 is consistent with the clinical observation that atherosclerosis usually takes several decades to cause clinically significant obstruction of the arteries involved. Similarly, angiographic and ultrasound studies have shown that plaques, once established, often remain unchanged over several years of observation.7,8

The low rate of SMC growth in atherosclerotic lesions likely results from the net effect of growth-stimulating and growth-inhibiting factors acting locally on the SMCs. Well-known stimulating factors include platelet-derived growth factor, basic fibroblast growth factor, epidermal growth factor, and interleukin-1.9 On the other hand, heparan sulfate proteoglycan secreted by endothelial cells has been said to be an important inhibitor of SMC growth,10,11 although recently this opinion has been challenged.12 Finally, transforming growth factor-\(\beta\) (TGF-\(\beta\)) exerts stimulatory or inhibitory effects on the SMCs, depending on the subtype of TGF-\(\beta\) receptor expressed by these cells.13

The human arterial intima also contains mast cells. In fatty streaks and in atheromas, the number of mast cells is increased,14,15 and in atherosclerotic lesions a substantial fraction of these cells are seen to be degranulated, reflecting their ongoing stimulation.15,16 We previously studied the interactions between rat serosal mast cells and rabbit aortic SMCs in vitro and found that, when stimulated, the mast cells induced intracellular accumulation of LDL cholesterol in the SMCs.17 On stimulation, rat serosal mast cells exocytose their...
cytoplasmic secretory granules and an array of soluble mediators of inflammation, including preformed mediators such as histamine, tumor necrosis factor-α, TGF-β, and newly formed mediators such as prostaglandin D2 and leukotriene C4.18 The main components of the granules are heparin proteoglycans, a fraction of which, after exocytosis, become solubilized and are released into the extracellular fluid. The residues, known as “granule remnants,” are globular particles (0.5 to 1.0 μm in diameter) composed solely of proteoglycans of the heparin type to which are tightly bound 2 neutral proteases, chymase and carboxypeptidase A (CPA).19

The heparin proteoglycans of rat serosal mast-cell granules are called “native heparin” or “macromolecular heparin.” They have an average molecular weight (average Mw) of 750 000 (range 500 000 to 1 000 000), each monomer comprising, on average, 10 heparin glycosaminoglycan chains with average Mw values of 75 000 (range 50 000 to 100 000).20–23 Structural analysis of the heparin proteoglycans shows that their disaccharide units have the composition typical of heparin (J.-p. Li, P. Kovanen, and U. Lindahl, unpublished results, 1995), thus differing from those of the heparan sulfate secreted by many other cell types, including the endothelial cells and SMCs of the arterial intima.11,22,23 Indeed, mast cells are the only cells in the mammalian body that synthesize heparin.24 Commercial heparin, when given intravenously to a rat or a rabbit before arterial injury, has been found to inhibit the rate of proliferation of SMCs in the neointima, leading to decreased growth of the neointima.25–27 This inhibitory effect of heparin on human SMCs has also been observed in culture.28–30 Since mast cells are the endogenous source of arterial heparin,16,31 the above observations with commercial heparin raise the possibility that mast cells, when stimulated to secrete their macromolecular heparin, may also participate in local regulation of SMC proliferation in the arterial intima. In the present investigation, we used an experimental model to study the effect of rat mast cells on rat aortic SMC proliferation in vitro.

Materials and Methods

Materials and Animals

Sodium [3H]thymidine (53 mCi/mg) was from Amersham International; RPMI 1640, Dulbecco’s PBS, FCS, penicillin, streptomycin, and L-glutamine were from Gibco; BSA, compound 48/80, N-benzoyl-tyro sine ethyl ester, phenylmethylsulfonyl fluoride, collagenase type IA, trypsin, heparanase I, RNase, ovalbumin, and high molecular weight (HMW) heparin (H-3393) were from Sigma; low molecular weight (LMW) heparin (Fragnim) was from Kabi Pharmacia; Sephacryl S-200 HR and Superdex 75 PC 3.2/30 column were from Pharmacia; recombinant human TGF-β1 (rTGF-β1), anti-TGF-β neutralizing antibody (chicken IgY, Chinese hamster ovary cell–derived rTGF-β1 as immunogen), and an ELISA kit for TGF-β1 were from R&D Systems; anti-TGF-β antibody (rabbit IgG, a peptide corresponding to amino acids 328 to 353 mapping at the carboxyl terminus of human TGF-1 as immunogen) used in Western blot was from Santa Cruz Biotechnology; and biotinylated goat anti-rabbit immunoglobulins and horseradish peroxidase conjugated streptavidin were from DAKO. The male Wistar rats (300 to 500 g) used in this study were from the Laboratory Animal Center of the University of Helsinki, Finland.

