Perivascular Mast Cells Promote Atherogenesis and Induce Plaque Destabilization in Apolipoprotein E–Deficient Mice

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Background—Mast cells are major effector cells in allergy and host defense responses. Their increased number and state of activation in perivascular tissue during atherosclerosis may point to a role in cardiovascular disorders. In the present study, we investigated the contribution of perivascular mast cells to atherogenesis and plaque stability in apolipoprotein E–deficient mice.

Methods and Results—We show here that episodes of systemic mast cell activation during plaque progression in mice leads to robust plaque expansion. Targeted activation of perivascular mast cells in advanced plaques sharply increases the incidence of intraplaque hemorrhage, macrophage apoptosis, vascular leakage, and CXCR2/VLA-4–mediated recruitment of leukocytes to the plaque. Importantly, treatment with the mast cell stabilizer cromolyn does prevent all the adverse phenomena elicited by mast cell activation.

Conclusions—This is the first study to demonstrate that mast cells play a crucial role in plaque progression and destabilization in vivo. We propose that mast cell stabilization could be a new therapeutic approach to the prevention of acute coronary syndromes. (Circulation. 2007;115: &NA;–&NA;)

Key Words: apoptosis ■ atherosclerosis ■ hemorrhage ■ inflammation ■ mast cells

Acute coronary syndromes, such as unstable angina and myocardial infarction, are commonly caused by erosion or rupture of vulnerable atherosclerotic plaques.1–3 Inflammatory cells are considered to play a key role in the pathogenesis of plaque rupture.4–6 Indeed, one of the inflammatory cell types, the mast cell,7–8 has been shown to accumulate in the rupture-prone shoulder region of human atheromas.9 Activated mast cells that contain proteases such as tryptase and chymase have been identified at the site of rupture in specimens of human coronary arteries.10–13 Human coronary artery specimens contain tumor necrosis factor-α–rich activated mast cells,14,15 which potentially aggravate the ongoing inflammatory response and may ultimately lead to plaque destabilization.16

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Clinical Perspective p 0000

Not only intimal inflammation but also inflammation of the arterial adventitia has been shown to influence the plaque vulnerability.17 Activated mast cells have been identified in the adventitia of vulnerable and ruptured lesions in patients with myocardial infarction,18–20 and, more importantly, their number was found to correlate with the incidence of plaque rupture and erosion.18 However, it remains to be clarified whether adventitial mast cells actively modulate lesion composition and are instrumental in causing plaque rupture or rather are part of the inflammatory cell recruitment secondary to plaque rupture.

In the present study, we demonstrate that systemic mast cell activation during atherogenesis leads to increased plaque progression. Moreover, we show that local activation of mast cells in the adventitia of advanced carotid artery plaques promotes macrophage apoptosis, microvascular leakage, and de novo leukocyte influx. Importantly, this culminates in a greatly enhanced incidence of intraplaque hemorrhage. Finally, mast cell stabilization by cromolyn was seen to prevent these pathophysiological events by inhibition of mast cell degranulation.

Methods

A detailed description of the Methods is given in the online-only Data Supplement.

Experimental Design

All animal work was performed in compliance with Dutch government guidelines. Male apolipoprotein E–deficient (ApoE–/–) mice,
obtained from the local animal breeding facility, were fed a Western-type diet that contained 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). After 4 weeks of Western-type feeding, all isoflurane-anesthetized animals were skin-sensitized on days 1 and 2 with dinitrofluorobenzene (DNFB; 0.5% v/v, Janssen Chimica, Beerse, Belgium) or vehicle control solution (acetone:olive oil 4:1, n = 7 per group) as described by Kraneveld et al.21 On day 5, the mice were challenged intravenously by injection of dinitrophenyl-albumin (DNP; 1 mg/animal), which was repeated once weekly for another 2 weeks to induce systemic adventitial mast cell activation. A separate group of mice received an intraperitoneal injection of the mast cell stabilizer cromolyn (50 mg/kg; Sigma, Zwijndrecht, the Netherlands)22,23 30 minutes before and after DNP challenge. After 8 weeks of diet feeding, the mice were anesthetized. In situ fixation through the left cardiac was performed24 and brachiocephalic artery lesions were analyzed.

