Altered Processing of Fibronectin in Mice Lacking Heparin

A ROLE FOR HEPARIN-DEPENDENT MAST CELL CHYMASE IN FIBRONECTIN DEGRADATION

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We have previously generated a mouse strain with a defect in its heparin biosynthesis by targeting the gene for N-deacetylase/N-sulfotransferase-2 (NDST-2). The NDST-2−/− mice show reduced levels of various mast cell mediators such as histamine and various heparin-bind- ing mast cell proteases, including chymases, tryptases, and carboxypeptidase A. In this work we have addressed the possible functional consequences of the lack of sulfated heparin. Peritoneal cells were harvested from normal and NDST-2−/− mice. After culturing the cells, conditioned media were collected and were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Several differences in the protein patterns were observed, including the presence of large amounts of a ~250-kDa protein in medium from NDST-2−/− mice that was absent in normal controls. Peptide microsequencing revealed identity of this protein with fibronectin. Western blot analysis showed the presence of fibronectin degradation products in cell cultures from normal mice, which were absent in cultures from NDST-2−/− animals. Further experiments showed that the degradation of fibronectin observed in cell cultures from NDST-2−/− mice was catalyzed by mast cell chymase in a strongly heparin-dependent manner. This report thus indicates a biological function for chymase/heparin proteoglycan complexes in fibronectin turnover.

Heparin is a highly sulfated glycosaminoglycan with well known anticoagulant properties commonly utilized in anti-thrombotic therapy. However, its exclusive synthesis by mast cells and its extravascular location within the secretory granules of mast cells strongly argues against a major role for heparin as a physiological anticoagulant. Heparin is synthesized as a (GlcA-GlcNAc)n backbone that undergoes several polymer modification reactions, including N-deacetylation, N-sulfation, C5 epimerization of selected GlcA residues to IdoA, 2-O-sulfation of IdoA/GlcA residues, and O-sulfation of glucosamine residues at the 6- and 3-positions (1). In its proteoglycan form, the heparin chains are attached through a Xyl-Gal-Gal linkage region to a serglycin protein core (2). To study the biological function of heparin proteoglycan, we recently developed a heparin-deficient mouse strain by targeting the gene for NDST-2,1 a biosynthetic enzyme involved in the initial stages of the heparin modification process (3). In the absence of functional NDST-2, heparin remains as the unmodified nonsulfated precursor. The NDST-2−/− mice develop normally and are fertile. However, their mast cells are severely affected (4, 5). The mast cells show drastically reduced levels of inflammatory mediators such as histamine and various mast cell proteases. In addition, the mast cell granules display an altered morphology as compared with the normal cells, with large apparently “empty” vacuoles instead of the normal electron-dense granule. Because the actual expression of mast cell proteases was not affected by the inactivation of NDST-2 it appears that, in the absence of mature heparin, the storage of these inflammatory mediators is impaired (4).

Large amounts of various proteases are stored in the mast cell secretory granules, constituting up to ~25% of the total cell protein in mast cells. The mast cell proteases are classified as either (i) tryptases, tetrameric serine proteases with trypsin-like substrate specificities, (ii) chymases, serine proteases with chymotrypsin-like substrate specificities, or (iii) carboxypeptidase A (6). In both the chymase and tryptase families, several related enzymes are expressed within the same species. In mouse, the connective tissue type of mast cells express the tryptases mouse mast cell protease 6 (mMCP-6) and mMCP-7 as well as the chymases mMCP-4 and mMCP-5 (7–12). Both the chymases and tryptases bind strongly to heparin proteoglycan within the secretory granules. In addition, both tryptases and chymases are dependent on heparin for their activity. The tryptases require binding to heparin for gaining its active tetrameric form (13), and the continuous presence of heparin is needed to maintain enzymatic stability (14). The chymases, on the other hand, are active in the absence of heparin. However, the presence of heparin leads to potentiation of chymase activity toward some substrates (15) and to protection from various macromolecular protease inhibitors (16).

