Mast Cell Chymase Induces Smooth Muscle Cell Apoptosis by a Mechanism Involving Fibronectin Degradation and Disruption of Focal Adhesions

Markus J. Leskinen, Ken A. Lindstedt, Yenfeng Wang, Petri T. Kovanen

Objective—Chymase released from activated mast cells has been shown to induce apoptosis of vascular smooth muscle cells (SMCs) in vitro. The proteolytic activity of chymase is essential for the proapoptotic effect, but the mechanism of chymase-induced apoptosis has remained unknown.

Methods and Results—Here we show by means of FACS analysis, immunohistochemistry, and Western blotting that mast cell-derived chymase induces SMC apoptosis by a mechanism involving degradation of an extracellular matrix component, fibronectin (FN), with subsequent disruption of focal adhesions. The FN degradation products induced SMC apoptosis of similar magnitude and with similar changes in outside-in signaling, as did chymase. Sodium orthovanadate, an inhibitor of tyrosine phosphatases, inhibited the chymase-induced SMC apoptosis. Focal adhesion kinase (FAK), one of the key mediators of integrin–extracellular matrix interactions and cell survival, was rapidly degraded in the presence of chymase or FN degradation products. Loss of phosphorylated FAK (p-FAK) resulted in a rapid dephosphorylation of the p-FAK–dependent downstream mediator Akt.

Conclusions—The results suggest that chymase-secreting mast cells can mediate apoptosis of neighboring SMCs through a mechanism involving degradation of pericellular FN and disruption of the p-FAK–dependent cell-survival signaling cascade. (Arterioscler Thromb Vasc Biol. 2003;23:238-243.)

Key Words: mast cell  smooth muscle cell  apoptosis  chymase  focal adhesion kinase

The fibrous cap of an atheroma is a dynamic structure consisting of arterial smooth muscle cells (SMCs) and a connective tissue matrix produced and maintained by the SMCs.1 The thickness of the cap determines plaque stability and so contributes to the risk of acute clinical complications of atherosclerosis, such as myocardial infarction and stroke. A thick overlying cap protects the lipid-rich core from rupturing, whereas a thin cap predisposes it to rupture. Accordingly, SMC apoptosis, followed by reduced production of the extracellular matrix, would tend to weaken the cap, increasing the risk of cap rupture.1 Indeed, in the fibrous caps of human atheromas, apoptotic SMCs2,3 are colocalized with infiltrates of inflammatory cells.4,5 These inflammatory cell infiltrates typically consist of macrophages,6 T-lymphocytes,6 and mast cells.7,8 Moreover, we have observed that in unstable coronary syndromes, the culprit lesions contain increased numbers of activated mast cells.9 Mast cells are filled with proinflammatory components, such as tumor necrosis factor-α and chymase, which, on mast cell activation and degranulation, are released. We have recently shown that chymase released from activated mast cells is able to induce apoptosis of vascular SMCs in vitro.10 The proteolytic activity of chymase is essential for the proapoptotic effect, but the exact mechanism of chymase-induced apoptosis has remained unknown. Chymase is a neutral serine endoprotease that cleaves the peptide or ester bonds at the carboxyl terminus of aromatic (Phe, Tyr, Trp) or branched-chain aliphatic (Leu, Ile, Val) amino acids. Accordingly, chymase has been shown to proteolyze several types of molecules present in the arterial intima, such as matrix metalloproteinases,11 transforming growth factor-β,12 angiotensin I,13 apolipoproteins,14 and several components of the extracellular matrix (ECM), such as fibronectin (FN)15 and vitronectin (VN).16

The ECM components FN,17,18 laminin,19 and VN20 are essential for the survival of several different cell types in that, when intact, they provide cells with survival signals. Extensive degradation of these ECM components leads to loss of the matrix survival signals, with subsequent induction of apoptosis in the cells of the tissue area involved.31 In the present report, we studied the effect of mast cell chymase on several ECM components and found that chymase-induced apoptosis involves degradation of FN by chymase. Moreover, the FN degradation products induced degradation of the focal adhesion kinase (FAK), one of the key mediators in the ECM-mediated outside-in cell-survival signaling.

