Mast Cell Infiltration in Acute Coronary Syndromes: Implications for Plaque Rupture

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Objectives. To define the role of mast cells in plaque destabilization.

Background. Inflammation is an essential feature of human coronary plaques. Macrophages and T lymphocytes are considered to contribute to destabilization of the plaques. The role of mast cells in this setting is less well studied. We therefore counted the mast cells in coronary atherectomy specimens from patients with chronic stable angina, unstable angina, and severe unstable angina.

Methods. Patients studied had chronic stable angina (group 1, n = 11), “stabilized” unstable angina (group 2; Braunwald’s class I and II, n = 11) and “refractory” unstable angina (group 3; Braunwald’s class III, n = 7). Samples of culprit lesions (n = 29) were obtained by directional atherectomy, snap-frozen and analyzed immunohistochemically. The numbers of mast cells and T lymphocytes per square millimeter squared were counted and the areas containing macrophages and smooth muscle cells were measured by computed planimetry.

Results. We found that the numbers of mast cells and T lymphocytes increased from group 1 through groups 2 to 3. Specimens from group 3 also contained the largest numbers of tumor necrosis factor alpha (TNF-α)-positive mast cells and of matrix metalloproteinase (MMP)-9 (92 kD gelatinase)-positive macrophages.

Conclusions. Unstable coronary syndromes are associated with increased numbers of mast cells in culprit lesions. Activated mast cells secrete neutral proteases capable of degrading the extracellular matrix and TNF-α, capable of stimulating macrophages to synthesize MMP-9. Our observations suggest that mast cells, in addition to macrophages, contribute to matrix degradation and, hence, to progression of coronary syndromes.

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in vitro (13). Since macrophages in unstable coronary plaques contain MMP-9 (14), we studied the atherectomy specimens for the presence of TNF-α positive mast cells (tryptase-positive cells) and MMP-9-positive macrophages (CD68-positive cells).

Methods

Coronary Atherectomy

Atherectomy specimens were obtained from patients who underwent directional coronary atherectomy (DCA) for a single de novo (primary) stenosis as the only intervention (at the Department of Cardiology, Academic Medical Center, University of Amsterdam, the Netherlands). The selection of patients for DCA was based on angiographic criteria, which included proximally located eccentric lesions and a vessel diameter of 3 mm or more. Culprit lesions were identified on the basis of their clinical and electrocardiographic manifestations. Twenty-nine consecutive patients (comprising 8% of the total angioplasty population, including percutaneous transluminal coronary angioplasty) met the following inclusion criteria for this study: (group 1) chronic stable angina, Canadian Society classification, classes I–III (n = 11); (group 2) “stabilized” unstable angina, Braunwald’s classification classes I and II (new onset, accelerating or angina pectoris at rest, but not within the preceding 48 hours) (n = 11); and (group 3) “refractory” unstable angina, Braunwald’s classification class III (angina pectoris at rest, acute within 48 hours; in our series these patients were dependent on intravenous treatment with nitrates and heparin) (n = 7). One patient in group 2 and three patients in group 3 had postinfarction angina. No patients with these patients were dependent on intravenous treatment with nitrates and heparin) (n = 7). One patient in group 2 and three patients in group 3 had postinfarction angina. No patients with

