Synthesis of Chondroitin Sulfate D and Heparin Proteoglycans in Murine Lymph Node-derived Mast Cells

THE DEPENDENCE ON FIBROBLASTS*

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Proteoglycans synthesized in cultured mast cells derived from horse serum-immunized lymph node cells were analyzed. Treatment of the 35S-proteoglycans extracted from these cells with either chondroitinase ABC or AC resulted in 95% ± 7% and 84% ± 7%, respectively (mean ± S.E., n = 3), of the radioactivity associated with disaccharides eluting in the included volume of PD-10. The 35S-proteoglycans were not hydrolyzed by nitrous acid elimination treatment. The chondroitinase ABC-generated disaccharides were analyzed by aminocyanol high performance liquid chromatography. 35S-Disaccharides eluted in a major peak at a retention time of 8.1 min, corresponding to the disaccharide of chondroitin 4-sulfate proteoglycan (ΔDi-4S), and a second peak at 12 min, corresponding to the disaccharide of chondroitin sulfate D proteoglycan (ΔDi-di5S). Further treatment with chondro-4-sulfatase did not affect the retention time of the disaccharide corresponding to ΔDi-di5S, whereas this peak disappeared after the digested proteoglycan was treated either by chondro-6-sulfatase or by both sulfatases. Therefore, this disaccharide was identified as chondroitin sulfate D. Quantification of the radiolabeled disaccharides showed that ΔDi-di5S contributed 20% ± 2% (n = 3) of the total sulfated disaccharides of the chondroitin sulfate of these cultured cells. The role of fibroblasts in inducing the shift of chondroitin sulfate D into heparin proteoglycan in these mast cells was also investigated by using three types of monolayers: mouse embryonic skin fibroblasts (MESF), rat embryonic skin fibroblasts (RESF), and 3T3 fibroblasts. 35S-Proteoglycans that were extracted from the lymph node-derived mast cells cultured for 30 days on MESF and on 3T3 fibroblast monolayers were 93% ± 4% and 30% ± 7% (n = 3) susceptible to nitrous acid elimination, respectively. No degradation by nitrous acid was observed in 35S-proteoglycans extracted from cells cultured on RESF monolayer. Since the MESF was found to be the most potent monolayer in the induction of heparin synthesis, the kinetics of changes in the synthesis of proteoglycan types were determined in lymph node-derived mast cells cultured on MESF for up to 30 days. It was found that the synthesis of chondroitin sulfate gradually declined whereas that of heparin starting between 4 and 7 days after plating gradually increased. From the 17th day on, only the synthesis of heparin was detected.

Oversulfated proteoglycans have become useful phenotypic markers for distinguishing subclasses of mast cells. Heparin proteoglycan was first described in the secretory granules of connective tissue mast cells derived from rat (1) and mouse (2) peritoneal cavity. Later it was also identified in human lung (3) and skin mast cells (4). Chondroitin sulfate E (ChS-E), an oversulfated proteoglycan of about M, 250,000, possessing disulfated disaccharides GlcUA → GalNAc-4, 6-diS04 (ΔDi-di5S) was identified in the secretory granules of cultured mouse bone marrow-derived or mouse fetal liver-derived, interleukin 3-dependent mast cells (2, 5, 6). These cultured mast cells are the analogues of ChS-E containing mucosal mast cells identified in vivo in the mucous membranes of human colon (7) and stomach (8) and later in human lung (9). ChS-E was also found in the secretory granules of cultured human bone marrow-derived mast cells (10). Another oversulfated chondroitin sulfate proteoglycan chondroitin sulfate B, (Chs-dSB), was identified in the secretory granules of rat bone marrow-derived mast cells (11). The different mast cell subclasses differ in a wide range of other properties. A most important aspect of this heterogeneity is the dependence of cultured mast cells on factors derived from T cells for differentiation and growth (5, 6, 12).

