Mast Cell Granule Heparin Proteoglycan Induces Lacunae in Confluent Endothelial Cell Monolayers

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The addition of rat mast cell granules to confluent bovine pulmonary artery endothelial cell monolayers resulted in the formation of numerous lacunae in the cultures. Several lines of evidence identified heparin proteoglycan as the component of the granule matrix responsible for the effect: presence of the activity in the proteoglycan fraction after chromatography of granule extracts, inhibition of granule activity by digestion with heparinase I, the failure of proteolysis of the proteoglycan fraction with proteinase K to significantly diminish its activity, and the failure of chymase and carboxypeptidase inhibitors to inhibit granule activity. The onset of hole formation was delayed for several hours after granule addition to the culture, and maximal hole formation occurred between 8 and 16 hours and was sustained as long as 24 hours. The lacunae formed by the separation of motile endothelial cells within the monolayer and was not attributable to cell contractile activity or cell loss. Time-lapse video recording showed that the holes were dynamic, individual holes expanding and regressing over a period of hours. Formation of lacunae occurred on gelatin and fibronectin surfaces alike. The presence of active chymase in the granules prevented the action of the proteoglycan. Heparin glycosaminoglycan as distinct from the proteoglycan did not similarly affect the endothelial monolayers but did block the action of granules added subsequently, indicating the likelihood of a heparin-reactive receptor or binding site. (*Am J Pathol* 1999, 154:1591–1600)

The predilection of mast cells for associating with and affecting blood vessels is well known.¹ Among the principal components of the secretory granules, histamine binds ionically to the granule matrix from which it is readily released on secretion² whereupon it is capable of inducing increased permeability of the microcirculation³ and contraction of endothelial cells⁴ as well as relaxation of vascular smooth muscle and contraction of other smooth muscle.⁵ Histamine is a small, readily diffusible molecule with a transient action on its target cells. In contrast, the macromolecular constituents of the granule matrix, principally heparin proteoglycan and several pro-

teases,¹ are likely to persist in the local extracellular compartment with the possibility of inducing additional reactions in blood vessels, such as angiogenesis, or delayed increases in permeability, such as are seen in the late-phase response that accompanies mast cell degranulation *in vivo*. To explore these possibilities, we have examined the effects on endothelial cells of mast cell granules stripped of histamine and found that granule matrices induce the formation of lacunae in confluent endothelial monolayers by a noncontractile mechanism. The granule component responsible for the formation of lacunae is heparin proteoglycan.

Materials and Methods

Bovine pulmonary artery endothelial cells (BPAE), CCL 209 from the American Type Culture Collection (Rockville, MD), were maintained in Eagle's minimal essential medium (MEM) with 2 mmol/L glutamine, 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin, pH 7.3. Cells were plated on dishes or flasks (Corning/Costar, Cambridge, MA) and cultured in a gas-flow, humidified incubator at 37°C using 5% CO₂ in air. Through passage 25, cultures were subcultured approximately every 7 days using 0.25% porcine trypsin (1:250; JRH Biosciences, Lenexa, KS) and plated at a 1:3 dilution.

Experiments evaluating formation of lacunae in confluent cultures were performed variously on cells grown in 35-mm dishes, Permax eight-chamber slides (Nunc, Naperville, IL), or 24-well, flat-bottom, multiwell plates (Corning/Costar, Corning, NY). The plastic surfaces were standardly coated with gelatin (Difco Laboratories, Detroit, MI); bovine fibronectin (Sigma) and Pronectin F (Protein Polymer Technologies, San Diego, CA) were also tested. All cultures were grown to confluence before initiating an experiment unless specifically indicated to the contrary.

For measuring lacunar areas, the cell monolayers were rinsed with PBS to remove serum protein and fixed in 3% *p*-formaldehyde in PBS. Cultures were stained with 0.4% sulforhodamine B in 1% acetic acid. Images were cap-

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tured by video camera, and an image analysis program (Optimas 5.0, Edmonds, WA, or ImagePro Plus, Media Cybernetics, Silver Springs, MD) were used to evaluate holes that formed in the monolayer. Thresholds for the lacunae were established interactively. The percentage of the area of the video frame occupied by the lacunae was determined for several fields per monolayer, and duplicate monolayers were assessed. Values are expressed as mean fractional area occupied by lacunae with standard error (SE) of the mean.