To determine the effect of local adventitial mast cell activation on advanced atherosclerotic lesions, carotid artery plaque formation was induced by perivascular collar placement in male ApoE-/- mice as described previously.24 Mice were anesthetized by subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health, Bladel, the Netherlands), fentanyl citrate (1.26 mg/kg, Janssen Animal Health, Sauderton, UK), and fluanisone (2 mg/kg, Janssen Animal Health). Five weeks after collar placement all animals were skin-sensitized as described above (control: n = 14; DNFB: n = 13). On day 5, the mice were challenged perivascularly by application of pluronic F-127 gel (25% wt/vol) or pluronic F-127 gel that contained DNP (50 μg/animal) at the lesion site. To measure de novo infiltration of circulating leukocytes into the lesions, some of the mice were injected intravenously with rhodamine-6G (0.67 mg/kg)25 to label circulating leukocytes. In a separate experimental set-up, 2 groups of mice (control: n = 11; DNFB: n = 10) received an intravenous injection that contained 25 mg/kg of cromolyn 30 minutes before local DNP or control challenge and twice during challenge by intraperitoneal injections with 50 mg/kg of cromolyn. Three days after challenge the animals were anesthetized and in situ perfusion-fixation was performed, after which the carotid artery lesions were analyzed.

Lesion analysis, cell culture experiments, vascular leakage, and perfusion studies were performed as detailed in the online-only Data Supplement.

**Statistical Analysis**

Data are expressed as mean±SEM. A 2-tailed Student t test was used to compare individual groups, whereas multiple groups were compared with a 1-way ANOVA and a subsequent Student-Newman-Keuls multiple comparisons test. Nonparametric data were analyzed with a Mann-Whitney U test. Frequency data analysis was performed by means of the Fisher exact test. Matched nonparametric data were analyzed with the Friedman test. A level of P<0.05 was considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Systemic Mast Cell Activation and Plaque Morphometry**

First, we delineated the effect of systemic mast cell activation during atherosclerotic lesion progression in ApoE-/- mice. In the adventitia of the brachiocephalic artery lesions, the percentage of activated mast cells at 7 days after mast cell activation was 62±9% in controls, 75±7% in DNP-challenged mice, and 54±10% in DNP-challenged mice that were treated with the mast cell stabilizer cromolyn (P<NS). Despite the fact that mast cells had only been activated 3 times during 8 weeks of lesion development, the brachiocephalic artery lesion size of DNP-challenged mice was significantly increased by >2-fold compared with controls (73±11×10³ μm² versus 35±11×10³ μm²; P<0.05) (Figure 1A and 1B). Interestingly, treatment of DNP-challenged animals with the mast cell stabilizer cromolyn prevented the DNP-induced plaque expansion (42±8×10³ μm²; P<0.05 compared with DNP-challenged mice). Relative macrophage content (monocyte and macrophage antibody, or MOMA-2, staining) did not differ between the groups (data not shown), and the amount of adventitial neutrophils was unchanged (20±1 in control mice, 19±5 in DNP-challenged mice, and 16±1 neutrophils/section in cromolyn-treated DNP-challenged mice; P=NS).

