Distribution of Inflammatory Cells in Atherosclerotic Plaques Relates to the Direction of Flow

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Background—The distribution of macrophages and smooth muscle cells (SMCs) within atherosclerotic plaques is highly variable. This is clinically relevant because these cell types have opposite effects on the stability of atherosclerotic plaques. The present study was designed to investigate whether local variations in arterial flow over the plaque surface could relate to differences in the distribution of SMCs and macrophages in plaques.

Methods and Results—Thirty-three entire carotid plaques were collected at autopsy and marked at their proximal (in relation to the direction of the blood flow) ends, and the cell composition of upstream parts (where high flow and high shear prevail) was compared with that of downstream parts (low flow and low shear stress). Seventy percent of plaques showed more SMCs in their downstream part, and 67% of plaques contained more macrophages in the upstream part. Immunostained macrophage areas were larger in upstream parts (P<0.011). Immunostained SMC areas were larger in downstream parts (P=0.031). Rupture sites of 6 of 9 ruptured plaques were in the upstream part.

Conclusions—Significant differences in cell composition between upstream and downstream parts of plaques indicate a role for arterial flow in the distribution of different cell types. The low-flow/low-shear downstream areas of plaques contain significantly more SMCs, which could provide the background for slowly progressive growth at distal ends of plaques. The significantly high number of macrophages in the upstream areas suggests a relationship between high flow/high shear and plaque instability. (Circulation. 1998;98:2000-2003.)

Key Words: plaque ■ stress ■ carotid arteries ■ atherosclerosis

Atherosclerotic plaques show marked variability with respect to the distribution of inflammatory cells, not only from one lesion to the other but also within one and the same plaque.1–3 This phenomenon is significant because plaque inflammation is widely considered to play a role in plaque destabilization and, eventually, plaque erosion and rupture.4–6 By the same token, plaques dominated by smooth muscle cells (SMCs) are considered stable. Indeed, coronary atherectomy specimens obtained from patients with chronic stable angina contain SMCs as the dominant cellular component, but in those obtained from patients with unstable angina or acute myocardial infarction, inflammation prevails.4,9,10

In view of these considerations, it is important to know which mechanisms could be responsible for these major variations in the cellular composition of atherosclerotic plaques. Hemodynamic factors, such as shear stress, are considered to play a role in plaque growth,11,12 but thus far the effect of these factors on the cellular composition of atherosclerotic plaques has not been studied. This may well be an interesting enterprise, because the geometry of a bulging plaque dictates differences in the impact of blood flow in relation to the direction of flow. In fact, the luminal endothelial lining on the upstream (proximal) sites of a plaque is under high shear stress, whereas at downstream (distal) sites, low shear stress prevails.13,14 We speculated that these differences may also have implications for local variation in the cellular composition of plaques.

To verify this hypothesis, we investigated the relationship between blood flow direction and the cellular composition of carotid plaques by quantitatively comparing SMC and macrophage contents of the upstream shoulder part of plaques with those of the downstream shoulder parts. The common carotid artery with its bifurcation site was chosen, because previous studies by Zarins et al12 and Ku et al15 have shown that these arterial sites may serve as a good model to investigate the relationship between fluid dynamics and atherosclerosis and also because the carotid bifurcation is a predilection site for plaque formation.

Methods

Tissue Sampling

Sixty-three left and right carotid artery bifurcations were studied in a consecutive autopsy series of 45 patients (mean age, 69 years; range, 26 to 89 years). In all cases, the postmortem interval was <24 hours. Excluded from the study were immunocompromised patients and patients who had died under septic conditions. Left and right
carotid arteries were carefully dissected from the surrounding tissues, and a segment was removed that contained 10 mm of the common carotid artery at the upstream end, the carotid bifurcation, and 10 mm of the internal and the external carotid arteries at the downstream ends. The arterial segment was opened longitudinally along its ventral side, fixed in 4% buffered formalin, and, if necessary, decalcified in EDTA for 4 days. Arteries with total luminal occlusion were excluded; none of the arteries showed recanalization. From the remaining samples, a longitudinal transmural tissue block of \( \frac{3}{4} \) mm was removed from both the internal and external carotid arteries. These tissue blocks were marked immediately with India ink at the upstream (proximal) site to ensure their topographic relation with the flow direction. They were then routinely processed for paraffin embedding and microscopic sectioning (Figure 1).