To evaluate cell movements, cultures were plated onto collagen-coated, 25-mm, 1 1/2 coverslips. Coverslips were coated with type 1 rat tail collagen (Upstate Biotechnology, Lake Placid, NY).⁶ Coverslips with adherent cells were placed either directly into a Leiden closed perfusion chamber (Medical Systems Corp., Greenvale, NY) or into a sterile Lexiglass holder for treatment before transfer to the perfusion chamber. The holder had 26-mm diameter wells 6 mm deep with a small eccentric semi-circular depression at the periphery for ease of adding and removing coverslips or media.

The perfusion chamber was equilibrated at 37°C before inserting a coverslip with its cells. The assembled chamber was placed on the microscope stage within a Nikon enclosed plexiglass housing maintained at 37°C with forced heated air. Culture medium, pH 7.3, fortified with 20 mmol/L Hepes buffer, was pre-equilibrated with 5% CO₂ in air and perfused through the chamber via R-3603 Tygon tubing at a constant rate of 0.019 ml/minute from a 60-ml syringe driven by an infusion pump (infusion/withdrawal pump, Harvard Apparatus, South Natick, MA). Cell activity was recorded with a 40× phase contrast objective on a Nikon inverted microscope and recorded with a 960 M Sony CCD video camera connected to a S-VHS Panasonic AG-6730 time-lapse videocassette recorder. Routinely, a time lapse ratio of 120/1 was used.

Isometric tension generated by the cells was monitored according to the method of Kolodney and Wysolmerski.⁷ Granules were added to the monolayers in the apparatus after an equilibration period, and tension was monitored for 18 to 20 hours thereafter. At the completion of the measurements, thrombin and cytochalasin D were added in sequence to evaluate the status of the monolayer as described by Kolodney and Wysolmerski.⁷

Normal peritoneal mast cells were isolated from male Sprague-Dawley retired breeders (Harlan, Indianapolis, IN) by washing the peritoneal cavity of the rats, after anesthesia with CO₂, decapitation, and exsanguination, with 10 ml of cold, heparinized balanced salt solution (BSS) containing 10 mmol/L phosphate, 154 mmol/L NaCl, 2.7 mmol/L KCl, and 0.68 mmol/L CaCl₂, with 0.5% albumin and 10 U of heparin/ml (Elkins-Sinn, Cherry Hill, NJ). The pooled cell washes from 8 to 10 animals were centrifuged for 8 minutes at 130 × *g* at 4°C. The supernatants were removed, and cells from four to six animals were resuspended in 4 ml of BSS with albumin and layered onto a Percoll suspension (7.5 ml of Percoll (Pharmacia Biotech, Piscataway, NJ), 1 ml of salt (1.54 mol/L NaCl, 27 mmol/L KCl, 6.8 mmol/L CaCl₂), 0.3 ml of water, and 0.14 ml of bovine albumin (Path-O-Cyte 4,

ICN, Costa Mesa, CA)) adjusted to pH 7.2 with 0.1 mol/L KH₂PO₄, and centrifuged at 225 × *g* for 20 minutes at 4°C. The mast cells sedimented to the bottom of the gradient at >90% purity. After removal of other cells and supernatant Percoll, the mast cells were resuspended in 7.5 ml of BSS with albumin and centrifuged at 150 × *g* for 5 minutes. The sedimented cells were resuspended in 5 ml of BSS with albumin. Cells were counted in a hemacytometer after fixation and staining with acidic toluidine blue.⁸