Preparation of Rat Aortic SMCs

Aortic SMCs were prepared from male Wistar rats as described previously.17,32 Briefly, the intima and media were dissected out from the thoracic aorta and cut into 1-mm pieces, which were first treated with 1 mg/mL collagenase for 1 hour to remove the endothelial cells, then washed with medium, and dispersed in a mixture containing 1 mg/mL collagenase and 0.5 mg/mL elastase in RPMI 1640 with 12.5% of FCS. After incubation at 37°C for 2 hours with occasional gentle agitation, the cell suspension was centrifuged at 800g for 5 minutes. The cell pellet was washed and resuspended in medium A (RPMI 1640 containing 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) containing 20% FCS. The cells were seeded at a density of 1×10⁶ cells/mL; incubated in the same medium; and, at confluency, subcultured (1:2) up to 9 times. The SMCs of 5 to 9 passages were used for the experiments.17,33

Growth Arrest of SMCs

To arrest the growth of SMCs, sparsely plated cultures (1×10⁴ to 5×10⁶ cells/mL) were washed and placed in medium A containing 0.4% FCS for 72 hours, as described by Castellot et al.34 Flow cytometric analysis showed that >80% of the cells had been arrested in the G1/G0 phase.

Isolation and Stimulation of Mast Cells

Serosal mast cells were isolated from the pleural and peritoneal cavities of rats as described previously.19 The mast cells were then stimulated to degranulate in culture medium or in PBS, ie, to trigger exocytosis of their cytoplasmic secretory granules. As stimulating agents, we used either compound 48/80,21 or a specific antigen (ovalbumin), if the mast cells had first been passively sensitized with hyperimmune serum containing anti-ovalbumin IgE.36 After stimulation, the mast cells were sedimented by centrifugation at 800g for 5 minutes. The supernatant, which contains all the material released from the stimulated mast cells, will be referred to in the text as “mast-cell releasate.” This releasate contains mainly the cytoplasmic secretory granules and histamine released from the granule compartment.19 The degree of mast-cell degranulation was determined by measuring the histamine content of the releasate and is expressed as μmol/L.37

Preparation of Granule Remnants and Granule Remnant–Free Releasate

To sediment the granule remnants, the mast-cell releasate was centrifuged at 13 000g for 10 minutes. After centrifugation, the supernatant was collected and used as granule remnant–free releasate. The sedimented granule remnants were then washed twice with water, resuspended in PBS, and used as granule remnants. The concentration of the granule remnants is expressed in terms of their protein content or of their content of Alcian blue–reactive material, which reflects their content of negatively charged sulfate groups. The ratio of Alcian blue–reactive material (μg) to protein (μg) in the remnants was 0.35±0.19 (mean±SD) in the 6 remnant preparations. The concentrations of the soluble heparin proteoglycans and chondroitin sulfate proteoglycans (“heparin-chondroitin sulfate proteoglycans”) in the granule remnant–free releasates are given in terms of their content of Alcian blue–reactive material.

Preparation of Purified Heparin Proteoglycans and Heparin Glycosaminoglycans From Mast-Cell Granule Remnants

Granule remnants of rat serosal mast cells contain only heparin proteoglycans (but not chondroitin sulfate proteoglycans) and neutral proteases (chymase and CPA). To obtain protease-free heparin proteoglycans from granule remnants, the neutral proteases were dissociated from the remnant heparin proteoglycans by incubating the remnants in 10 mmol/L phosphate buffer supplemented with 2 mol/L KCl, pH 7.0. The mixture containing the solubilized remnants (heparin proteoglycans and proteases) was then applied to a
Sepharcl S-200 HR column (10 × 600 mm) and eluted with the same buffer as was used for dissociation. Fractions containing Alcian blue–reactive material were collected, dialyzed extensively against water, concentrated with a Centricon 10 filter (Amicon), and used in the experiments.20,28 These fractions were devoid of any protease activity, as determined by a sensitive method involving analysis of the proteolytic products of angiogenulin I by reverse-phase high-performance liquid chromatography29 and will be referred to in the text as mast cell–derived “heparin proteoglycans.” Heparin glycosaminoglycans were prepared from such heparin proteoglycans by incubating the latter for 14 hours in 0.5 mol/L NaOH at 25°C to hydrolyze the core protein of the proteoglycans.20 The sample was then lyophilized to 80 μL, and its heparin glycosaminoglycans (average M, 75 000) and the protein hydrolysates (peptides and amino acids) were separated on a Superdex 75 PC 3.2/30 column with 150 mmol/L NaCl in 5 mmol/L Tris-HCl buffer, pH 7.4. The fractions containing Alcian blue–reactive material were collected and used for the experiments.