The interrelationship between these two cell subclasses of mast cells has been extensively investigated. We have shown that signals derived from the mouse embryonic skin fibroblasts (MESF) induce a phenotype change in cultured mast cells derived from immune lymph nodes (LNMC) into connective tissue-like mast cells (13, 14). These phenotypic changes were confirmed later in other culture systems in which Chs-E containing mouse bone marrow-derived mast cells cultured on mouse skin-derived 3T3 fibroblast cell line synthesized a significant amount of granular heparin proteoglycan (15). These studies have been extended by Kitamura and co-workers (16, 17), who provided evidence in vivo which

*This work was supported by the Fund for Basic Research administered by the Israel Academy of Sciences and Humanities (to H. G.) and by the German-Israeli Foundation for Scientific Research and Development (to E. R.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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indicates that the mast cell can acquire phenotypic characteristics depending upon the environmental signals. Therefore, T cell growth-dependent mast cells may function as precursors of both types of cells. Most recently, mice mesenteric lymph node-derived mast cell committed precursors have been reported to acquire a serosal (connective tissue) mast cell phenotype when cultured on monolayers of 3T3 fibroblast cell line or MESF or when cultured on methylocellulose in the presence of medium conditioned by these monolayers (18). On the other hand, these mouse lymph node-derived mast cell committed precursors acquire a mucosal mast cell phenotype when cultured in the presence of mouse interleukin 3.

In a previous study on mouse LNMC cultured in the presence of medium conditioned by the WEHI-3B myelomonocytic cell line, which releases mouse interleukin 3, we have analyzed the granular proteoglycan of these cells (19). We found that chondroitinase ABC-digested proteoglycan yielded two disaccharides that co-migrated on ascending thin layer chromatography with ΔDi-4S and ΔDi-diSz standards. By using this criterion, without any further analysis, it was assumed that the LNMC granular proteoglycan is ChS-E. The cells were therefore considered cultured analogues of mucosal mast cells.

In the present study we have reanalyzed the disaccharide composition of the glycosaminoglycans of this LNMC-derived proteoglycan using additional methods. It was found that these glycosaminoglycans contain disulfated disaccharides GlcUA-2-So4 → GalNAc-6-So4, termed chondroitin sulfate D (ChS-D) rather than ChS-E as described previously (19). In order to investigate whether these ChS-D-containing mast cells change their phenotype depending upon their microenvironment (in the same way that mouse bone marrow-derived mast cells depend on 3T3 fibroblasts), further work has been done. The role of three different fibroblast monolayers in triggering synthesis of heparin proteoglycan in these mast cells was investigated. Out of the three types of monolayers (MESF, RESF, and 3T3 fibroblasts), the MESF monolayer was found to be far superior in causing such an induction.

**MATERIALS AND METHODS**

**Preparation of LNMC**—The procedure for producing a 98% homogenous population of LNMC has been described previously (13). Briefly, BALB/c female mice 1–2 months old were injected intraperitoneally with 0.2 ml of horse serum once a week for 5 weeks. Two to seven days later 15 x 10^6 mesenteric lymph nodes from immunized mice were cultured in the presence of Dulbecco’s modified Eagle’s medium supplemented with 15% (v/v) horse serum and 10% conditioned medium (cultured medium) in tissue culture tubes (Falcon, Becton Dickinson). The conditioned medium was prepared by culturing mesenteric lymph node cells derived from cercaria-infected mice (13). After 14 days of incubation, 98% of the homogenous population of LNMC was identified. Then, 3 x 10^6 LNMC were cultured for various periods of time in 3 ml of cultured medium on different types of fibroblast monolayers prepared on 35-mm Petri dishes (Falcon).

**Preparation of Fibroblast Monolayers**—MESF was prepared from skin of 19- to 20-day-old BALB/c mouse embryos as described previously (20). RESF was prepared from Sprague-Dawley rat embryos (19–20 days) by the same procedures described for the preparation of MESF (21, 22). The Swiss-albino mouse skin-derived 3T3 fibroblast cell line was obtained from the American Type Culture Collection, Rockville, MD. The fibroblasts were x-irradiated with a cytostatic dose of 5000 rad and then cultured in Waymouth medium (20) in concentrations ranging from 0.06 x 10^6 to 1 x 10^6 cells/plate. (The fibroblasts remained viable yet did not divide. The cells continued to synthesize proteoglycans, which were analyzed in monolayers prior to co-culture and in fibroblasts separated from the co-culture after 30 days.

