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Communication

Secretry Granales of Heparin-containing Rat Serosal Mast Cells also Posses Highly Sulfated Chondroitin Sulfate Proteoglycans*

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Rat serosal mast cells, which synthesize only heparin proteoglycans as detected by intrinsic labeling with [35S]sulfate, were analyzed for the presence of intracellular chondroitin sulfate proteoglycans by chemical and immunochemical means. Rat serosal mast cells met >99% purity were treated with Zwittergent 3-12 and 4 M guanidine HCl, and the extracted nonradiolabeled proteoglycans were purified by density gradient centrifugation. As assessed by quantification of the unsaturated disaccharides released from the proteoglycans by chondroitinase ABC treatment, 10⁶ rat serosal mast cells contain 2.4-4.5 µg of chondroitin sulfate proteoglycans. Analysis of the chondroitinase ABC digests by high performance liquid chromatography revealed the unsaturated disaccharides ADi-4S, ADi-diSE and ADi-diSB which were derived from GlcA-GalNAc-4-SO₄, iduronic acid-2-SO₄-GalNAc-4-SO₄ and GlcA-GalNAc-4,6-diSO₄ respectively. The molar ratio of the monosulfated to disulfated disaccharides was 2:1 with ADi-diSB > ADi-diSB. When analyzed with a mouse anti-chondroitin sulfate monoclonal antibody and fluorescein-labeled F(ab')₂ goat anti-mouse IgG, ~91% of permeabilized and chondroitinase ABC-treated cells in the mast cell preparations exhibited intracellular fluorescence, and the pattern of staining indicated that the chondroitin sulfate molecules were located in the secretory granules. The specificity of the monoclonal antibody for the unsaturated double bond created by chondroitinase ABC treatment of the proteoglycan in situ was established by the absence of fluorescence when the chondroitinase ABC step was omitted or when heparinase digestion was substituted for chondroitinase ABC. Furthermore, the ability of the anti-chondroitin sulfate monoclonal antibody to mediate fluorescence in situ was markedly reduced by absorption with solid-phase chondroitin sulfate proteoglycan that had been chondroitinase ABC-treated, but not by absorption with undigested proteoglycan or with solid-phase heparin. The highly sulfated chondroitin sulfate proteoglycans of rat serosal mast cells are the same type synthesized by the rat mucosal mast cell subclass. The simultaneous presence of both heparin and chondroitin sulfate glycosaminoglycans in rat serosal mast cells in the absence of detectable chondroitin sulfate synthesis suggests that this chondroitin sulfate-containing proteoglycan is a remnant of an earlier stage of development and implies an ontologic relationship between the two mast cell subclasses.

Distinct subpopulations of mast cells in the rat have been identified by the differences in the types of proteoglycans (1, 2) and serine proteases (3, 4) that they synthesize and store in their secretory granules, by their differential histochemical staining properties (5), by their relative histamine content per cell (6, 7), by the presence or absence of T-cell dependence for maintenance (8), and by the relative ability of various agonists to elicit release of preformed intragranular mediators (7). Mast cells obtained from the serosal surfaces of the rat peritoneal cavity incorporate [35S]sulfate into a protease-resistant Mᵥ 750,000 heparin proteoglycan (1), whereas T-cell-dependent mast cells isolated from the mucosa of the small intestine of Nonpromyelorygulys brasiliensis-infected rats incorporate [35S]sulfate into a protease-resistant Mᵥ 150,000 chondroitin sulfate proteoglycan (2). When the chondroitin sulfate proteoglycans from rat mucosal mast cells are incubated with chondroitinase ABC and the generated unsaturated disaccharides are analyzed by high performance liquid chromatography (HPLC), the three disaccharides with 4-SO₄, ADi-diSE, and ADi-diSB are detected with the concentration of ADi-4S = ADi-diSB > ADi-diSB. Although rat serosal mast cells do not synthesize detectable levels of 35S-labeled chondroitin sulfate proteoglycans in vivo, they synthesize chondroitin sulfate glycosaminoglycans that are rich in Di-diSE onto the exogenous glycosaminoglycan acceptor, p-nitrophenyl-β-D-xylodside, during short-term culture (9). We now demonstrate by chemical techniques that rat serosal mast cells have chondroitin sulfate proteoglycans and that their chondroitin sulfate glycosaminoglycans contain the same types of unique disulfated disaccharides that are detected by 35S-labeling of rat mucosal mast cells. Furthermore, using a novel monoclonal anti-chondroitin sulfate antibody for intracellular immunofluorescence staining, we have determined that the chondroitin sulfate molecules are in essentially all rat serosal mast cells and are located intragranularly.

