Mast Cells of Two Types Differing in Neutral Protease Composition in the Human Aortic Intima

Demonstration of Tryptase- and Tryptase/Chymase-Containing Mast Cells in Normal Intimas, Fatty Streaks, and the Shoulder Region of Atheromas

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Mast cells participate in a variety of pathological processes, including allergic reactions, and appear to play a fundamental role in defending their host against parasitic infections. Because of their sentinel function against outside invaders, mast cells are found mostly in body surfaces facing the environment, i.e., in the skin and mucosal surfaces.

Studies in mice and rats have indicated that mast cells are derived from hematopoietic precursor cells originating in the bone marrow and circulating in the blood. After migrating into specific tissue sites, the precursors enter a final differentiation program in response to signals generated by the surrounding cells.

In normal intimas, fatty streaks, and the shoulder region of atheromas, the mast cells amounted to 3% of all nucleated cells. The ratios of mast cells to T lymphocytes and to macrophages, respectively, were 2:1 and 1:4 in normal intimas, 1:3 and 1:10 in fatty streaks, and 1:5 and 1:20 in the shoulder region of atheromas. Thus, among the blood-borne cells in the human aortic intima, mast cells compose a significant cell population, and in terms of their protease content, these intimal mast cells are heterogeneous. The presence of mast cells in fatty streaks (the site of foam cell formation) and in the shoulder region, i.e., the growing edge, of atheromas supports the hypothesis that mast cells play a role in both the early and late stages of atherogenesis.

Mast cells are also present in the arterial intima, the site of atherogenesis. The idea of a connection between mast cells and atherosclerosis was first put forward by Constantinides. He suggested that mast cells, being a source of endogenous heparin, normally protect the arterial wall from atherosclerosis, but that in atherosclerotic lesions they have lost this protective effect. Indeed, in both fatty streaks and atheromas a marked decrease (up to 90%) in the number of mast cells was found. However, attempts to verify the role of mast cells in the development of atherosclerotic lesions have so far been unsuccessful, owing to the lack of suitable experimental models. Recently, studies on the role of mast cells in atherogenesis have been made by more direct methods. These studies have taken advantage of novel in vitro and in vivo models that used rat serosal mast cells, mouse macrophages, and isolated human lipoproteins.

The results disclosed a series of metabolic events by which mast cells can induce the formation of macrophage foam cells resembling those typical of atherosclerotic lesions. In mast cell-induced foam cell formation, major roles could be attributed to the heparin proteoglycan and chymase of the mast cell granules, which on mast cell stimulation are expelled into the extracellular fluid, where they can act on lipoproteins. These observations prompted us to ask whether similar metabolic events might occur in the human arterial intima. As a first step,
we examined aortic intimas for the presence of the two mast cell–specific major proteases chymase and tryptase. Indeed, we found that a fraction of the mast cells contain chymase. Moreover, with the sensitive immunohistochemical methods now available,16 we were also able to obtain a reliable estimate of the mast cell numbers in various types of atheromatous lesions of the human aorta.

Methods

Reagents

A biotinylated anti-chymase monoclonal antibody B7 and an alkaline phosphatase–conjugated anti-tryptase monoclonal antibody G3 were purchased from Chemicon. Anti-macrophage monoclonal antibody HAM 56 and an anti–T-cell monoclonal antibody UCHL 1 were obtained from Dakopatts. Peroxidase-conjugated streptavidin, biotinylated anti-mouse IgG, and avidin-biotin-peroxidase complexes were from Vector Laboratories. Fast blue RR, naphthol AS-MX phosphate, 3-amino-9-ethylcarbazole, and 3,3′-diaminobenzidine were purchased from Sigma Chemical Co, safranin from Merck, and Alcian blue from Hopkin and Williams.