In the present study, the aim was to study the consequences of the lack of functional heparin. Peritoneal cells, a cellular population containing both macrophages and lymphocytes as well as mast cells of the connective tissue subtype were cultured, and the proteins secreted by normal and NDST-2−/− cells were compared. Although several differences were noticed, the most striking finding was the presence of a ~250-kDa protein band in conditioned media from NDST-2−/− cells, that was absent in media from normal cells. The protein was identified as fibronectin. Subsequent analyses showed that, in nor-

1 The abbreviations used are: NDST-2, N-deacetylase/N-sulfotransferase; PBS, phosphate-buffered saline; mMCP, mouse mast cell protease; rMCP, rat mast cell protease; PAGE, polyacrylamide gel electrophoresis.
mal cells, fibronectin is degraded in a heparin-dependent manner by mast cell chymase, whereas very little degradation is seen in the NDST-2−/− cells. This report thus indicates that heparin in complex with chymase may be involved in the turnover of fibronectin.

**MATERIALS AND METHODS**

**Reagents**—Protramine (M, ~4500) grade IV, cycloheximide, peptatin, N-ethylmaleimide were purchased from Sigma. PefablocSC, a serine protease inhibitor, was purchased from Pentapharm Ltd. (Basel, Switzerland). Rat chymase (rMCP-1) and heparin proteoglycan were purified from peritoneal mast cells as described previously (17). α-Antichymotrypsin and α1-antitrypsin were purchased from Calbiochem (La Jolla, CA), and donkey anti-rabbit Ig conjugated to horseradish peroxidase was from Amersham Pharmacia Biotech. Fibrinogen purified from human plasma and rabbit anti-human fibronectin antiserum were kindly provided by Staffan Johansson (Dept. of Medical Biochemistry and Microbiology, Uppsala University).

Cells—Peritoneal cells from NDST-2+/+ mice and corresponding NDST-2−/− mice (4) (females, 12–15 weeks old) were collected by peritoneal washing with 10 ml of cold phosphate-buffered saline (PBS), pH 7.4. Cells were centrifuged (300 × g, 4 °C, 10 min) and cultured in serum-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Life Technologies, Inc.). The Dulbecco’s modified Eagle’s medium was supplemented with 2 mM L-glutamine (Life Technologies, Inc.) and penicillin-streptomycin (100 IU/ml, 100 mg/ml; Life Technologies, Inc.). The cells were distributed in 24-well plates (Nunc; ~2 × 10^6 cells in 0.4 ml/well). Cells were incubated at 37 °C overnight in a humidified atmosphere of 5% CO2.

**Protein Microsequencing**—Samples from conditioned media were recovered from peritoneal cell cultures and were subjected to SDS-PAGE on 10% polyacrylamide gels under reducing conditions. Gels were silver-stained according to Morrissey (18). Bands of interest were subsequently excised, trypsin-digested and subjected to microsequencing using a Q-tof (Micromass, Manchester, UK) electrospray tandem mass spectrometer.

**Western Blot Analysis**—After culturing peritoneal cells for ~20 h, conditioned media were collected. To 400 μl of conditioned medium, 3× SDS-PAGE sample buffer (200 μl) was added. Cell extracts were prepared by adding 300 μl of 3× SDS-PAGE sample buffer to the culture dishes after removal of conditioned media. Samples (40 μl) of these mixtures were subjected to SDS-PAGE on 6% polyacrylamide gels under reducing conditions in a Laemmli system. Proteins were subsequently transferred onto nitrocellulose membranes, followed by blocking with 5% milk powder in PBS for 2 h at 20 °C. Next, the membranes were incubated with rabbit anti-human fibronectin, diluted (1:200) in 5% milk powder/TBS/0.1% Tween 20, at 4 °C for 20 h. After washing the membranes extensively with TBS/0.1% Tween 20, the membranes were incubated with donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech; 1:3000 dilution in TBS/0.1% Tween 20). After 45 min of incubation at 20 °C, the membranes were washed extensively with TBS/0.1% Tween 20, followed by washing with TBS without detergent. The membranes were developed with the ECL system (Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer.