**Local Adventitial Mast Cell Activation and Plaque Morphology**

Because mast cell density and activation is particularly high in the adventitia of human type V/VI atherosclerotic lesions,
and as we cannot exclude a systemic inflammatory response when mast cells are activated as described above, we assessed the influence of focal and acute adventitial mast cell activation on advanced atherosclerotic plaques in ApoE−/− mice. The hapten challenge was therefore applied perivascularly by administration of a DNP-loaded pluronic F-127 gel at the collar-induced carotid artery lesion.24 The hapten challenge was therefore applied perivascularly by administration of a DNP-loaded pluronic F-127 gel at the collar-induced carotid artery lesion.24

Morphometric analysis of the lesions did not reveal any differences in plaque size between control and DNP-challenged animals at 3 days after challenge (54±8×10^3 μm^2 versus 59±9×10^3 μm^2, respectively) (Figure 2A), which was also not affected by cromolyn treatment during challenge of the animals (control: 54±7×10^3 μm^2 versus DNP-challenged: 66±11×10^3 μm^2). Medial surface area was slightly increased in the DNP-challenged mice (42±4×10^3 μm^2 versus 34±2×10^3 μm^2 in controls; P=0.04), which did not occur after acromelin treatment (DNP-challenged: 36±3×10^3 μm^2 versus controls: 35±3×10^3 μm^2).

Resting and activated mast cells in the adventitia of the lesions were detected by toluidin blue (Figure 2B). Alcian blue/saphranin O staining revealed that the majority of the adventitial mast cells were connective tissue–type mast cells (98.6%).28 Both the number of adventitial mast cells in DNP-challenged mice (7.5±1.4 versus 4.8±0.7 MC/mm^2 adventitial tissue in the control mice; P<0.05) (Figure 2C) and the percentage of degranulated adventitial mast cells were significantly increased (74.7±3.9% versus 44.6±5.7% in control animals; P<0.001) (Figure 2D) at 3 days after perivascular challenge. Cromolyn treatment completely abolished the DNP-induced mast cell recruitment (3.1±0.5 MC/mm^2 adventitial tissue, P<0.01, compared with DNP-challenged mice that had not received cromolyn) (Figure 2C), and the amount of activated mast cells returned to basal levels (32.4±4.9% in controls and 30.4±7.4% in DNP mice that received cromolyn) (Figure 2D). In the adventitia of these lesions, the total number of neutrophils did not differ between any of the groups (Figure 2E, P=NS), which indicated that cromolyn did not affect neutrophil infiltration.

Strikingly, further analysis of the plaque morphology revealed massive intraplaque hemorrhages, characterized by the presence of intimal erythrocytes, in 7 of 26 plaques of DNP-challenged animals (Figures 3A and 3F), whereas we observed no such phenomena in controls (Figure 3B) (0 of 27, P=0.004). CD31-positive microvessels were detected, generally in close proximity of hemorrhages (Figure 3C). A Perl’s iron staining confirmed these findings (6 of 26 compared with 0 of 27 for control mice; P=0.01) (Figure 3D, 3E, and 3F). Iron staining was found to correlate with the presence of intraplaque hemorrhage (P<0.0001), colocalized with ceroid-rich regions (data not shown) and was confined mostly to the central atheroma. Of 6 iron-positive lesions, 5 arteries revealed iron staining also in the media (P=0.02) and enhanced medial thickening. Importantly, none of the cromolyn-treated DNP-challenged mice showed intraplaque hemorrhage or iron staining (P=0.01 compared with DNP-challenged animals that had not received cromolyn) (Figure 3F).
In Vivo Apoptosis

As mast cell degranulation was reported to promote apoptosis of vascular smooth muscle cells and endothelial cells, we stained sections for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Figure 4A). Indeed, we observed a significant increase in TUNEL-positive area in DNP-challenged lesions (3.3±0.5% TUNEL-stained area compared with 0.6±0.2% in control animals; P=0.002). Although the iron-negative lesions of the DNP-challenged animals displayed enhanced levels of TUNEL-positive area (2.6±0.6% TUNEL-positive area; P=0.02 compared with controls), the degree of apoptosis was even more pronounced in the iron-positive sections (4.8±0.6% TUNEL-positive area; P=0.01) (Figure 4B). These data were confirmed by scoring of the TUNEL-positive nuclei in the plaque (6.1±2.0% versus 2.1±0.6% in the controls; P=0.04) (Figure 4C). The majority of the apoptotic cells were located in the central atheroma rather than in the SMC-rich lesion cap (P=0.04) (Figure 4C), which suggests that adventitial mast cell degranulation preferentially induces macrophage apoptosis. Strikingly, no differences were observed between controls and DNP-challenged mice that had been treated with cromolyn (1.5±0.7% versus 2.4±0.9% TUNEL-stained area, respectively; P=0.4), which suggests that cromolyn prevented the adventitial mast cell induced macrophage apoptosis.