EXPERIMENTAL PROCEDURES

Chemical Characterization of Chondroitin Sulfate in Rat Serosal Mast Cells—Rat serosal mast cells were obtained by lavage of the

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1 The abbreviations used are: HPLC, high performance liquid chromatography; ADi-4S, ADi-diSE, ADi-diSB, ADi-diSB, the unsaturated disaccharides derived from GlcA-GalNAc-4-SO₄, GlcA-GalNAc-4,6-diSO₄, iduronic acid-2-SO₄-GalNAc-4-SO₄, GalC-A-GalNAc-4,6-diSO₄, respectively; HBS, Hanks' balanced salt solution.

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peritoneal cavity of each rat with 30 ml of Tyrode's buffer containing 1 mg/ml gelatin. The mast cells were concentrated to >99% purity by one isopyknic (1) and two isokinetic (10) centrifugations. Each preparation of 1.1 - 1.3 x 10^6 highly purified mast cells from 100 rats was suspended in 150 μl of 1% (w/v) Zwittergent 3-12 (Behring Diagnostics) for ~15 s and then in 12 ml of 4 M guanidine HCl containing 0.1 M NaCl and CaCl₂ (final density 1.4 g/ml) (11). The mast cell extracts were centrifuged for 48 h at ~100,000 × g, and the bottom halves of the gradients were dialyzed sequentially against 1 M sodium acetate followed by 0.1 M ammonium bicarbonate; the dialyzed samples were lyophilized and resuspended in 2 ml of HBSS containing 0.25% (v/v) of protease inhibitors (17). The permeabilized mast cells was also estimated by its integrated absorbance relative to that of the antibody used (5/29/2-B-6) was of the IgG, subclass and was obtained from goat anti-mouse IgG (Cooper Biomedical, Malvern, PA). Following appropriate dilutions, the mast cells were spread on cytocentrifuge slides and treated with HBSS, 0.25% (v/v) of protease inhibitors (17) and incubated with 1 unit of chondroitinase ABC for 30 min at 37°C; the digestion was stopped by washing the gel with HBSS. Samples (100 μl) of the 5/29/2-B-6 antibody (6.6 μg of IgG/ml) were mixed for 1 h at room temperature with 25 μl of packed chondroitin sulfate proteoglycan-Sepharose (containing an estimated 12 μg of chondroitin sulfate glycosaminoglycans), 25 μl of packed chondroitin sulfate proteoglycan-Sepharose that had been treated with chondroitinase ABC, or 15.7 μl of packed heparin-agarose (containing an estimated 12 μg of heparin glycosaminoglycans). The supernatants were recovered by centrifugation and the capacities of the retained disulfated disaccharides were analyzed in three separate experiments, with the results being 2.3:1.0 and 2.0:1.0. In each case, the findings were comparable to those of packed heparin-agarose (con-}

RESULTS

Chemical Characterization of Chondroitin Sulfate in Rat Serosal Mast Cells—As assessed by the carboxylreduction reaction, the density gradient-purified extracts contained ~25 μg of uronic acid per 10^6 rat serosal mast cells. Incubating 200 μg of this material with chondroitinase ABC resulted in a net material with chondroitinase ABC resulted in a net 25 mg of packed chondroitin sulfate proteoglycan-Sepharose that had been treated with chondroitinase ABC, or 15.7 μl of packed heparin-agarose (containing an estimated 12 μg of heparin glycosaminoglycans). The supernatants were recovered by centrifugation and the capacities of the retained disulfated disaccharides were analyzed in three separate experiments, with the results being 2.3:1.0, 1.4:1.0, and 2.0:1.0. In each case, the findings were comparable to those of packed heparin-agarose (con-}

FIG. 1. HPLC of a chondroitinase ABC digest of density gradient-purified proteoglycans from rat serosal mast cells. The retention times of the disulfated disaccharides were analyzed in three separate experiments, with the results being 2.3:1.0, 1.4:1.0, and 2.0:1.0. In each case, the findings were comparable to those of packed heparin-agarose (con-}

The retention times of the standard disaccharides ADi-4S (4S), ADi-diS(B), and ADi-diS(E) are indicated.
Chondroitin Sulfate Proteoglycans in Rat Serosal Mast Cells

**FIG. 2.** Fluorescence (A, B) and paired Nomarski interference microscopy (A', B') photomicrographs of permeabilized rat serosal mast cells stained with anti-glycosaminoglycan monoclonal antibodies. Cells were treated with chondroitinase ABC and then incubated with either anti-chondroitin sulfate (A) or anti-keratan sulfate (B) monoclonal antibodies before being stained with fluorescein-conjugated F(ab')2 goat anti-mouse IgG. The arrows in panel A' identify two cells that failed to fluoresce and are not seen in panel A. Magnification, ×1043.