Autopsy Material and Treatment of Tissue

The autopsy series comprised 35 subjects (23 males, 12 females) 13 to 67 years old. The causes of death were cardiovascular disease (n=11), violent deaths, ie, accidents, homicides, and suicides (n=19), and poisoning with self-administered alcohol and drugs (n=5). The autopsy material (abdominal aorta) was obtained within 24 hours of death (range, 2 to 24 hours; mean, 13 hours). There was no correlation between the mast cell number in the sample and the time interval between death and tissue sampling. The samples included macroscopically normal aorta, fatty streaks, and raised atheromatous lesions. Complicated lesions with ulceration were excluded from the study. The samples were fixed in Carnoy's fluid (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 24 hours and then embedded in paraffin.

Immunocytochemistry

For immunocytochemistry the sections (2 to 4 μm thick) of tissue fixed in Carnoy's fluid were deparaffinized in xylene and rehydrated in a graded series of ethanol, and endogenous peroxide was inhibited by incubation in 0.6% H2O2 in methanol. Then, either a simultaneous or sequential double-labeling method with anti-tryptase and anti-chymase monoclonal antibodies was performed as described by Irani et al.16 In the simultaneous method the slides were incubated overnight at 4°C with a mixture of the anti-tryptase antibody G3 (1:600) and anti-chymase antibody B7 (1:650). Tryptase-containing cells were stained blue by fast blue RR and naphthol AS-MX phosphate. The slides were then mounted in Proceedings of the National Academy of Sciences of the United States of America 1969:60:1011-1015. For this purpose the slides were incubated overnight at 4°C with either HAM 56 (1:50) or UCHL 1 (1:50). The slides were then incubated with a biotinylated second-step antibody and the avidin-biotin-peroxidase complex, and the peroxidase was visualized with diaminobenzidine and hydrogen peroxide. The tissues were counterstained with Mayer's hematoxylin to visualize the nuclei and mounted. In control slides, the primary antibody was omitted.

Alcian Blue/Safranin Method for Staining Mast Cells

The paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. The slides were then rinsed with tap water, stained in Alcian blue solution for 15 minutes (Alcian blue 0.9 g, safranin 0.045 g, ferric ammonium sulfate 1.2 g in 250 mL of acetic buffer, pH 1.42), rinsed in tap water, and dehydrated in tert-butyl alcohol.20 Finally, the slides were cleared in xylene and mounted in Pertex (Histolab).

Microscopy

The antibody-positive cells and the hematoxylin-stained cells were counted at ×200 magnification. Magnification ×400 was used for identifying the subtypes of mast cell. The cells were counted or analyzed in normal intimas, in fatty streaks, and in the shoulder, fibrous cap, and core of atheromas.21

Results

Chymase and Tryptase in Aortic Intimal Mast Cells

Fig 1 shows a typical example of the mast cells in an aortic fatty streak after simultaneous double-label staining. In this particular lesion, seven cells were stained blue; ie, they were G3-positive mast cells that contained tryptase (Fig 1A). When the same section was then stained for chymase, four of the cells stained brown; ie, they were B7-positive chymase-containing mast cells (Fig 1B). Staining for chymase did not disclose any new immunoreactive cells. This result shows that all intimal mast cells contain tryptase and that a proportion of them contain chymase as well.

In the simultaneous double labeling, some mast cells stained so intensely blue that subsequent detection of brown color (chymase) was difficult (see Fig 1B). For unequivocal detection of chymase, sequential double-labeling experiments were performed in which the mast cells were first incubated with B7 and stained for chymase and then incubated with G3 and stained for tryptase. In this method, the chymase-containing mast cells stain intensely brown, and the subsequent blue staining for tryptase in these cells is almost completely blocked.16 As shown in Fig 2, with this method some cells stained intensely brown and some stained blue; thus, mast cells that contained chymase were clearly distinguished from those that did not.

Percentage of Chymase-Containing Mast Cells in Aortic Samples

To determine the proportion of chymase-containing mast cells in the aortic samples, the sequential method was used for staining mast cell proteases (see above), and the percentage of mast cells that contained chymase (brown) was calculated. The percentage of chymase-containing mast cells was highly variable in normal aortic intimas, in fatty streaks, and in atheromas, the
average in each group being 40% (Fig 3). This variability did not depend on age or sex.