Mast Cell Releasate Induces Macrophage Apoptosis

Releasates of MC/9 cells and of mast cell freshly isolated from the peritoneal cavity (peritoneal mast cells) were analyzed after stimulation with compound 48/80 for their composition. MC/9 releasate was previously shown to contain histamine and was demonstrated to have β-hexosaminidase activity (1.3% of total release) as well as to contain tryptase (1.2% of total release). Peritoneal mast cells releasate also showed β-hexosaminidase activity (16% of total release) and contained chymase, tryptase, vascular endothelial growth factor, and histamine (7.8%, 3.5%, 18.7%; and 5.5% of total release, respectively).

Supernatant from MC/9 mast cells induced apoptosis of RAW 264.7 macrophages by up to 5-fold (21.8±0.7% of apoptotic cells versus 4.4±0.3% for control medium) (Figure 5A). To pinpoint the culprit mast cell constituent, we assessed the effect of tryptase (leupeptin), chymase (soy bean trypsin inhibitor) as well as of histamine receptor antagonists in mast cell–induced apoptosis. Soy bean trypsin inhibitor, leupeptin, and the H1-receptor antagonist triprolidine were able to inhibit mast cell–induced macrophage apoptosis, whereas the H2- and H3-receptor antagonists cimetidine and thioperamide were ineffective (Figure 5A). None of the inhibitors affected endogenous apoptosis or H2O2-induced apoptosis of RAW 264.7 cells, which thus excluded that the inhibitors used are pro- or ant apoptotic by themselves (data not shown). As both the protease inhibitors and the H1-receptor antagonist completely inhibited macrophage apoptosis, we verified whether histamine acts synergistically on tryptase-induced apoptosis or vice versa. Incubation with histamine for 16 hours strongly increased macrophage apoptosis (9-fold; P<0.01), whereas subsequent posttreatment with tryptase (6 hours) led to an additive (18-fold) increase in macrophage apoptosis compared with treatment with histamine only. Tryptase treatment for 6 hours appeared to be ineffective. Conversely, priming of macrophages with tryptase for 16 hours slightly enhanced RAW 264.7 cell apoptosis (13±3% compared with 4±2% for untreated cells; P<0.05), but did not sensitize macrophages
for histamine-induced apoptosis (data not shown). In analogy to the MC/9 experiments, the supernatant of peritoneal mast cells and that of primary cultured bone marrow–derived mast cells, both degranulated with compound 48/80, induced peritoneal macrophage apoptosis (Figure 5B). In agreement with previous studies,\textsuperscript{12,13} supernatant of degranulated MC/9 cells induced vascular smooth muscle cell apoptosis after 48 hours (data not shown), although vascular smooth muscle cells appeared to be less susceptible to mast cell–induced apoptosis than macrophages.