Mast cell granule isolation was based on the method of Krüger et al.⁹ A total of 2 × 10⁶ to 3 × 10⁶ cells were suspended in 1 ml and sonicated using a 40-Hz water bath sonicator (Sonagen Ultrasonic Cleaner, Branson Instruments, Stanford, CT) for two 15-second bursts and checked for the extent of cell disruption; 95% loss of intact cells served as the criterion for adequate sonication. The disrupted cells were centrifuged twice at 130 × *g* for 8 minutes at 4°C. The granule-containing supernatants were pooled and carefully layered on top of a concentrated suspension of Percoll (4.5 ml of Percoll, 0.45 ml of salt solution (1.54 mol/L NaCl, 27 mmol/L KCl, 6.8 mmol/L CaCl₂, 0.1 mol/L Hepes) and 0.063 ml of Path-O-Cyte 4) and centrifuged at 27,000 × *g* for 20 minutes at 4°C. Granules separated into two bands; those in the topmost band, designated A granules, had lost their membranes during sonication. The lower, denser band (C granules) consisted of granules that had retained their membranes and thus maintained their native high density. The two bands were removed from the gradient separately and diluted in PBS, and each was centrifuged at 1800 × *g*, removing much of the Percoll. After a second wash and centrifugation in a Beckman Microfuge B at 10,000 × *g*, membranes were stripped from the C granules by suspending them in 0.1% Triton X-100 in PBS for 10 minutes on ice. The small amount of tryptase present in the granules was released from the granules on removal of the membranes.¹⁰ Granule histamine and any residual tryptase were eluted from the granules with PBS. The stripped granules were centrifuged for 3.5 minutes at 10,000 × *g*, washed once in PBS, and resuspended in a final volume of 0.5 ml of PBS. Aliquots were removed for protein determination and assay of chymase activity. The remaining sample was treated for 30 minutes with phenylmethylsulfonyl fluoride (PMSF), 50 μg/ml, at 37°C to inhibit the chymase activity at least 95%, confirmed by assay of residual activity. The granules were stored at 4°C.

Heparin proteoglycan was obtained from isolated granules that were solubilized in 3 mol/L NaCl, 10 mmol/L Hepes, pH 7.2, on ice for 10 minutes and passed over a 30 × 1 cm Superose 6 or 12 HR column eluting with the same high salt solution using a Pharmacia FPLC system. Samples containing heparin were pooled, and 2 vol of 10 mmol/L Hepes, pH 7.5, were added, followed by 4 vol of ethanol. The precipitate was allowed to form overnight at 4°C and was collected by centrifugation at 27,000 × *g*. The ethanol was removed, and the pellet containing the heparin proteoglycan was dissolved in PBS.

Heparin proteoglycan was isolated from rat skin essentially according to the procedure of Horner.¹¹ Subcuta-

neous tissue was removed by scraping the pelts with a scalpel blade. The pooled material was weighed and suspended in 3 vol of 3 mol/L KCl, 0.1 mol/L Hepes, pH 7.4 and homogenized in a Waring blender. The crude homogenate was stirred continuously for 1 hour at 40°C, large pieces of tissue were removed, and the remaining sample was centrifuged at $45,400 \times g$ for 30 minutes at 4°C. The lipid pellicle at the surface was removed from the sample, and the remainder of the suspension was filtered through glass wool. The sample was diluted to 1 mol/L KCl with water, and a 10% volume of 3% cetylpyridinium chloride (CPC) in 1 mol/L KCl at room temperature was added. The mixture was stirred for 20 minutes and then centrifuged at $1800 \times g$ for 10 minutes. The supernatant was removed, and the pellet was resuspended in 3% CPC in water, stirred for 20 minutes, and again centrifuged. Pellets were combined and washed several times at 37°C with ethanol saturated with sodium thiocyanate. The precipitate was dissolved in PBS followed by concentration of the sample in a Centricon 10 concentrator. The sample was sterilized by filtration before addition to the cell culture. Heparin concentration was determined with the *N*-sulfated hexosamine assay. The ratio of *N*-sulfated hexosamine to uronic acid of the proteoglycan so isolated was 0.85, identifying the principal glycosaminoglycan present as heparin. If heparan sulfate is assumed to be the principal contaminant contributing uronic acid to the preparation, it can be estimated that the heparin content is 70% to 80% depending on the *N*-sulfate content of the contaminating heparan sulfate.

The heparin preparation used in the experiments to test the efficacy of heparin glycosaminoglycan to induce the same effects as heparin proteoglycan and to test for possible blocking of heparin proteoglycan was obtained from Sigma. Analysis of several lots of the preparation (grade 1-A from porcine intestinal mucosa), including that currently available (H 3393), indicate a high *N*-sulfate to uronic acid ratio (>90% of theoretical), 3 to 3.3 anionic charges per disaccharide by both chemical analysis and acridine orange titration,¹² and a protein content of less than 1%.