**Separation of LNMC from Fibroblasts**—The cells from each plate were dispersed by 0.1% trypsin, washed twice, and resuspended with 30 ml of Dulbecco’s modified Eagle’s medium containing 15% horse serum. The cell suspension was then plated into three tissue culture dishes (100 x 20 mm) (Falcon). After a 1-h incubation at 37°C, CO2 incubator, most of the fibroblasts adhered to the plates whereas the mast cells remained in suspension and were enriched up to a purity of 94% ± 6% (mean ± S.E., n = 6) as determined by toluidine blue staining.

**Proteoglycan Analysis** (3, 9) —Proteoglycans were characterized in each cell type separately both prior to the co-culture and after separation from the co-culture. The LNMC-associated proteoglycan was characterized prior to co-culture in cultures that contained more than 94% mast cells and in mast cells separated and enriched from the co-cultures (as described above) after the different co-culture periods. The fibroblasts were separated from all three monolayers prior to the co-culture, as a base line, and after the fibroblasts were separated from the co-culture at the times indicated.

The procedure for proteoglycan analysis was as follows. Cells were incubated for 4 h at 37°C in fresh culture medium containing 100 μCi/ml [35S]sulfate (4000 Ci/mmol) (Du Pont-New England Nuclear). The radiolabeled cells were sedimented at 400 x g, resuspended in 0.2 ml of 0.05 M sodium acetate containing 1% (w/v) Zwittergent 3-12 detergent (Calbiochem), 0.1 M sodium EDTA, 5 mM benzamidine HCl, and 1 mM sodium iodoacetamide followed by the addition of 0.6 ml of 0.1 M sodium acetate and 4 μg bovine serum albumin and 30 μg of heparin carrier (Sigma). Total [35S]sulfate incorporation into macromolecules was assessed by chromatography of a sample diluted to 0.5 ml in 0.1 M Tris HCl, 0.1 M sodium sulfate, 4 μg guanidine HCl, pH 7.4 (TSG buffer), on a PD-10 gel filtration column (Pharmacia LKB Biotechnology Inc.) equilibrated and eluted in TSG buffer. Half-milliliter fractions were collected, and the radioactivity was determined in a β-scintillation counter. 35S-labeled proteoglycans were purified by separation over a 45 × 0.8-cm Sepharose 4B column (Pharmacia) equilibrated and eluted in phosphate-buffered saline, pH 7.4. One-milliliter samples were collected and radioactivity peak separated, dialyzed overnight against water, and lyophilized. Samples were resuspended in phosphate-buffered saline containing 0.2% trypsin (Sigma) and incubated for 4 h at 37°C. [35S]Proteoglycans were then purified further by CsCl density gradient ultracentrifugation under dissociative conditions by adding CsCl to a starting density of 1.4 g/ml in TSG buffer to the cell extracts and centrifugation at 95,000 x g for 48 h at 20°C. CsCl gradients were divided into three fractions. The bottom fraction, which contained 80% of the [35S]labeled macromolecules, was dialyzed for 1 day against water and lyophilized. The purified proteoglycans were resuspended in 500 ml of TSG buffer and applied to PD-10 columns. Twenty 0.5-ml fractions were collected, and the radioactivity was quantified. 35S-Proteoglycans eluted at the PD-10 void volume (V0) were collected, dialyzed for 1 day against water, and lyophilized. The presence of [35S]labeled heparin and [35S]labeled chondroitin sulfate was assessed by determining the susceptibility of the proteoglycans to degradation by nitrous acid elimation and chondroitinase ABC and AC digestion. Nitrous acid susceptibility was determined as described previously (2). Briefly, [35S]labeled glycosaminoglycans were incubated for 24 h at 37°C in the presence of 1,2-dimethoxyethane and butyl nitrite (Eastman Kodak). Authentic [3H]heparin (Du Pont-New England Nuclear) was used as control. Elimination products were chomatographed on the Sephadex G-25/PD-10 columns, with degradation being assessed by determining the shift in [3H]radioactivity from the V0 to the included volume. For the determination of glycosaminoglycan susceptibility to degradation by chondroitinase ABC or AC, proteoglycans of the purified radiolabeled proteoglycans were resuspended in 1 ml of Tris-HCl buffer, pH 8.0, composed of 50 mM Tris-HCl, 50 mM NaCl, 35 mM sodium acetate, and 0.5% bovine serum albumin with or without 0.2 unit of either chondroitinase ABC or AC (Sigma) for 4 h at 37°C (2). Cold chondroitin-6-sulfate and chondroitin-4-sulfate (5 μg each) (Sigma) were used as carriers to reduce contamination. Portions of the purified radiolabeled proteoglycans were digested with bone marrow-derived mast cell-derived [35S]labeled chondroitin sulfate E proteoglycan was used as control. Digests were chromatographed on the Sephadex G-25/PD-10 columns, with degradation being assessed by determining the shift in [3H]radioactivity from the V0 to the included volume.

