Accumulation of Activated Mast Cells in the Shoulder Region of Human Coronary Atheroma, the Predilection Site of Atheromatous Rupture

Maija Kaartinen, MD; Antti Penttilä, MD, PhD; Petri T. Kovanen, MD, PhD

Background Rupture in the shoulder region of a coronary atheroma is considered to be a sequel to local extracellular matrix degradation in this highly vulnerable site. Such degradation could be triggered by mast cells, which are filled with neutral proteases and are present in coronary atheromas. However, the distribution and phenotype of mast cells within coronary atheromas have not been studied.

Methods and Results Specimens of normal and atherosclerotic human coronary intima from 32 autopsy cases with ages ranging from 13 to 67 years were stained with monoclonal antibodies against the two major proteases of mast cells, tryptase and chymase. Of the tryptase-containing mast cells, a variable proportion (average, 40%; range, 0% to 100%) also contained chymase. In the normal coronary intimas, mast cells amounted to 0.1% of all nucleated cells. In the fatty streaks, this proportion was higher by 9-fold, and in the cap, core, and shoulder regions of atheromas by 5-, 5-, and 10-fold, respectively. Electron and light microscopic studies of mast cells in the shoulder region of atheromas revealed degranulation of mast cells, a sign of their activation, and moreover, that the proportion of activated mast cells was much higher (85%) in this region than in the normal intima (18%).

Conclusions The far higher proportion (50-fold) of activated mast cells in the shoulder region of atheromas supports the hypothesis that mast cells, a cell type capable of triggering matrix degradation, actively participate in the destabilization and ensuing rupture of coronary atheromas and thus may trigger an acute coronary event. (Circulation. 1994;90:1669-1678.)

Key Words • mast cells • atherosclerosis • chymase • tryptase • coronary disease

Macrophages, T lymphocytes, endothelial cells, smooth muscle cells, and thrombocytes are the cell types considered to be important in the atherosclerotic process. The arterial wall also contains mast cells, and during the atherosclerotic process their number has been noted to vary. In the three reports available on human aortic intimal mast cells, a far smaller number of mast cells was observed both in fatty streaks and in atheromas compared with normal intimal areas, whereas in the atherosclerotic human coronary intima, the number of mast cells was found to be greater.

The role of mast cells in the development of atherosclerosis is unknown. However, recent studies in vitro and in vivo in animal models have demonstrated that the granules contained in these cells may participate in the development of macrophage foam cells. In mast cell–induced foam cell formation, major roles could be assigned to heparin proteoglycans and neutral protease chymase present in the granules. Thus, in the series of metabolic events culminating in foam cell formation, low-density lipoproteins (LDL) are first bound to the heparin proteoglycan matrix of the exocytosed mast cell granules, then proteolyzed by granule chymase, and ultimately the proteolytically modified LDL particles are carried into macrophages by phagocytosis of the LDL-loaded granules.

Human mast cells, like their counterparts in other mammals, contain proteoglycans and proteolytic enzymes, the neutral proteases being chymase and tryptase. Human mast cells contain about 10 times the quantity of neutral proteases as do polymorphonuclear leukocytes, which have always been considered a very rich source of these enzymes; accordingly, mast cells are considered to be the major source of neutral proteases in the loose connective tissue of many organs. According to their neutral protease content, human mast cells have been divided into two phenotypes: those that contain tryptase and those that contain tryptase and chymase. It has not hitherto been shown which phenotype(s) is present in the human coronary intima; neither has it been known how mast cells are distributed within an atheroma. Answers to these two unresolved questions were sought in the present work by staining human coronary samples with monoclonal antibodies against tryptase and chymase.

Methods

Reagents

A biotinylated anti-chymase monoclonal antibody B7 and an alkaline phosphatase-conjugated anti-tryptase monoclonal antibody G3 were purchased from Chemicon. Unconjugated anti-tryptase monoclonal antibody G3 was a kind gift from Dr. L.B. Schwartz, Medical College of Virginia, Richmond. Peroxidase-conjugated streptavidin was from Vector Laboratories; anti-macrophage monoclonal antibody HAM 56, anti-T-cell monoclonal antibody UCHL 1, and peroxidase-conjugated...
goat anti-mouse immunoglobulin were from Dakopatts; and 3-amino-9-ethylcarbazole and 3,3’-diaminobenzidine were from Sigma.