Figure 5. In vitro apoptosis of macrophages induced by mast cell degranulation. A, Supernatant from degranulated MC/9 mast cells induced apoptosis of RAW 264.7 macrophages in a dose-dependent fashion (5\texttimes{} and 50\texttimes{} dilution of mast cell supernatant, upper left; *\textit{P}<0.05, **\textit{P}<0.01 compared with Dulbecco’s modified Eagle’s medium control). MC/9 mast cell induced apoptosis of RAW 264.7 cells (**\textit{P}<0.01 compared with with Dulbecco’s modified Eagle’s medium control) was inhibited by the chymase inhibitor soybean trypsin inhibitor (SBTI) and by the trypsin inhibitor leupeptin (##\textit{P}<0.01 compared with with MC/9 supernatant, upper right). Compound 48/80, used to degranulate the MC/9 mast cells, did not exert any effect on macrophage apoptosis. Mast cell–induced apoptosis was completely abolished by the H\textsubscript{1}-receptor antagonist triprolidine (##\textit{P}<0.01 compared with the mast cell supernatant–induced apoptosis), but not by the H\textsubscript{2}-receptor antagonist cimetidine and the H\textsubscript{3}-receptor antagonist thioperamide (lower left). Incubation of RAW 264.7 cells with 100 \textmu{}mol/L of histamine induced macrophage apoptosis, which was even enhanced after 6 hours of preincubation with tryptase, whereas tryptase itself did not induce apoptosis after 6 hours (lower right). B, Supernatant from compound 48/80–activated MC/9 cells, peritoneal mast cells, and bone marrow–derived mast cells induced apoptosis of freshly isolated peritoneal macrophages (*\textit{P}<0.05). C, Also, supernatant from MC/9, peritoneal mast cells, and bone marrow–derived mast cells was able to induce proliferation of H5V endothelial cells (**\textit{P}<0.05, ***\textit{P}<0.0001). PMC indicates peritoneal mast cells; BMMC, bone marrow–derived mast cells.
Furthermore, we determined whether mast cell supernatant from MC/9 cells, peritoneal mast cells, and bone marrow–derived mast cells, degranulated with compound 48/80, was able to induce endothelial cell proliferation. Indeed, murine endothelial H5V cells showed an increase in proliferation up to 200% when exposed to supernatant of either the activated mast cell line or the primary cultured mast cells ($P<0.05$) (Figure 5C).

Cromolyn Effects on Leukocytes

To determine whether cromolyn was mast cell specific, we systemically challenged C57Bl6 mice and analyzed effects of cromolyn on peritoneal cell composition. Cromolyn treatment did not affect relative neutrophil (Figure 6A), monocyte (Figure 6B), or T-cell content (Figure 6C). However, the number of peritoneal CD117$^+$ mast cells was significantly reduced by treatment with cromolyn ($P<0.001$) (Figure 6D).

Similar results were obtained after cromolyn treatment of locally challenged C57Bl6 mice (online-only Data Supplement Figure I). In line with these data, after perivascular DNP challenge of mast cell–deficient Kit(W-sh/W-sh) mice,30 cromolyn did not affect neutrophil and monocyte levels in the peritoneal cavity (online-only Data Supplement Figure II). Myeloperoxidase activity of peritoneal neutrophils also remained unaltered by cromolyn treatment after systemic (Figure 6E) and local challenge (Figure 6F) in C57Bl6 mice and did not differ in cromolyn-treated Kit(W-sh/W-sh) mice after perivascular challenge (Figure 6G).

Analysis of DNFB-sensitized skin segments of C57Bl6 mice revealed that cromolyn treatment did not affect the percentage of neutrophils and eosinophils. The percentage of mast cells was significantly enhanced in these mice after DNP challenge ($P<0.01$), an effect that was reduced by cromolyn administration (online-only Data Supplement Figure IIIA). In Kit(W-sh/W-sh) mice, cromolyn did not alter the levels of skin neutrophils and eosinophils after DNFB sensitization, whereas mast cells were, as expected, undetectable in these mice (online-only Data Supplement Figure IIIB).