**Density of Mast Cells in the Aortic Intima**

To establish the density of mast cells in the aortic intima, the surface areas of intimal cross sections were measured, and the numbers of all tryptase-positive cells in the measured areas were counted in simultaneously double-labeled specimens. As shown in Fig 4A, the number of mast cells varied substantially from case to case. Both in normal intimas and in fatty streaks, the density averaged 15 mast cells/mm². In atheromatous lesions, the density of mast cells was much lower, averaging only 3 cells/mm².

However, the distribution of mast cells within an atheroma was extremely uneven (Fig 4B): in the shoulder region the average mast cell count was 8/mm² and in the fibrous cap 1/mm², whereas in the core region occasional mast cells were found in only 2 of 19 samples. Fig 5 shows the typical localization of mast cells to the shoulder region of an atheroma. The density of mast cells in normal intimas, fatty streaks, and atheromas did not correlate with age or sex. In two cases (a 13-year-old girl and a 37-year-old man) no mast cells could be found in any of the specimens, and in one case (a 34-year-old man), mast cells were found only in the fatty streak and the atheroma but not in the normal intima.
Alcian Blue/Safranin Versus Sequential Double-Labeling Method

The proportion of chymase-containing cells was calculated as a percentage of the total number of mast cells.

**Immunocytochemical Staining**

For comparison with the mast cell numbers found in the human aortic intima in previous studies that used metachromatic staining (toluidine blue/methylene blue), sections of aortic samples were treated with an orthochromatic stain (Alcian blue/safranin), which, like the metachromatic stains, colors granule proteoglycans. Adjacent sections (2 to 4 µm thick) were stained with the monoclonal antibodies against tryptase and chymase by the sequential method. Comparison of the mast cell counts in serial sections revealed that in most of the samples the orthochromatic staining was less sensitive (Fig 6). Thus, in normal intimas (n=34) the proportion of mast cells that stained with Alcian blue/safranin averaged only 45% of the numbers visualized with the monoclonal antibodies. In fatty streaks (n=35) and in the shoulder regions of atheromas (n=19) the Alcian blue/safranin stained even fewer cells, since in these lesions only 25% and 10%, respectively, of the protease-positive cells could be detected. (In the cap and core regions of atheromas, the numbers of mast cells were too small to allow reliable comparison of the two methods.) Accordingly, counts of the Alcian blue/safranin-stained mast cells appeared to show that the fatty streaks contained significantly fewer mast cells than normal intimas (6.5±8.5 cells/mm² in normal intimas and 3.6±3.5 in fatty streaks, mean±SD). However, the immunocytochemical method showed that there was no difference in mast cell density between normal intima (14.6±12.5 cells/mm²) and fatty streaks (14.3±11.3 cells/mm²). Finally, counting of mast cells in the shoulder regions of atheromas by the Alcian blue/safranin method appeared to reveal almost complete loss of these cells (0.8±1.2 cells/mm²), whereas the immunocytochemical method showed a smaller decrease in their number (7.6±5.4 cells/mm²). In those samples in which no intimal cells stained with Alcian blue/safranin, staining of mast cells was observed in the adventitia, demonstrating the success of the staining procedure.

**Mast Cells, T Lymphocytes, and Macrophages as Percentages of the Total Number of Intimal Cells**

We next determined the numbers of mast cells, macrophages, and T lymphocytes, the three intimal cell types that originate in the circulation and migrate into the intima, relative to the total number of intimal cells.

For this purpose, serial sections were stained with one of the following monoclonal antibodies: G3 for mast cells, UCHL 1 for T lymphocytes, and HAM 56 for macrophages. The nuclei of the intimal cells were stained with Meyer's hematoxylin. It was found that the mast cells amounted to 3% of the total number of cells in normal intimas and fatty streaks and about 1% in atheromas (Fig 7). Correspondingly, the T lymphocytes amounted to 2%, 8%, and 7% and the macrophages to 12%, 23%, and 42%. Calculations of the ratio of mast cells to T lymphocytes disclosed that in the normal aortic intima the ratio was 2:1, in fatty streaks 1:3, and in atheromas 1:5. Similar calculations revealed that in normal aortic intimas there was 1 mast cell to 4 macrophages, in fatty streaks 1 mast cell to 10 macrophages, and in atheromas 1 mast cell to 50 macrophages. In the shoulder region of atheromas there was 1 mast cell to 5 T lymphocytes and 1 to 20 macrophages.