Separation of LMW and HMW Substances Present in Granule Remnant–Free Releasate
To separate the soluble LMW substances (histamine; also prostaglandins, leukotrienes, and cytokines) from the soluble HMW substances (heparin-chondroitin sulfate proteoglycans; average M, 750 000), a granule remnant–free releasate was prepared and applied to a Centricon 10 filter (Amicon) with a M, 10 000 cutoff. The releasate was then centrifuged at 3000g until the flow through the filter ceased.35 The fractions of the releasate retained by the filter, which contained the HMW substances (M, >10 000), were washed off with PBS to the original volume, while the ultrafiltrates, which contained the LMW substances (M, <10 000), were used as such.

Determination of DNA Synthesis
Mast cell–derived products were usually collected in PBS. Purified heparin proteoglycans were reconstituted for bioassay by exhaustive dialysis against distilled water and diluted at least 20-fold with culture medium before being added to the SMCs. Aortic SMCs, 1 × 10⁴ to 5 × 10⁴ cells/mL, were seeded, and when they reached subconfluence, the growth of the SMC monolayers was arrested, and they were incubated with the various mast cell–derived products for 16 hours or as indicated in the figure legends. After incubation, the cells were released from the G0 block by addition of FCS (final concentration: 20%, vol/vol), and incubation was continued for 26 hours. The rate of DNA synthesis was then determined by measuring the incorporation of [3H]thymidine into the trichloroacetic acid (TCA)–precipitable material of the SMCs.44 For this purpose, 2 μCi/mL [3H]thymidine was added to the culture medium, followed by incubation for an additional 2 hours. At the end of the labeling period, the cells were detached with 0.25% trypsin and collected in tubes. A 10-μL sample of the cell suspension was used for counting the cell number. The remaining cells were washed 3 times with cold PBS and incubated in 1 mL of cold 0.61 mol/L (10%) TCA solution for 30 minutes at 4°C. The TCA solution was then discarded, and the cells were washed 3 times with 0.61 mol/L (10%) TCA solution. The residual TCA-precipitated label was extracted with 0.2 mol/L NaOH, and the radioactivity of the extract was determined with a WinSpectral 1414 liquid scintillation counter (Wallac). [3H]Thymidine incorporation into the DNA of SMCs is expressed in terms of dpm/10⁴ cells.

Flow Cytometric Analysis of the Cell Cycle
The cell-cycle distribution of SMCs was determined by flow cytometric analysis of propidium iodide–labeled cells.40 Briefly, the cells were collected and fixed in 90% methanol on ice for 10 minutes. After fixation, the cells were washed twice with PBS, resuspended in RNase solution in PBS (100 U/mL), and incubated at 37°C for 30 minutes. The RNase-treated cells were then stained with propidium iodide (20 μg/mL) and analyzed by FACScan (Becton Dickinson). Cells having lower DNA content than G0/G1 cells (hypodiploidy) were considered apoptotic.41

Determination of TGF-β1 in SMC Culture Medium
Cultures of SMCs were treated with mast cell–derived heparin proteoglycans. After incubation, the culture media received the following protease inhibitors: phenylmethylsulfonyl fluoride (final concentration 1 mmol/L), aprotinin (2 μg/mL), leupeptin (2 μg/mL), and pepstatin A (2 μg/mL). The medium was then collected and centrifuged at 800g for 5 minutes. The quantity of TGF-β1 in the supernatant was determined directly by an ELISA kit as recommended by the manufacturer (R&D systems) or concentrated with a Centricon 10 filter (Amicon; M, 10 000 cutoff) for immunoblotting of active TGF-β1, as described by Taipale et al.42

Other Assays
Granule remnant protein was determined by the procedure of Lowry et al.,43 with BSA as standard. The glycosaminoglycan content of proteoglycans was determined by assaying Alcian blue–reactive material with commercial heparin as standard.44 Chymase activity was determined spectrophotometrically with N-benzoyl-tyrosine ethyl ester as substrate.45 The activities of chymase and CPA in the purified heparin proteoglycans were determined by analyzing the proteolytic products of angiogenulin I with reverse-phase high-performance liquid chromatography.35

Statistical Analysis
Data, shown as mean±SD, were analyzed with Student’s t test for determination of the significance of differences, which were considered to be statistically significant at a P value <0.05.