Autopsy Material and Tissue Preparation

The autopsy series for light microscopy comprised 32 cases (20 male and 12 female) aged 13 to 67 years. The causes of death were cardiovascular diseases (n=10); violent deaths, ie, accidents, homicides, and suicides (n=18); and poisonings (n=4). The unopened left coronary artery was removed from the heart, and tubelike specimens, each about 3 mm long, were cut to include the bifurcation and the adjacent portions of the left main and left anterior descending branches. These specimens were fixed in Carnoy’s fluid (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 24 hours and embedded in paraffin. The mean interval between death and the start of fixation was 13 hours (range, 2 to 24 hours). Similar specimens for electron microscopy were taken from the bifurcation of the left coronary artery, fixed in a solution containing 2% glutaraldehyde and 1.5% paraformaldehyde, and postfixed in osmium tetroxide. The samples were then dehydrated and embedded in epoxy. We used light microscopy to evaluate the atherosclerotic involvement of the coronary arteries. For this purpose, the sections were stained with hematoxylin and eosin, as well as with elastica-van Gieson stain (Weigert's hematoxylin, metanil yellow, acid fuchsin, and picric acid) for collagen and elastin.

Immunocytochemistry

For immunocytochemistry, fixed serial sections (2 to 4 μm) were dewaxed in xylene and rehydrated in graded series of ethanol, and endogenous peroxide activity was inhibited by incubation in 0.6% hydrogen peroxide in methanol. The sections were then stained either by a single indirect immunoperoxidase method with anti-tryptase or by a sequential double-labeling method with conjugated anti-chymase and conjugated anti-tryptase, as described by Irani et al. In the single-staining method, the slides were incubated overnight at 4°C with anti-tryptase G3 (1.5 μg/mL), then peroxidase-conjugated goat anti-mouse IgG was added, and finally peroxidase was visualized with 3-amino-9-ethylcarbazole and hydrogen peroxide. In the sequential double-labeling method, the tissue sections were first incubated with the biotinylated anti-chymase B7 (4 μg/mL) overnight at 4°C, and the chymase-containing cells were stained indirectly with immunoperoxidase, by use of peroxidase-conjugated streptavidin and 3-amino-9-ethylcarbazole. The tissue sections were then incubated overnight at 4°C with the alkaline phosphatase-conjugated anti-tryptase antibody G3 (0.7 μg/mL), followed by fast blue RR and naphthol AS-MX phosphate to stain the tryptase-containing cells.

The macrophages and T lymphocytes in the arterial intima were detected by the avidin-biotin-complex method with an anti-macrophage monoclonal antibody, HAM 56, and an anti-T-cell monoclonal antibody, UCHL 1. The slides were incubated overnight with HAM 56 (1:50) or UCHL 1 (1:50) at 4°C and then incubated with a biotinylated second-step antibody and the avidin-biotin-peroxidase complex. The peroxidase was visualized with diaminobenzidine and hydrogen peroxide. The tissues were then counterstained with Mayer’s hematoxylin and mounted.

Morphometric Analysis and Microscopy

For light microscopy, the intimal areas (normal intimas; fatty streaks; and cap, core, and shoulder regions of atheromas) in each section of a coronary artery were measured by planimetry using CUE 2 Planimorphometry Soft Wear program at the Department of Meat Technology, University of Helsinki, and immunopositive macrophages, and hematoxylin-stained cells were counted in the measured areas at ×100 magnification. Magnification ×400 was used for identifying the subtypes of mast cells, and magnification ×1000 was used to observe degranulation of mast cells. For electron microscopy, ultramicrotome sections of the epoxy-embedded coronary samples were stained with uranyl acetate and lead citrate and viewed with a Jeol JEM-1200EX transmission electron microscope at the Department of Electron Microscopy, University of Helsinki.

Statistical Analysis

Within-case comparison was made of numbers of mast cells, T lymphocytes, and macrophages in normal sites versus atherosclerotic sites (fatty streak, cap, core, and shoulder of atheroma). The response was the number of cells in the sample, and the explanatory factors were case and the site where the sample was taken (normal and atherosclerotic). The analysis depended on the form of the response variable: Poisson regression analysis was used to model number of cells (mast cells, T lymphocytes, and macrophages) per tissue area, and logistic regression analysis was used to model the proportions of mast cells, T lymphocytes, and macrophages in relation to total cell count. Ratios of mast cells to T lymphocytes and macrophages were analyzed by paired t test. The proportions of chymase-containing mast cells in relation to all mast cells could not be modeled, and the data are simply presented as mean±SD. Differences were considered statistically significant when P<.05.