To evaluate possible loss of cells from the monolayer, BPAE cells were plated in MEM, 10% FCS containing [³H]thymidine (ICN Biomedical, Costa Mesa, CA) at 0.05 μ Ci/ml media. Cultures were maintained and re-fed in medium containing the radiolabeled thymidine for 6 days. Radioactive medium was then removed, and the monolayer was washed three times and placed in MEM, 10% FCS. Granules were added to the cultures, and 4 or 8 hours later the supernatant was removed, and the culture was washed with PBS. The wash was added to the supernatant and centrifuged at $225 \times g$ for 5 minutes. The radioactivity of an aliquot of the supernatant was measured in Cytoscint scintillation fluid (ICN). The pellet was dissolved in 0.45 ml of 0.5 N NaOH and neutralized with HCl, and its radioactivity was measured. The remaining cell sheet on the plate was also solubilized in 1 ml of 0.5 N NaOH for 5 minutes, and the plate was washed with another 0.5 ml of NaOH. The combined sample was neutralized with HCl, and its radioactivity was measured.

Uronic acid was assayed by the method of Bitter and Muir.¹³ Hexosamine-*N*-sulfate was determined as described by Lagunoff and Warren¹⁴ or in the case of the heparin glycosaminoglycan by selective *N*-sulfate hydrolysis¹⁴ and assay of the resulting free amino groups with fluorescamine. Chymase activity was determined using the substrate *N*-succinyl-leu-leu-val-try-7-amido-4-methylcoumarin at 4 μ g/ml in 0.01 mol/L Hepes, 10 mmol/L CaCl₂, and 0.1% Triton X-100, pH 7.5.⁸ Tryptase was assayed as previously described.¹⁰ Carboxypeptidase activity was assayed based on the hydrolysis of hippuryl-DL-phenyllactic acid (hip-phe; Sigma). The substrate was 11.4 mmol/L in BSS, pH 7.5. Ten microliters of sample was added to 0.25 ml of the hip-phe for 10 minutes at 37°C. The reaction was stopped with 0.25 ml of 20% trichloroacetic acid at room temperature. After a 10-minute centrifugation, 0.3 ml of supernatant was removed and added to 0.7 ml of 0.1 mol/L borate, pH 10.6. To this solution, 0.10 ml of freshly prepared fluorescamine reagent (3 mg of fluorescamine in 20 ml of acetone) was added. Fluorescence was measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer at nominal excitation/emission wavelengths of 395/475 nm.

Lactic dehydrogenase (LDH) was assayed using the method described by Riley.¹⁵ Phenol red was omitted from the culture media used for these experiments as the dye interfered with the assay. Fluorescence was measured at 360/480 nm. Percent LDH release was calculated as the activity in the medium divided by the total activity in Triton-X-100-treated cultures.

Results

When mast cell granule matrices stripped of histamine were added to confluent monolayers of endothelial cells, lacunae appeared in the monolayer after a delay of several hours (Figure 1). The fractional area of the monolayer occupied by lacunae was dependent on the quantity of granules added to the cultures and the length of exposure time (Figure 2). Formation of lacunae induced by mast cell granules was not significantly different on plastic dishes coated with gelatin or fibronectin (Figure 3). Recombinant mouse TNF- α when added to BPAE monolayers in 35-mm dishes at concentrations of 1, 10, and 100 U/ml failed to induce lacunae in 24 hours, and antibody to TNF- α did not inhibit the effect of the granules.

Inhibition of granule chymase with PMSF did not modify the effect of granules on endothelial monolayers, nor did inhibition of granule carboxypeptidase with carboxypeptidase inhibitor from potato tuber (Sigma; Table 1). As tryptase¹⁰ and both β -glucuronidase and β -hexosaminidase^{10,16,17} are, like histamine, lost from the granule matrices under the conditions used in the preparation of the granules, these granule components were not studied.

To test the possibility that granule binding of a serum factor was responsible for lacunae formation, experiments were conducted in culture medium with FCS replaced by serum albumin. Under these circumstances the effect of granules was lost. However, when granule

Figure 1. Phase micrographs of endothelial cells. **A:** Control cells, 13-day culture, after 7 hours of perfusion. **B:** Granule-treated cells, 8-day culture, 9 hours after the addition of granules ($13.6 \mu\text{g}$ of granule heparin/ 2×10^5 ECs) and 7 hours of perfusion. Images are single frames captured from time-lapse video tape recordings (S-VHS). The mast cell granules are evident in **B**. Magnification, $\times 490$.

chymase activity was inhibited with $\alpha 1$ -antiprotease, the induction of lacunae could be demonstrated in the absence of whole serum (Table 1), indicating that the effect of serum was to inhibit the chymase rather than provide an essential factor. Consistent with this interpretation, when granules were suspended in 10% FCS, chymase in the granules exhibited nearly complete loss of activity over a period of several hours (Figure 4). All subsequent experiments were performed with granules treated with PMSF to inhibit the chymase and obviate any variability arising from different rates or extents of inactivation of the enzyme by serum.