Results
Effect of Mast-Cell Degranulation on SMC Proliferation
To study the effect of activated mast cells on proliferation of SMCs, we prepared monolayers of subconfluent SMCs from rat aortic media and arrested them at the G0 phase with 0.4% FCS medium. Rat serosal mast cells were then isolated and passively sensitized with rat serum containing anti-ovalbumin IgE. The sensitized mast cells (ie, mast cells with IgE bound to their high-affinity IgE receptors) were added to the SMCs and stimulated to degranulate in the coculture system by adding ovalbumin (the antigen) to the incubation medium. The degree of degranulation of the sensitized mast cells was ascertained by measuring the concentration of histamine in the culture medium. As shown in Figure 1A, in dishes containing both sensitized mast cells and antigen (column b), the amount of histamine in the culture medium was higher, revealing degranulation of the mast cells. Sixteen hours after mast cell degranulation, the growth-arrested SMCs were stimulated to degranulate with 20% FCS, and their proliferation was monitored by determining the amount of [3H]thymidine incorporated into the DNA of the cells. In the dishes in which mast cells had been cultured to degranulate, as shown in Figure 1B, incorporation of [3H]thymidine by the SMCs was strongly inhibited as compared with control SMCs that had been cultured without mast cells (b versus a, P<0.0001). No such inhibition was observed when only the antigen was added, without mast cells (c versus a; P>0.05). Furthermore, when the coculture system included sensitized mast cells without the antigen (column d) or with nonsensitized mast cells and the antigen (column e), there was only slight release of histamine (because of spontaneous degranulation; panel A), and the effect on SMC proliferation was also slight (d versus a and e versus a, P<0.05 for both).
Similar results were observed when the cells were counted after coculture of SMCs and degranulating mast cells for 72 hours (data not shown). To relate the degree of mast-cell degranulation (determined as release of histamine) to the inhibitory effect on SMC proliferation, mast cells were stimulated to degranulate with a commercial noncytotoxic mast cell–specific agent, compound 48/80, with respect to the compound concentrations. We found that the concentration of histamine increased rapidly as the concentration of compound 48/80 increased. Most importantly, incorporation of $[^3H]$thymidine into the DNA of the SMCs was inversely related in a dose-dependent manner to the degree of mast-cell degranulation, half-maximal inhibition being achieved with releasate containing 30 μmol/L histamine (data not shown).

Heparin Proteoglycans Are the Main Inhibitors of SMC Proliferation in the Mast-Cell Releasate

The inhibitory effect of commercial heparin on the growth of vascular SMCs is well established.46–49 Mast-cell granule remnants contain “native” or “macromolecular” heparin, which is a heparin proteoglycan of very high molecular weight (average $M_r$ 750 000). We isolated granule remnants from mast-cell releasate, added them to SMCs, and found that incorporation of $[^3H]$thymidine into the DNA of SMCs was gradually inhibited as the concentration of the granule remnants in the incubation medium increased. Half-maximal inhibition was achieved with 2 mg/mL remnant heparin proteoglycans (data not shown).

We next studied the effect of granule remnants on $[^3H]$thymidine incorporation into SMCs after the release of the SMC from the G₀ phase. In this case, FCS was added to the cultures (final concentration: 20%, vol/vol) to release growth-arrested SMC from the G₀ block in the absence or presence of granule remnants. As shown in Figure 2A, in the absence of granule remnants, $[^3H]$thymidine incorporation into the DNA of SMCs was gradually inhibited as the concentration of the granule remnants in the incubation medium increased. Half-maximal inhibition was achieved with 2 μg/mL remnant heparin proteoglycans (data not shown).

Figure 1. Effect of activated mast cells on $[^3H]$thymidine incorporation into DNA of SMCs in coculture. Serosal mast cells were isolated and passively sensitized with IgE-containing serum as described in Materials and Methods. The sensitized cells ($1 \times 10^5$) were then washed with PBS 3 times and added to subconfluent monolayers of growth-arrested SMCs with or without stimulation with the IgE-specific antigen (89 nmol/L ovalbumin; 4 μg/mL). Antigen, sensitized mast cells, and unsensitized mast cells plus antigen were used as controls. After incubation at 37°C for 16 hours, the cocultured SMCs were stimulated to proliferate by addition of FCS (final concentration: 20%, vol/vol), and $[^3H]$thymidine incorporation into the DNA of the SMCs was determined as described in Materials and Methods (B). Histamine in the culture medium was determined to quantify the degranulation of mast cells (A). Values are mean±SD of triplicate incubations. Similar results were obtained in 2 other independent experiments.

Figure 2. Kinetics of granule remnant–mediated inhibition of SMC proliferation. Growth-arrested SMCs were incubated at 37°C for the indicated time periods in medium A containing 20% FCS with or without 2 μg heparin/mL of granule remnants. After incubation, $[^3H]$thymidine incorporation into the DNA of the SMCs was determined. For comparison, $[^3H]$thymidine incorporation into the DNA of SMCs in the absence of FCS (0.4% FCS) was also determined (A). In parallel dishes, the cell number was counted (B). Values are mean±SD of triplicate incubations.
granule remnants would still inhibit SMC proliferation, the remnants were added to the cultures at various time points after addition of FCS. As shown in Figure 3, granule remnants could be added as much as 16 hours after addition of serum with little loss of their antiproliferative effect, but this effect was progressively lost when the remnants were added 20 or more hours after the cells had been released from the G0 phase. In accord with the observations shown in Figure 2A, that the SMCs started to enter the S-phase of the cell cycle at 8 to 12 hours, granule remnants may block the G0/S transition or early S-phase events. Therefore, in an additional experiment (not shown), we examined how long the inhibitory effect would last after removal of the granule remnants.

