Activated Mast Cells Induce Endothelial Cell Apoptosis by a Combined Action of Chymase and Tumor Necrosis Factor-α

Hanna M. Heikkilä, Soili Lättilä, Markus J. Leskinen, Jukka K. Hakala, Petri T. Kovanen, Ken A. Lindstedt

Objective—Activated mast cells (MCs) induce endothelial cell (EC) apoptosis in vitro and are present at sites of plaque erosions in vivo. To further elucidate the role of MCs in endothelial apoptosis and consequently in plaque erosion, we have studied the molecular mechanisms involved in MC-induced EC apoptosis.

Methods and Results—Primary cultures of rat cardiac microvascular ECs (RCMECs) and human coronary artery ECs (HCAECs) were treated either with rat MC releasate (ie, mediators released on MC activation), rat chymase and tumor necrosis factor-α (TNF-α), or with human chymase and TNF-α, respectively. MC releasate induced RCMEC apoptosis by inactivating the focal adhesion kinase (FAK) and Akt-dependent survival signaling pathway, and apoptosis was partially inhibited by chymase and TNF-α inhibitors. Chymase avidly degraded both vitronectin (VN) and fibronectin (FN) produced by the cultured RCMES. In addition, MC releasate inhibited the activation of NF-κB (p65) and activated caspase-8 and -9. Moreover, in HCAECs, human chymase and TNF-α induced additive levels of apoptosis.

Conclusions—Activated MCs induce EC apoptosis by multiple mechanisms: chymase inactivates the FAK-mediated cell survival signaling, and TNF-α triggers apoptosis. Thus, by inducing EC apoptosis, MCs may contribute to plaque erosion and complications of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2008;28:309-314)

Key Words: atherosclerosis ■ mast cell ■ apoptosis ■ chymase ■ plaque erosion

The most important pathological processes underlying the sudden onset of acute coronary syndromes, including unstable angina and acute myocardial infarction, are focal erosions and ruptures of a vulnerable coronary plaque.1 Although erosions of coronary plaques account for as much as 30% of the fatal acute myocardial infarctions and sudden coronary deaths,2 the molecular mechanisms of plaque erosion have remained enigmatic. One possible cause of plaque erosion is apoptosis of the involved endothelial cells (ECs).3 Indeed, patients with unstable angina or myocardial infarction have increased levels of circulating EC-derived apoptotic cells.4,5 EC apoptosis is especially seen in downstream areas of atherosclerotic plaques subjected to aberrant hemodynamic forces including turbulent blood flow.6

The vulnerable sites of atherosclerotic plaques also contain an increased number of mast cells (MCs) that show signs of activation by degranulation, which is a prerequisite for the release of preformed mediators, both insoluble and soluble.7,8 We have previously shown that activated rat serosal MCs induce apoptosis of rat cardiac microvascular ECs (RCMECs) in vitro, by a mechanism that involves the presence of both insoluble tumor necrosis factor-α (TNF-α) in the granule-remnant fraction and soluble TNF-α in the granule-remnant free supernatant.9 However, TNF-α alone could not explain the observed effects,9 suggesting that other components of the MC releasate may have been involved. Chymase, the neutral serine protease of MCs, has been shown to induce apoptosis of smooth muscle cells (SMCs),10 myocytes,11 and epithelial cells.12 Recently, we have shown that the MC-derived proteases, chymase and tryptase, when incubated with arterial segments induce plaque erosion ex vivo.13 Furthermore, we have also shown that activated MCs are present at sites of such erosions,5,13 and that the number of MCs at plaque erosions correlates with the number of parietal microthrombi.13 Moreover, targeted activation of perivascular MCs in advanced plaques of apolipoprotein E–deficient mice sharply increases the incidence of intraplaque hemorrhage and vascular leakage, suggesting MC-mediated EC damage.14