**FIG. 3.** Immunofluorescence of permeabilized, chondroitinase ABC-treated rat serosal mast cells stained with dilutions of unabsorbed anti-chondroitin sulfate 5/29/2-B-6 monoclonal antibody (○), 5/29/2-B-6 after absorption with heparin-agarose (■), or 5/29/2-B-6 after absorption with nontreated (○) or chondroitinase ABC-treated (■) rat chondrosarcoma chondroitin sulfate proteoglycan coupled to Sepharose 4B. The binding of undiluted, unabsorbed antibody is indicated by the asterisk. Data are from one representative experiment.

μg (mean ± range, n = 2) of chondroitin sulfate.

*Intracellular Detection by Immunofluorescence Microscopy of Chondroitin Sulfate in Rat Serosal Mast Cells—*Incubating permeabilized, chondroitinase ABC-treated rat serosal mast cells with a saturating amount of the anti-chondroitin sulfate 5/29/2-B-6 monoclonal antibody and then with fluoresceinated F(ab')2 goat anti-mouse IgG resulted in the staining of 91 ± 4.5% (mean ± S.D., n = 3) of the cells; the intensity of staining ranged from dim to extremely bright (Fig. 2A). The fluorescence was intracellular and granular in all positive cells. The specificity of the 5/29/2-B-6 monoclonal antibody for the unsaturated double bond created in the chondroitin sulfate glycosaminoglycan after treatment with chondroitinase ABC was confirmed *in situ* by the finding that permeabilized cells not treated with chondroitinase ABC failed to exhibit fluorescence. Permeabilized, chondroitinase ABC-treated mast cells incubated with the anti-keratan sulfate antibody also did not stain (Fig. 2B). No fluorescent staining was detected when the permeabilized cells were treated with large amounts of heparinase before the interaction with the anti-chondroitin sulfate monoclonal antibody. This excluded the possibility that a heparinase contaminant in the chondroitinase ABC preparation was creating a determinant from heparin that was recognized by the anti-chondroitin sulfate monoclonal antibody.

The specificity of the antibody-mediated fluorescence was also confirmed by absorption with solid-phase proteoglycans of an amount of 5/29/2-B-6 antibody that gave maximal, but just saturating, fluorescence staining of permeabilized, chondroitinase ABC-treated rat serosal mast cells. The relative amount of residual affinity-absorbed antibody was determined in each case by measuring its ability to mediate staining of rat serosal mast cells. When the 5/29/2-B-6 antibody was absorbed with chondroitinase ABC-digested chondrosarcoma chondroitin sulfate proteoglycan coupled to Sepharose, a 1:8 dilution of the absorbed antibody preparation stained only 3.7 ± 2.1% (mean ± S.D., n = 3) of the rat serosal mast cells (Fig. 3). Sixty-nine ± 11% (mean ± S.D., n = 3) of the cells stained after incubation with an identical dilution of unabsorbed 5/29/2-B-6 antibody. An equal amount of the 5/29/2-B-6 antibody that had been absorbed with non-chondroitinase ABC-treated proteoglycan and diluted 1:8 stained 57 ± 8.5% (mean ± S.D., n = 3) of the rat serosal mast cells, whereas a 1:8 dilution of antibody absorbed with heparin-agarose under identical conditions stained 70 ± 10% (mean ± S.D., n = 3) of the cells. Further dilution of the latter three antibody
preparations to 1:32 resulted in the staining of 35 ± 15%, 20 ± 5.7%, and 32 ± 17% (mean ± S.D., n = 3) of the cells, respectively, which was comparable to the activity of a 1:2–1:4 dilution of the monoclonal antibody following absorption with chondroitinase ABC-digested solid-phase chondroitin sulfate proteoglycan (Fig. 3).