According to the systemic challenge in C57Bl6 mice, serum levels of IL-2, IFN$\gamma$, IL-4, and tumor necrosis factor-$\alpha$ were not significantly different between control and DNP-challenged animals, although a trend toward reduced IL-4 levels was observed ($P=0.07$ compared with control mice) (online-only Data Supplement Figure IV). As mentioned previously, we cannot exclude the possibility that systemic inflammatory responses after systemic mast cell activation may indirectly have influenced atherogenesis. Therefore, we next focused on mast cell activation locally at the lesion site. As anticipated, after local challenge serum IFN$\gamma$ remained undetectable, whereas serum IL-2, IL-4, and tumor necrosis factor-$\alpha$ did not differ between the groups (online-only Data Supplement Figure V). Similar results were obtained in Kit(W-sh/W-sh) mice (online-only Data Supplement Figure VI).

Microvascular Leakage In Vivo

Apart from promoting macrophage apoptosis, mast cells have been thought to induce vascular leakage.31,32 We indeed observed that after intradermal injection of $5\times10^5$ activated MC/9 cells in mice, vascular leakage as judged by Evans Blue spot size was significantly enhanced compared with PBS and was demonstrated to only be inhibited by the H1-receptor antagonist triprolidine (online-only Data Supplement Figure VII, $P<0.001$). More importantly, carotid artery lesions, perivascularly challenged with DNP and injected with Rhodamine 6G to label circulating leukocytes, contained a higher number of Rhodamine-positive cells than controls.
and DNP-challenged animals. B, In vivo perfusion of DNP-challenged atherosclerotic plaques resulted in increased adhesion of Rhodamine-positive leukocytes (**P = 0.0009; left). Ex vivo perfusion of the carotid arteries revealed an increased adhesion of Calcein-labeled MonoMac-6 cells to DNP-challenged arteries, which could be blocked with anti-CXCR2 and anti-VLA-4 antibody treatment but not with Met-RANTES (right; *P = 0.02 compared with vehicle control, #P < 0.05 compared with DNP-challenged mice). C, Suggested mechanism of adventitial mast cell degranulation on atherosclerotic lesions.

**Figure 7.** Increased leukocyte adhesion by perivascular mast cell activation. A, Influx of newly recruited Rhodamine-positive leukocytes into DNP-challenged atherosclerotic plaques is increased compared with the control plaques (**P = 0.0002; left). The middle and right panels show representative pictures of Rhodamine-positive leukocytes, depicted by the white arrows, in control and DNP-challenged animals. B, In vivo perfusion of DNP-challenged atherosclerotic plaques resulted in increased adhesion of Rhodamine-positive leukocytes (**P = 0.0009; left).

**In Vivo Intravital Microscopy/Ex Vivo Perfusion**

To establish whether adventitial mast cell activation affects the adhesion of leukocytes to atherosclerotic plaques from the luminal side, circulating leukocytes were Rhodamine-6G-labeled in vivo and adhesion of these cells to DNP- and control-challenged atherosclerotic lesion was monitored by intravital microscopy. The adhesion of labeled leukocytes to plaques was 2.5-fold increased after perivascular mast cell activation (49 ± 6 versus 19 ± 4 leukocytes/microscopic field for DNP versus vehicle control plaques, respectively; P = 0.0009) (Figure 7B). Ex vivo perfusion studies on the adhesion of Calcein-labeled monotypic MonoMac-6 cells confirmed these findings (25 ± 6 cells/field in DNP-challenged arteries versus 9 ± 2 cell/field in control arteries; P = 0.02) (Figure 7B). Strikingly, antibody blockade of either the KC receptor CXCR2 or the β1-integrin and vascular cell adhesion molecule-1 receptor VLA-4 on the MonoMac-6 cells completely inhibited the mast cell-mediated monocyte adhesion to the atherosclerotic plaque, whereas blockade of CCR1, CCR3, and CCR5 with Met-RANTES and isotype control antibodies had no effect. None of the treatment modalities significantly reduced monotypic cell adhesion to control atherosclerotic plaques in the area of interest.