The disaccharides of the chondroitin sulfate disaccharides were analyzed by aminoacyl HPLC by a method described previously (23) and by their mobilities on ascending thin layer chromatography on precoated cellulose acetate plates (19). Chondroitin-6-sulfatase (0.002 unit/μg of carrier), chondroitin-4-sulfatase (0.002 unit/μg of carrier), or

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both sulfatases sequentially in a specific order were added to some samples after the chondroitinase ABC digestion (2). The same treatment was performed with \(^{35}\)S-ChS-E proteoglycan (2) derived from cultured mouse bone marrow-derived mast cells and for ChS-D proteoglycan derived from shark cartilage (kindly provided by Dr. Seno, Ochanomizu University, Tokyo, Japan). For HPLC analysis, \(^{35}\)S-labeled proteoglycans were digested by chondroitinase ABC and were extracted in 80% ethanol, cooled to 4 °C for 2 h, and then centrifuged in Beckman Microfuge at 8000 x g for 5 min. The supernatant was decanted, dried over nitrogen, and resuspended in the HPLC solvent, which was 70% acetonitrile/methanol (3:1, v/v) and 30% 0.5 M ammonium acetate/acetic acid, pH 5.3, with an apparent final pH of 7.0. A 4.6 x 250-mm Partisil-10 PAC aminocono column with a 4.6 x 25-mm precolumn containing the same packing (Whatman) was used for separating disaccharides. Eluates were collected for 0.5-min intervals and quantified by \(\beta\)-scintillation counting. The following disaccharides were used for standards: ADi-6S and ADi-4S (Miles Laboratories), \(^{35}\)SADi-diS\(_S\) (mouse bone marrow-derived mast cells) (2), \(^{35}\)SADi-diS\(_S\) (rat basophilic leukemia cells) (11), and ADi-diS\(_S\) (shark cartilage). These standards, which were run each time, were detected either by continuous monitoring of ultraviolet absorbance at 232 nm or by determining the \(^{35}\)S radioactivity. The retention times of the disaccharide standards were: ADi-6S, 5.8 min; ADi-4S, 8.1 min; ADi-diS\(_S\), 12 min; ADi-diS\(_B\), 14.2 min; ADi-diS\(_B\), 15.9 min; and free \(^{35}\)S, 24-26 min. The disaccharides that were liberated by chondroitinase ABC digestion and were treated further by the sulfatases were characterized by their mobility relative to disulfated and monosulfated disaccharide standards on HPLC.

Histamine Analysis—Histamine was assayed by the enzymatic method modified by Taylor et al. (24).