**Discussion**

The results of the present study show that the cell population of the human aortic intima includes mast cells, thus confirming earlier observations. In sharp contrast to the earlier reports, however, we found no decrease in mast cell numbers in fatty streaks; moreover, we detected a highly specific pattern of mast cell distribution in atheromas. In the earlier studies, mast cells were detected by the conventional method of staining granule proteoglycans with cationic dyes. The better ability of the current immunocytochemical method to detect mast cells may reflect a higher sensitivity of this staining procedure. Additionally, it may show differences in the functional state of the mast cells, reflecting differences in granule composition between stimulated and unstimulated mast cells. This latter notion is supported by the finding that in isolated rat serosal mast cells (the rodent model of a connective tissue-type mast cell that corresponds to human mast cells, UCHL 1 for T lymphocytes, and HAM 56 for macrophages. The nuclei of the intimal cells were stained with Meyer's hematoxylin. It was found that the mast cells amounted to 3% of the total number of cells in normal intimas and fatty streaks and about 1% in atheromas (Fig 7). Correspondingly, the T lymphocytes amounted to 2%, 8%, and 7% and the macrophages to 12%, 23%, and 42%. Calculations of the ratio of mast cells to T lymphocytes disclosed that in the normal aortic intima the ratio was 2:1, in fatty streaks 1:3, and in atheromas 1:5. Similar calculations revealed that in normal aortic intimas there was 1 mast cell to 4 macrophages, in fatty streaks 1 mast cell to 10 macrophages, and in atheromas 1 mast cell to 50 macrophages. In the shoulder region of atheromas there was 1 mast cell to 5 T lymphocytes and 1 to 20 macrophages.

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cells containing both tryptase and chymase\(^7\)), stimulation causes preferential secretion of granule proteoglycans, with relative conservation of granule proteases.\(^{14,22}\)

In the normal intima, 45% of the protease-positive cells, ie, mast cells, stained positively for proteoglycans also. The current methods do not allow us to determine how much of this weaker staining is due to a lower sensitivity of the method for staining proteoglycans and how much is due to a selective loss of proteoglycans caused by mast cell activation. In fatty streaks and the growing edges (shoulders) of atheromas, the proportions of mast cells that are proteoglycan-positive were much smaller than in normal intimas, only 25% and 10%, respectively. This lower proportion of proteoglycan-positive mast cells in atherosclerotic lesions, with their high densities of T lymphocytes and macrophages (see Fig 7), which are capable of activating mast cells,\(^{23,24}\) strongly suggests that the mast cells in these areas have been more extensively stimulated than those in the normal intima. This observation would also explain why, in earlier studies, the numbers of mast cells were found to be lower in atherosclerotic lesions. On the basis of the above observations, a reformulation of the original concept of the role of mast cells in atherosclerosis appears necessary. Originally, one mechanism triggering atherosclerosis was considered to be disappearance of mast cells from intimal areas susceptible to atherosclerosis.\(^{12}\)

We suggest that the mast cells in these areas do not disappear but rather are stimulated and play an active role in the formation and progression of atherosclerotic lesions.

What factors might be responsible for the presence of mast cells in the aortic intima? 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 of either mucosal or connective tissue type.\(^5\) As the factor responsible for the local development of mast cells, one possible candidate is the stem cell factor (SCF). Thus, in all experimental systems 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\(^{25-27}\); moreover, in the adult human system, of the many cytokines and growth factors studied, only SCF promoted the development of mast cells.\(^{28}\)

Considering that SCF induces the development of mast cells and regulates their numbers in different anatomic sites, this growth factor appears likely to be at least one of the agents leading to mast cell development in the human aortic intima. Similarly, interindividual variation in intimal SCF level could be one factor responsible for the considerable variation in mast cell density (0 to 47 mast cells/mm\(^2\)) observed among individuals.