Immunochemistry

After the DCA procedure, atherectomy tissues were carefully oriented in a drop of Tissue Tek embedding fluid in such a way that the largest tissue area was available for cutting sections, and immediately frozen in liquid nitrogen and stored at −80°C. For immunochemistry, frozen sections were cut serially at 5 μm, fixed in acetone and air-dried. Details of the monoclonal antibodies used in this study are summarized in Table 1. Mast cells were stained by a single indirect immunoperoxidase method, as previously described (11,15). For detection of T lymphocytes and anti-MMP-9 reactivity, a routine three-step indirect immunoperoxidase assay was used. In both methods, horseradish peroxidase activity was visualized with 3-amino-9-ethyl carbazole, and the sections were counterstained with hematoxylin for histomorphologic evaluation of the atherosclerotic plaque. After treatment with hematoxylin-eosin and elastic van Gieson stains, all specimens were screened for the presence of thrombus, atheroma (extracellular lipid core), fibrous tissue (of fibrocellular of fibrosclerotic type) and media. Anti-TNF-α reactivity in mast cells was established with an anti-TNF-α/antitryptase immunodouble staining method based on antibodies from different species (TNF-α: rabbit; tryptase: mouse). Smooth muscle cells and macrophages were detected simultaneously in the same sections, using an immunodouble staining method based on the application of two antibodies of different IgG subclasses. Briefly, a cocktail of antismooth muscle α-actin, 1A4 (IgG2a), and anti-CD68, EBM-11 (IgG1), was applied, followed by a second cocktail of secondary antibodies consisting of goat antimouse IgG2a/biotin and goat antimouse IgG1/alkaline phosphatase (both Southern Biotechnology Associations, Birmingham, AL) and a final step with strep/β-galactosidase (Boehringer, Mannheim, Germany). β-Galactosidase activity was developed as turquoise (in smooth muscle cells), using X-gal (Boehringer) as substrate and ferri-ferro cyanide as chromogen and then, by washing with alkaline phosphatase, fuchsin was developed as red (in macrophages), using a NEW fuchsin detection kit (Dako, Glostrup, Denmark).

Morphometry

The total area of each tryptase- and CD3-stained section was assessed planomorphometrically. The numbers of immunopositive mast cells and T lymphocytes were counted at × 100 magnification throughout the entire section and expressed as numbers of cells per square millimeter. Because of the large numbers and close apposition of the macrophages and of the smooth muscle cells in the immunostained sections, it was not possible to count these individual cells separately. Therefore, in the atherectomy sections that were immunodouble-stained with antiCD68 and with anti-α-actin, the tissue areas occupied by immunostained cytoplasm of either macrophages or smooth muscle cells were quantified planimetrically. The total area of each double-stained section was outlined on a video screen connected to a personal computer provided with image analysis software. The surface area occupied by immunostained

Table 1. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cell Specificity</th>
<th>Working Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antitryptase</td>
<td>Mast cells</td>
<td>1.5 μg/ml</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Leu 4 (CD3)</td>
<td>T lymphocytes</td>
<td>0.3 μg/ml</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>EBM-11*</td>
<td>Macrophages</td>
<td>4 μg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>1A4*</td>
<td>α-Smooth muscle actin</td>
<td>2 μg/ml</td>
<td>Dako</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>TNF-α</td>
<td>0.1 μg/ml</td>
<td>Otsuka</td>
</tr>
<tr>
<td>Anti-MMP-9</td>
<td>MMP-9</td>
<td>2 μg/ml</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

* = double staining. Dako (Glostrup, Denmark); Becton Dickinson (San Jose, CA); Chemicon (Temecula, CA); Otsuka Pharmaceutical Co., Ltd (Tokushima, Japan).
Plaque Histology

Macrophages, particularly those of foam cell type, were also observed being 96 mast cells/mm². Likewise, the number of T lymphocytes and mast cells were completely absent. In a few instances, however, we found tightly packed macrophage-rich areas and smooth muscle cell-rich areas. Furthermore, mast cells and T lymphocytes were often found in clusters, with a predilection for the border zone between macrophage-rich areas and smooth muscle cell-rich areas. In a few instances, however, we found tightly packed macrophages infiltrating large areas from which T lymphocytes and mast cells were completely absent.

Analyses of the cellular composition of each of the histologically defined components of the plaque led to the following observations. Most inflammatory cells, including macrophages, T lymphocytes and mast cells, were present in the fibrocellular tissue component of the specimens. In contrast, sections containing fibrosclerotic intimal tissue and media were virtually always devoid of inflammatory cells. Macrophages, particularly those of foam cell type, were also present in atheromas. In these areas, mast cells and T lymphocytes were seldom seen. Fragments of thrombus contained variable quantities of macrophages and some T lymphocytes and mast cells.