For this purpose, SMCs were kept growth arrested by incubating them in the presence of 0.4% FCS with or without granule remnants (2 μg/mL heparin proteoglycans). After incubation for 48 hours, any granule remnants were removed, and fresh medium containing 20% FCS was added to release the cells from G0 and stimulate them to proliferate. We found that the control SMCs (not exposed to granule remnants) began to incorporate [3H]thymidine into their DNA between 8 and 12 hours and peaked at 26 hours, similarly to those shown in Figure 2A. In contrast, the SMCs exposed to granule remnants showed only a slow increase in the rate of [3H]thymidine incorporation into the DNA within the period of observation (26 hours), revealing that after removal of the granule remnants, their inhibitory effect persisted for at least this length of time.

We also studied the effect of mast-cell granule remnants on the cell-cycle distribution by flow cytometric analysis. As shown in Table 1 (experiment A), when the concentration of granule remnants increased, the proportion of SMCs in the G0/G1 phase and in the G2/M phase gradually increased, while the proportion of SMCs in the S phase gradually decreased, suggesting an inhibition or a delay of the G0→S transition and an extension of the G2/M phase of the cell cycle in the SMCs. When considered together with the result from the [3H]thymidine incorporation assay shown in Figure 2A, an inhibition rather than a delay of the G0→S transition of SMCs by mast-cell granule remnants is likely.

To further establish that the proteoglycans in the granule remnants were responsible for the inhibitory effect, we purified heparin proteoglycans from the remnants by size-exclusion chromatography. The purified proteoglycans were extensively dialyzed and added to SMC cultures. As shown in Table 1, experiment B, when SMCs were treated with the purified heparin proteoglycans, the proportions of the cells in the G0/G1 and G2/M phases increased, and the proportion in the S-phase decreased, as found with native granule remnants (experiment A). If cell proliferation was measured by [3H]thymidine incorporation into the DNA of SMCs, the same amount of the purified heparin proteoglycans inhibited the incorporation of [3H]thymidine into the DNA of the SMCs by 68% (P<0.005; n=6; data not shown).

As described in Materials and Methods, we prepared mast-cell releasates by stimulating mast cells to degranulate and then removing the cells by centrifugation. The releasates contained all of the material released from the stimulated mast cells. We next separated the releasates by centrifugation into granule remnants (sediment) and granule remnant–free releasate (supernatant). Granule remnants of rat serosal mast cells are composed, in addition to the heparin proteoglycans, of 2 neutral proteases, chymase and CPA. CPA, when purified and added to SMC culture, had no effect on the cell

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**Figure 3.** Sensitivity of SMC proliferation to inhibition with granule remnants at different time points after release from G0. Growth-arrested SMCs were released from G0 block with 20% FCS (0 hour). After incubation for the indicated time periods, 2 μg heparin/mL of granule remnants was added, and incubation was continued for up to 26 hours. [3H]thymidine incorporation into the DNA of the SMCs was then determined during an additional 2-hour period. Values are mean±SD of triplicate incubations.

**Table 1.** Effect of Mast-Cell Granule Remnants and the Remnant-Derived Heparin Proteoglycans on Cell Cycle Distribution of SMCs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Proportion of Cells, %</th>
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<tbody>
<tr>
<td></td>
<td>G0/G1</td>
</tr>
<tr>
<td>A. Granule remnants, μg heparin/mL</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>52.8±1.5</td>
</tr>
<tr>
<td>2.5</td>
<td>52.5±1.9</td>
</tr>
<tr>
<td>5</td>
<td>55.1±1.7†</td>
</tr>
<tr>
<td>10</td>
<td>57.1±1.1‡</td>
</tr>
<tr>
<td>B. Heparin proteoglycans, μg heparin/mL</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>52.4±1.7</td>
</tr>
<tr>
<td>20</td>
<td>57.9±2.0†</td>
</tr>
</tbody>
</table>

Growth-arrested SMCs were incubated for 16 hours with indicated concentrations of mast-cell granule remnants and the remnant-derived heparin proteoglycans. After incubation, the cells were stimulated to proliferate by addition of FCS (final concentration: 20%, vol/vol), and incubation was continued for another 26 hours. The cell-cycle distribution of the SMCs was examined by flow cytometric analysis as described in Materials and Methods. Values shown in experiment A are mean±SD of 2 separate experiments, each performed in triplicate incubations, and those shown in experiment B are mean±SD of triplicate incubations of a single experiment.