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Methods

Materials and Animals
Compound 48/80, bovine pancreatic α-chymotrypsin, and trypsin were from Sigma. Cathepsin G and elastase from human neutrophils were from Calbiochem. Sephacryl S-200 HR and the HiTrap heparin-Sepharose column were from Pharmacia. Male Wistar rats (300 to 500 g) were from the Laboratory Animal Center of the University of Helsinki. The rats were treated in accordance with institutional guidelines, which had been approved by the institutional ethics committee.

Culture and Growth Arrest of Rat Aortic SMCs
Aortic SMCs were prepared from male Wistar rats, as described previously.22 The cells were seeded into 25-cm² Falcon polystyrene tissue-culture flasks at a density of 2 × 10⁴ cells/cm² in 5 mL of RPMI 1640 culture medium supplemented with 2 mmol/L L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% FCS. After 3 days, the SMCs reached confluence when subcultured (1:2) up to 9 times. SMCs of the 5th to 9th passages were used for the experiments. To obtain growth-arrested SMCs, the cells were cultured to a subconfluent cell density (1.3 × 10⁴ cells/cm²) in 12-well tissue culture plates in 2 mL of the above culture medium containing 10% FCS, after which they were growth-arrested in 0.4% FCS-containing medium for 48 hours.23

Isolation and Purification of Mast Cell Chymase
Serosal mast cells were isolated from the pleural and peritoneal cavities of rats and stimulated to exocytose their chymase-containing cytoplasmic granules.24 Chymase from the exocytosed granules, ie, granule remnants, was purified to apparent homogeneity as described previously25 and stored at −70°C until used.

Production of Chymase-Generated FN Degradation Products
To produce FN degradation products, commercial FN (Sigma, 48000 Da) was incubated at 37°C either for 30 minutes with proteolytically active mast cell granule remnants containing 40 BTEE units/mL of chymase (Figure 2) or for 2.5 minutes with 20 BTEE units/mL of purified chymase (Figures 3 and 4). After incubation, chymase was inactivated by either removing the granule remnants by centrifugation (20 minutes, 15 000g) or by adding PMSF (1 mmol/L). The absence of contaminating active chymase in the FN degradation products was verified by a chromogenic substrate (BTEE) and a highly sensitive reverse-phase high-performance liquid chromatography (HPLC) method.13 Briefly, angiotensin I, a peptide highly susceptible to chymase degradation, was incubated with the sample for 30 minutes, and the peptides were then isolated and analyzed on reverse-phase HPLC. Angiotensin I-derived bands were visualized through enhanced chemiluminescence (Amersham Pharmacia Biotech) with either horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (anti-FAK, anti-Akt, and 4G10), HRP-conjugated goat anti-rabbit IgG (anti–p-FAK), or HRP-conjugated rabbit anti-sheep IgG (anti–p-Akt). The immunoblots were quantitated by densitometric scanning with a Gel Doc 2000 gel documentation system (Bio-Rad).

Flow Cytometric Analysis of Apoptosis
The cellular DNA content was determined by flow cytometric analysis of PI-labeled cells.10 Briefly, the SMCs attached to the culture dishes were detached with trypsin and combined with the cells floating in the culture medium. The cells were then fixed with methanol, incubated with 100 U/mL of RNAsae, and, finally, stained with PI (final concentration, 25 μg/mL). After staining, the cells were analyzed with a FACScan (Becton Dickinson) using CellFit 2.02 software. Fluorescence was measured from 10 000 events in list mode, and gating on FL2-A versus FL2-W was used to remove doublets. Apoptotic nuclei were identified as a subgenome DNA peak and were distinguished from cell debris by both their forward light scatter and the fluorescence of PI.