Quantitative Immunocytochemistry

For each patient in each group, the numbers of mast cells and T lymphocytes per square millimeter (upper panel) and the percentages of immunostained macrophages and smooth muscle cell areas (lower panel) are given in Figure 2. The number of mast cells per square millimeter clearly tended to increase from group 1 through groups 2 and 3. The mast cell densities ranged from 0 to 10 cells/mm². The values represent means obtained by counting all the mast cells in the total area from samples ranging in size from 2 to 16.5 mm². As stated above, the distribution of mast cells in these samples was highly uneven. In the clusters, the densities of mast cells typically varied between 30 and 50 cells/mm²; the highest density observed being 96 mast cells/mm². Likewise, the number of T

Table 2. Plaque Histology in Atherectomy Specimens

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Total Area (mm²)</th>
<th>Thrombus</th>
<th>Atheroma</th>
<th>Fibrous Tissue</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>6.10 ± 2.67</td>
<td>3/11 (27%)</td>
<td>4/11 (36%)</td>
<td>11/11 (100%)</td>
<td>4/11 (38%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>6.67 ± 4.01</td>
<td>8/11 (72%)</td>
<td>8/11 (72%)</td>
<td>11/11 (100%)</td>
<td>4/11 (38%)</td>
</tr>
<tr>
<td>Group 3</td>
<td>5.73 ± 2.58</td>
<td>6/7 (86%)</td>
<td>6/7 (86%)</td>
<td>7/7 (100%)</td>
<td>2/7 (29%)</td>
</tr>
</tbody>
</table>

Group 1: chronic stable angina; group 2: unstable angina, Braunwald’s classes I and II; group 3: unstable angina pectoris in patients requiring intravenous treatment with nitrates and heparin, Braunwald’s class III. Hematoxylin-eosin and elastic van Gieson staining were used.

Results

Statistical Analysis

The grouping based on the severity of symptoms was used as an explanatory variable. Poisson regression analysis was used to model number of cells (mast cells, TNF-α-positive mast cells, T lymphocytes and those macrophages that were MMP-9-positive) per tissue area (per square millimeter) as response variable. The usage of this model was based on the assumption that the numbers of cells per tissue area were distributed according to a Poisson distribution. The areas of samples were used as weight in Poisson regression model. Logistic regression analysis was applied when the proportion of macrophage or smooth muscle cell areas of total area were as-used responses. Because the proportion of area is always between zero and one, the choice of logistic regression analysis is plausible (16). Differences were considered statistically significant when p < 0.05.

Plaque Histology

Table 2 shows the histologic findings for each of the three groups. A thrombus was present in 27%, 72% and 86% of the atherectomy samples, respectively. Interestingly, these frequencies coincided with the presence of an extracellular lipid core (atheroma). All atherectomy samples contained fibrous tissue and, on average, one third of the samples in each group contained variable quantities of macrophages and some T lymphocytes. Inflammatory cells were identified in most areas containing large quantities of macrophages, T lymphocytes and mast cells, were present in the fibrocellular tissue component of the specimens. In contrast, sections containing fibrosclerotic intimal tissue and media were virtually always devoid of inflammatory cells. Macrophages, particularly those of foam cell type, were also present in atheromas. In these areas, mast cells and T lymphocytes were seldom seen. Fragments of thrombus contained variable quantities of macrophages and some T lymphocytes and mast cells.

Qualitative Immunocytochemistry

Figure 1 illustrates an atherectomy section stained immunochemically for macrophages, smooth muscle cells, mast cells and T lymphocytes. Inflammatory cells were identified in all three groups (group 1: chronic stable angina; group 2: unstable angina, Braunwald’s class I and II; and group 3: unstable angina pectoris in patients requiring intravenous treatment with nitrates and heparin, Braunwald’s class III). Analysis of the cellular composition and distribution in the atherectomy samples, using immunodouble staining, revealed striking zonal distributions of macrophages and smooth muscle cells: most areas containing large quantities of macrophages were almost devoid of smooth muscle cells, whereas areas rich in smooth muscle cells contained few macrophages, T lymphocytes or mast cells. Comparison of serial sections of each atherectomy specimen revealed that macrophages, T lymphocytes and mast cells were almost always colococalized in the same tissue areas. Furthermore, mast cells and T lymphocytes were often found in clusters, with a predilection for the border zone between macrophage-rich areas and smooth muscle cell-rich areas. In a few instances, however, we found tightly packed macrophages infiltrating large areas from which T lymphocytes and mast cells were completely absent.