*P<0.01; †P<0.05; ‡P<0.001.
TABLE 2. Soluble Heparin Proteoglycans Are the Only Inhibitor of SMC Growth in Granule Remnant–Free Releasate

<table>
<thead>
<tr>
<th>Experiment and Additions</th>
<th>[^{[3]H}]Thymidine Incorporation into the DNA of SMCs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(10^4 \text{ dpm}/10^6 \text{ cells} \times % \text{ of control} )</td>
</tr>
<tr>
<td>A. Fractions of granule remnant-free releasate</td>
<td></td>
</tr>
<tr>
<td>Control (no addition)</td>
<td>17.53(\pm)4.73</td>
</tr>
<tr>
<td>Granule remnant-free releasate</td>
<td>10.12(\pm)1.17*</td>
</tr>
<tr>
<td>LMW substances</td>
<td>16.00(\pm)4.34*</td>
</tr>
<tr>
<td>HMW substances</td>
<td>10.27(\pm)2.00*</td>
</tr>
<tr>
<td>B. Effect of heparinase on the inhibitory effect of HMW substances</td>
<td></td>
</tr>
<tr>
<td>Control (no addition)</td>
<td>7.79(\pm)1.71</td>
</tr>
<tr>
<td>Heparinase-treated HMW substances</td>
<td>3.67(\pm)1.37(\dagger)</td>
</tr>
<tr>
<td>Heparinase-treated HMW substances</td>
<td>7.80(\pm)1.85(\dagger)</td>
</tr>
</tbody>
</table>

Growth-arrested SMCs were incubated for 16 hours with indicated mast-cell products (100 \(\mu\)g/mL in experiment A; 10 \(\mu\)g heparin/mL in experiment B). After incubation, the cells were stimulated to proliferate with 20% FCS, and \[^{[3]H}\]thymidine incorporation into the DNA of the cells was determined as described in Materials and Methods. Each microliter of the product was derived from 3 separate experiments. Values of experiment A are means\(\pm\)SD of 6 incubations, and that of experiment B, mean\(\pm\)SD of 9 incubations.

*\(P<0.01\); \(\dagger P<0.05\); \(\ddagger P<0.001\).

remnants, but not isolated heparin proteoglycans derived from them, induced apoptosis of SMCs. As shown in Table 3, when growth-arrested SMCs were cultured in the presence of mast-cell granule remnants, the percentage of apoptotic cells was significantly higher than that of the control cells (\(P<0.001\)). If the cells were counted at the end of the incubation, the number of granule-remnant–treated SMCs was 29% less than in control cultures. The low percentage of apoptotic cells (2.33%) contrasts with the 29% reduction in the final number of SMCs after the 42-hour (total duration) incubation with granule remnants. Therefore, we infer that the apoptotic cell death cannot explain the observed effect of granule remnants on SMC number. Apoptosis of the SMCs in granule remnant–containing cultures was also identified by observing the condensation of the cytoplasm, compaction of the chromatin, and fragmentation of the nucleus into discrete masses scattered throughout the cell cytoplasm when the cells were stained with May-Grünwald Giemsa stain (data not shown).

Mast Cell–Derived Heparin Proteoglycans Are More Potent Inhibitors of SMC Proliferation Than Commercial Heparins

We next compared the inhibitory effects of the purified heparin proteoglycans obtained from mast-cell granule remnants and of commercial heparin of 2 types, LMW heparin (LMW heparin; average \(M_\text{w} 5000\) ) and HMW heparin (HMW heparin; average \(M_\text{w} 15000\) ). As shown in Figure 4A, for equal concentrations of heparin (0–20 \(\mu\)g/mL Alcian blue–reactive material), the mast cell–derived heparin proteoglycans had a stronger inhibitory effect on \[^{[3]H}\]thymidine incorporation by SMCs than either of the 2 commercial heparins (\(P<0.05\)). This difference was even more pronounced when the data were considered on the basis of molarity rather than mass. Thus, in a separate experiment (panel B), mast cell–derived heparin proteoglycans (average \(M_\text{w} 75000\) ) at a concentration of 27 mmol/L (corresponding to 20 \(\mu\)g/mL heparin proteoglycans, 0.4 \(\mu\)g/mL HMW heparin, and 0.13 \(\mu\)g/mL LMW heparin) exhibited an inhibitory activity that was \(\approx\)20 times that of either commercial heparin (95% inhibition versus 5% inhibition). We next hydrolyzed the core protein of the heparin proteoglycans and purified heparin glycosaminoglycans from the hydrolysates. It was found that the purified heparin glycosaminoglycans efficiently inhibited...
[\textsuperscript{3}H]thymidine incorporation into SMCs. This inhibitory effect was weaker than that of intact heparin proteoglycans but stronger than that of either LMW or HMW commercial heparins (each \(P<0.05\), \(n=3\); data not shown).