**Purification of Mast Cells**—Peritoneal cells from normal mice (females, 12–15 weeks old) were collected by peritoneal washing with 10 ml of cold PBS, pH 7.4. Mast cells (connective tissue type) of ~95% purity (the majority of the contaminating cells were red blood cells), as judged by staining with toluidine blue, were prepared by density gradient centrifugation on metrizamide (19).

**Inhibition of Fibronection Degradation**—Peritoneal cells from normal mice were collected and cultured as described above. Different inhibitors were added to the cells during the culture period: protamine (0.25 μg/ml, 2.5 μg/ml, 25 μg/ml); cycloheximide (5 μg/ml); α1-antichymotrypsin (5 μg/ml, 45 μg/ml); α1-antitrypsin (5 μg/ml, 45 μg/ml); N-ethylmaleimide (1 mM); EDTA (10 mM); pepstatin (10 μg/ml); and PefablocSC (2 mM).

**Stimulation of Chymase-catalyzed Fibronection Degradation by Heparin**—Fibrinogen (0.2, 2, and 20 μg) purified from human plasma was incubated in the presence of purified rMCP-1 and 50 ng of rat heparin proteoglycan in PBS (total volume, 100 μl). Samples (30 μl) were taken after 15 and 40 min of incubation, mixed with 15 μl of 3× SDS-PAGE sample buffer and subjected to SDS-PAGE under reducing conditions in a Laemmli system, utilizing 6% polyacrylamide gels, followed by Western blot analysis as described above. To equalize the amount of fibronectin in each lane, samples containing 20 μg of fibronectin were diluted 100-fold in SDS-PAGE sample buffer before electrophoresis, and samples containing 2 μg of fibronectin were diluted 10 × in SDS-PAGE sample buffer before analysis (samples containing 0.2 μg of fibronectin were analyzed without dilution).

**Determination of Cleavage Sites in Fibronection**—Fibronection (40 μg) purified from human plasma was incubated with 100 ng of purified rMCP-1 and 250 ng of rat heparin proteoglycan in PBS (total volume 50 μl). Samples (25 μl) were taken after 90 min of incubation, mixed with 12.5 μl of 3× SDS-PAGE sample buffer, and subjected to SDS-PAGE under reducing conditions in a Laemmli system, using 6% polyacrylamide gels. After electrophoresis, degradation products were transferred to a polyvinylidene difluoride membrane (Bio-Rad; equilibrated ~10 min in methanol) by using a Mini Trans-Blot Cell (Bio-Rad). After transfer (~1 h, 90 mA), the membrane was stained with Coomassie brilliant Blue for ~5 min and destained in 50% methanol/10% acetic acid. The membrane was dried at 1 h on Whatman paper. N-terminal sequence analysis of recovered fragments was carried out using an ABI 476A (Applied Biosystems, Foster City, CA) automatic protein sequenator.

**RESULTS**

Total peritoneal cell populations from both NDST-2+/+ and NDST-2−/− mice were cultured overnight. To elucidate any differences among the proteins expressed by these cells, conditioned media as well as cell solubilisates were subjected to SDS-PAGE analysis followed by silver staining (Fig. 1). The cell fractions from both normal and NDST-2−/− mice displayed highly similar protein patterns. In contrast, several differences were seen among the proteins present in the conditioned media (Fig. 1). In particular, high amounts of a ~250-kDa band are seen in media from NDST-2−/− cells but are largely absent in media from normal cells. Material corresponding to this band was recovered and subjected to microsequencing after trypsin digestion. Several tryptic peptides were obtained, and their
sequences are displayed in Table I. The sequences of all these peptides matched the sequence of mouse fibronectin. Thus, large amounts of soluble fibronectin are present in conditioned media from NDST-2−/− cells but are largely absent in media from normal cells.