**Light Microscopy**

Serial sections 6 \( \mu \)m thick were cut parallel to the long axis of the arterial segment, and 2 sections were stained with hematoxylin-eosin and an elastic–van Gieson stain for screening. Arterial segments that appeared to contain diffuse atherosclerosis or fatty streaks, as well as plaque-free segments, were all excluded from the study. Ruptured or eroded plaques were excluded from morphometric evaluation; these plaques were studied to determine at which site of the plaque (upstream or downstream) rupture or erosion had occurred. The remaining segments, containing raised plaques with intact upstream and downstream shoulders and cap parts, were used for further investigation (Figure 1).

**Immunohistochemistry**

Adjacent serial sections were stained for SMCs with an anti-\( \alpha \)-actin antibody (SMA, clone 1A4, DAKO, dilution 1:200). Macrophages were stained with an anti-CD68 antibody (clone PG-M1, DAKO, dilution 1:100). A 3-step indirect streptavidin-biotin technique with peroxidase was used, in which final visualization of the peroxidase activity was performed with diaminobenzidine as chromogen. Nuclei were faintly counterstained with hematoxylin. In negative controls, the primary antibody was replaced by an irrelevant mouse monoclonal antibody of the same subclass.

**Morphometry**

Surface areas of the upstream (proximal) shoulder and the downstream (distal) shoulder of plaques were planimetrically quantified in tissue sections with image-analysis software running on a PC connected with a video-mounted microscope. The shoulder parts were defined as the plaque area reaching from the adjacent normal intima (upstream or downstream) to the outer sides of the lipid core (atheroma) at both ends (Figure 1). These areas were outlined manually, and the percentage of immunostained surface was measured automatically with gray-scale detection. In this way, we calculated the anti-CD68 (macrophages) and anti-\( \alpha \)-actin (SMC) immunopositive areas as a percentage of the total area of each shoulder part in square millimeters. The ratios of macrophage-positive areas and SMC-positive areas were calculated for each plaque individually. Results were recorded as mean \( \pm \) SD.

**Statistical Analysis**

For comparison of morphometric data between different plaque areas, which were not compatible with a normal frequency distribution, a paired Student’s \( t \) test with the logarithmic transformation of individual values was used (\( \pm \) SD). Values of \( P < 0.05 \) were considered significant.

**Results**

Six carotid arteries (10%) were occluded. Histopathological analysis of the carotid artery samples resulted in 41 plaque-free segments (36%), 31 segments containing diffuse atherosclerosis (27%), and 9 segments showing rupture or erosion (8%). The site of rupture or erosion was upstream in 6 of the 9 plaques. Each of the remaining 33 carotid artery segments (29%) contained an entire atherosclerotic plaque, and these were used for further study (Figure 2). Immunohistochemistry revealed large variations in the numbers of SMCs and macrophages among plaques of different patients but also
Macrophage and SMC Area Quantification in the Upstream and Downstream Shoulder Parts of 33 Atherosclerotic Plaques in the Carotid Artery

<table>
<thead>
<tr>
<th></th>
<th>Upstream</th>
<th>Downstream</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean measured area, mm²</td>
<td>2.49±1.81</td>
<td>2.38±1.19</td>
<td>...</td>
</tr>
<tr>
<td>ln(Mac %)</td>
<td>1.55±1.14</td>
<td>0.81±1.95</td>
<td>0.011</td>
</tr>
<tr>
<td>ln(SMC %)</td>
<td>2.56±1.09</td>
<td>2.97±1.08</td>
<td>0.031</td>
</tr>
<tr>
<td>ln(Mac %/SMC %)</td>
<td>−1.02±1.51</td>
<td>−2.67±2.53</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Mac indicates macrophage.

within one and the same plaque. Some plaques contained only SMCs, whereas others were almost totally infiltrated by macrophages. On average, however, SMC areas were larger than macrophage areas.

The results of area quantification of SMCs and macrophage areas in the immunostained sections are shown in the Table. In 23 of the 33 sections (70%) stained with anti-α–actin, the SMC areas of the downstream (distal) shoulder were larger than those of the upstream shoulder part, with an upstream/downstream ratio [ln(U/D)] of −0.41. In the downstream (distal) shoulder, the SMC areas were significantly larger than in the upstream (proximal) shoulder (P=0.031) (Figure 2). In 22 of 33 cases (67%), anti-CD68–stained sections showed larger macrophage areas in the upstream (proximal) shoulder part of the plaque, with a U/D ratio [ln(U/D)] of 0.74. Macrophage areas were significantly larger in the upstream shoulder areas of plaques (P=0.011) (Figure 2).