Immunoblotting
Growth-arrested SMCs were exposed to chymase or chymase-generated FN degradation products and lysed in sodium orthovanadate buffer (150 mmol/L NaCl, 10 mmol/L Tris, pH 7.6, 1% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 2 mmol/L benzamidine, 2.5 μmol/L aprotonin, 2.5 μmol/L leupeptin, and 1 mmol/L sodium orthovanadate). For each sample, 40 μg protein was electrophoresed on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-FAK, anti-Akt (1:1000), anti–p-FAK (1:500) (both from BD Transduction Laboratories), anti-p-FAK (Tyr379, 1:1000), anti-p-Akt (Ser473, 0.5 μg/mL), or anti-phosphotyrosine antibody 4G10 (0.5 μg/mL (all three from Upstate Biotechnology). Immunoreactive bands were visualized through enhanced chemiluminescence (Amersham Pharmacia Biotech) with either horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (anti-FAK, anti-Akt, and 4G10), HRP-conjugated goat anti-rabbit IgG (anti–p-FAK), or HRP-conjugated rabbit anti-sheep IgG (anti–p-Akt). The immunoblots were quantitated by densitometric scanning with a Gel Doc 2000 gel documentation system (Bio-Rad).

Other Assays
Protein was determined by the standard Lowry procedure, with BSA as standard.

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

Results
We have recently shown that mast cell chymase can induce apoptosis of rat arterial SMCs by a mechanism involving its proteolytic activity.10 To additionally study whether chymase-mediated apoptosis can occur under physiological conditions, ie, in the presence of natural inhibitors present in tissue fluids, we incubated SMCs and chymase in the presence of serum. For comparison, several other neutral proteases were also tested for their ability to induce SMC apoptosis under similar conditions. As shown in the Table, in the absence of serum, all of the proteases tested induced apoptosis of the growth-arrested rat SMCs. However, in serum (10% FCS), only the heparin proteoglycan-bound chymase present in exocytosed mast cell granules was able to retain a part (~4%) of its apoptotic potential (Table). Thus, under conditions mimicking those in physiological tissues, ie, in the presence of protease inhibitors, chymase in its natural form, ie, when bound to heparin proteoglycans, retained a part of its proteolytic activity (20 versus 3.8 BTEE units/mL) and was able to induce SMC apoptosis.

After treating SMCs with proteolytically active chymase, the typical cell spreading of the SMCs (as seen in Figure 1A) was found to be disturbed, and the cells showed a rounded-up

Immunocytochemical Detection of FN and Phosphorylated FAK
Growth-arrested SMCs were cultured in the presence or absence of chymase for 2 hours and fixed in 4% paraformaldehyde for 10 minutes at room temperature. SMC-derived ECM was prepared by sequential extraction with sodium deoxycholate and hypotonic buffer in the presence of protease inhibitor, as described by Hedman et al.,26 except that the SMCs were cultured on Thermanox cover-slips. FN was detected using a polyclonal antibody (Santa Cruz Biosciences) and visualized with an Alexa Fluor (546)-labeled goat anti-rabbit antibody (Molecular Probes).
morphology (Figure 1B), suggesting that chymase may induce apoptosis by disrupting the interactions of cells with the ECM. Because FN is known to be an important ECM component involved in SMC spreading and also to be highly susceptible to chymase-mediated proteolysis, we next studied the effect of chymase on SMC-derived FN. Untreated SMCs showed extensive cell spreading and positive perinuclear staining of FN, reflecting ongoing synthesis of FN. In addition, a light diffuse positive staining was seen in their pericellular vicinity, indicating secretion of synthesized FN (Figure 1C). In contrast, SMCs treated with chymase were retracted and showed intense punctate plasma membrane-associated staining of FN (Figure 1D). Furthermore, the light diffuse staining of pericellular FN observed in the untreated SMCs was lost in the chymase-treated SMCs, indicating extensive degradation of the extracellular FN. By first detaching and removing the cultured SMCs with detergents, we could visualize the SMC-derived intact FN matrix, which appeared as a typical fibrillar layer attaching to the coverslips on which the SMCs had been cultured (Figure 1C, inset). In sharp contrast, when such intact FN-matrix was treated with chymase, the fibrillar staining was rapidly lost and intense punctate staining appeared, reflecting extensive degradation of the FN chains (Figure 1D, inset). To additionally analyze the effect of chymase on SMC spreading, we stained the SMCs with an antibody specifically recognizing activated p-FAK, a key component in the formation of focal adhesion complexes responsible for cell spreading. As shown in Figure 1E, normal spreading of SMCs involves the active formation of focal adhesion complexes, appearing as focal contacts in the plasma membrane of the SMCs (Figure 1E, arrows). In contrast, as shown in Figure 1F, treatment of SMCs with proteolytically active chymase disrupted the focal adhesion complexes, leading to a loss of the focal contacts in the plasma membranes of the SMCs. Interestingly, after chymase treatment, p-FAK appeared to accumulate in the nuclei of the treated SMCs (Figure 1F, arrows).