Results

To evaluate the atherosclerotic involvement of the coronary arteries, the specimens were stained with hematoxylin and eosin, and with elastica-van Gieson stain for collagen and elastin. The normal intima demonstrated variable thickness and moderate overall cellularity (Fig 1A). Fatty streaks were characterized by subendothelial and/or deep intimal collections of foam cells (containing numerous vacuoles) (Fig 1B). Coronary atheromas appeared as intimal areas in which, in addition to foam cells, there were accumulations of extracellular lipid (large nonstaining areas interspersed with typical cholesterol clefts). These areas contained very few cells or were acellular, a sign of cell loss (necrotic lipid core) (Fig 1C). With elastica-van Gieson staining, fibrous capsules were clearly demarcated from the necrotic lipid core. By these criteria, in all 32 coronary samples we found areas in which the intima appeared normal; in 25 samples there were also fatty streaks and in 19 samples also atheromas. In the atheromas, the various cell types to be studied were counted separately in the cap, core, and shoulder regions. A photomicrograph of a coronary atheroma and a schematic representation showing the locations of the various regions of the atheroma are shown in Fig 2.

To demonstrate the presence in the coronary intimas of mast cells containing either tryptase alone or tryptase and chymase, the coronary sections were stained by the sequential double-labeling method. Fig 3 shows a typical example of the mast cells in a fatty streak, the G3-positive tryptase-containing mast cells staining blue and the B7-positive mast cells that contained both tryptase and chymase staining brown.
Fig 1. Light microscopic views of different segments of a ring from the bifurcation area of the left coronary artery displaying normal structure (A), a fatty streak lesion (B), and an atheroma (C). In the atheroma, the cap and necrotic lipid core regions can be distinguished. The specimen was from a 47-year-old woman who died of myocardial infarction. I indicates intima; M, media; and A, adventitia. Arrows point to foam cells, and arrowheads to cholesterol crystals. The sections were stained with elastica-van Gieson. Original magnifications: A, ×200; B, ×400; C, ×200.

Fig 2. Light microscopic view of a cross section of the origin of the left anterior descending coronary artery (A) and schematic representation (shadow picture) (B) showing the locations of the regions in which the various cell types were counted separately. The section was stained with elastica-van Gieson. Original magnification ×20.
To determine the proportion of chymase-containing mast cells in the coronary intimas, the percentage of mast cells that contained chymase was calculated (Fig 4). In normal intimas, in fatty streaks, and in shoulder regions of atheromas, the proportion of mast cells containing chymase averaged 35% to 40%, whereas in the cap and core regions of atheromas, the percentages were slightly higher: 53% in the cap and 45% in the core region. The percentage of chymase-containing mast cells in each of the regions studied was extremely variable, ranging from 0% to 100%.

To establish the density of mast cells in the intima of coronary arteries, tryptase was detected in intimal cells with the monoclonal antibody against tryptase. The intimal areas were then measured by planomorphometry, and the tryptase-positive cells and all nucleated cells in the measured areas were counted. Three portions of left coronary arteries were examined: the left main, the bifurcation, and the proximal part of the left descending coronary artery. The densities of mast cells in the various portions of the coronary intimas were similar within a case, but the mast cell density varied from case to case. As shown in Fig 5, in the normal intima only a few mast cells could be found, the average density being 1 mast cell/mm². In atherosclerotic lesions, the number of mast cells was higher: 5 mast cells/mm² in fatty streaks, 2 mast cells/mm² in both the cap and core regions of atheromas, and 6 mast cells/mm² in the shoulder region of atheromas. In the 32 cases studied, mast cells were present in 50% of the normal intimas, in 84% of the fatty streaks, in 50% of the core, in 65% of the cap, and in 95% of the shoulder regions in the atheromas. Fig 6 shows the edge of an atheromatous shoulder with seven subendothelial mast cells.