In normal arteries, EC survival and function largely depend on the existing hemodynamic forces.15 High laminar shear stress protects ECs from ensuing apoptosis16–18 by inducing the expression of antiapoptotic genes19 and integrins.20 The interactions between EC integrins and the extracellular matrix (ECM) components of the arterial wall are especially critical for the proper function of the endothelium, because ECs lacking this interaction rapidly undergo apoptosis.21–23 The most important integrins for EC survival are αvβ3 and α5β1 integrins, known as the primary receptors for the ECM components vitronectin (VN) and fibronectin (FN), respectively.24,25 Because integrins induce their cellular effects via
focal adhesions, ie, complex ECM adhesion sites in the plasma membrane, the integrity of the focal adhesions is important for EC survival. Focal adhesion kinase (FAK) is a central molecule of focal adhesions26 and its activation, ie, phosphorylation, is induced by binding of integrins to VN27 and FN.28,29 Activation of FAK leads to downstream activation of other signaling proteins such as Akt and nuclear factor κB (NF-κB).30,31

In the present study, we show that activated MCs induce EC apoptosis through a novel dual mechanism involving parallel and additive effects of chymase and TNF-α. By avidly degrading the pericellular matrix components VN and FN, and by inactivating the focal adhesion-mediated EC survival-signaling pathways, chymase renders the otherwise resistant ECs susceptible to apoptosis. Subsequently, TNF-α released together with chymase triggers apoptosis in the protease-sensitized ECs.

Methods
Please see the online supplemental data section at http://atvb.ahajournals.org for a detailed Methods section.

Culture of RCMECs and Human Coronary Artery ECs
RCMECs were isolated from male Wistar rat (300 to 500 g) myocardium as described in the detailed Methods section online, and cultured in M199 culture media (Cat. M7528, Sigma) supplemented with 15% FBS, 2 mmol/L L-glutamine, 250 U/mL PS and 2.5 μg/mL amphotericin B. Experiments were performed with ECs of 2nd to 6th passages in serum-free conditions.

Human coronary artery ECs (HCAECs; PromoCell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium MV (PromoCell) supplemented with 5% fetal calf serum (PromoCell), growth factors (0.4% endothelial cell growth supplement/heparin, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortison; PromoCell), and antibiotics (100 U/mL PS, 50 ng/mL amphotericin B; Cambrex) according to instructions of the supplier. Experiments were performed with cells of 7th passage in serum-free conditions.

Statistical Analysis
Data, shown as means±SDs, were analyzed by the Student 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
To further elucidate the mechanisms involved in MC-mediated EC apoptosis,9 RCMECs were treated with rat MC releasate, in the absence or presence of a neutralizing TNF-α antibody and a chymase inhibitor (TRINH). The commercial agent (compound 48/80) used here to induce MC degranulation did not affect the observed effects in the treated RCMECs (Figure 1). The MC releasate induced a 4-fold increase in the level of apoptotic cells (2.7±0.84 versus 0.7±0.22), which was partially inhibited in the presence of a neutralizing TNF-α antibody (1.7±0.34) or a chymase inhibitor (TRINH; 1.0±0.44; Figure 1). When both inhibitors were added together, the level of apoptosis was similar to the level seen in control cells (0.9±0.38 versus 0.7±0.22 in control; Figure 1). However, the difference in the level of inhibition of apoptosis between the individual and combined treatments was significant (P<0.01) only between the neutralizing TNF-α antibody (1.7±0.34) and the combination of a neutralizing TNF-α antibody and the chymase inhibitor, TRINH (0.9±0.38). These results suggest that the MC releasate contains multiple components, notably chymase and TNF-α, that are capable of inducing EC apoptosis.