**DISCUSSION**

Through the use of chemical and immunologic approaches, this study establishes that rat serosal mast cells contain chondroitin sulfate proteoglycans that are similar to the novel proteoglycans that can be 35S-labeled ex vivo in rat mucosal mast cells. These highly sulfated chondroitin sulfate proteoglycans are rich in the disulfated disaccharides Di-diSB (iduronic acid-2-SO3−GalNAc-4-SO3−) and Di-diSE (GlCα−GalNAc-4,6-diSO3−) (Fig. 1). Quantification of the chondroitin sulfate glycosaminoglycans by measurement of the unsaturated disaccharides released by chondroitinase ABC treatment of the density gradient-purified proteoglycans revealed that 105 rat serosal mast cells contain 2.4 ± 0.5 μg of chondroitin sulfate proteoglycans, compared to a reported heparin proteoglycan content of more than 20 μg per 106 cells. Upon isolation and radiolabeling with [35S]sulfate in vitro, rat serosal mast cells synthesize proteoglycans that contain heparin glycosaminoglycans but not chondroitin sulfate glycosaminoglycans (1), although biosynthesis of over-sulfated chondroitin sulfate can be observed in the presence of p-nitrophenyl-β-D-xlyoside (9). Thus, either the rat serosal mast cell synthesizes chondroitin sulfate proteoglycans at an earlier stage of its differentiation or there is a preferential suppression of chondroitin sulfate synthesis when the cells are placed in short-term tissue culture.

Using a mouse IgG, monoclonal antibody prepared to chondroitinase ABC-treated bovine nasal cartilage proteoglycan, we determined by intracellular fluorescence microscopy that at least 91% of the permeabilized and chondroitinase ABC-treated cells in the rat serosal mast cell preparations contained chondroitin sulfate proteoglycans (Fig. 2A). The pattern of staining of the cells with the anti-chondroitin sulfate monoclonal antibody indicated that these proteoglycans were stored in the secretory granules of the cells. The in situ specificity of this monoclonal antibody for chondroitin sulfate glycosaminoglycans was limited to permeabilized, chondroitinase ABC-treated rat serosal mast cells (Fig. 2A). No fluorescence was observed in permeabilized, non-chondroitinase ABC-treated cells or in permeabilized, heparinase-treated cells. Solid-phase absorption of the antibody with chondroitinase ABC-digested chondroitin sulfate proteoglycan effectively abrogated subsequent binding to permeabilized, chondroitinase ABC-treated rat serosal mast cells (Fig. 3). However, solid-phase absorptions with equivalent amounts of either non-chondroitinase ABC-digested chondroitin sulfate proteoglycan or heparin did not reduce in situ binding to permeabilized and chondroitinase ABC-treated mast cells, as assessed by dilution analysis of absorbed antibody preparations compared with unabsorbed antibody (Fig. 3). Non-specific absorption of IgG molecules and non-specific binding of the secondary, fluorescein-conjugated antibody to the secretory granules were ruled out because the mouse IgG, monoclonal antibody that is reactive with keratan sulfate glycosaminoglycans did not bind to permeabilized and chondroitinase ABC-treated rat serosal mast cells (Fig. 2B). Although ~91% of chondroitinase ABC-treated rat serosal mast cells exhibited intracellular fluorescence when stained with the anti-chondroitin sulfate monoclonal antibody, there was considerable heterogeneity in the intensity of staining from cell to cell. This variation could be due to differential loss of the chondroitin sulfate proteoglycans during the permeabilization and enzymatic steps, to differences in the susceptibility of the chondroitin sulfate proteoglycans in situ to degradation by chondroitinase ABC because of varied interactions with other cationic proteins in the secretory granules, or it could reflect different stages of mast cell maturation.

The presence of both heparin and chondroitin sulfate glycosaminoglycans in highly purified, normal ex vivo serosal mast cells is a novel finding. In an earlier study we demonstrated that the rat basophilic leukemia-1 cell line, which by a number of criteria is homologous to rat mucosal mast cells (18), synthesizes both nitrous acid-degradable and chondroitinase ABC-degradable glycosaminoglycans (19, 20). As assessed by HPLC analysis of chondroitinase ABC-generated unsaturated disaccharides, the chondroitin sulfate of the rat basophilic leukemia-1 proteoglycan is composed of Di-4S and Di-diSB (19). The presence of heparin and chondroitin sulfate that is rich in Di-diSB, as well as Di-diSB, in freshly isolated rat serosal mast cells demonstrates that the classification of subpopulations of rat mast cells by their proteoglycan phenotype may be more accurately defined by the ratios of the constituent glycosaminoglycans rather than by the absolute amount of a single glycosaminoglycan.

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