Mast Cells in Neovascularized Human Coronary Plaques Store and Secrete Basic Fibroblast Growth Factor, a Potent Angiogenic Mediator

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Objective—Intraplaque neovascularization and hemorrhage may facilitate plaque progression. We studied expression of basic fibroblast growth factor (bFGF), a potent angiogenic mediator, by mast cells (MCs) in human coronary plaques with increasing degrees of atherosclerosis.

Methods and Results—Normal and atherosclerotic coronary segments were collected from 30 autopsied subjects. Immunohistochemical methods were used to detect MCs, bFGF, and microvessels. Both adventitial and intimal MCs showed intracytoplasmic granular staining for bFGF, and bFGF-positive extracellular granules were observed close to the MCs. Increased numbers of bFGF-positive MCs were detected in neovascularized areas of plaques, and there was a positive correlation between numbers of bFGF-positive MCs and microvessels in both the intima and adventitia. In plaques, the highly neovascularized areas contained increased numbers of bFGF-positive MCs compared with the adjacent nonvascularized areas, where only few MCs were present. Importantly, the proportion of intimal MCs expressing bFGF increased with increasing severity of atherosclerosis.

Conclusions—The present work reveals a novel source of bFGF in human coronary arteries, the intimal and adventitial MCs. The association of bFGF-positive MCs with microvessels and with the severity of atherosclerosis suggests that coronary MCs, by releasing bFGF, may play a role in angiogenesis and progression of coronary plaques.

Key Words: basic fibroblast growth factor • mast cells • neovascularization • adventitia • atherosclerosis

The intima of normal human coronary arteries lacks capillaries, and the intimal cells obtain oxygen and nutrients from the arterial lumen by diffusion. When atherosclerosis develops, however, the intima becomes thicker and neovascularization may develop in the deep parts of atherosclerotic lesions in response to hypoxia. The factors involved in this development of neovascularization include various cytokines and growth factors. Basic fibroblast growth factor (bFGF or FGF-2) is a well-characterized angiogenic growth factor, and its target cells include endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts. The mast cell (MC) is a type of inflammatory cell implicated in angiogenesis. In human coronary atheromas, MCs appear near intimal neovessels, and in the adventitia, MCs are often located close to the vasa vasorum. These localizations of coronary MCs suggest that in coronary atheromas they may contribute to neoangiogenesis. Increased numbers of bFGF-positive MCs have been found in tissues characterized by neovascularization, fibrosis, and inflammation, such as human fibrotic lung and cutaneous hemangiomas. In MCs, bFGF is located predominantly in the heparin-containing granules. The emerging concept of intraplaque neovascularization and hemorrhage as factors contributing to plaque progression prompted us to search for bFGF in MCs in human coronary arteries showing various degrees of atherosclerosis.

Methods

Autopsy Material
The autopsy series comprised 30 cases (24 men and 6 women) aged 34 to 78 years. The proximal left coronary artery was removed, cut into successive segments 5 mm long, fixed in neutral buffered formalin, and embedded in paraffin. Sections (2 to 4 μm) were stained with hematoxylin-eosin and elastic-van Gieson and evalu-
ated for atherosclerotic involvement according to the guidelines of the American Heart Association (AHA). Ten segments with normal intimas (AHA types 0 and I), 10 with fatty streaks or intermediate lesions (types II and III), and 10 with advanced lesions (types IV through VI) were subjected to further analyses.

**Immunohistochemistry**

The tissue sections were deparaffinized and rehydrated, and endogenous peroxidase activity was quenched with 0.6% H2O2 in methanol. A mouse monoclonal antibody (148.6.1.1, a kind gift from Dr. C. Hart, ZymoGenetics, Seattle, Wash) was used for detection of bFGF. This antibody was raised against human recombinant bFGF, and its specificity was determined by Western blot analysis and with an affinity support column containing immobilized human recombinant bFGF.17 A previously described method served for detection of bFGF in MCs.11 Briefly, the sections were treated with hyaluronidase for antigen retrieval. To exclude nonspecific binding, the sections were incubated in a blocking solution containing 3% normal horse serum, 0.5% BSA, and 0.3% Triton X-100 in PBS. They were then incubated overnight with the anti-bFGF antibody (0.05 μg/mL) diluted in PBS containing 2% normal horse serum. The avidin-biotin complex method (ABC-AP Kit, Vector Laboratories) was used to detect the primary antibody with Vector Blue as the chromogenic substrate. The sections were then incubated overnight with the anti-trypsin antibody (AA1, 0.5 μg/mL, Dako, Glostrup, Denmark), with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories) and finally with fluorescein-conjugated streptavidin (10 μg/mL, Dako). Colocalization of bFGF and MCs, macrophages, and T lymphocytes was also studied by comparison of 2 sequential sections, 1 stained for bFGF and the other stained each time for a different one of these 3 types of inflammatory cells.