RESULTS

Disaccharide Composition of the Glycosaminoglycan Side Chains of LNMC Proteoglycans—Purified \(^{35}\)S-labeled proteoglycans extracted from LNMC were either enzymatically digested with chondroitinase ABC or AC or chemically degraded by nitrous acid elimination. The percentages of disaccharides liberated by each procedure were quantitated by Sephadex G-25/PD-10 chromatography. In the untreated control sample, all radioactivity of \(^{35}\)S-proteoglycans was filtered in the void volume of the PD-10 column (Fig. 1a). Treatment with either chondroitinase ABC or AC resulted in 95% and 7% (n = 3), of the total radioactivity being associated with disaccharides eluting in the included column volume (Fig. 1, b and c). The \(^{35}\)S-proteoglycans were not degraded by nitrous acid elimination treatment (Fig. 1d). The chondroitinase ABC-generated unsaturated disaccharides derived from the LNMC proteoglycans were analyzed first by their mobilities on ascending thin layer chromatography. These disaccharides migrated in the position of ADi-4S and of an oversulfated disaccharide corresponding to either ADi-diS\(_B\) or ADi-diS\(_S\). These two oversulfated disaccharides migrated to the same position on this thin layer chromatography and cannot be separated under these conditions. The further addition of chondro-4-sulfatase to the digestion mixture depleted the detectable ADi-4S; however, it did not affect the migration of the oversulfated disaccharide. Treatment with chondro-6-sulfatase markedly depleted the oversulfated \(^{35}\)S-labeled disaccharide. \(^{35}\)SADi-diS\(_S\), which was used as control, was susceptible to either one of the sulfatases. Since by using such thin layer chromatography the oversulfated disaccharides could not be separated by migration from ADi-diS\(_S\), the chondroitinase ABC-generated disaccharides were analyzed by aminocono HPLC. \(^{35}\)S-Disaccharides eluted in a major peak at a retention time of 8.1 min, corresponding to ADi-4S, and a second peak at 12 min, corresponding to ADi-diS\(_S\) (Fig. 2a). Treatment of the chondroitinase ABC-digested proteoglycans with chondro-4-sulfatase did not affect the retention time of the disaccharide corresponding to ADi-diS\(_S\) (Fig. 2c). However, when these chondroitinase ABC-digested proteoglycans were treated with chondro-6-sulfatase, the peak corresponding to ADi-diS\(_S\) disappeared, and a new peak eluted at a retention time of 3.8 min (Fig. 2b). Double digestion of the chondroitinase ABC products with chondro-6-sulfatase and chondro-4-sulfatase resulted in the disappearance of the peak corresponding to ADi-4S and ADi-diS\(_S\) and the appearance of the new peak at retention time of 3.8 min (Fig. 2d). \(^{35}\)SADi-diS\(_B\), which was used as control, eluted at a retention time of 15.9 min, and this peak disappeared after treatment with either sulfatase or with both. These results are in accordance with the results obtained from the TLC and therefore indicate that the oversulfated disaccharide derived from LNMC proteoglycan is identical to the chondroitin sulfate \(\Delta\) purified from shark cartilage (23). The relative amount of oversulfated disaccharides was determined by comparing total peak cpm values (Fig. 2a) while considering the \(^{35}\)S:molecule ratio for ADi-4S and ADi-diS\(_S\), 1:1 and 2:1, respectively. It was calculated that ADi-diS\(_S\) contributed 20% ± 2% (n = 3) of the total sulfated disaccharides of the chondroitin sulfate proteoglycan of these cultured cells.

Proteoglycan Characterization of the Three Fibroblast Monolayers—Purified \(^{35}\)S-labeled proteoglycans extracted from the various fibroblast monolayers were analyzed prior to co-culturing with LNMC. As in the LNMC, the proteoglycans were subjected to enzymatic degradation with chondroitinase ABC and AC and to degradation by nitrous acid elimination. Proteoglycan degradation was determined and quantitated by liberation of disaccharides from the macromolecules as detected following each procedure by Sephadex G-25/PD-10 chromatography (see Table I). The analysis of the proteoglycans degradation revealed that \(^{35}\)S-proteoglycans extracted from LNMC alone; b, after treatment with chondroitinase ABC; c, after treatment with chondroitinase AC; d, after treatment with nitrous acid. The results shown are from one representative experiment out of three.

FRACTION No.

Fig. 1. Sephadex G-25/PD-10 filtration of \(^{35}\)S-labeled proteoglycans extracted from LNMC. a, LNMC alone; b, after treatment with chondroitinase ABC; c, after treatment with chondroitinase AC; d, after treatment with nitrous acid. The results shown are from one representative experiment out of three.
from each of the three fibroblast monolayers were not susceptible to nitrous acid elimination. Chondroitinase ABC and chondroitinase AC treatments of these proteoglycans revealed that most of their proteoglycans are dermatan sulfate rather than chondroitin sulfate.

In order to determine whether the ChS-D-containing mast cells change their phenotype depending upon their microenvironment, they were co-cultured with the various types of fibroblasts and then characterized.