Western blot analysis, utilizing a polyclonal anti-fibronectin antibody, confirmed the presence of fibronectin in media from NDST-2−/− cells. Again, very little intact fibronectin antigen was found in normal cell culture media. However, several bands of lower molecular weight that reacted with the anti-fibronectin antiserum were observed, indicating that fibronectin was degraded in cell cultures from normal cells (Fig. 2A). Also cellular extracts displayed reduced amounts of fibronectin in the normal as compared with NDST-2−/− cells. Further, reconstitution of the mast cell-depleted peritoneal cells with purified mast cells resulted in increased fibronectin degradation in a dose-dependent fashion (Fig. 3). Purified mast cells did not show any detectable expression of fibronectin (Fig. 3).

Fibronectin degradation was not inhibited by EDTA, N-ethylmaleimide, nor pepstatin, inhibitors of metalloproteases, thiol proteases, and aspartyl proteases, respectively. In contrast, complete inhibition of fibronectin proteolysis was obtained by PefablocSC, a specific inhibitor of serine proteases (Fig. 4A). These results strongly suggest that fibronectin is degraded by mast cell serine proteases. To distinguish between trypsin and chymases, the effect of the macromolecular weight protease inhibitors α1-antichymotrypsin and α1-antitrypsin was investigated. Tryptase is known to be insensitive to both of these inhibitors, whereas chymase is inhibited to some extent by α1-antichymotrypsin and α1-antitrypsin (16). Results presented in Fig. 4B show that the degradation of fibronectin was completely inhibited by α1-antichymotrypsin but not by α1-antitrypsin, indicating that mast cell chymase is the enzyme mainly responsible for fibronectin degradation in the normal cells. Because chymase is essentially absent in NDST-2-deficient mast cells (4), the lack of fibronectin degradation in the NDST-2-deficient cells is thus in agreement with this notion.

Chymases have previously been shown to be dependent on heparin for optimal proteolysis of certain substrates (17, 21, 22). To investigate whether the degradation of fibronectin is dependent on heparin, we tested the effect of protamine, a heparin antagonist, on the degradation of fibronectin in cell cultures from normal mice. Addition of protamine to the cell cultures caused inhibition of fibronectin proteolysis in a dose-dependent fashion, with essentially complete inhibition obtained after addition of 10 μg of protamine to the cells (Fig. 4C). This indicates that chymases are highly dependent on heparin for optimal degradation of fibronectin in the cell cultures.

Further studies were performed in a purified system to study the effect of heparin on chymase-catalyzed fibronectin degradation and to determine major cleavage sites in fibronectin. Chymase (rMCP-1; rat homologue to the major chymase in mouse peritoneal mast cells, mMCP-4; Ref. 23) was incubated with different concentrations of human fibronectin, in either the absence or the presence of heparin. Rapid degradation of fibronectin was observed, and the rate of chymase-catalyzed fibronectin proteolysis was enhanced by heparin proteoglycan (Fig. 5). The enhancing effect of heparin was most prominent at

| TABLE I  |
|------------------|
| Tryptic peptide sequences obtained by microsequencing of ~250-kDa band present in conditioned media from NDST-2−/− peritoneal cells |
| I  | Thr-Leu-Thr-Gly-Leu-Gln-Pro-Gly-Thr-Asp-Tyr-Lys |
| II | Tyr-Asn-Ile-Ile-Val-Glu-Ala-Leu-Gln-Asn-Gln-Arg |
| III | Tyr-Gly-Thr-Gly-Gly-Arg |

Fig. 2. Degradation of fibronectin by NDST-2−/− peritoneal cells. Peritoneal cells from NDST-2+/+ and NDST-2−/− mice were cultured ~20 h. A, after culture, the conditioned media (A) and cell fractions (B) were subjected to Western blot analysis using an anti-fibronectin antiserum.