To study the effect of MC releasate on the levels and activities of FAK and Akt, two central players in the cell-survival signaling pathways,31 RCMECs were treated with MC releasate. As shown in Figure 2, MC releasate efficiently induced both dephosphorylation and degradation of FAK (Figure 2A and 2B), whereas the releasate had a minor effect on the phosphorylation of Akt compared with the compound 48/80 control (Figure 2C), and no effect on the Akt protein level (Figure 2D). To determine which of the two apoptosis-inducing components in the mast cell releasate, ie, chymase or TNF-α, are involved in the observed effect on
cell-survival signaling, RCMECs were treated with purified rat chymase and recombinant rat TNF-α. Chymase alone induced both dephosphorylation and degradation of FAK (Figure 3A and 3B), whereas Akt was dephosphorylated but not significantly degraded (Figure 3C and 3D). Whereas equivalent amounts of purified chymase induced a similar degree of FAK dephosphorylation and degradation as the MC releasate (Figure 2A and 2B), TNF-α did not have a significant effect on phosphorylation or degradation of either FAK or Akt.

Because chymase induces apoptosis of SMCs and epithelial cells by degrading fibronectin (FN), and because also vitronectin (VN) is necessary for focal adhesions and cell survival signaling, we next studied the effect of MC chymase on purified VN and FN, as well as on VN and FN produced by the RCMECs. As shown in Figure 4, chymase avidly degraded purified VN (Figure 4A) and FN (Figure 4B). In addition, incubation of cultured RCMECs with chymase induced a significant degradation of VN and FN present in their extra- and pericellular matrix (Figure 4C and 4D).

As the cellular effects of αvβ3 integrin-mediated outside-in survival signaling in ECs is controlled by NF-κB, a transcription factor also known to regulate the delicate balance of pro-inflammatory and anti-inflammatory genes in the atherosclerotic process, we studied the effect of MC releasate on NF-κB. Here we show, by means of immunostaining of the p65 subunit of NF-κB and by EMSA, that MC releasate induces the nuclear translocation of NF-κB (see supplemental Figure I). In control cells, p65 was present both in the nucleus and in the cytoplasm of RCMECs (supplemental Figure IA, top left), whereas when treated with MC releasate the nuclear staining was lost (supplemental Figure IA, top right). Furthermore, RCMECs treated with lipopolysaccharide (LPS), an efficient inducer of NF-κB activity, showed a strong nuclear staining of p65 (supplemental Figure IA, bottom left), whereas LPS-activated RCMECs treated with MC releasate did not (supplemental Figure IA, bottom right). In addition, by EMSA analysis we could show that the chymase and TNF-α–containing granule remnants (GRs) present in the MC releasate, abolished the nuclear translocation of p65 (supplemental Figure IB).

Furthermore, to analyze the onset of apoptotic death in the MC releasate-treated RCMECs, the levels of initiator caspase-8 and -9 were measured with a fluorometric assay. As shown in Figure 5A and 5B, MC releasate induced the
activities of both caspase-8 and -9, which were partially inhibited by their specific inhibitors, Z-IETD-FMK and Z-LEHD-FMK, respectively. Furthermore, a combined inhibition of caspase-8 and -9 resulted in an additive reduction in the degree of apoptosis (Figure 5C).

Finally, to investigate whether chymase and TNF-α could induce apoptosis also in human ECs, isolated ECs from human coronary arteries (HCAECs) were treated with recombinant human chymase and TNF-α. Indeed, as shown in Figure 6, both chymase and TNF-α alone were able to induce apoptosis in HCAECs (33.4±2.13% and 29.9±2.17% as compared with 16.7±1.65% of apoptotic cells in controls), and the level of apoptosis was significantly increased being additive when chymase and TNF-α were added together (49.2±2.16% apoptotic cells).

Discussion

In the present study, we show that activated mast cells (MCs), by a combined action of chymase and TNF-α, induce apoptosis in both RCMCs and HCAECs in vitro. The molecular mechanisms of MC-mediated apoptosis involve degradation of VN and FN, with subsequent inactivation of the FAK, Akt, and NF-κB signaling pathways, and activation of both caspase-8 and -9.