**Effect of Mast Cell–Derived Heparin Proteoglycans on SMC Proliferation Is Not TGF-\(\beta\) Dependent**

Commercial heparin has been reported to release TGF-\(\beta\) from serum, and this in turn may inhibit the proliferation of SMCs.\(^{50,51}\) To study whether the inhibitory effect of the mast cell–derived heparin proteoglycans on SMC proliferation is TGF-\(\beta\) dependent, we first tested the effects of commercial rhTGF-\(\beta\) on SMC proliferation under the conditions used in this study. As shown in Figure 5A, 50 ng/mL rhTGF-\(\beta\) inhibited the synthesis of DNA by SMCs by 48% (b versus a, \(P<0.05\)). This inhibitory effect was blocked by 65% in the presence of 100 \(\mu\)g/mL anti–TGF-\(\beta\) neutralizing antibody (c versus b, \(P<0.05\)). Incubation of SMCs with mast cell–derived heparin proteoglycans in medium containing 20% FCS inhibited the synthesis of DNA by SMCs by 80% (panel B, b versus a, \(P<0.0005\)). This inhibitory effect was not counteracted even when increasing concentrations of anti–TGF-\(\beta\) neutralizing antibody were present in the culture medium (c through f versus b, each \(P<0.05\)). In addition, we determined by ELISA the amount of TGF-\(\beta\) in medium of mast-cell heparin proteoglycan–treated SMC culture in the presence of 20% FCS. As shown in Table 4, compared with the amount of TGF-\(\beta\) in the control culture medium, mast-cell heparin proteoglycans did not significantly increase the quantity of either active or total TGF-\(\beta\) in the culture medium. Furthermore, we failed to detect the presence of active TGF-\(\beta\) by immunoblotting (detection limit, 0.5 ng/lane) in 25-fold concentrated SMC culture medium, in which 20% FCS and 20 \(\mu\)g/mL mast cell–derived heparin proteoglycans were included (data not shown).

**Discussion**

The present study revealed a novel property of mast cells, namely, that they are capable of inhibiting SMC growth. The main inhibitory factor was identified as the heparin proteoglycans secreted by these cells. This applies to both the insoluble heparin proteoglycans of the granule remnants and the soluble heparin proteoglycans released from the exocytosed granules. The mast cell–derived heparin proteoglycans, also called “macromolecular heparin,” were much more efficient than their commercial counterparts; on a molar basis,

<table>
<thead>
<tr>
<th>Addition</th>
<th>Active TGF-(\beta), pg/well</th>
<th>Total TGF-(\beta), pg/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58±10</td>
<td>1288±55</td>
</tr>
<tr>
<td>Heparin proteoglycans</td>
<td>58±17*</td>
<td>1064±189*</td>
</tr>
</tbody>
</table>

Growth-arrested SMCs were incubated for 16 hours with 20 \(\mu\)g/mL mast cell–derived heparin. After incubation, the cells were stimulated to proliferate by addition of FCS (final concentration: 20%, vol/vol), and incubation was continued for another 26 hours. At the end of the incubation, cell culture medium was collected and the amount of TGF-\(\beta\) in the medium was determined by ELISA, as described in Materials and Methods. Values are mean±SD of quadruplicate incubations.

*NS, vs control.

**Table 4. Effect of Mast Cell–Derived Heparin Proteoglycans on the Quantity of Active and Total TGF-\(\beta\) in SMC Culture Medium**
their inhibitory effect on SMC proliferation was at least 20-fold that of the commercial heparins. In a structural analysis of the heparin proteoglycans, the composition of the disaccharide units was found to be typical of heparin (J.-p. Li, P. Kovanen, and U. Lindahl, unpublished results, 1995). Therefore, the observed differences in inhibitory potential between the heparin proteoglycans and commercial heparins must depend on factors other than the composition of the individual glycosaminoglycan chains. The ability of intact heparin proteoglycans (average \( M, 750 000 \)) to inhibit SMC growth was greater than that of the heparin glycosaminoglycan chains (average \( M, 75 000 \)) prepared from the heparin proteoglycans, which, again, was greater than that of the 2 types of commercial heparin (HMW heparin, average \( M, 15 000 \), and LMW heparin, average \( M, 5000 \)). Accordingly, the most important factors contributing to the observed inhibition are the large size of the mast cell–derived heparin chains and their attachment to a core protein (creating “macromolecular” heparin). The same principle of size dependency was recently observed to apply also to the ability of heparins of various types to prevent collagen-induced platelet aggregation.52 It is of interest that endothelial heparan sulfate proteoglycans, with an overall size of average \( M, \approx 1000000 \) and with heparan sulfate chains of average \( M, 60000 \) (ie, closely resembling in size the mast cell–derived heparin proteoglycans used in this study), are much more potent inhibitors of SMC growth than is commercial heparin.11