Fig. 3. Fibronectin degradation is mast cell-dependent. Non-fractionated peritoneal cells (Control), mast cell-depleted cells, and mast cell-depleted cells reconstituted with different numbers of mast cells (5 × 10^5, 20 × 10^5, and 100 × 10^5) and different numbers of purified NDST-2−/− mast cells (20 × 10^5 and 100 × 10^5) were incubated ~20 h. After incubation, the conditioned media were subjected to Western blot analysis using an anti-fibronectin antiserum. MC, mast cells.
low fibronectin concentrations (Fig. 5). Fibronectin fragments were blotted onto nitrocellulose (Fig. 6) and subjected to N-terminal sequence analysis. N-terminal sequences were obtained for fragments 1–4 (Fig. 6) and are listed in Table II. The N-terminal of fragment 5 was blocked. Because the N-terminal of human fibronectin is known to be blocked (24), fragment 5 may correspond to the original N-terminal portion of fibronectin. Comparison of the obtained N-terminal sequences with that of intact fibronectin revealed three major cleavage sites for chymase (Table III). Fragments 2 and 4 contained the same N-terminal sequence, indicating that fragment 4 had undergone further processing in the C-terminal portion. In accordance with the known substrate specificities of chymotrypsin-like enzymes, all cleavage sites contain an aromatic amino acid residue at the P1 position and show no obvious homologies in the P2–P5 or P1’–P5’ regions. The cleavage sites are all located in the first three type III repeats of
fibronectin, on the C-terminal side of the collagen/gelatin-binding domain (Fig. 7).

DISCUSSION

The recently developed heparin-deficient mouse strain provides an excellent tool to elucidate the biological function of this polysaccharide. In addition, because the heparin-deficient mice also are essentially completely devoid of the various heparin-binding proteases that are expressed by the connective tissue subtype of mast cells, these mice may also be valuable for studying functional aspects of the mast cell proteases (4).

The biological function(s) of the mast cell proteases is not understood. However, the secretion of these enzymes during mast cell degranulation suggests involvement in inflammatory reactions. Indeed, several studies have pointed to important pro-inflammatory properties of both tryptases (25–28) and chymases (29, 30) in vivo. Although their involvement in inflammatory responses appears clear, the exact mechanism by which they act is not certain. It has been proposed that tryptases can modulate inflammation by activating protease activated receptor-2 on the surface of various cell types, leading to cellular activation (31). In addition, various other proteins of potential importance in the inflammatory response, including high molecular weight kinogen (32), fibrinogen (33, 34), and complement factor C3 (35), have been shown to be substrates for tryptase. Several substrates for various chymases have been identified (22, 36–44), although it is not clear whether any of these proteins are substrates for chymase in vivo. In particular, it is worth noting that several known substrates for both tryptases and chymases are connective tissue components or are involved in connective tissue turnover (21, 40, 45–48). One possible biological function of the mast cell proteases, although not proven in vivo, may thus be to carry out or assist in connective tissue turnover.