It is well known that MCs contain a broad variety of mediators, by which they are capable of orchestrating both innate and acquired immune reactions. In addition to their extremely potential repertoire of effector molecules, the strategic location of MCs at sites such as skin and vascular and mucosal barriers support an evolutionary role for them in primary host defense, ie, in killing invading parasites and bacteria, as well as infected and damaged cells. Because a “clean” process of apoptotic death, in contrast to “dirty” necrotic death, would be the most feasible and physiological mechanism of removing such intruders, one could reason that MCs, as a part of the normal host defense, would possess means to induce proapoptotic mechanisms in vivo. Here we describe a novel cell-mediated proapoptotic mechanism, in which activated MCs use a unique sequence of multiple events to induce apoptosis in ECs in vitro. This sequence of events involves the parallel and additive action of two simultaneously acting effector molecules, chymase and TNF-α, secreted by the activated MCs. The proteolytically active chymase degrades the pericellular matrix components VN and FN and inhibits the FAK- and NF-κB–mediated antiapoptotic signaling mechanisms, sensitizing the normally apoptosis-resistant ECs to proapoptotic stress. Thus, based on our data it seems evident that the chymase-induced inhibition of both FAK- and Akt-phosphorylation occurs through indirect mechanisms, ie, via degradation of the extracellular matrix components VN and FN that are necessary for the maintenance of focal adhesions, followed by an increased degradation of the downstream molecules involved in focal adhesion signaling by intracellular proteases.

The observed MC-induced activation of both caspase-8 and -9, the initiator caspases of the extrinsic and intrinsic pathways of apoptosis, respectively, also suggests an interaction between the two death pathways. Thus, chymase may trigger the intrinsic pathway of apoptosis with the concomitant activation of caspase-9. Subsequently, in the absence of integrin-maintained antiapoptotic pathways, TNF-α may induce EC apoptosis via the extrinsic pathway, ie, by activation of caspase-8. Interestingly, the ability of TNF-α to induce apoptosis in ECs is significantly increased when protein or RNA synthesis is inhibited. Thus, by simultaneously suppressing antiapoptotic and inducing apoptotic mechanisms, MCs may actively disturb the balance between cell survival and death in neighboring ECs.

In our experiments, the chymase-mediated effect on RCMC apoptosis seems to rely on its ability to proteolytically degrade ECM proteins VN and FN. It has been shown that FN adhesive fragment is buried in intact FN, but on generation by matrix metalloproteinase (MMP) 2 becomes exposed. In addition, specific FN fragments have been shown to induce apoptosis of SMCs, epithelial cells, and periodontal ligament cells. However, some FN fragments have been shown to rather promote human retinal EC adhesion and proliferation indicating that the FN fragments have different effects depending on how they are generated. Also, blocking peptides of αvβ3 integrins have been shown to induce apoptosis in ECs, indicating that also VN is an important mediator of cell survival. Moreover, RGD-containing peptides can also enter cells through endocytic pathways and thereby induce apoptosis by direct activation of caspase-3.

In addition, it has been shown that unligated integrins can induce apoptosis of adherent cells by a mechanism involving the direct activation of caspase-8. However, our present results show that the major effect of MC-induced RCMC apoptosis is attributable to the proteolytic degradation of the matrix components, VN and FN, which intactness is necessary for the maintenance of focal adhesions and cell survival. The chymase-generated VN and FN peptide fragments have only a minor direct effect on RCMC apoptosis (data not shown).