We also determined the numbers of mast cells relative to the total numbers of intimal cells (Table 1). The mast cells amounted to about 0.1% of the total number of cells in the normal intima. In the fatty streaks and in the
The shoulder region of the atheromas, the proportion of mast cells was about 10 times that in the normal intima, on average 1%. In the cap and core regions of atheromas, fewer mast cells (0.5%) were present. For comparison, the numbers of T lymphocytes and macrophages in relation to the total cell number were also determined in the same areas as mast cells (Table 1). We found that the proportions of T lymphocytes and macrophages were higher in fatty streaks and higher still in atheromas. However, most of these cells were found in the core regions of the atheromas, in contrast to the mast cells, which were most prevalent in the shoulder region. Table 2 shows the ratios of mast cells to T lymphocytes and macrophages in the various intimal samples. In the shoulder region of atheromas, the favored site for mast cells, the ratios of these cells to T lymphocytes and macrophages were, on average, 1:4 (26%) and 1:8 (12%), respectively.

To investigate whether intimal mast cells were activated, signs of degranulation were sought in the various samples, using both electron and light microscopy. We found degranulated mast cells in the normal intima, the fatty streaks, and the various regions of atheromas. An electron micrograph of one typical mast cell with a few extracellular granules is shown in Fig 7. Most of the cytoplasmic granules are intact and electron-dense (dark), but several are electron-transparent (light), showing that they have been involved in the degranulation process and have lost some of their contents. Moreover, three granules (arrows) are located extracellularly, demonstrating recent activation and degranulation of this mast cell. To study the proportions of mast cells that are activated in the various regions of atheromas, we turned to light microscopy and observed at high magnification (×1000) every mast cell (484 in total) present in the specimens studied. At this magnification, extracellular...
TABLE 1. Percentages of Mast Cells, T Lymphocytes, and Macrophages of the Total Numbers of Intimal Cells

<table>
<thead>
<tr>
<th>Region</th>
<th>Mast Cells, %</th>
<th>T Lymphocytes, %</th>
<th>Macrophages, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal intima (n=32)</td>
<td>0.1 (0-0.8)</td>
<td>0.6 (0.2-2.3)</td>
<td>3.0 (0.5-7.4)</td>
</tr>
<tr>
<td>Fatty streak (n=25)</td>
<td>0.9 (0-4.8)</td>
<td>8.5 (0.4-35.4)</td>
<td>12.9 (1.6-39.2)</td>
</tr>
<tr>
<td>Atheroma (n=19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cap</td>
<td>0.5 (0-1.7)</td>
<td>8.7 (0.2-23.9)</td>
<td>15.7 (1.5-61.3)</td>
</tr>
<tr>
<td>Core</td>
<td>0.5 (0-3.0)</td>
<td>16.1 (1.6-47.7)</td>
<td>29.7 (4.2-86.7)</td>
</tr>
<tr>
<td>Shoulder</td>
<td>1.1 (0-4.0)</td>
<td>11.7 (0.8-41.5)</td>
<td>21.5 (1.0-55.0)</td>
</tr>
</tbody>
</table>

Values are means and ranges. Statistical differences between normal intima and the various types of atherosclerotic intima were highly significant (P<.0001) for each cell type.

Discussion

The results of the present study reveal a new cell type, the mast cell, among the immunologically active cells in the shoulder region of coronary atheromas. What factors might be responsible for the presence of mast cells in this region and in the coronary intima in general? Recent studies in mice, rats, and primates have demonstrated that tissue mast cells originate from precursors that arise in the bone marrow and migrate to specific tissue sites, where they differentiate locally into mast cells. In these animal models, a key factor mediating the migration of mast-cell precursors to specific tissue sites and their local differentiation into mature mast cells appears to be the stem cell factor (SCF), which has also been called mast cell growth factor. Indeed, in every experimental system studied so far, the development of mast cells of connective tissue type (to which the intimal mast cells belong, according to their location) appears to depend on the presence of SCF; thus, in all likelihood, this growth factor is responsible for the development of mast cells in the human coronary intima also. This would imply that the observed wide interindividual variation in the number of intimal mast cells is a reflection of variations in the local production of SCF.

