
A Link Between Inflammation and Cerebrovascular Events

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A central role for inflammation in the pathogenesis of atherosclerosis has been demonstrated by multiple histopathologic, experimental, and clinical studies (1–9). However, the challenge of recognizing vulnerable plaque noninvasively at early stages, and before the onset of an acute clinical event, still remains.

Carotid plaque disruption and distal embolization of atheromatous plaque debris is the most common pathogenic mechanism for cerebral ischemia from carotid atherosclerotic disease. Our group and other investigators have shown that histomorphologic composition of the atherosclerotic plaque, rather than the degree of stenosis, is a major determinant for increased risk of plaque rupture and subsequent thrombosis in the carotid territory (10–12).

It is thus clear that the clinical debate should move from whether to treat to whom to treat. Recently, several biological markers have been proposed to identify unstable, vulnerable plaques, including high-sensitivity C-reactive protein (hsCRP), serum matrix metalloproteinases (MMPs), and soluble adhesion molecules. Elevated levels of hsCRP were shown to be predictive of coronary or peripheral atherosclerosis and to constitute an independent predictor of advanced carotid plaques in hyperlipidemic subjects (13). Several MMPs are overexpressed in atherosclerotic lesions by inflammatory cells, therefore playing a role in the progression and destabilization of the disease (14–16). Recently, pregnancy-associated plasma protein-A (PAPP-A), a novel, high-molecular, zinc-binding metalloproteinase described during pregnancy, is highly expressed in both ruptured and eroded coronary unstable plaques from patients
who died suddenly from cardiac causes (17). Pregnancy-associated plasma protein-A has been found to be an independent predictor for the presence and extent of coronary artery disease in stable patients (18) and cardiovascular events in high-risk patients affected by acute coronary syndromes (19). Moreover, Beaudeux et al. (20) found that elevated serum PAPP-A levels are associated not only with the echocentricity of atherosclerotic carotid lesions but also with an enhanced inflammatory state in asymptomatic hyperlipidemic subjects.

However, little is known about PAPP-A cellular expression in carotid atherosclerotic disease and if this protein could be a marker of vulnerability in the carotid artery distribution. The aim of this study was to evaluate PAPP-A expression in stable and unstable carotid atherosclerotic plaques collected from patients undergoing surgical carotid endarterectomy.

METHODS

Case selection. Seventy-two carotid atherosclerotic plaques from patients undergoing carotid endarterectomy at Mayo Clinic and Foundation, Rochester, Minnesota (25 patients) and at San Donato Hospital, San Donato Milanese, Italy (47 patients) formed the study population. Nineteen plaques were obtained from patients who suffered a nonlacunar stroke, 24 from patients who suffered transient ischemic attack (TIA), and 29 plaques from asymptomatic patients.

A routine preoperative evaluation in all patients included clinical assessment of risk factor profile, a cerebral computed tomographic (CT) scan study, a Duplex examination, and a selective angiographic examination of extracranial and intracranial carotid arteries and their branches.

Major stroke was defined as a clinical syndrome characterized by rapidly developing focal or at times global symptoms without significant clinical improvement within seven days in the distribution of symptomatic carotid artery, not hemorrhagic, and with no cause other than vascular origin, assessed by brain CT study as a cortical or deep white matter or basal ganglia lesion bigger than 1 cm. Transient ischemic attack was defined as recent (<120 days before surgery) occurrence of any sudden focal neurological deficit that cleared completely within 24 h (12). Asymptomatic patients had never suffered from neurological symptoms and had no cerebral lesions assessed by CT.

Patients were excluded from the study if: 1) they had a probable cardiac embolus source (rhythm disorders, mitral valve stenosis, prolapose or calcification, mechanical cardiac valves, recent myocardial infarction, left ventricular thrombus, atrial myoxma, endocarditis, dilated cardiomyopathy, patent foramen ovale); or 2) symptoms that could be attributed to nonatherosclerotic disease (aneurysm, fibromuscular dysplasia); or 3) stenosis >50% of the circle of Willis.