Under physiological conditions, the mechanism of MC activation, which is a prerequisite for the release of its powerful mediators, is strictly regulated. However, under pathological conditions, such as atherosclerosis, the regulation of MC activation may become uncontrolled, leading to undesired and excessive tissue destruction. Thus, the role of activated MCs in the process of plaque erosion and rupture may result from a vicious circle, in which an ongoing ancient remodeling process is aiming at repair, but rather initiates an uncontrolled process of pathological destruction. Thus, by secreting chymase and TNF-α,
activated MCs induce apoptosis of ECs in vitro by a mechanism involving degradation of VN and FN with subsequent inactivation of FAK, Akt, and NF-κB and activation of caspase-8 and -9. In excess, this cellular derangement may contribute to focal erosion of atherosclerotic plaques with its clinical sequelae.

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Disclosures

None.

References


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Methods

Animals

Male Wistar rats (300 – 500 g) were obtained 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 of rat cardiac microvascular ECs (RCMECs) and human coronary artery ECs (HCAECs)

RCMECs were isolated from rat myocardium and cultured as previously described with some modifications. Briefly, rat myocardium was dissected for perfusion with an enzyme solution [0.5 mg/ml collagenase type 2 (Cat. 4176, Worthington); 1 mg/ml dispase II (Cat. 165859, Roche); 10 μg/ml trypsin (Cat. T-4665, Sigma) with 0.5 mg/ml bovine serum albumin (BSA) in perfusion buffer containing 110 mM NaCl, 2.68 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 11 mM glucose, 2 U/ml heparin] for 20 min and for additional 10 minutes supplemented with BSA (40 mg/ml BSA in perfusion buffer). To facilitate the recovery of separated cells, the perfused tissue was cut into small pieces (approximately 1mm x 1mm) in enzyme solution and then filtered through a nylon mesh (150 μm pore size). The myocardial perfusate and the filtered suspension were combined and further treated with 2 mg/ml trypsin, 6 mM CaCl₂ and 40 mg/ml BSA in perfusion buffer for 30 min at 37°C, after which the cells were collected by centrifugation (200g, 5 min), and resuspended in M199 culture medium (Cat. M7528, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), 10% rat
serum, 2 mM L-glutamine (Cambrex), 625 U/ml penicillin-streptomycin (PS, Cambrex) and 2.5 μg/ml amphotericin B (Sigma) in two 25 cm² culture bottles. After 30 min, non-attached cells were washed away and the attached ECs were cultured in M199 media as described above at 37°C under 5 % CO₂. After one day, the culture media were supplemented with 15% FBS, 2 mM L-glutamine, 250 U/ml PS and 2.5 μg/ml amphotericin B. Experiments were performed with ECs of 2-6th passages in serum-free conditions.

The RCMECs isolated with this method were identified as ECs by their morphology, positive staining with *Griffonia simplicifolia I* lectin and uptake of DiI-labeled acetylated LDL (data not shown). At 2-4th passage, contamination with other cardiac cells was found to be less than 1%.

HCAECs (PromoCell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium MV (PromoCell) supplemented with 5 % fetal calf serum (PromoCell), growth factors (0.4 % endothelial cell growth supplement/heparin, 10 ng/ml epidermal growth factor, 1 μm/ml hydrocortison; PromoCell) and antibiotics (100 U/ml PS, 50 ng/ml amphotericin B; Cambrex) according to instructions of the supplier. Experiments were performed with cells of 7th passage in serum-free Endothelial Cell Growth Medium MV or Endothelial Cell Basal Medium containing growth factors and antibiotics like above.
Preparation of serosal mast cells (MCs), MC releasate and granule remnants

Serosal MCs were isolated from pleural and peritoneal cavities of rats and stimulated to degranulate with compound 48/80 (1 μg/ml, Sigma) in PBS as described earlier. Briefly, “MC releasate”, which contains all the material released from the stimulated MCs, was collected after stimulation as the supernatant obtained by centrifugation at 200g for 5 minutes. A sediment containing ”granule remnants”, i.e. exocytosed granules, was obtained by further centrifugation of the MC releasate at 13 000g for 10 minutes. For experiments with ECs (~3x 10^5), the releasate from ~5x 10^4 MCs was used in FACS and Western blotting experiments, from ~9x 10^4 MCs in caspase assays, and from ~5x 10^5 MCs in NF-κB experiments. Thus, in this manuscript the sources of chymase and their chymase activities added per 10^6 RCMECs are as follows; Releasate (Rel), 30-50 BTEE units; Granule remnants (GR), 20-35 BTEE units; and purified chymase, 15 BTEE units.