**Discussion**

Activated mast cells have been shown to accumulate in the arterial adventitia during plaque progression and are abundantly present in the adventitia of vulnerable and ruptured lesions. To date, it remains to be clarified whether these
adventitial mast cells contribute to plaque progression and whether these cells are instrumental in plaque rupture. To address these key questions, we recruited and activated mast cells in the adventitia of atherosclerotic lesions in ApoE<sup>-/-</sup> mice. We demonstrate that systemic mast cell activation during atherogenesis leads to increased plaque progression and that focal activation of mast cells in the adventitia of advanced plaques increases the incidence of intraplaque hemorrhage. Inhibition of mast cell degranulation by the mast cell stabilizer cromolyn prevented these pathophysiological events.

Systemic mast cell activation was seen to aggravate spontaneous plaque progression in the brachiocephalic artery of ApoE<sup>-/-</sup> mice, an effect that was not observed after prior mast cell stabilization with cromolyn. More importantly, as most mast cells are present in type V/VI human atherosclerotic lesions, we have addressed the effect of focal mast cell activation on preexisting collar-induced carotid artery plaques, which are more easily accessible for local intervention. These atherosclerotic plaques, formed proximal to the collar, were previously shown to be shear stress–induced and absolutely lipid-dependent, thus representing a valid model of true atherosclerosis.<sup>24,33</sup>

The DNP challenge led to a striking and acute increase in the incidence of intraplaque hemorrhage within 3 days after challenge. Also, iron deposits were observed in the media of these lesions, which suggests that culprit factors likely entered the intima after adventitial release and crossing of the media. Lesions with intraplaque hemorrhage tended to contain relatively more adventitial mast cells than those lacking hemorrhages, a finding that is in line with the observation by Laine et al.<sup>18</sup> that the adventitia of ruptured lesions in human coronary artery species contained increased levels of mast cells. Importantly, the total number of mast cells detected in the adventitia of mouse carotid artery plaques corresponded with that observed in adventitial tissue of human plaques,<sup>18,19</sup> which indicated that the mouse model offers a realistic representation of the human situation. Intraplaque hemorrhage seemed to colocalize with ceroid-rich regions in close proximity to microvessels. Interestingly, Kolodgie et al.<sup>35</sup> that phagocytosis of accumulated erythrocytes by activated macrophages leads to ceroid production and further plaque expansion, which may promote the formation of rupture-prone lesions. Treatment of mice with cromolyn during DNP challenge normalized the extent of mast cell degranulation in the adventitia and prevented intraplaque hemorrhage. Importantly, cromolyn treatment was demonstrated to be mast cell–specific at the dosage used in our animal models and did not exhibit any side effects on other cell types such as neutrophils and macrophages. These data imply that inhibition of perivascular mast cell degranulation may help to maintain plaque stability.

Mast cells were reported to induce apoptosis of cardiomyocytes,<sup>36</sup> vascular smooth muscle cells,<sup>12,13,27</sup> and endothelial cells<sup>38</sup> in vitro, which could translate to reduced plaque stability.<sup>37,38</sup> Indeed, we observed a highly significant increase of intimal apoptotic nuclei in the DNP-challenged mice. To our surprise, apoptosis was mainly localized in the central atheroma, which implied that the majority of apoptotic cells are of macrophage rather than of vascular smooth muscle cell origin. To date, mast cell degranulation has not been linked to macrophage apoptosis. Macrophage apoptosis may very well result in an enlarged necrotic core of the lesions and in the release of tissue factor–rich apoptotic microbodies,<sup>39</sup> which thereby decrease plaque stability and promote thrombosis. Our in vitro findings concurred with the in vivo data, in that macrophages displayed a high susceptibility to mast cell–induced apoptosis, whereas vascular smooth muscle cells appeared to be less sensitive. Protease inhibitors were able to prevent the mast cell–induced macrophage apoptosis, and an H<sub>2</sub>-receptor antagonist could completely blunt macrophage apoptosis. Both tryptase and chymase were suggested to be proapoptotic by themselves<sup>12,13,27</sup> but also to potentiate the proapoptotic action of histamine,<sup>40</sup> a hypothesis that was confirmed by our in vitro data.