Discussion

Several risk factors for atherosclerosis, including hemodynamic factors, may be implicated in determining the cellular composition of plaques. To the best of our knowledge, this is the first study that shows marked topographic variation in cellular composition within atherosclerotic plaques related to the direction of the blood stream. Planimetric quantification of the macrophage contents in carotid plaques showed statistically significantly larger macrophage-rich areas in the upstream shoulder than in the downstream shoulder of the same atherosclerotic lesion. This phenomenon may render the upstream part of an atherosclerotic plaque more vulnerable to erosion or rupture than the downstream part. Indeed, 6 of 9 rupture sites were upstream. Differences in fluid mechanics at the luminal site of different regions of the plaque could be responsible for differences in plaque architecture.

The carotid artery has been used by several investigators to study the relationship between flow dynamics and plaque formation. Zarins et al and Ku et al showed that the upstream sites of plaques are preferentially under high flow/high shear stress, whereas downstream parts are under low flow/low shear stress. Plaque growth, moreover, has been shown to occur predominantly in regions of low shear stress. A recent angiographic study of femoral arteries also revealed that plaque growth in the downstream direction occurs significantly more frequently than in the upstream direction.

It is known from in vitro studies of endothelial cells under shear stress conditions that high shear stress induces increased expression of endothelial adhesion molecules, such as ICAM and VCAM, resulting in enhanced leukocyte adherence, including monocytes and lymphocytes. Conversely, other investigators found a relationship between low shear and macrophage infiltration due to prolonged and intimate contacts between mononuclear cells and the endothelial lining. However, in the in vivo situation of human arteries, shear stress may not be the only rheological factor interfering with leukocyte adherence and influx. Recently, Tropea et al studied the differences in monocyte binding upstream and downstream to artificial coarctations in lipid-fed New Zealand White rabbits. They demonstrated that monocyte adhesion and VCAM-1 expression were increased upstream of the stenosis, which resulted in enhanced intimal thickening and accumulation of macrophages at these sites.

Because the common carotid artery conveys blood with high flow and high kinetic energies, one may assume that at sites of atherosclerotic plaques, similar mechanisms are involved, as described by Tropea et al. This, then, could account for the large macrophage-rich areas in the upstream shoulder of the lesions. Nevertheless, the dominant overall cell type in most plaques appeared to be the SMC. In individual plaques, however, the downstream shoulders showed (on average) larger SMC-rich areas than their upstream counterparts. This is of interest because an increase of shear stress activates endothelium-derived nitric oxide (NO) synthase and NO production, and a chronic increase in NO has an inhibitory effect on SMC protein synthesis and SMC proliferation. In areas with low shear stress, such as the downstream parts, there is no step-up in NO production. In fact, it has been shown that low shear stress increases endothelin production, which acts as a stimulating factor for the production of extracellular matrix components by SMCs and for SMC proliferation. Platelet adherence in low-flow areas, with release of platelet-derived growth factor and basic fibroblast growth factor, could provide another stimulus for SMC growth. SMC growth with connective tissue production is generally considered to be the mechanism responsible for a gradually progressive growth of atherosclerotic plaques. These phenomena, therefore, could provide an explanation for the differences in SMC content between the upstream and the downstream parts of plaques and the slowly progressive growth downstream of the main lesion.

Obviously, shear stress cannot be the only factor involved. It is likely that a balance between local hemodynamic variables, such as pulse pressure, wall stress, and turbulence, all play a role in the eventual component makeup of an atherosclerotic plaque. And because this study is based on specimens obtained at autopsy, without much clinical information, a variety of intrinsic and environmental risk factors for plaque development and growth could have been involved, which could be reflected in variability in macrophage and SMC contents in plaques of different patients. Conversely, one may anticipate that such factors affect the overall composition of plaques rather than inducing local changes. However, it appears from this study that the overall effect of the variables involved in human plaque formation results in increased macrophage infiltration at the upstream site and increased SMC growth at the downstream sites.
Our observations in human carotid arteries are of clinical relevance. Plaque instability leading to plaque rupture is considered to be a disbalance between reparative activities (SMC growth and collagen synthesis) and degrading activities induced by macrophages. Therefore, the large amounts of macrophages in the upstream parts of plaques could indicate a relationship between high flow/high shear stress and plaque instability.

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References
5. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation. 1994;89:36–44.
8. Lendon CL, Davies MJ, Born GVR, Richardson PD. Atherosclerotic plaque caps are locally weakened when macrophages density is increased. Atherosclerosis. 1991;87:87–90.
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