Other materials in cell incubations

To analyze the effect of the individual granule remnant components, namely chymase and TNF-α, RCMECs were incubated (1) with rat MC releasate in the absence or presence of an inhibitor of TNF-α (1.2 μg/ml neutralizing TNF-α antibody, Cat. MAB510, R&D Systems, UK) or an inhibitor of chymase (100 μg/ml TRINH, Sigma), or (2) with purified rat chymase (3 BTEE units /ml) and rat recombinant TNF-α (20 ng/ml; Cat. PRP16, Serotec, UK). In experiments with HCAECs, recombinant human chymase (10 BTEE units /ml; kindly provided by Dr Takashi Kamimura, Teijin Institute for Biomedical Research, Teijin Limited, Tokyo, Japan) and TNF-α (20 ng/ml; Cat. 210-TA,
R&D Systems) were used. The activities of both rat and human chymase were
determined with BTEE (N-benzoyl-L-tyrosine ethyl ester, Cat. B-6125, Sigma) as a
substrate (one unit hydrolyzes 1 μmol of BTEE per minute at +25°C, pH 7.8).

**Degradation of purified vitronectin (VN) and fibronectin (FN)**

4 μg of purified VN (Promega) or FN (Sigma) were exposed to rat chymase (3 BTEE
units) at 37°C in 20 μl volume of reaction buffer (50 mM Tris-HCl, 0.15 M NaCl pH 7.5)
for indicated times (see figure), reactions terminated by adding SDS-PAGE loading
buffer, and the samples run on 4-20 % SDS-polyacrylamide gel and stained with
Coomassie Blue (SimplyBlue SafeStain, Invitrogen).

**Determination of apoptotic ECs**

The cellular DNA content was determined by flow cytometric analysis of propidium
iodide (PI)-labeled cells 7,8. Briefly, ECs attached to culture dishes were detached with
trypsin and combined with the cells floating in the culture medium. The cells were then
fixed with cold 90 % methanol or 70 % ethanol, incubated with 100 units/ml of RNase,
and stained with 25-50 μg/ml PI. After staining, the cells were analyzed by
FACSCalibur, FACScan or BD LSR II (BD Biosciences, San Jose, CA, USA).
Fluorescence was measured from ≥ 10 000 events, and gating on FL2-A versus FL2-W
was used to remove doublets. Apoptotic nuclei were identified as subgenomic DNA, and
were distinguished from cell debris on the basis of forward light scatter and fluorescence
of PI.
Nuclear fragmentation was measured also with Cell Death Detection ELISAPLUS Kit (Roche, Cat. 1774425). Briefly, treated cells were lysed and DNA-histone-complexes from the cytoplasm detected with ELISA method according to manufacturer’s instructions.

