Macrophages and mast cells are involved in carotid plaque instability

A. MARZULLO1), M. M. CICCONE1), CLAUDIA COVELLI2), GABRIELLA SERIO2), D. RIBATTI3)

1) Department of Pathology
2) Department of Cardiology
3) Department of Human Anatomy and Histology
University of Bari Medical School, Bari, Italy

Abstract
The aim of this study was to evaluate the role of macrophages and mast cells and of microvascular density in atherosclerotic plaques collected from 63 consecutive symptomatic and asymptomatic patients undergoing carotid endarterectomy for carotid disease. Results have shown no statistically significant differences between the two groups as concerns: (i) the degree of stenosis; (ii) the extension of the lipidic core; (iii) the thickness of the fibrous cup; (iv) the inflammatory infiltrate; (v) the degree of calcification; (vi) the intraplaque hemorrhage. Otherwise, statistically significant difference was found in microvascular density, in the number of CD68-positive macrophages and tryptase-positive mast cells in plaques from symptomatic patients, as compared to asymptomatic patients. Overall, this study indicate that although advanced symptomatic and asymptomatic carotid plaques present similar histomorphological characteristics, the degree of macrophage and mast cell infiltration and differences in microvascular density could help to discriminate between symptomatic and asymptomatic patients.

Keywords: angiogenesis, carotid plaque, inflammation, macrophages, mast cells.

Introduction
There are two types of carotid artery plaque: one stable, which is unlikely to produce symptoms and a second unstable with high risk of embolization and related acute cerebrovascular events. The mechanisms that underlie plaque instability involve biologic factors that are intrinsic to plaque structure and biomechanical factors that favor plaque breakdown.

Several studies have compared the morphological aspects of carotid plaques removed from symptomatic and asymptomatic patients with the aim to better define the factors involved in plaque destabilization [1–3]. Plaque rupture is more frequent in symptomatic than asymptomatic patients and in symptomatic patients [2–4] in which the fibrous cap is thinner with a greater inflammatory infiltrate [1, 2, 5, 6].

The aim of this study was to evaluate the role of major cardiovascular risk factors and histomorphological characteristics, in particular of macrophages and mast cells and of microvascular density, in atherosclerotic plaques collected from 63 consecutive symptomatic and asymptomatic patients undergoing carotid endarterectomy for carotid disease.

Materials and Methods
Study population
Atherosclerotic plaques were collected from 63 consecutive patients undergoing carotid endarterectomy for carotid disease. Patients’ biographical, clinical and instrumental data were analyzed in order to identify cardiovascular risk factors and associated disorders. Patients were divided in two groups: symptomatic patients (defined according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) Classification) [7] presenting with cerebrovascular TIAs or stroke within the last three months, and asymptomatic patients, exhibiting progressive ipsilateral internal carotid artery stenosis. The study was approved by the local ethic committee, and written informed consent was obtained from all patients.

Tissue collection
All plaques were assessed macroscopically for signs of luminal ulceration, intraplaque hemorrhage, and for the presence of a soft or ruptured necrotic core. The plaques were washed in normal saline solution, fixed in 10% neutral formalin for 24–48 hours, and representative segments of 5 mm length were cut, numbered sequentially to reconstruct the entire plaque length from proximal common carotid artery to distal segment of internal carotid artery, and included in paraffin blocks.

Histology
Four µm thick sections were stained with Hematoxylin–Eosin, Masson trichromic stain and van Gieson stain to evaluate the following histological parameters: (i) the degree of stenosis (estimated > or <70%); (ii) the extension of the lipidic core (estimated as evident, scarce or absent); (iii) the thickness of the fibrous cup (estimated >165 µm or <165 µm); (iv) the
inflammatory infiltrate (estimated as diffuse, scarce or absent); (v) the degree of calcification (estimated as diffuse, scarce or absent); (vi) intraplaque hemorrhage (estimated as present or absent).

**Immunohistochemistry**

Immunohistochemical analysis was performed on four µm thick sections prepared from paraffin blocks. All plaques specimens were examined by immunohistochemistry for the presence of endothelial marker CD31, macrophage marker CD68 and mast cell marker tryptase. Three murine monoclonal antibodies (MAbs) against CD31 (MAb 1A10 Novocastra), CD68 (PGM 1 Dako, Glostrup, Denmark), and tryptase (MAb AA1, Dako, Glostrup, Denmark) were used. Briefly, sections were collected on 3-amino-propyl-triethoxysilane coated slides, deparaffinized by the xylene-ethanol sequence, rehydrated in a graded ethanol scale and in TRIS-slushes, deparaffinized by the xylene-ethanol sequence, rehydrated in a graded ethanol scale and in TRIS-buffered saline (TBS, pH 7.6), and incubated overnight at 4°C with MAbs, after prior antigen retrieval by enzymatic digestion with Ficin (Sigma, St. Louis, MO, USA) for 30 minutes at room temperature for trypstase, and high temperature antigen retrieval using 1 mM EDTA retrieval solution (pH 8.0) for CD31 and CD68. The immunoreaction was performed with alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako) and Fast Red as chromogen for trypstase, and with the Streptavidin-peroxidase complex (LSAB2, Dako) and 3,3′-diaminobenzidine tetrahydrochloride (Dako) 5% as chromogen for CD31 and CD68, followed by Hematoxylin counterstaining. A preimmune serum (Dako) replacing the primary antibody served as negative control.