**Phenotypic Changes in LNMC Cultured on the Various Fibroblast Monolayers—** A comparative analysis was performed among MESF, RESF, and 3T3 fibroblast monolayers on the induction of heparin proteoglycan synthesis in the LNMC. Confluent fibroblast monolayers of all three types were treated prior to the initiation of co-culture with LNMC with a cytostatic dose of x-irradiation (5000 rads). Fibroblast monolayers irradiated as controls showed no proliferation activity, and yet the cells were viable as determined by trypan blue dye exclusion. The amount of \(^{35}\text{S}\) sulfate incorporation into the LNMC macromolecules was determined. LNMC cultured for 30 days on MESF monolayer incorporated 17 times greater \(^{35}\text{S}\) sulfate into macromolecules than the amount observed for LNMC that were not plated on monolayer (Table II), as assessed by PD-10 chromatography. The other two fibroblast monolayers, RESF and 3T3, were found to be less effective in the induction of \(^{35}\text{S}\) sulfate incorporation.

LNMC co-cultured on the various monolayers for 15 and 30 days were separated from the fibroblasts to a purity of more than 90%. \(^{35}\text{S}\)-Proteoglycans were then extracted from the mast cells and subjected to chondroitinase AC and nitrous acid treatments. Proteoglycans extracted from LNMC that were separated from co-culture with MESF after 30 days were 93% ± 4% (n = 3) susceptible to nitrous acid elimination (Fig. 3) whereas only 30% ± 7% (n = 3) of the \(^{35}\text{S}\)-proteoglycans extracted from LNMC separated from co-culture with 3T3 fibroblasts was degraded by nitrous acid. No degradation by nitrous acid was observed in \(^{35}\text{S}\)-proteoglycans extracted from cells separated from co-culture with RESF monolayer.

Although co-cultured LNMC proteoglycans were extracted for analysis from highly purified mast cells, the composition of the MESF fibroblast monolayer's proteoglycan was reanalyzed after the fibroblasts were separated from co-culture with the LNMC. It was found that the post-co-culture MESF proteoglycan composition was identical to that found prior to the co-culture (Table I). More than 80% of the post-co-culture MESF proteoglycan was identified as dermatan sulfate. The amount of incorporation into macromolecules was of the same order as that found prior to the co-culture. During the co-culture period, the fibroblasts maintained their normal histologic appearance with no apparent changes.

Since the MESF was found to be the most potent monolayer in the induction of heparin synthesis in these cells, the kinetics of the shift in proteoglycan types synthesized were determined in LNMC grown on this monolayer (Fig. 4). As can be seen, the synthesis of chondroitin sulfate D gradually declined whereas that of heparin began 4–7 days after plating and gradually increased. The ratio of 4:1 between ChDi-4S and ChDi-6S is preserved for the rest of the culture period.

**TABLE I**

| Types of fibroblasts | % Digestion (mean ± S.E., n = 3) | Chondroitinase ABC | Chondroitinase AC |
|----------------------|----------------------------------|--------------------|--------------------|
| MESF                 | 80 ± 9.2                         | 7 ± 0.3            |
| RESF                 | 72 ± 4.2                         | 16 ± 1.3           |
| 3T3                  | 84 ± 10                          | 20 ± 0.0           |

**TABLE II**

| Incorporation of \(^{35}\text{S}\) sulfate into proteoglycan synthesized by mast cells grown on various types of fibroblast monolayers |
|-------------------------------------------------------------------------------------------------------------------------------|
| | \(^{35}\text{S}\) Sulfate incorporation (mean ± S.E.; n = 3) |
|---------------------------------------------------------------|
| LNMC                | 10,185 ± 1,400                        |
| LNMC co-cultured with MESF                                   | 175,200 ± 30,600                      |
| LNMC co-cultured with RESF                                    | 36,150 ± 3,800                        |
| LNMC co-cultured with 3T3 fibroblast monolayers               | 31,460 ± 5,200                        |

**Fig. 3.** Synthesis of heparin proteoglycan (ACase) by LNMC co-cultured with various fibroblast monolayers. A, LNMC alone; B and C, LNMC cultured for 15 days and 30 days, respectively, on MESF; D and E, LNMC cultured for 15 days and 30 days, respectively, on 3T3 fibroblasts; F and G, LNMC cultured for 15 days and 30 days, respectively, on RESF. Results are expressed as the mean ± S.E. of three experiments.
During the last decade oversulfated proteoglycans have become important phenotypic markers that distinguish different mast cell subsets. Although chondroitin sulfates (ChS-E in mouse bone marrow derived mast cells (2) and ChS-D in rat bone marrow derived mast cells (11)) characterize mast cells of a mucosal phenotype, heparin characterizes mast cells of a connective tissue phenotype (1, 2).