To additionally differentiate between direct effects of chymase and possible additional effects of chymase-generated FN degradation products on SMC apoptosis, we treated commercial plasma-derived FN with heparin proteoglycan-bound chymase (granule remnants). The formed FN degradation products were separated from the granule remnants by sedimenting the remnants by centrifugation, after which the aliquots of the protease-negative supernatant were added to growth-arrested SMCs. In contrast to untreated intact FN, which had no significant effect on the level of SMC apoptosis, the FN degradation products were found to induce apoptosis in a dose-dependent manner and to a similar extent as chymase (Figure 2).

Because ECM components are known to regulate cell survival through outside-in signaling, ie, by activating protein tyrosine kinases in the cell-survival signaling pathway, we next studied the effect of chymase and chymase-generated FN degradation products on the tyrosine phosphorylation status in SMCs. As shown in Figure 3, purified chymase induced rapid changes in the level of tyrosine phosphorylation in SMCs. As shown in Figure 1F, treatment of SMCs with proteolytically active chymase disrupted the focal adhesion complexes, leading to a loss of the focal contacts in the plasma membranes of the SMCs. Interestingly, after chymase treatment, p-FAK appeared to accumulate in the nuclei of the treated SMCs (Figure 1F, arrows).

Additional data from Table 1 are shown in Figure 2. Table 1 shows the results of the addition of different proteases to SMCs. The table presents the mean ± SEM of triplicate incubations. Similar results were obtained in another independent experiment.

Table 1. Effect of Different Proteases on SMC Apoptosis

<table>
<thead>
<tr>
<th>Additions</th>
<th>Proportion of Apoptotic Cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−FCS</td>
</tr>
<tr>
<td>No addition</td>
<td>1.09±0.22</td>
</tr>
<tr>
<td>Purified chymase</td>
<td>12.1±0.80</td>
</tr>
<tr>
<td>Heparin proteoglycan-bound chymase</td>
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<tr>
<td>α-Chymotrypsin</td>
<td>12.2±1.7</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>5.5±1.2</td>
</tr>
<tr>
<td>Elastase</td>
<td>11.4±0.3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>12.6±2.0</td>
</tr>
</tbody>
</table>

P<0.05 vs control (no addition).