DNP challenge substantially increased intimal influx of erythrocytes. We demonstrate here that mast cell degranulation enhances microvascular leakage (Figure 7C). Circulating leukocytes were seen to extravasate possibly through microvessels rendered permeable by mast cells in response to mast cell–derived chemotactic stimuli, as judged by the increased presence of rhodamine-labeled leukocytes in the DNP-treated plaque. In vitro studies showed endothelial proliferation in response to mitogenic factors, possibly vascular endothelial growth factor secreted by mast cells,<sup>41</sup> which could result in enhanced outgrowth of microvessels and transform the lesion into even more leakage-prone plaques. In neovascularized areas of human coronary atheromas, it was demonstrated that mast cells colocalized with intraplaque microvessels.<sup>42</sup> Furthermore, mast cells that contained basic fibroblast growth factor, a potent angiogenic factor, were located near microvessels in the intima and adventitia of human coronary artery lesions,<sup>43</sup> a finding that suggests that mast cells might indeed play a role in neoangiogenesis, vascular leakage, and plaque progression.

Furthermore, we demonstrated that perivascular mast cell activation increased leukocyte adhesion to the proximal area of atherosclerotic plaques in a CXCR2– and vascular cell adhesion molecule–1–dependent manner. Murine mast cells have previously been shown to release the CXCR2 ligand KC<sup>44</sup> (or its human ortholog interleukin-8)<sup>45,46</sup> suggesting that the arrest-triggering response directly originates from activated mast cells. This is also conceivable, as tissue-derived chemokines, such as interleukin-8, can be abluminally internalized and transcytosed to the luminal side of endothelial cells, where the chemokines can exert their effects.<sup>47</sup> Previous studies in ex vivo perfused arteries have revealed that monocyte arrest on early atherosclerotic endothelium is also mediated by VLA-4 and triggered by KC via CXCR2.<sup>48</sup> In conjunction with our results, this finding indicates interesting mechanistic parallels in the contribution of these molecules to atherogenic recruit-
ment at different stages (eg, between initial arrest and into advanced plaques destabilized by mast cells).

In conclusion, we are the first to provide in vivo proof that mast cells contribute significantly to atherosclerotic plaque progression. Moreover, we show that perivascular mast cells can promote macrophage apoptosis, increase leukocyte influx, and enhance microvascular leakage in preexisting atherosclerotic plaques, effects that result in a sharply increased risk of intraplaque hemorrhage and plaque destabilization. Thus, our present in vivo findings concur with previous in vitro findings and point to a significant role for activated mast cells in plaque stability and acute coronary syndromes. We propose that mast cell stabilization can be an effective new therapeutic approach in the prevention of acute coronary syndromes.

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Disclosures
None.

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Mas cells are major effector cells in allergy and host defense responses. Their increased number and activation status in perivascular tissue during atherosclerotic lesion progression, and particularly in the adventitia of ruptured plaques, points toward a role for mast cells in cardiovascular disorders, as well. In this study, we show that episodes of systemic mast cell activation during plaque progression in apolipoprotein E–deficient mice lead to robust plaque expansion. Targeted activation of perivascular mast cells in advanced atherosclerotic plaques sharply increased the incidence of intraplaque hemorrhage and also resulted in enhanced macrophage apoptosis and vascular leakage in the lesions, both features of reduced plaque stability. Furthermore, perivascular mast cell activation caused increased recruitment of leukocytes to the plaque, which amplified plaque inflammation. Importantly, treatment with the mast cell stabilizer cromolyn did prevent all the adverse phenomena elicited by mast cell activation, without exhibition of any side effects. This is the first experimental study to demonstrate that mast cells play a crucial role in plaque progression and destabilization in vivo, and we propose mast cell stabilization as a promising new therapeutic modality in the prevention of acute coronary syndromes.
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