Analyses of the cellular composition of each of the histologically defined components of the plaque led to the following observations. Most inflammatory cells, including macrophages, T lymphocytes and mast cells, were present in the fibrocellular tissue component of the specimens. In contrast, sections containing fibrosclerotic intimal tissue and media were virtually always devoid of inflammatory cells. Macrophages, particularly those of foam cell type, were also present in atheromas. In these areas, mast cells and T lymphocytes were seldom seen. Fragments of thrombus contained variable quantities of macrophages and some T lymphocytes and mast cells.
lymphocytes per millimeter squared gradually increased from group 1 through group 2 and group 3. The areas occupied by macrophages and smooth muscle cells in groups 1, 2 and 3 did not differ significantly. Finally, we examined the relationships between the number of mast cells and the numbers or areas of other types of intimal cells in the atherectomy samples. As expected, there was a positive correlation between the numbers of T lymphocytes and mast cells, and a negative correlation between the areas occupied by smooth muscle cells and the numbers of mast cells (not shown). However, there were no significant correlations between the areas occupied by macrophages and the numbers of mast cells.

Anti-TNF-α/antitryptase doublestaining showed that, in all three groups, most of the mast cells contained TNF-α and a fraction of the macrophages contained MMP-9. The quantities of both TNF-α-positive mast cells and the MMP-9-positive macrophages were highest in group 3 (Fig. 3). Thus, the number of TNF-α-positive mast cells per square millimeter was 0.49 ± 0.55 (mean ± SD) in group 1, 1.10 ± 1.56 in groups 2, and 2.56 ± 2.79 in group 3. The corresponding numbers for MMP-9-positive macrophages were 1.84 ± 2.08, 2.18 ± 1.61 and 9.64 ± 4.07.

Discussion

In previous studies we have shown that mast cells occur in the shoulder region of stable atherosclerotic plaques as well as at sites of plaque rupture (6,11). The present study demonstrates that the number of mast cells tends to increase with the clinical severity of the coronary syndromes. The lesions obtained from patients with unstable angina of Braunwald’s class III contained significantly more mast cells than those obtained from patients with chronic stable angina. These findings thus strongly suggest that mast cells contribute to the process of plaque destabilization. Then, the important question arises whether mast cells (and T lymphocytes and macrophages) enter the lesion before or after the plaque rupture. Mast cells infiltrate not only the sites of coronary arteries at which

Figure 1. a, Frozen section of part of an atherectomy specimen obtained from a patient with unstable angina (group 2). The section is immunodouble-stained for macrophages (anti-CD68) in red and for smooth muscle cells (anti-alpha actin) in blue. The boxed area is enlarged in b through d. Original magnification ×30. b, Inflammatory area containing closely packed macrophages (red) and sparse smooth muscle cells (blue). c, Adjacent section stained for mast cells (antitryptase). d, Adjacent serial section stained for T lymphocytes (anti-CD3). Original magnification b through d, ×90.
erosion or rupture has occurred, but also sites of coronary plaques susceptible to erosion or rupture (11), that is, they invade before an actual intimal event. The same applies also for macrophages and T lymphocytes (11). Hence, there is much evidence to suggest that the infiltrates of inflammatory cells are present in the lesions prior to erosion or rupture, rather than part of an inflammatory response to rupture initiated by some other process.

Mast cell degranulation. To initiate matrix degradation, mast cells have to be stimulated to secrete their neutral proteases. The best understood immunologic stimulus leading to degranulation of human mast cells is their activation when the IgE molecules on their surfaces bind a relevant antigen (17). The development of cardiovascular disease is associated with elevated levels of IgE, suggesting that IgE-mediated events play a role in the pathogenesis of these diseases (18,19).

A second pathway leading to degranulation of mast cells in the arterial intima—which appears more likely in the setting of atherosclerosis—is their stimulation by other immunologically activated cells, such as T lymphocytes (20) or macrophages (21). Indeed, mast cells were found to be accompanied by T lymphocytes and by macrophages at the sites of coronary arteries where erosion or rupture had occurred (6). Probably even more important, the degree of mast cell stimulation (degranulation) was shown to be highest at those sites where the numbers of other inflammatory cells were highest (6). In the present study we did not determine the degree of mast cell degranulation, as degranulation may have been caused artificially by mechanical trauma associated with the atherectomy procedure. However, the present study did show that, in unstable angina, mast cells were accompanied by T lymphocytes and macrophages, and that the more severe the symptoms of angina pectoris, the greater were the number of the T lymphocytes surrounding the mast cells. Recently, in a similar retrospective study of DCA specimens of patients with various ischemic coronary syndromes, we showed that the increase in the numbers of macrophages and T lymphocytes with the severity of the angina is accompanied by an increase in human leukocyte antigen-DR expression on the cells (5). This indicates that active inflammation plays a role in the progression of angina pectoris. A major conclusion derived from the present study is that mast cells play an integral part in this active inflammatory process. Moreover, the study implies that local conditions include the active inflammation (secretion of cytokines) necessary for mast cell stimulation.