Growth-arrested SMCs were exposed for 24 hours to 10 BTEE U/mL of chymase, corresponding to 36 nmol/L or equivalent concentrations of the following proteases: α-chymotrypsin and trypsin from bovine pancreas, and cathepsin G and elastase from human neutrophils. After incubation, the percentage of apoptotic cells was measured by flow cytometry. Data shown are mean±SEM of triplicate incubations. Similar results were obtained in another independent experiment.
FN degradation products resulted in a dephosphorylation pattern similar to that obtained with chymase, additionally suggesting that both chymase and the FN degradation products induced SMC apoptosis via a similar outside-in signaling mechanism (Figure 3B). The observed reduction in tyrosine phosphorylation of a 130-kDa molecule (Figure 3A, upper arrow) and the loss of focal adhesion complexes (see Figure 1F) both suggest that FAK itself may be a target of dephosphorylation. As shown in Figure 4, both chymase (panel A) and the FN degradation products (panel B) induced a time-dependent, strong reduction in the level of phosphorylated active FAK (p-FAK). However, both chymase and the FN degradation products also induced a similar time-dependent reduction in the protein level of FAK, suggesting an extensive proteolytic degradation of this molecule. The FN degradation products showed a somewhat delayed and less strong effect in FAK degradation, revealing that degradation of pericellular FN by chymase was more efficient in inducing apoptosis than addition of soluble anti-adhesive FN degradation products. To additionally verify the inhibition of the p-FAK–dependent signal-transduction cascade, we analyzed the degree of phosphorylation and the protein levels of a downstream mediator, Akt. As shown in Figure 4, both chymase (panel C) and the FN degradation products (panel D) induced Akt dephosphorylation, whereas neither had a significant effect on the level of Akt protein.

Discussion

In this study, we show that mast cell chymase induces apoptosis of SMCs under conditions mimicking the in vivo situation and that this effect seems to involve degradation of the ECM component, FN, with subsequent disruption of focal adhesion complexes. Thus, chymase in its natural form was capable of inducing SMC apoptosis in the presence of serum. Moreover, chymase-generated FN degradation products induced SMC apoptosis similar to that observed with chymase. In addition, we found that the FN degradation products induced similar outside-in tyrosine phosphorylation signaling in SMCs, as did chymase. In particular, FAK, one of the key mediators of focal adhesions and cell-ECM interactions, was rapidly degraded, ie, inactivated, in the presence of chymase or FN degradation products, leading to a dephosphorylation of Akt, a downstream mediator of the FAK-dependent survival signaling cascade.

The ECM exerts profound control over cells, regulating their proliferation, differentiation, and apoptosis. These effects are primarily mediated by the integrins, which regulate the activities of various cytoplasmic kinases, growth factor receptors, and ion channels. The major FN-binding integrin, α5β1, has also been shown to mediate cell survival through outside-in signaling by activating protein tyrosine kinases, notably the FAK.

Interestingly, chymase and chymase-generated FN degradation products...
products, when added to fresh cultures of SMCs, induced rapid degradation of FN, supporting that chymase may interfere with the FN-αβ1 integrin signaling pathway in the SMCs. Moreover, 2 separate antiapoptotic pathways associated with FN-integrin interactions and FAK-mediated downstream signaling have been demonstrated. First, after serum withdrawal, FN has been shown to promote survival signals in fibroblasts and endothelial cells by suppressing p53-mediated apoptosis through the FAK, Ras/rac1/Pak1/ MAPK kinase 4 (MKK4), and c-Jun NH2-terminal kinase pathways. Second, binding of the αβ1 integrin to FN in CHO cells upregulates the expression of antiapoptotic genes, such as bcl-2, through an FAK-SHC-Ras-phosphatidylinositol 3-kinase (PI3-kinase)-Akt pathway. Thus, although cell-specific differences may exist, the two antiapoptotic pathways described above show similarities in that they involve the activation of FAK and Ras. Additional experiments will reveal which downstream signaling pathways are involved in the chymase-mediated apoptosis of SMCs.