**Western blot analysis**

RCMECs were treated with either MC releasate or chymase and TNF-α for 4 hours, and the ECs were lysed in ice-cold sodium orthovanadate buffer for 30 minutes on ice. Proteins were separated on a 10-12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% defatted milk in 10 mM Tris, 100 mM NaCl, 0.1% tween 20, pH 7.5 for 1 hour, and then further incubated with specific antibodies against FAK (1:1000, Cat.610087, mouse monoclonal IgG, BD Biosciences Pharmingen, San Jose, CA, USA), p-FAK (1:500, Cat.36-7900, Zymed Laboratories, South San Francisco, CA, USA, or 1:1000, Cat. #07-012, Upstate Biotechnology, Lake Placid, NY, USA, both rabbit polyclonals), Akt (1:1000, Cat. 610860, mouse monoclonal IgG, BD Biosciences Pharmingen), p-Akt (1:1000, Cat. #4058 and #9271, Cell Signaling technology, MA, USA, rabbit monoclonal IgG and rabbit polyclonal, respectively), fibronectin (1:5000, Cat. F3648, rabbit polyclonal, Sigma, MI, USA), vitronectin (1:1000, Cat. Ab45139, rabbit monoclonal IgG, Abcam, Cambridge, UK) and β-actin (1:80 000, Cat. Ab8226, mouse monoclonal IgG, Abcam, Cambridge, UK). The primary antibodies were detected with horseradish peroxidase -conjugated goat anti-mouse IgG (1:2000, Cat. 0447, Dako, Glostrup, Denmark) for Akt and β-actin, rabbit-anti-mouse (1:2000, Cat. 0260, Dako) for FAK and
goat anti-rabbit (1:2000 and 1:3000, Cat. 0448, Dako) for p-FAK, FN, VN and p-Akt, respectively, and an enhanced chemiluminescence detection system (Amersham). The immunoblots were quantitated with a Gel Doc 2000 gel documentation system (Bio-Rad).

**Immunocytochemistry**

RCMECs were cultured on Thermanox coverslips (Nunc, Rochester, NY, USA) in the presence or absence of MC releasate and 5 μg/ml lipopolysaccharide (LPS, Sigma) for 6 hours. The treated cells were fixed in 1 % paraformaldehyde for 10 minutes, and cell membranes permeabilized in methanol for 8 minutes. Nonspecific binding sites were blocked in blocking buffer [3 % BSA, 3 % normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA), and 0.1 % Tween-20 in phosphate-buffered saline (PBS)], and NF-κB p65 detected by incubations with primary antibody (1:500, Cat. sc-372, rabbit polyclonal IgG, Santa Cruz Biotechnology, Heidelberg, Germany) and biotinylated secondary antibody (1:200, Cat. E0432, Dako) in blocking buffer described above, respectively, and by further incubation with FITC-conjugated streptavidin (1:100, Cat. F0422, Dako) in PBS containing 3% BSA and 0.1% tween-20.

**Electrophoretic mobility shift assay (EMSA)**

RCMECs (1x 10^6) were exposed to MC granule remnants (from ~4x 10^5 MCs) or to 5 μg/ml lipopolysaccharide (LPS, Sigma) for 4 hours and nuclear proteins were extracted as previously described. EMSA was performed as described previously. Briefly, nuclear proteins (20 μg) were incubated with 25 000 cpm of 32P-labeled NF-κB oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3', Cat. sc-2505,
Santa Cruz Biotechnology) and samples were run on 4% polyacrylamide gel. Specificity was verified by the addition of 100-fold molar excess of unlabeled oligonucleotide probe (competitor) or mutated unlabeled oligonucleotide probe (5’- AGT TGA GGC GAC TTT CCC AGG C – 3’, Cat. sc-2511, Santa Cruz Biotechnology) before addition of radioactive oligonucleotide probe.

**Caspase activity assays**

RCMECs were exposed to MC releasate for 6 hours and enzymatic activities of caspase-8 and -9 were measured using a fluorometric assay according to the manufacturer’s recommendations (R&D Systems). Data obtained was adjusted for protein content measured by BCA protein assay. Caspase-8 and -9 inhibitors (Z-IETD-FMK and Z-LEHD-FMK, respectively, R&D Systems) were used at 10 μM concentrations.

**Other assays**

Protein was determined according to the standard procedure by Lowry\textsuperscript{12} or with BCA protein assay (Pierce), with BSA as standard.