In the present study we have reanalyzed the proteoglycans of LNMC which have already been partially characterized in the past. These mast cells differ from other murine-derived cultured mast cells in their tissue of origin (mesenteric lymph nodes versus bone marrow) and in their morphology. We have previously described preliminary analysis of the granular proteoglycans derived from murine LNMC cultured and prepared in a way similar to that described in this work (19). In this present study ascending thin layer chromatography was used for the separation of chondroitinase ABC-digested proteoglycans extracted from the LNMC. This enzyme digestion revealed two products that migrated in the positions of ADi-diSs and of oversulfated disaccharides that could be either ΔDi-diSa or ΔDi-diSs (25). In that study the chondroitinase ABC products of the LNMC proteoglycans were not treated further by sulfatases, and it was presumed that the oversulfated disaccharides were ADi-diSa. This assumption was based on the identification of that proteoglycan in cultured mouse bone marrow-derived mast cells, which have been well characterized before (2). In the present work, chondroitinase ABC-treated samples were digested further by sulfatases before they were loaded onto the ascending thin layer chromatography plate for analysis. In this TLC procedure we have identified the oversulfated disaccharide as ADi-diSa and not ADi-diSs since chondro-4-sulfatase could not digest the oversulfated material that chondro-6-sulfatase did. To verify our observation and in order that ΔDi-diSs would be separated from ΔDi-diSa we have analyzed further the composition of the LNMC disaccharides by HPLC. In the HPLC, as in the TLC, chondro-6-sulfatase or both sulfatases together but not chondro-4-sulfatase alone was effective in cleaving the sulfated material located on the hexosamine of the disaccharide. Therefore, we concluded that the proteoglycans derived from these LNMC contain disulfated disaccharides GlcUA-2-SO₄ + GalNAc-6-SO₄ (ChS-D). Mouse lymph node-derived mast cell committed precursors, which could not be found in the bone marrow, could be differentiated into connective tissue mast cells in response to conditioned medium derived from fibroblasts (18). This strengthens the idea that the ChS-D-containing LNMC represent a distinct subclass of mast cells. However, it has to be investigated further whether ChS-D-containing LNMC differ by other criteria from the ChS-E-containing mast cells. Another interesting point is whether mast cells have the biochemical machinery to change ChS-D into ChS-E and vice versa.

The present work and studies described previously by us and by others (13–17) emphasize the important role played by fibroblasts in regulating and determining the phenotypic properties of mast cells. Here we have investigated further the fibroblast effect on the synthesis of granule constituents in LNMC.

The procedures for the preparation of RESF and MESF monolayers were well established in the literature (21, 22, 26, 27). These irradiated embryonic skin monolayers of rat and mouse used for maintaining the mast cell differentiation appear as a network of cytoplasm extensions and fibers. Unlike epithelial or macrophage monolayers, these monolayers can be mechanically peeled off as a whole sheet. Additionally, the whole cell population could be destroyed by detergent such as Triton X, leaving behind an extracellular matrix made of continuous fibrous mesh which detached as
an integral structure. In electron microscope profiles, such monolayer cultures, bundles of fibrilles, and fibers showed the cross-banding typical of collagen I. In scanning electron microscopy, these skin-derived monolayers appeared as a continuous uninterrupted layer. Thus, we view the monolayer as the in vitro reflection of connective tissue, where the cell commonly recognized as fibroblast (29) is the chief cell. These skin monolayers were particularly rich in dermatan sulfate, more than 90% of the MESF-labeled proteoglycan (Table I), suggesting that the fibroblasts grown were of embryonic dermis. No mast cells or mast cell proteoglycans were identified in such cytostatically irradiated monolayers in over 40 days of culture.