With $\text{TNF-}\alpha$, chymase, tryptase, and carboxypeptidase eliminated as the granule constituents responsible for the effects of granules on endothelial cells, attention was directed to heparin. A range of concentrations of heparin glycosaminoglycan (Sigma) did not reproduce the effects of granules on endothelial monolayers, but when a 3 mol/L salt extract of granules was chromatographed on Superose 12 (Figure 5), activity on endothelial monolayers was found in the highest molecular weight fraction that contained heparin proteoglycan (Table 2). Proteinase K digestion of the active fraction with re-chromatography did not significantly diminish the activity (Table 3). Heparin proteoglycan isolated from rat skin also proved active in inducing lacunae in endothelial cell monolayers

Figure 2. Effect of granule quantity and time on formation of lacunae. Cells were plated on eight-well Permanox slides coated with fibronectin. Cultures, 4 day, were fixed at intervals after exposure to different amounts of granules and stained, and the retraction fraction was measured. Values are means with SEs of the means for six separate determinations.

(Figure 6). Additional evidence that the active component of the granules was heparin proteoglycan was provided by inhibition of activity on treatment of granules with heparinase I (Sigma H2519; Figure 7). Although heparin glycosaminoglycan did not induce lacunae, the glycosaminoglycan was able to prevent the formation of lacunae on the subsequent addition of granules (Table 4).

To characterize the process of formation of the lacunae, the morphology of endothelial cells in culture was examined using extended time-lapse video recording. This technique allowed continuous examination of cell behavior after addition of granules. In the recordings, there was no morphological evidence of an increased rate of cell death. Consistent with this observation there was no measurable increase in release of LDH into the medium. Cell loss from the monolayer was specifically examined by labeling cells with [^3H]thymidine and then measuring both soluble and particulate radioactivity. No significant differences were found between the radioactivity in the fractions from cells exposed to granules and from control cells (Table 5).

Videos of monolayers after exposure to granules provided no evidence of contractile activity that might be pulling the cells apart, such as is the case with thrombin, and specific measurements of the isometric tension of the monolayer in three experiments demonstrated no increase in tension; in two of the three experiments, there was a slow, partial loss of tension over a period of several hours beginning before the time of appearance of obvious lacunae (Figure 8). Endothelial cells exposed to

Figure 3. Comparison of lacunae formation in monolayers cultured on gelatin and fibronectin. Cells were plated on eight-well Permanox slides coated with fibronectin or gelatin, grown to confluency for 4 days, and treated with granules for 8 hours. Values are means with SEs of the means for six separate determinations.

granules and later treated with thrombin and cytochalasin D in sequence responded normally.

When ECs were cultured at subconfluent densities, the trypsinized cells initially rounded up; these condensed cells adhered to the collagen-coated glass, spread, and began to move about at a leisurely pace. From time to time the cells divided. ECs came together and parted during this phase, unlike epithelial cells, which after initial contact form stable adherens junctions resulting in tight cell clusters of immobile cells.¹⁷ After 7 days of culture following a 1/3 split, confluent endothelial cells continued to show movement in the time-lapse recordings; this activity increased after exposure to granules, and lacunae formed after a several-hour-long lag period. The lacunae appeared as microscopic gaps between cells. The gaps were dynamic, opening and closing at different locations in the field of observation over time (Figure 9). The larger

Figure 4. Inhibition of granule chymase activity by fetal calf serum. Isolated mast cell granules were suspended in MEM containing different concentrations of fetal calf serum. Samples were removed at intervals and assayed for chymase activity.

gaps often exceeded the area occupied by three or four cells. There was no evidence that any cells came loose from the substrate to create the lacunae. An occasional mitotic or apoptotic event led to loss of cell adherence, reversibly and irreversibly, respectively, in control and granule-treated cultures alike. Any gaps in the monolayer resulting from either of these two processes were promptly filled in by a combination of expansion of adjacent cells and cell movement.