**Statistical analysis**

Data, shown as means ± standard deviations (SDs), were analyzed by the Student’s $t$ test for determination of the significance of differences, which were considered to be statistically significant at a $P$ value of less than 0.05.
References


Detailed Figure Legends

Figure 1. MC releasate-induced EC apoptosis is mediated by chymase and TNF-α

RCMECs (~3x 10^5) were treated with MC releasate (from ~5x 10^4 MCs) with and without inhibitors for TNF-α (1.2 μg/ml neutralizing TNF-α antibody, anti-TNF-α) and chymase (100 μg/ml TRINH) for 4 hours, and the level of apoptosis was measured by flow cytometry. Compound 48/80 was used as a control for the releasate. Control; cells incubated in serum-free media. Data shown are means with SDs, * = P <0.05, ** = P <0.01, *** = P <0.001. Similar results were obtained in three independent experiments.

Figure 2. MC releasate induces FAK degradation and Akt dephosphorylation

RCMECs (~3x 10^5) were treated with MC releasate (from ~5x 10^4 MCs) for 4 hours, and the levels of p-FAK (A), FAK (B), p-Akt (C), Akt (D) and β-actin were detected by Western blotting. Compound 48/80 was used as a control for the releasate. Data shown are means with SDs, * = P <0.05, ** = P <0.01, *** = P <0.001. Similar results were obtained in three independent experiments.

Figure 3. Chymase induces FAK degradation and Akt dephosphorylation
RCMECs were treated with purified rat chymase (chy; 3 BTEE units /ml) and recombinant rat TNF-α (20 ng/ml) for 4 hours, and the levels of p-FAK (A), FAK (B), p-Akt (C), Akt (D) and β-actin were detected by Western blotting. Data shown are means with SDs, * = P <0.05, ** = P <0.01, *** = P <0.001. Similar results were obtained in two independent experiments.

**Figure 4. Chymase degrades vitronectin (VN) and fibronectin (FN)**

Purified VN (A) and FN (B) were treated with chymase (3 BTEE units / sample) for different times as indicated in the figure, run on 4-20 % SDS-polyacrylamide gel and stained with Coomassie Blue. RCMECs were treated for 4 hours with chymase (3 BTEE units/ml) and the level of VN and FN degradation was measured by Western blot analysis (C and D). Data shown are means with SDs, ** = P <0.01. Similar results were obtained in two independent experiments.

**Figure 5. MC releasate induces caspase-8 and caspase-9 activation**

RCMECs (~3x 10^5) were treated for 6 hours with MC releasate (from ~9x 10^4) with and without inhibitors for caspase-8 and -9 (Z-IETD-FMK and Z-LEHD-FMK, respectively), after which caspase activities were measured with a fluorometric assay (A and B) and apoptosis was measured with FACS (C). Data shown are means with SDs, ** = P<0.01. The results are representative of two independent experiments.
Figure 6. Chymase and TNF-α induce apoptosis of HCAECs

HCAECs were treated for 16 hours with recombinant human chymase (10 BTEE units /ml) and TNF-α (20 ng/ml), and apoptosis was measured with flow cytometry. Data shown are means with SDs, ** = P <0.01, *** = P <0.001. Similar results were obtained in three independent experiments.

Supplemental Figure I. MC releasate induces inhibition of NF-κB activity

RCMECs (~3x 10^5) were treated for 6 hours with MC releasate (Rel; from ~5x 10^5 MCs) with or without 5 μg/ml lipopolysaccharide (LPS), and NF-κB p65 was detected with immunofluorescent staining (A). RCMECs (1x 10^6) were treated for 4 hours with MC granule remnants (GR; from ~4x 10^5 MCs) or LPS, and NF-κB DNA-binding activity was measured with EMSA (B). Specificity of the 32P-labeled NF-κB oligonucleotide probe was confirmed with 100-fold molar excess of unlabeled control (COLD) and mutated (MUT) oligonucleotide probes.
Figure I

A. Cont               Rel

LPS                  LPS + Rel

B. Cont  GR  LPS  LPS  LPS  LPS

NF-κB binding activity

COLD MUT