Monoclonal antibody HAM56 (0.7 μg/mL) was used for detection of macrophages, and UCHL1 (4 μg/mL) was used to detect T lymphocytes (both from Dako). ECs of the intimal neovessels were detected with a cocktail of monoclonal antibodies against CD31 (8 μg/mL, Dako) and CD34 (0.3 μg/mL, Novocastra, Newcastle on Tyne, UK), and adventitial microvessels were detected with an antibody against von Willebrand factor (2 μg/mL, Dako). The ABC method was used, combined with diaminobenzidine or 3-amino-9-ethylcarbazole as chromogenic substrates.

To determine the numbers of degranulated MCs, we used a method extensively used in our laboratory over many years in studies of human coronary MCs,19,20; coronary sections were first incubated with anti-trypsin antibody, and the antibody was then detected using the ABC method (Vector Laboratories) combined with the peroxidase substrate diaminobenzidine which gave a dark brown color. This staining allowed clear definition of MC boundaries and detection of extracellularly located granules against the light-blue hematoxylin background. A MC having 1 or more tryptase-positive extracellular granules in its close vicinity was classified as a degranulated MC. The sections were examined using light microscopy at high magnification (objective ×100) and with the aid of immersion oil. The quantification of degranulated MCs was performed by a single investigator (P.L.) who was blinded to the other results of the study.

For determination of numbers of microvessels in the most neovascularized areas of plaques, the "hotspots," we adopted a technique proposed by Weidner et al19 and widely used to stain and count microvessels on surgical samples of solid tumors. The areas of highest neovascularization are found by scanning the intimal sections (stained for ECs) by light microscopy at low magnification (objective ×10). The numbers of microvessels per microscopic field are counted using objective ×40. Any brown staining of an EC or an EC cluster that is clearly separate from adjacent microvessels is considered a single countable microvessel. Identification of a vessel lumen is not necessary for a structure to be defined as a microvessel, and identification of red cells in the tissue is not used to define a vessel lumen. For comparison of the numbers of bFGF-positive MCs in the hotspots and in other areas of plaques, we used serially cut adjacent sections, one stained for ECs and the other double-stained for tryptase and bFGF.

As a negative control, we replaced the primary antibody with nonimmune mouse IgG of the same isotype as the primary antibody and found no staining of MCs, indicating no significant binding of IgG to heparin. As a further control, we preincubated the sections in 2 mol/L NaCl, which has been reported to release bFGF from heparin.20 After such preincubation, positive staining of MCs by the anti-bFGF antibody was abolished, indicating specificity of the staining for bFGF rather than for heparin.

**Morphometry**

Imaging was conducted with a Nikon E600 fluorescence microscope equipped with a charge-coupled device camera. The adventitial and intimal areas were measured by image analysis software (Image-Pro Plus 4.1, Media Cybernetics). The bFGF-positive adventitial and intimal MCs, CD31/34-positive intimal microvessels, and von Willebrand factor-positive adventitial microvessels were immunostained and counted in 2 to 4 nonconsecutive sections (a minimum of 40 μm apart). We evaluated a total of 680 and 1710 MCs for bFGF expression in the intimal and the adventitial areas, respectively.

**Statistical Analysis**

The Jonckheere-Terpstra test was used to study the presence of a trend in the numbers of MCs or microvessels in coronary segments with atherosclerosis of increasing severity. Wilcoxon signed rank paired test was applied in comparisons between numbers of bFGF-positive MCs in the intimal areas with the highest numbers of microvessels and in the adjacent nonvascularized areas. It was also used when the proportion of MCs positive for bFGF in the adventitia was compared with that in the intima. Spearman correlation coefficient was calculated between the numbers of bFGF-positive MCs and microvessels. Statistical significance was defined as P<0.05.

**Results**

**Immunohistochemistry**

To study for the presence of bFGF in inflammatory cells in coronary arteries, we stained serially-cut tissue sections for bFGF and MCs, macrophages, and T lymphocytes. In the adventitia, strongly bFGF-positive cells were frequently detectable, whereas the surrounding connective tissue was virtually free of bFGF. Figure 1 shows typical findings in the adventitial layer of atherosclerotic coronary arteries with several microvessels. Figure 1A and 1B shows consecutive sections stained for the MC-specific marker tryptase and for bFGF, respectively. Comparison of these microscopic fields reveals that the 5 cells strongly positive for bFGF are MCs. We then used a double-staining method to confirm the presence of bFGF in MCs. Figure 1C and 1D shows a single section of an atherosclerotic coronary artery. Comparison reveals that all adventitial cells positive for bFGF are MCs. To study cellular architecture of the MCs and to classify the cells into resting and activated (ie, degranulated) MCs, we used light microscopy at high magnification (objective ×100). The results in Figure 1E and 1F show that MCs, whether stained for tryptase or bFGF, could be classified into resting and degranulated ones. Moreover, these results are compatible with the idea that MC granules are bFGF-positive. To confirm this proposition MCs were double-labeled for tryptase and bFGF. Figure 1G and 1H demonstrates the colocalization of tryptase and bFGF in extracellular granules of MCs, revealing that coronary MCs are able to exocytose bFGF.