Extracellular matrix degradation. Together with previous data, the present observations support the concept that mast cells play a role in increasing the activity of the enzymes required for extracellular matrix digestion (the MMPs). Recent immunocytochemical and in situ hybridization studies of human atherosclerotic plaques have revealed that the macrophages and smooth muscle cells of the plaques synthesize

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**Figure 2.** Graphs showing the numbers per square millimeter of mast cells and T lymphocytes (upper panel) and the percentages of immunostained macrophage and smooth muscle cell areas (lower panel) in the atherectomy specimens of each group (group 1: chronic stable angina; group 2: unstable angina, Braunwald’s classes I and II; and group 3: unstable angina pectoris in patients requiring intravenous treatment with nitrates and heparin, Braunwald’s class III). Individual data points, means and p values are shown.

**Figure 3.** Graph showing the numbers per square millimeter of TNF-α-positive mast cells and of MMP-9-positive macrophages in atherectomy specimens for each patient group (see Fig. 2). Individual data points, means and p values are shown.
MMP-1, MMP-3 and MMP-9 (92-kDa gelatinase) (14,22,23). Synthesis of the MMPs is regulated by cytokines secreted by the various cell types present in the plaques (24). We recently showed that TNF-α, a strong proinflammatory cytokine present in the mast cells of coronary plaques (12), stimulates the synthesis of MMP-9 by macrophages in vitro (13). The present findings that the number of TNF-α-containing mast cells and MMP-9-containing macrophages both increase as the lesions become more severe strongly suggest that one mechanism by which mast cells render the plaque unstable is by stimulating local synthesis of MMP-9. As we have found previously in stable coronary plaques (12), we now found in unstable plaques that the macrophages also contain TNF-α (not shown), suggesting the presence of autocrine and paracrine loops in macrophages for the synthesis of MMP-9. However, the MMPs are synthesized and secreted as inactive proforms (proMMPs) that can then be converted into active forms by cleavage at special sites. In vitro studies have shown that the tryptase and chymase derived from mast cells can effectively activate proMMPs: tryptase can activate prostromelysin (proMMP-3) (25), and chymase can activate the interstitial procollagenase (proMMP-1) (26). Because MMP-3 can activate several other types of proMMPs, the mast cell tryptase secreted by stimulated mast cells could well be among the agents that trigger the extracellular activation of several proMMPs in human atherosclerotic plaques. MMP activity has been measured in coronary atherectomy samples by using zymography (27). Interestingly, the greatest activity resided in the 92-kD band, revealing the presence of active MMP-9 in the samples.

Unique proinflammatory properties of mast cells. The number of macrophages did exceed by far the number of mast cells in the plaques. However, the mast cells are a completely different type of inflammatory cells and they possess unique proinflammatory properties (28). Regarding matrix degradation in the plaque, the best example is probably the activation of the MMPs by mast cell-derived tryptase or chymase which is plasmin-independent. In other situations the presence of plasmin or expression of the membrane-type MMPs appears to be obligatory for MMP activation (29). The unique molecular aspect of mast cell-dependent MMP activation is that the two MMP-activating mast cell neutral proteases, tryptase and chymase, are fully active upon secretion from their parent mast cells. Moreover, they both have activity against the various components of the extracellular matrix and can initiate matrix degradation directly. Thus, both tryptase and chymase have been shown to degrade pericellular matrices (fibronectin) (30,31).

Coronary atherectomy specimens provide a unique source of plaque tissues because they make it possible to correlate features of plaque biology with the clinical status of the patient. However, only part of the entire plaque is excised and, thus, in studies based on examination of these specimens, sampling bias has to be considered. Despite these limitations, we propose that mast cells may initiate matrix degradation by multiple mechanisms and therefore, actively participate in local weakening of unstable coronary lesions. Thus, a new possibility emerges for prevention of the progression of coronary plaques to unstable lesions: inhibition of mast cell degranulation.

References
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