Discussion

The studies we report here originated in speculation that mast cell granule matrix constituents in addition to histamine might have an effect on endothelium. When isolated mast cell granules depleted of histamine were added to confluent monolayers of bovine pulmonary artery endothelial cells, we observed that holes developed at multiple sites in the monolayer where endothelial cells sepa-

Table 1. Effect of Protease Inhibitors on Activity of Mast Cell Granules

Inhibitor (enzyme)	% Inhibition	Fractional retraction		
		Control (no granules)	Granules	Inhibited granules
10% Fetal calf serum				
PMSF (chymase)	98	0.055 $\pm$ 0.006	0.188 $\pm$ 0.014	0.190 $\pm$ 0.02
Potato inhibitor (carboxypeptidase)	98	0.032 $\pm$ 0.002	0.153 $\pm$ 0.010	0.166 $\pm$ 0.022
0.1% Bovine serum albumin				
α 1-Antiprotease (chymase)	95	0.016 $\pm$ 0.009	0.009 $\pm$ 0.002	0.376 $\pm$ 0.024

Granules were incubated at 37°C for 2.5 hours with the inhibitors (0.3 mmol/L PMSF, 0.2 mg/ml potato inhibitor, and 3.2 mg/ml α 1-antiprotease). Granules were washed and assayed for residual activity before adding to cultures. Cultures were incubated for 16 hours, and fractional retraction was measured. Values are means for six determinations with SEs of the mean.

Figure 5. Superose separation of a 3 mol/L salt extract of granules. Isolated mast cell granules were extracted in 3 mol/L NaCl and chromatographed on Superose 12, eluting in 3 mol/L salt. Column elution was monitored with absorbance at 280 nm. Fractions were analyzed for uronic acid and protein.

rated from one another. This response differed morphologically from the contractile response of monolayers to vasoactive agents such as thrombin, and the cells did not exhibit the increase in contractile tension characteristic of the response to thrombin.^{7,19}

Rat mast cell secretory granules contain, in addition to histamine and serotonin, high molecular weight heparin proteoglycan, chymase, carboxypeptidase A, tryptase, β -hexosaminidase, β -glucuronidase, TNF- α ,²⁰ FGF-2,^{21,22} and VEGF.²³ The mast cell granule matrix is formed by ionic bonding between basic proteins of modest molecular weight and heparin proteoglycan.^{2,16} The compact intracellular granule structure is transformed to a more diffuse organization during the process of secretion. This change can be mimicked by stripping isolated granules of their encompassing membranes with Triton X-100 or

Figure 6. Formation of lacunae induced by rat skin heparin proteoglycan. Monolayers grown to confluence for 6 days in 24-well plates coated with gelatin were treated with granules or rat skin heparin proteoglycan. After 19 hours of incubation, cultures were fixed and stained, and the fraction of the monolayer area occupied by holes was measured. Values are means with SEs of the means for three measurements.

Table 2. Activity of Mast Cell Granule Fractions from Superose 12 Column

Sample	Protein (μ g)	Heparin (μ g)	Retraction fraction
Initial 3 mol/L extract	8.0	9.8	0.115
Column Fx 20	0.08	7.2	0.140
Column Fx 33	1.4	ND	0.006
Column Fx 53	1.7	ND	0.000

Granules were extracted in 3 mol/L NaCl and chromatographed on Superose 12 in 3 mol/L salt (see Figure 5). An aliquot of the original extract and aliquots from the peak fractions from the column were diluted 1/50 into media to test the effect on 10-day cultures. The cultures were fixed after 4 hours. Values for heparin and protein are μ g/ 2×10^5 cells. ND, not done.

Table 3. Effect of Proteinase K Treatment on Granule Proteoglycan Activity

Proteoglycan (μ g/ 10^5 cells)	Control	Proteinase K treated	Probability A	Probability B
0	0.024 $\pm$ 0.012			
5	0.118 $\pm$ 0.055	0.108 $\pm$ 0.043	0.019	0.44
10	0.181 $\pm$ 0.048	0.129 $\pm$ 0.009	0.0004	0.19
20	0.281 $\pm$ 0.064	0.189 $\pm$ 0.064	0.0009	0.17

Heparin proteoglycan from granules was chromatographed on Superose 12, and the leading fraction was tested for activity on ECs with and without proteinase K treatment as described in the text. Values are retraction fraction means for at least four determinations with SEs. Probability A is for the null hypothesis using the *t*-test for means with unequal variances, proteoglycan versus no proteoglycan. Probability B is for the null hypothesis, proteinase K versus control.

hypotonic solutions and then exposing them to 0.1 mol/L sodium chloride at pH 7.5.²⁴ The structural change is accompanied by the loss of histamine, tryptase, β -glucuronidase, and β -hexosaminidase.