**Microvascular density**

To estimate the microvascular density, 4 to 6 200× fields covering almost the whole of each of four sections per sample of carotid plaques of symptomatic and asymptomatic patients were examined with a 144-intersection point square reticulum (0.78 mm²) inserted in the eyepiece and the microvascular density was calculated by point counting as the ratio between the number of points falling in microvessel profiles and the total number of test points. This parameter, which represents an unbiased estimator of the microvascular density, was simultaneously assessed without knowledge of the final pathological diagnosis by two investigators with a double-headed light microscope (Axioplan II, Zeiss, Oberkochen, Germany). For each analyzed section, the two estimates were then averaged to provide the final value of the parameter.

**Cell counts**

Tryptase-positive mast cells and CD68-positive macrophages counts were carried out on transversal sections of carotid plaques of symptomatic and asymptomatic patients. The counts were performed on a Zeiss Axioskop light microscope, using a micrometer grid fitted in a ×10 eyepiece at a ×200 objective magnification. Five to 10 contiguous, non-overlapping rectangular areas (each area measured 0.0117 mm²) of three sections per sample for each case, were examined. Mean, median and standard deviation were determined for each group of samples.

**Statistical analysis**

Results were expressed as mean ± standard deviation (SD). All morphometric data and cellular counts were compared between asymptomatic and symptomatic plaques using paired Student’s t-test. χ² analysis was used to compare data between both groups.

**Results**

A total of 63 patients, 64% males and 36% females, were studied. 79% suffered from hypertension, 64% from dysplipidemia, 56% from diabetes mellitus. There were no significant differences between the symptomatic and asymptomatic patients in terms of age, sex, cardiovascular risk factors (hypertension, dysplipidemia, diabetes mellitus).

No statistically significant difference was found as concerns: (i) the degree of stenosis (>70% in 72% of symptomatic patients vs. 63% of asymptomatic patients; <70% in 28% of symptomatic patients vs. 27% of asymptomatic patients, χ²=0.07); (ii) the extension of the lipidic core (evident in 59% of symptomatic patients vs. 45.4% of asymptomatic patients; scarce in 36.4% of symptomatic patients vs. 36.4% of asymptomatic patients; absent in 4.6% of symptomatic patients vs. 18.2% of asymptomatic patients, χ²=1.74); (iii) the thickness of the fibrous cup (>165 µm in 54.5% of symptomatic patients vs. 72.7% of asymptomatic patients; <165 µm in 45.5% of symptomatic patients vs. 27.3% of asymptomatic patients, χ²=0.39); (iv) the inflammatory infiltrate (diffuse in 45.5% of symptomatic patients vs. 27.3% of asymptomatic patients, scarce in 40.9% of symptomatic patients vs. 54.5% of asymptomatic patients; absent in 13.6% of symptomatic patients vs. 18.2% of asymptomatic patients, χ²=1); (v) the degree of calcification (diffuse in 18.2% of symptomatic patients vs. 27.3% of asymptomatic patients; scarce in 63.6% of symptomatic patients vs. 54.5% of asymptomatic patients, absent in 18.2% of symptomatic patients vs. 18.2% of asymptomatic patients, χ²=0.366); (vi) the intraplaque hemorrhage (present in 77.3% of symptomatic patients vs. 81.8% of asymptomatic patients; absent in 22.7% of symptomatic patients vs. 18.2% of asymptomatic patients, χ²=0.022).

Statistically significant difference was found in microvascular density (high in 63.6% of symptomatic patients vs. 18.2% of asymptomatic patients; low in 36.4% of symptomatic patients vs. 81.8% of asymptomatic patients, χ²=4.38).