Mast cell chymases have rather broad substrate specificities. Thus, it is likely that most proteins contain peptide sequences that potentially could be recognized as substrates by mast cell chymase provided that they are exposed. Accordingly, chymase may have a general degradative role in the tissue after release from the mast cells. However, the cleavage preferences of the chymases may not solely be related to the structure of the actual active site but could also be influenced by other factors. One such factor may be the association of chymases with the strongly negatively charged heparin proteoglycan (49). Because chymases are present as tight complexes with heparin proteoglycan both within the mast cell granules and after exocytosis, it is relevant to consider the complex of chymase with heparin as the physiological form of chymase. Therefore, effects on substrate cleavage properties imposed by heparin proteoglycan are likely to reflect the situation in vivo. We demonstrated recently that the chymase-catalyzed cleavage of thrombin, a heparin-binding protease, was strongly potentiated by heparin proteoglycan (15). Blocking of the heparin-binding site of thrombin abolished the stimulatory effect of heparin on thrombin degradation. These results led to a model of chymase action where the association of chymase with heparin proteoglycan was suggested to direct the substrate specificity preferentially toward heparin-binding proteins. Various proteins containing a heparin-binding region may be “captured” by the heparin chains in the chymase/heparin complex, thereby facilitating contact between chymase and susceptible regions in its potential heparin-binding substrates. The present report suggests that fibronectin is a substrate for chymase/heparin proteoglycan complexes. Because fibronectin is a known heparin-binding protein (24), the degradation of fibronectin by chymase in complex with heparin proteoglycan is thus consistent with the suggested model.

The degradation of fibronectin in peritoneal cell cultures from NDST-2−/− mice was essentially completely inhibited when protamine, a heparin antagonist, was present. Protamine is a polycationic protein that has been shown to compete with chymase for binding to heparin proteoglycan (50). The addition of protamine would therefore result in release of chymase from heparin proteoglycan, without affecting the actual active site of chymase. Our results suggest that free chymase is less effective in degrading fibronectin than when bound to heparin proteoglycan, supporting a vital importance of the association of chymase with heparin proteoglycan for optimal rate of chymase-catalyzed fibronectin proteolysis.

The suggested mechanism for the enhancement of fibronectin degradation by heparin implies that heparin increases the likelihood that fibronectin molecules reach contact with chymase. Thus, heparin enhances the apparent affinity of fibronectin with heparin, which would be reflected by a decreased $K_m$ for the rate of proteolysis. Hence, if the rate of fibronectin proteolysis follows Michaelis-Menten kinetics, the stimulatory effect of heparin would be largest at low fibronectin concentrations. When increasing amounts of fibronectin is available, the rates of fibronectin proteolysis would reach the same $V_{max}$ both in the absence or presence of heparin. Our results show that the stimulatory effect of heparin is largest at lower fibronectin concentrations, in agreement with an effect of heparin on the $K_m$ for the rate of fibronectin proteolysis.

Peritoneal mast cells are known to express at least two different chymases, mMCP-4 and -5 (4). Because both mMCP-4 and -5 are highly basic and thus capable of interaction with heparin, either or both of these chymases may be responsible for the observed degradation of fibronectin. However, because no reagents that specifically block either mMCP-4 or -5 activity are available, we can at present not distinguish whether fibronectin is primarily degraded by mMCP-4 or mMCP-5.

Fibronectin is a multi-functional protein, built up of several distinct domains capable of interacting with various ligands, including collagens, integrins, heparin/heparan sulfate, and
fibrin (Ref. 24 and Fig. 7). Accordingly, fibrinectone is known to play a role in diverse biological processes, such as cell binding, cell migration, matrix assembly, embryonic development, and connective tissue remodeling (24, 51). Altered fibrinectone processing has been noted in numerous pathological conditions, including cancer, arthritis, thrombosis, and wound healing. For instance, inflammatory conditions are often associated with the generation of fibronectin degradation products that are not found under normal conditions (52). Such fibrinectone fragments may reflect tissue degradation because of the release of proteolytic enzymes during disease. The present report raises the possibility that mast cell chymase may be involved in the formation of such fibronectin degradation products. Degradation of fibronectone could, for example, affect the ability of inflammatory cells to attach to matrix and may disrupt the organization of the extracellular matrix, thereby facilitating movement of cells within the tissue. Importantly, it has been suggested that some of the fibrinectone fragments display activities that are cryptic in the intact molecule (53–55). Thus, proteolysis of fibrinectone by chymase could unleash fibrinectone activities that potentially may be important components in the regulation of e.g. inflammatory responses.

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