Our initial prime candidates for the granule component acting on endothelial cells were TNF- α and chymase. Both were readily eliminated as the active component of the granules, and it was further possible to demonstrate that the effects of granules in the absence of serum were expressed only when chymase was inhibited. Carboxypeptidase, the other known tightly bound component of the granule matrix, was excluded by demonstrating that extensive inhibition did not diminish the effect of the granules.

The next approach to the identification of the active component of the granule matrix was to solubilize granules in 3 mol/L NaCl and separate the constituents. Under these conditions, activity was found in the high molecular

Figure 7. Effect of heparinase I on granule activity. Granules were treated with heparinase I as described in the text, and control and heparinase-treated granules were assessed for activity. Monolayers in 24-well plates coated with gelatin were grown to confluence for 6 days, and the retraction fraction was measured 4 hours after the addition of granules. Values are means with SEs of the means for six separate determinations.

weight, heparin-containing fractions that appear at or near the void volume of Superose 12 or Superose 6 columns well separated from the granule proteins. The heparin in this fraction is present as a proteoglycan with

Table 4. Inhibition of Granule Activity by Heparin Glycosaminoglycan

Heparin glycosaminoglycan added	Granules added	Retraction fraction
None		0.008 ± 0.004
50 µg/ml		0.008 ± 0.004
None	22 µg/ml	0.119 ± 0.056
50 µg/ml	22 µg/ml	0.024 ± 0.015

Endothelial cell monolayers (7-day cultures) were treated with heparin glycosaminoglycan for 30 minutes at 37°C, and then granules (7.2 µg of heparin/2 × 10⁵ cells) were added. The cultures were incubated for 19 hours and then fixed. Values are means for six determinations with SEs.

Table 5. Loss of DNA from Cultures Treated with Mast Cell Granules

Time (hours)	Granules	Medium supernatant (10 ³ cpm)	Medium precipitate (10 ³ cpm)	Monolayer (10 ³ cpm)	% loss of [³ H]thymidine
0	—	0.4	0.3	80.3	0.9
4	—	2.8	2.6	80.7	6.3
4	+	3.4	3.4	81.3	7.7
8	—	5.7	3.4	78.3	10.4
8	+	5.2	6.6	82.5	12.5

Endothelial cells were grown in the presence of [³H]-thymidine (0.05 µCi/ml) for 6 days. The cells were washed in cold medium and incubated with granules (7 µg of heparin per 2 × 10⁵ cells) for 4 or 8 hours. The medium was centrifuged, and the radioactivity of the supernatant and precipitate and the cell monolayer was measured.

Figure 8. Contractile tension of the monolayer was measured according to Kolodney and Wysolmerski,⁷ in dynes, before and after the addition of granules (10 µg of heparin/10⁵ ECs). After 20 hours, thrombin and cytochalasin D were added successively. Two of three experiments gave results like those shown. In one experiment, the granules had no apparent effect on the tension.

a molecular weight estimated between 750 and 1000 kd.^{11,25} Heparin proteoglycan prepared from isolated mast cells contained ~1% protein, an amount consistent with the protein intrinsic to the proteoglycan. In an effort to exclude the possibility that traces of another protein or polypeptide avidly bound to heparin contributed to the activity, the heparin proteoglycan isolated from granules was treated with proteinase K and reisolated. The exposure to proteinase K neither reduced the size of the proteoglycan as estimated on Superose 6 nor significantly reduced its activity on endothelial cells. Partially purified heparin proteoglycan prepared from rat skin by differential CPC precipitation was also active on monolayers. Additional evidence supporting the identification of heparin as the critical component of granules was provided by the elimination of much of the granule activity with heparinase I.