Stimulated mast cells also secrete factors that could potentially act as stimuli of SMC growth. For example, histamine, which is released by mast cells on their activation, is known to stimulate SMC proliferation when added to cultured SMCs.53 Nevertheless, in the present experiments, the net effect of stimulated mast cells on SMC proliferation was always (irrespective of the degree of mast-cell stimulation) inhibitory. The failure of the released histamine to counteract the effect of the released heparin proteoglycans was found to be due to its low concentration. In the incubation media of maximally stimulated mast cells, this was in the micromolar range (maximally 100 \( \mu \text{mol/L} \)), whereas, with commercial histamine, the concentration required for stimulation of SMC growth has been found to be in the millimolar range (100 \( \text{mmol/L} \)).53 The fraction of mast-cell releasate containing the LMW substances (including histamine) was without any significant effect on SMC growth even in the absence of heparin proteoglycans (see Table 2). This conclusion regarding the physiological effects of degranulating rat mast cells applies to the other LMW substances, such as TGF-\( \beta \), present in the mast-cell releasate and known to inhibit or stimulate the growth of rat aortic SMCs in culture.54,55

What could be the mechanism by which the mast cell–derived heparin proteoglycans inhibit the growth of SMCs in response to serum in vitro? In principle, the heparin proteoglycans could activate inhibitory factors present in the serum or exert their effects directly on the SMCs. Previous reports with commercial heparin have provided evidence for actions of both types. Regarding the first type of action, Grainger et al50 reported that the heparin-induced inhibition of SMC growth in response to FCS could be due to release of active TGF-\( \beta \) from the added FCS by the heparin. We found that proliferation of cultured rat aortic SMCs in response to FCS was inhibited when commercial TGF-\( \beta \) was added to the incubation medium, revealing that, under the culture conditions used, the rat aortic SMCs were responsive to TGF-\( \beta \)-induced growth inhibition. However, in contrast to the findings reported with commercial heparin, the mast cell–derived heparin proteoglycans appeared to inhibit SMC growth independently of TGF-\( \beta \), since addition of anti–TGF-\( \beta \) neutralizing antibody to the FCS-containing culture medium failed to block the inhibitory effect of the added heparin proteoglycans (see Figure 5B). In addition, the heparin proteoglycans did not increase the quantity of active TGF-\( \beta \) in the culture medium (see Table 4). Regarding a possible direct inhibitory effect, binding and uptake of heparin mediated by both receptor-dependent and receptor-independent endocytic pathways was required for its inhibitory effect on rat aortic SMCs.56,57 The conclusion that heparin uptake by SMCs is essential for the inhibitory action of heparin was also reached in a study with heparin-sensitive and heparin-resistant SMCs, in which it was found that upregulation of heparin binding to the SMCs was strongly linked to subsequent internalization and degradation of heparin and was required for the antiproliferative effect of heparin.46,47 We have previously shown by electron microscopy and by fluorescent microscopy that exoyosited mast-cell granule remnants are phagocytosed by SMCs in culture17 and are also phagocytosed by the SMCs in the atherosclerotic human arterial intima in vivo.16 In vitro studies revealed that this phagocytosis was mediated by the scavenger receptors of the SMCs, the negatively charged heparin proteoglycans of the remnants being responsible for receptor recognition.58 In the present study, we found that maleylated albumin, a compound able to block scavenger receptors,59 was without effect on SMC growth, but when added together with granule remnants, it counteracted their growth-inhibitory effect (data not shown). Taken together, the present and previous observations with mast cell–derived heparin proteoglycans strongly suggest that they directly inhibit the SMC growth response to serum. Regarding the intracellular regulatory events of SMC proliferation, we found that the heparin proteoglycans of rat serosal mast cells blocked the \( G_0 \rightarrow S \)–phase transition and the exit from the \( G_2/M \) phase of the cell cycle. The precise mechanism by which heparin proteoglycans act on the intracellular signaling pathways regulating cell proliferation remains to be studied.

The rate of proliferation of the SMCs in the human arterial intima is controlled by a multitude of factors present in the blood or produced locally in the vessel wall.60–62 The present findings reveal a new potential source of local growth regulators, ie, activated mast cells. The number of mast cells and the degree of their degranulation are known to be increased in atherosclerotic lesions.14,15,63 On the basis of the present in vitro findings, we propose that heparin proteoglycans secreted by activated mast cells in the arterial intima tend to locally inhibit SMC growth.

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References


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