Morphological analysis revealed a higher neovascularization in plaques from symptomatic patients, as compared to asymptomatic patients. New-formed blood vessels were located in the intima, interfacing the necrotic core, and at the shoulder area. There were also vessels within the necrotic core and in the adventitia (Figure 1). Moreover, we found a significant increased number of CD68-positive macrophages and tryptase-positive mast cells in plaques from symptomatic patients, as compared to asymptomatic patients (Figure 1).
Macrophages and mast cells are involved in carotid plaque instability

Figure 1 – Biopsy samples of atherosclerotic plaques from asymptomatic (A, C, E) and symptomatic patients (B, D, F), stained with antibodies anti-CD31 to mark endothelial cells (A, B), anti-CD68 to mark macrophages (C, D), and anti-tryptase to mark mast cells (E, F). Note the higher density of blood vessels, macrophages and mast cells in specimens from symptomatic vs. asymptomatic patients. Original magnification: A–F, ×200.

Morphological evidence concerning microvascular density, CD68-positive macrophages and tryptase-positive mast cells have been confirmed by morphometric analysis (Table 1).

Table 1 – Microvascular density, tryptase-positive mast cells and CD68-positive macrophages counts in carotid plaques of asymptomatic and symptomatic patients

<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic</th>
<th>Symptomatic</th>
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<tr>
<td>Microvascular density</td>
<td>12±4</td>
<td>28±5*</td>
</tr>
<tr>
<td>Mast cells</td>
<td>6±2</td>
<td>44±7*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>12±5</td>
<td>36±6*</td>
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*p<0.001 vs. asymptomatic.

Discussion

Only few studies have correlated the presence of major risk factor with the histological characteristics of atherosclerotic plaques [1, 8, 9]. In this study, by comparing symptomatic and asymptomatic patients undergoing carotid endarterectomy for carotid disease, no significant differences between the two groups in terms of age, sex, cardiovascular risk factors (hypertension, dyslipidemia, diabetes mellitus), and as concerns histomorphological characteristics of the plaque (degree of stenosis; extension of the lipidic core, thickness of the fibrous cap, inflammatory infiltrate, degree of calcification, intraplaque hemorrhage) were recognizable, while statistically significant difference was found in microvascular density, the number of CD68-positive macrophages and tryptase-positive mast cells.

Previous studies have shown that macrophage infiltration is greater in symptomatic plaques than in asymptomatic plaques. Bassiouny HS et al. [5] demonstrated that symptomatic plaques exhibit three times more macrophages infiltrating the fibrous cap compared with asymptomatic plaque and Husain T et al. [10] confirmed that macrophage accumulation within the cap of carotid atherosclerotic plaques is associated with the onset of cerebral ischemic events. Moreover, macrophage infiltration may modulate lesion progression through the release of free radicals and other mitogenic and tissue necrosis factors [11, 12]. Finally, macrophages may induce a prothrombotic effect by inhibiting tissue plasminogen activators and enhancing the thrombotic complications associated with atherosclerotic plaques [13].

As concerns the presence of mast cells, the close association of mast cells with intimal neovascularization has been observed in the deep regions of human coronary atheromas [14, 15].

Angiogenesis of an atherosclerotic arterial segment is associated with infiltration of macrophages, T-cells, and mast cells and each type of these inflammatory cells is capable of contributing to angiogenesis [16]. All these three types of inflammatory cells are present in the areas of neovascularization of the atherosclerotic plaque, revealing local inflammatory reaction [17]. Accordingly, both macrophages and mast cells contribute to the higher angiogenic response occurring in the atherosclerotic plaque of our symptomatic patients as compared to the asymptomatic ones. Both type of cells are able to secrete several angiogenic cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), and various proteases [18].

Increased plaque vascularity may contribute to lesion progression, being a source of nutrient, inflammatory cell recruitment, and intraplaque hemorrhage, which tends to weaken the plaque and predispose to plaque rupture with ensuing clinical sequel, such as stroke [18]. Moreover, the prevalence of neovascularization in carotid arteries of symptomatic patients could help to explain the more rapid development of unstable lesions with subsequent thrombosis.

These data indicating an increase in microvascular density and macrophage and mast cells populations in symptomatic vs. asymptomatic patients indicates that antiangiogenic therapy could have a beneficial effect of the progression of asymptomatic plaque. For example, one of the most successful mode of therapy against plaque destabilization is statin treatment and Koutouzis M et al. [19] demonstrated that patients treated with statin have reduced a intraplaque angiogenesis in their carotid endarterectomy specimens.

Conclusions

Overall, the results of this study indicate that although advanced symptomatic and asymptomatic carotid plaques present similar histomorphological characteristics, the degree of macrophage and mast cell
infiltration and differences in microvascular density could help to discriminate between symptomatic and asymptomatic patients.

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References

Corresponding author
Domenico Ribatti, Professor, Department of Human Anatomy and Histology, University of Bari Medical School, Piazza Giulio Cesare 11, Policlinico, 70124 Bari, Italy; Phone +39.080.5478326, Fax +39.080.5478310, e-mail: ribatti@anatomia.uniba.it

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