Porcine intestinal heparin glycosaminoglycan was inactive in producing lacunae at concentrations up to 100 µg/ml, an order of magnitude greater than that of the effective concentrations of heparin proteoglycan. However, the glycosaminoglycan was able to block the effect of granules, indicative of a binding site for the proteoglycan that can be occupied by either heparin proteoglycan or heparin glycosaminoglycan but activated only by the proteoglycan.

Binding of heparin to cell surfaces has been reported from several laboratories,^{26–29} and a putative binding protein has been isolated from endothelial cells.³⁰ The

Figure 9. Sequence of phase micrographs of a perfused endothelial cell monolayer. Granules were added at 11:40 and the cultures perfused from 14:00. Frames were captured at ~40-minute intervals as indicated by the video clock on the time-lapse recording. Holes in each image were painted black in Photoshop. The **black arrows** identify a shrinking hole and the **white arrows** an expanding hole. Magnification, $\times 260$.

relationship of that protein to the binding site responsible for the effects of heparin proteoglycan is not known.

To examine the formation of the lacunae, we used time-lapse video recording. This technique revealed that the lacunae were transient. A hole would open between cells, enlarge, and then close, and another hole would open at another site. There was no measurable loss of cells from the monolayer to account for the holes, nor was there an increase in the isometric tension of the monolayer to account for the holes. The dynamic character of

the lacunae suggested that their formation might be associated with increased motility of the endothelial cells. Direct observation of endothelial motility with time-lapse video recording demonstrated an obvious increase in motility of the cells after contact with the granules. Quantitative measurements of cell motility will be reported separately. Mast cell granules have previously been demonstrated by Azizkhan et al³⁰ to increase motility of preconfluent endothelial cells. In their experiments, which used a different method of measuring motility, the effect

of granules was mimicked by heparin glycosaminoglycan, in contrast to the negative findings with respect to lacunae in our studies using the glycosaminoglycan.

Exogenous heparin in several systems enhances the effects of FGF-2,³² and FGF-2 is known to enhance motility of preconfluent endothelial cells.³³ The occurrence of lacunae in the absence of serum does not eliminate a role for FGF-2 as the endothelial cells themselves remain a potential source of the growth factor.³⁴ The failure of heparin glycosaminoglycan to induce lacunae argues against such an effect, as most reported experiments on the cooperation of heparin with FGF-2 have used the glycosaminoglycan form. We have tested the effect of exogenous FGF-2 on confluent endothelial cells and found that it increases motility but does not induce the formation of lacunae.

Although the effects of mast cell granules on bovine pulmonary endothelial cells are striking and reproducible, there remain several important questions concerning the phenomenon. One such question relates to *in vivo* correlates of the lacunae. Although access of granule proteoglycan to endothelial cells could be severely constrained by the basal lamina that separates mast cells from the endothelium of even the smallest vessels, it is conceivable that the proteolytic armamentarium of the granules could allow their penetration of the basement membrane before the onset of inhibition by serum protease inhibitors necessary for the expression of the action of the heparin proteoglycan. Gaps such as those we observed in tissue culture have been described *in vivo* associated with the delayed increase in permeability seen in the late-phase reaction after mast cell secretion.³⁵ Although the late-phase response is associated with and believed to be dependent on an influx of neutrophils, the mediator responsible for the formation of the interendothelial gaps associated with the increase in vascular leakage 4 to 6 hours after the initiation of the reaction has not been identified.³⁴ Our studies suggest that heparin proteoglycan should be considered a candidate for that mediator. Another potential role for mast cell granule proteoglycan suggested by our studies is the reactivation of contact-inhibited endothelial cells as a first step in setting the stage for angiogenesis.

A second question concerns the mechanism of gap formation. As we have ruled out a concerted contractile activity pulling the cells apart, the most likely basis for the observed separation of ECs is a loosening of cell-cell junctions. Adherens junctions, which are well developed in confluent ECs are dependent on cadherin-5 (VE-cadherin).^{36,37,38} Preliminary immunofluorescence examination of the junctions in ECs exposed to granules using specific antibody to cadherin-5 revealed no loss of this protein from the apposing cell junctions except where the cells bordered lacunae. Detailed studies are in progress to evaluate the effects of heparin proteoglycan on EC adherens junctions with particular attention to the state of phosphorylation of the cadherin³⁹ and associated proteins⁴⁰ of the junctions. Examination of tight junctions may also be necessary.

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