We next sought bFGF in the medial and intimal layers. Figure 2A shows an overview of an advanced coronary...
plaque with an accumulation of bFGF-positive cells in the intima. One area, when enlarged (Figure 2B and 2C), reveals that the bFGF-positive cells are MCs. This plaque is richly neovascularized, and the close association of bFGF-positive MCs with microvessels is evident. In contrast to the intimal and adventitial layers, bFGF-positive MCs are very rarely found in the media. When observed, they were usually associated with medial microvessels. Such microvessels appeared to penetrate the media, connecting adventitial vasa vasorum with intimal microvessels, present in the deep areas of advanced atherosclerotic plaques.

Among the 3 types of inflammatory cells studied, bFGF-positive staining appeared only in MCs, whereas macrophages and T lymphocytes were bFGF-negative (not shown). This finding applied to all segments studied, irrespective of the degree of atherosclerosis.

We then searched for signs of intimal hemorrhage by staining the sections with Prussian blue for detection of iron and hemosiderin. In the 30 coronary segments studied, 5 contained atherosclerotic lesions with deposition of iron, of which 1 was an intermediate and 4 were advanced lesions. Figure 3 shows an advanced plaque in which hemorrhage is present in a superficial and a deep area of the intima. Extensive neovascularization is present in the deep area of iron deposition, and this area is infiltrated with bFGF-positive MCs.

### Quantitative Measurements

We then counted the bFGF-positive MCs and microvessels in the adventitial and intimal layers of the 30 coronary segments available. In segments with normal intimas (types 0 and I), early to intermediate lesions (types II and III), and advanced lesions (types IV through VI), numbers of adventitial bFGF-positive MCs were $17 \pm 9$ (mean $\pm$ SD), $32 \pm 17$, and $46 \pm 20$ cells/mm$^2$, respectively. When severity of atherosclerosis increased, a significant trend appeared in the numbers of bFGF-positive MCs ($P<0.001$). Similarly, the numbers of adventitial microvessels were 26, 45, and 69 microvessels/mm$^2$ in normal intimas, early to intermediate lesions, and in advanced lesions, respectively ($P<0.001$ for trend).
In the intima, numbers of intimal bFGF-positive MCs tended to increase with increasing severity of atherosclerosis from 0.5 to 2.0 cells/mm² (P=0.10), and numbers of intimal microvessels increased significantly from 0 to 7.8 microvessels/mm² (P<0.001 for trend). Moreover, the numbers of bFGF-positive MCs and microvessels correlated positively in both adventitia (R=0.63, P<0.001) and intima (R=0.49, P<0.01).

We next calculated the proportion of MCs positive for bFGF and found that in all but 2 patients, the proportion of bFGF-positive MCs was higher in the adventitia than in the intima. Indeed, in the intima, the fraction of MCs expressing bFGF was 45±18% (mean±SD), whereas in the adventitia it was 78±20%. In the segments with normal intima and early-to-intermediate lesions, proportions of adventitial bFGF-positive MCs were similar: on average, 71% of the MCs. By contrast, in the segments with advanced lesions, 92% of the MCs were positive for bFGF. Importantly, in the intima, a highly significant trend appeared toward an increase in proportion of bFGF-positive MCs with increasing severity of atherosclerosis in segments with normal intima (types 0 and I), in early to intermediate lesions (types II and III), and in advanced lesions (types IV through VI); proportions of MCs positive for bFGF were 34±16% (mean±SD), 39±11%, and 60±17%, respectively (P<0.001).

MC degranulation (ie, exocytosis of MC granules) is a sign of MC activation and a prerequisite for mediators stored in the granules to influence their microenvironment. We examined the sections using light microscopy at high power (objective ×100) to determine numbers of degranulated intimal MCs. The proportion of degranulated MCs increased with an increasing degree of atherosclerosis (Figure 4A; P<0.01 for trend).

We then focused on colocalization of intimal neovessels and bFGF-positive MCs, and found neovascularization in 11 of the 30 coronary segments (Figure 4B). Of these 11 lesions, 7 were advanced plaques and 4 were fatty streaks or intermediate lesions. To learn whether the bFGF-positive MCs were localized specifically in the most neovascularized areas or were more scattered in the plaque, we compared the numbers of bFGF-expressing MCs in the areas of highest neovascularization (“hotspots”; see Methods) with the adjacent nonvascularized areas. The spatial density of bFGF-positive MCs was significantly higher in vascularized than in nonvascularized areas (on average, 2.5 and 0.1 cells per microscopic field, respectively; P<0.05).