Each of the three types of monolayers induced [35S]sulfate incorporation into LNMC macromolecules. However, LNMC cultured on MESF incorporated [35S]sulfate during a 4-h period 4.7–5.6-fold more than LNMC cultured on RESF or 3T3 fibroblast monolayers, and 17-fold more than LNMC not plated on monolayer (Table II). MESF induced the most dramatic change on the LNMC as expressed by the shift from ChS-D synthesis into almost 100% heparin synthesis (Fig. 3). The RESF monolayer did not induce any change in the type of proteoglycan synthesized by the cells (Fig. 3). However, it induced a 3-fold increase in the incorporation of [35S]sulfate into proteoglycans synthesized by the LNMC, which may represent an increase in the rate of proteoglycan synthesis (Table I). In LNMC separated from co-culture with the 3T3 cell line monolayer, there was a shift in proteoglycan synthesis from 0 to 30% heparin synthesis, with the rest of the proteoglycan remaining ChS-D (Fig. 3). The later results are in agreement with what was reported previously for bone marrow-derived mast cells that were grown on a 3T3 fibroblast monolayer and synthesized only 30–40% granular heparin and 60–70% chondroitin sulfate proteoglycans (15) and with what was reported previously on heparin synthesis in heterogeneous populations of LNMC and MESF (29). Thus, it can be concluded that the MESF is much more potent as compared with the 3T3 fibroblast line in inducing the conversion of chondroitin sulfate-containing mast cells into heparin-containing mast cells. MESF may represent a heterogeneous population of fibroblasts that contain subclasses of cells that are very effective in producing the inducible signal(s) whereas the 3T3 cell line that we used may represent only a selection of fibroblast clones that either do not produce such signal(s) or have lost such properties following transformation. The inability of the RESF to trigger heparin synthesis in mouse LNMC could be explained by the species difference.

The conversion of ChS-D-containing LNMC into heparin-containing mast cells was time dependent (Fig. 4). Chondroitin sulfate D gradually declined whereas heparin synthesis appeared between 4 and 7 days after plating and gradually increased. Fifty percent of the total [35S]-proteoglycans extracted after 15 days was found to be heparin. Whether the same cell synthesizes both types of proteoglycans has to be explored. However, we assume that the shift takes place within the cells and not in populations of cells since it has already been demonstrated by histologic staining in mouse bone marrow-derived mast cells co-cultured with 3T3 fibroblasts that the same cell contains both heparin and chondroitin sulfate proteoglycans (15). The shift of connective tissue mast cells into mucosal type was investigated under in vivo conditions. Somoda and his collaborators (30) have shown the development of mucosal mast cells after injection of a single connective tissue type mast cell in the stomach of genetically mast cell-deficient w/w mice. This may indicate that the shift from ChS-D into heparin-containing mast cells could be reversible and dependent on the appropriate microenvironment.

A strong correlation was observed among the fibroblast density, duration of exposure to monolayer, and histamine content in LNMC. Mast cells that were exposed to optimal concentrations of MESF contained up to 3 μg of histamine per 10^6 cells and were able to degranulate more than 90% of their histamine content (13).

Mast cell-fibroblast interactions in the co-cultures also include influences exerted on the fibroblasts. Although we did not find evidence for such effects of mast cells on the fibroblasts in this study, other works did. Morphologic studies revealed that the fibroblasts became vacuolated and less adherent to plastic culture dishes when co-cultured with mast cells (32). Moreover, mast cell degranulation induced fibroblast contraction and displacement. Fibroblasts lost their contact inhibition and increased their growth rate when co-cultured with proliferating interleukin 3-stimulated mast cells (33). This finding may be explained by the latest observation that mast cells release several cytokines in response to immunologic stimuli (34–36), one of which may well be a fibroblast growth factor.

In conclusion, we find that LNMC contain chondroitin sulfate D as their major oversulfated granular proteoglycan. These mast cells may represent a new unfamiliar mast cell subclone. Like the mouse bone marrow derived mast cells, when co-cultured with fibroblast monolayers, the LNMC shift the synthesis of their granular oversulfated proteoglycan into 100% heparin and change their histology to a more mature mast cell histology. The results indicate that the degree of granule constituent synthesis induced by MESF is a function of the duration of exposure to and the concentration of the factor(s) produced by the fibroblasts (Fig. 5). These fibroblasts may be considered as a source of cells for further investigating the signal(s) that induce mast cell phenotype changes.

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