We found mast cells only in the intimal and adventitial layers of the aortic wall and not in the medial layer. The stroma cells of the aortic intima and media are smooth muscle cells, and those of the adventitia are fibroblasts. The smooth muscle cells of the media are in a contractile state, whereas the intimal smooth muscle cells are phenotypically modified and functionally resemble fibroblasts, in that they divide and actively synthesize extracellular matrix.\(^{29,30}\) Accordingly, the phenotypic state of such smooth muscle cells is referred to as “synthetic.” Thus, it appears that of the two types of smooth muscle cell present in the aortic wall, only those in the synthetic state offer a parenchymal environment favorable for the development of mast cells. Since SCF is a product of the stroma cells that create the parenchymal environment in tissues to which mast cell precursors are attached,\(^{31}\) we are left with the challenging question of whether transformation of smooth muscle cells from the contractile to the synthetic state, a process regarded as highly atherogenic,\(^{32}\) is accompanied by expression of SCF in these cells.

In the human aortic intima, as in all other human tissues studied so far, mast cells show phenotypic dichotomy in terms of their neutral protease content.
Thus, in some mast cells, chymase is expressed and in others, it is not. Tissues with high densities of mast cells, such as mucosal surfaces and skin, display a typical phenotypic pattern of neutral protease expression. Thus, in mucosal surfaces most mast cells lack chymase, whereas in the skin most of them contain this enzyme. Interestingly, the human aortic intima showed extreme interindividual variation in mast cell phenotype pattern; ie, in some cases none, in other cases all, and in still others a highly variable fraction of the mast cells contained chymase. What factors could account for the great interindividual variation in chymase expression in this single anatomic location? Studies on human mast cell development have shown that in culture systems containing mast cell precursors, addition of human recombinant SCF alone induces strong expression of tryptase and only weak expression of chymase (estimated chymase content about 1/450 of that found in skin mast cells). 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 in developing mast cells. Our finding of distributed intimal mast cells of two phenotypes in apparently random fashion and often close to each other suggests local differences in the production of such cofactors, reflecting the presence of functionally different microenvironments in the intimal parenchyma.

In terms of lipoprotein metabolism, mast cells containing chymase are of special interest. Thus, recent extensive studies in vitro on the interaction between rat peritoneal macrophages in a medium enriched with low-density lipoproteins (LDLs) induced 50-fold enhancement of LDL uptake by macrophages, with formation of foam cells. This process, called the "granule-mediated uptake of LDL," consisted of initial binding of the LDL particles to the heparin proteoglycan matrix of the mast cell granules and subsequent sequential proteolysis of the bound LDL by the matrix-bound neutral proteases chymase and carboxypeptidase A. The proteolyzed LDLs become unstable and fuse into larger lipid droplets containing up to 100 LDL particles each. Such fusion of LDL on the granule remnant surface enhances the capacity of the granule remnants to bind and carry LDL. The fused LDL particles are ultimately ingested by the macrophages as they phagocytose the granule remnants that carry them.

In addition, stimulated mast cells were shown to block the removal of cholesterol from the macrophage foam cells. In this process, chymase of exocytosed granules proteolyzed high-density lipoprotein 3 particles, thereby lessening their ability to induce efflux of cholesterol from foam cells. The dual action of chymase, both promoting uptake and inhibiting release of cellular cholesterol, makes this enzyme a potentially powerful inducer of foam cell formation in the intimal areas in which chymase-containing mast cells and macrophages coexist. The most recent observations concerning the antioxidative actions of exocytosed chymase and histamine indicate that activated mast cells may have antiatherogenic potential as well. Thus, the ultimate role of intimal mast cells in the development of atherosclerotic lesions will then depend on the relative magnitudes of the atherogenic and antiatherogenic actions.

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