The observation that chymase-degraded FN, in contrast to untreated FN, induces SMC apoptosis is compatible with the idea that FN contains proapoptotic epitopes that under normal conditions are inactive but become activated when the conformation of FN is disturbed through proteolytic cleavage. Indeed, FN has been shown to contain antiadhesive epitopes (FN domain III14-2), and peptides derived from such epitopes can induce apoptosis of human umbilical vein endothelial cells by blocking αβ1 integrin-mediated cell-survival signaling. Recently, another antiadhesive and proapoptotic site (YTIYVIAL) in the presence of physiological inhibitors, such as bcl-2, through an FAK-SHC-Ras-phosphatidylinositol 3′-kinase (PI3-kinase)-Akt pathway. Thus, although cell-specific differences may exist, the two antiapoptotic pathways described above show similarities in that they involve the activation of FAK and Ras. Additional experiments will reveal which downstream signaling pathways are involved in the chymase-mediated apoptosis of SMCs.

In this work, we were able to exclude the 70-kDa N-terminal FN fragments from being responsible for the apoptotic effect (data not shown). This raises the possibility that the chymase-generated C-terminal FN fragment previously described by Tchougounova et al is among the ~180-kDa degradation products observed to induce apoptosis in this work.

Furthermore, the different subdomains of the FN molecule have been shown to differentially regulate focal adhesions and MMP expression via αβ1 and αββ integrins. Interestingly, MMP-2 has also been suggested to be able to induce apoptosis, and the mechanism may well be similar to that of chymase. Moreover, by specifically activating matrix-degrading metalloproteinases (MMPs), secreted chymase could additionally augment the proteolytic degradation of the ECM of the atherosclerotic plaques, with subsequent induction of SMC apoptosis.

Recently, collagen fragments were also shown to induce integrin (αβ)-mediated disassembly of focal adhesions in SMCs by activation of calpain I with subsequent degradation of FN. Whether calpain is involved in chymase and FN degradation product-induced degradation of FAK with subsequent disassembly of focal adhesions and SMC apoptosis is presently not known. Interestingly, the chymase-treated SMCs showed an accumulation of p-FAK positive staining in their nuclei (see Figure 1F), suggesting a transport of p-FAK degradation products from the focal adhesion sites to the nuclei of apoptotic SMCs. This result differs from that obtained with human umbilical vein endothelial cells, in which FAK cleavage fragments generated during apoptosis remained in the cytosolic fraction, but is similar to the result obtained with apoptotic glioblastoma cells, in which N-terminal FAK fragments accumulated in the nuclei. Future experiments will reveal the relevance of nuclear accumulation of such p-FAK degradation products in SMC apoptosis.

Among the proteases tested in this study, only heparin proteoglycan-bound chymase retained a part of its proteolytic activity in the presence of natural protease inhibitors (10% FCS) and thus partly retained its ability to induce apoptosis under conditions mimicking those present in the extracellular compartment of tissues. This confirms the finding that chymase, when bound to the heparin proteoglycan chains of the exocytosed mast cell granules, is capable of exerting proteolytic activities even in the presence of physiological inhibitors, such as α1-antitrypsin, α2-macroglobulin, and α1-antichymotrypsin. Thus, compared with other neutral proteases, mast cell-derived granule-bound chymase may be unique in that, after being secreted into the extracellular fluid, it retains a part of its activity even in the presence of natural protease inhibitors and so may possess the ability to degrade ECM components in vivo.

A fraction of the chymase-containing mast cells found in human atherosclerotic plaques have exocytosed their chymase-containing granules into the extracellular space, suggesting that such activated mast cells could participate in the induction of SMC apoptosis in atherosclerotic plaques. Interestingly, chymase has also been shown to induce apoptosis of cardiomyocytes in vitro, but the mechanisms for such chymase-mediated apoptosis of myocytes are still unknown. However, because myocytes, like SMCs, require the presence of intact FN for matrix attachment and cellular differentiation, it is possible that the apoptotic mechanism is similar to that described in the present report. Thus, activated mast cells, by secreting the neutral protease chymase, may mediate apoptosis of neighboring muscle cells, such as SMCs and myocytes, by a mechanism involving rapid degradation of FN followed by a disassembly of focal adhesion complexes and inhibition of the FAK-Akt cell-survival signaling pathway.

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