Discussion
The present work reveals a novel source of bFGF in human coronary arteries, the intimal and adventitial MCs. Importantly, in coronary lesions with increasing severity of atherosclerosis, the fraction of MCs positive for bFGF increased. In normal intima only 34% of the MCs expressed bFGF, compared with 60% in advanced plaques. Factors that regulate bFGF expression in intimal MCs remain unknown. Interestingly, in cultured murine MC lines, transforming growth factor β and tumor necrosis factor α, factors present also in human coronary intima, have been found to regulate bFGF mRNA expression.21 In addition to bFGF, MCs secrete a variety of other angiogenic factors such as tryptase, heparin, histamine, vascular endothelial growth factor (VEGF), and nerve growth factor (NGF).7,22–24 The roles of the various angiogenic mediators of MCs and their interactions are poorly understood. Interestingly, the major MC protease tryptase also has strong angiogenic effects.22 Thus, in an in vitro model, coculture of human microvascular ECs with human MCs greatly augmented vascular tube formation, and antibodies against tryptase caused ~80% suppression of this process. An in vivo model of angiogenesis, the chick chorioallantoic membrane assay, revealed that addition of antibodies against bFGF and VEGF reduced the angiogenic response to MCs and their granules by 50% and 30%, respectively.25 Recently, the direct mitogenic effect of NGF on cultured ECs has been demonstrated,23 thus raising interest on this MC-derived growth factor and other neurotrophins as molecules for reparative angiogenesis in ischemic tissues.26 NGF has also been reported to be present in advanced human coronary...
atherosclerotic tissues. In such coronary arteries the numbers of MCs and of NGF receptors were increased, whereas the levels of NGF were decreased.27

We found a significant correlation between numbers of bFGF-positive MCs and microvessels both in adventitia and in the intima. Moreover, in the neovascularized lesions, the intimal areas with the highest numbers of microvessels contained the highest numbers of MCs. According to the prevailing concept, intraplaque neovascularization may have effects on progression of atherosclerosis.13,14 Thus, the neovessels in atherosclerotic plaques express leukocyte adhesion molecules and are therefore able to sustain the influx of leukocytes into the plaque.28 Importantly, the neovessels may also act as a port of entry for circulating MC progenitors to the deep areas of the plaques. In addition, rupture of the fragile walls of the neovessels may allow erythrocytes to leak into the plaque. Indeed, glycoporphin, an erythrocyte-specific marker, has been detected in human coronary atheromas, and the cholesterol-rich erythrocytes have been suggested to contribute to the formation of the atheromatous lipid core of the plaque.14 Moreover, the extravasated erythrocytes lead to local iron deposition and thereby lead to increased oxidative stress in the atheroma.29 Besides being associated with plaque neovessels, bFGF may contribute to plaque remodeling by stimulating the proliferation of SMCs. Because bFGF lowers the capacity of SMCs to synthesize collagen,30 MC-derived bFGF may potentially have plaque-weakening effects. These effects may be particularly important in the rupture-prone shoulder areas, where accumulation of MCs has been found.18

In conclusion, we demonstrate a close association between bFGF-containing MCs and coronary intimal and adventitial microvessels and severity of coronary atherosclerosis. Yet in several plaques, no MCs were found in the neovascularized areas. It is evident that the hypoxia-initiated process of neovascularization in advanced atherosclerotic plaques results from a complex interplay between various cytokines and growth factors derived from both MCs and other cells present in the plaques. Obviously, the bFGF secreted by MCs is only one of the many potential candidates contributing to this process. Because bFGF is not only an angiogenic mediator but also a chemoattractant for MCs, the bFGF released from MCs may recruit new MCs to the neovascularized areas.31 If so, rather than being the initiators of the process, the newly recruited MCs would amplify and sustain the ongoing angiogenic process originally triggered by hypoxia present in the atherosclerotic lesions. In all, the detection of bFGF, in addition to the multitude of other proangiogenic factors in coronary MCs, further emphasizes the potentially powerful role of MCs in the regulation of neoangiogenesis in atherosclerotic plaques, and also calls for in vivo studies in experimental animals aimed at pharmacological regulation of MC activity during atherogenesis.

Limitations of the Study
This human study is limited by its correlative nature. Because it is not possible in humans to study the actual histological development of a single plaque, we chose the strategy to study autopsy material, which included all different graded stages of atherosclerosis (AHA classes I through VI). It was done in an attempt to have a grasp of the dynamic nature of one aspect of this human disease.

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