Interindividual variation was also observed in the phenotypic pattern of coronary intimal mast cells in terms of their neutral protease content. Thus, whereas all mast cells contained tryptase, the presence of chymase was highly variable: in some individuals, all of the mast cells contained chymase; in others, all the mast cells lacked it; and in the rest, a variable proportion of the mast cells contained chymase. What factor(s) could account for this great interindividual variation in chymase expression? In vitro studies on human mast cell development have shown that in culture systems containing mast cell precursors, addition of SCF induces strong expression of tryptase and
Only weak expression of chymase. Only if the system includes a stroma cell layer will the developing mast cells express chymase in significant amounts. Thus, it appears that, in addition to SCF, some cofactors produced by stroma cells are necessary for the expression of chymase. Our finding of the great interindividual variation in chymase expression suggests great local differences in the production of such cofactors, reflecting the presence of functionally different microenvironments in the intimal parenchyma. Since the parenchymal environment of the intima is created by smooth muscle cells, which also are phenotypically heterogeneous, being either contractile or synthetic, we are left with a challenging question of whether the
phenotypic modulation of smooth muscle cells is accompanied by expression of factors that may modify the phenotype of the mast cells.

In the late lesions, ie, the atheromas, the density of mast cells was highest in the shoulder region. What may be the role of mast cells in these specific regions? Recent clinical and pathological studies of coronary arteries of patients suffering myocardial infarction have demonstrated that atheromas typically rupture in the shoulder region, an intimal area that is characterized by high circumferential stress. Indeed, the site of rupture/erosion beneath coronary thrombi is heavily populated with activated macrophages, which may synthesize and secrete several proteases capable of degrading various components of the extracellular matrix. Experiments in vitro have demonstrated that human mast cell tryptase is able to activate prostromelysin, a metalloproteinase synthesized by the macrophages of human atherosclerotic plaques, which when activated effectively degrades the extracellular matrix. Moreover, activated stromelysin can activate procollagenase, the precursor of another matrix-degrading metalloproteinase secreted by macrophages. Recently, we found that chymase is able to directly cleave and activate procollagenase. Thus, both tryptase and chymase of mast cells in the shoulder region of coronary atheromas may initiate a sequence of events leading to degradation of the extracellular matrix and so contribute to destabilization of the shoulder region.

Both tryptase and chymase are already in active form while in the cytoplasmic secretory granules of mast cells. However, before these neutral proteases can activate the extracellular matrix-degrading proenzymes, they must first be released from mast cells. What may be the physiological triggers of mast cell degranulation in the intimal environment? The best-understood immunologic stimulus of human mast cells is their activation upon binding of a relevant antigen (allergen) to the IgE molecules present on the mast cells. Recent data from a retrospective study of a defined human population initially enrolled under a Lipid Research Clinic protocol indicated that IgE may be an independent marker of cardiovascular disease in men, suggesting that IgE-mediated events may play a role in the pathogenesis of cardiovascular disease, including myocardial infarction. However, to link this hypothesis with a mast cell–mediated mechanism, the presence of IgE and allergens on intimal mast cells must still be demonstrated, and functional models must be found for testing the hypothesis. A second, and perhaps even more likely, pathway of mast cell involvement is their stimulation by other immunologically activated cells, such as T lymphocytes and macrophages. The present study shows that the shoulder regions of coronary atheromas, with their increased densities of mast cells, also contain high densities of T lymphocytes and macrophages, and

### Table 3. Number of Activated Mast Cells in Relation to the Total Number of Mast Cells in Normal and Atherosclerotic Coronary Intima

<table>
<thead>
<tr>
<th>Mast Cells</th>
<th>Normal Intima</th>
<th>Fatty Streak</th>
<th>Cap</th>
<th>Core</th>
<th>Shoulder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated/total*</td>
<td>12/66</td>
<td>73/140</td>
<td>24/68</td>
<td>86/93</td>
<td>100/117</td>
</tr>
<tr>
<td>Percent activated</td>
<td>18%</td>
<td>52%</td>
<td>35%</td>
<td>92%</td>
<td>85%</td>
</tr>
</tbody>
</table>

* Mast cell activation was determined by examining mast cells in all sections used in this study. Light microscopy was used at ×1000 magnification. Total number of mast cells denotes the sum of mast cells with and without signs of activation.
others have shown that in atheromas most of the T lymphocytes and probably also the macrophage foam cells are in an activated state. Thus, the conditions necessary for local mast cell stimulation appear to prevail in the shoulder region of coronary atheromas. Indeed, in our studies of mast cells with electron and light microscopy, we did find signs of degranulation, and most importantly, we found that the density of activated mast cells in the shoulder regions of the atheromas was 50-fold higher than in normal coronary intima. The present findings therefore strongly suggest that the mast cell, a powerful neutral protease-containing effector cell, may play a part in the often fatal final event of coronary atherosclerosis, atheromatous rupture.

Acknowledgments

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