Angiographic carotid stenosis was measured utilizing the method from the North American Symptomatic Carotid Endarterectomy Trial (NASCET) (21) by two independent physicians; intraobserver and interobserver reliability was more than 98%. The time interval range between symptom onset and carotid endarterectomy was from 3 to 22 months for patients with stroke/TIA and 4 to 21 months for asymptomatic patients.

The study was approved by both institutional review boards, and all patients gave verbal consent to the study.

Plasma protein assay of PAPP-A. Blood samples were obtained in fasting individuals by venipuncture the day before the surgical procedure, placed on ice, and centrifuged within 30 min at 1,600 × g for 5 min. All samples were analyzed by personnel who had no knowledge of the subjects' clinical data (C.O.); PAPP-A levels were determined by means of a biotin-tyramide-amplified enzyme immunoassay with a limit of detection of 0.03 mIU/l and intra-assay and inter-assay coefficients of variation of 10% and 15%, respectively. Pregnancy-associated protein-A polyclonal antibodies were used for capture (22), and a combination of monoclonal antibodies was used for detection (23) as previously described. The assay was calibrated against the World Health Organization's international reference standard 78/610, which is the standard for pregnancy-associated proteins.

Histologic sampling and light microscopy. Carotid plaques were removed en bloc during surgery to preserve plaque structure entirely. Sixty-five of those plaques were utilized for histologic examination, and six plaques for ribonucleic acid molecular assay. For histology, surgical samples were fixed for 24 h in 10% buffered formalin immediately upon removal, a method previously described by our group (12). After decalcification, specimens were sectioned transversely every 5 mm and paraffin embedded. Hematoxylin-eosin and Movat pentachrome stains were performed for morphologic study. Each segment was sequentially numbered to reconstruct the entire plaque length. For each plaque, 3 to 10 sections were examined according to the plaque length (mean, 5 sections per specimen).

Plaques were evaluated for acute thrombosis associated with plaque rupture, minimum cap thickness, and cross-sectional area of lipid-necrotic core. The latter was calculated manually, tracing the perimeter of the different components of the vessel. Plaque rupture was defined as

The following table provides the abbreviations and acronyms used in the text:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>FITC</td>
<td>Streptavidin-fluorescin conjugate</td>
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<tr>
<td>hsCRP</td>
<td>High-sensitivity C-reactive protein</td>
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<tr>
<td>ICM</td>
<td>Immunofluorescence confocal microscopy</td>
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<td>MMPs</td>
<td>Serum matrix metalloproteinases</td>
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<td>NASCET</td>
<td>North American Symptomatic Carotid Endarterectomy Trial</td>
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<tr>
<td>PAPP-A</td>
<td>Pregnancy-associated protein-A</td>
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<tr>
<td>RT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>TIA</td>
<td>Transient ischemic attack</td>
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complete disruption of a fibrous cap over a lipid core, with acute thrombus in contact with the lipid pool. Thrombus was defined as platelets or fibrin on the plaque surface, and characterized by lamination with or without red and white cells interspersed (24).

Immunohistochemistry. To characterize plaque’s cell population and PAPP-A expression, single-label immunohistochemistry was performed. Five micron sections were deparaffinized, and endogenous peroxidase activity was blocked with H2O2. Primary antibodies (anti-human CD3, dilution 1:100, Dako, Glostrup, Denmark; anti-human neutrophil CD15, dilution 1:100, Sigma, St. Louis, Missouri) were applied for 1 h at room temperature followed by a secondary antibody incubation (biotinylated goat anti-mouse, dilution 1:40 or goat anti-rabbit, dilution 1:20) for 30 min at room temperature. Then, avidin biotin amplification (ABC kit) was applied for 30 min at room temperature. Incubation with 0.1% 3',3'-diaminobenzidine and H2O2 at room temperature for 5 to 10 min produced a brown reaction pigment. A light hematoxylin counterstaining was used to visualize all nuclei in the tissue sections. Control sections were incubated with a mixture of irrelevant monoclonal reagents with a similar isotype.

Two pathologists (A.M. and E.B.) blinded to the clinical findings evaluated all histocytologic components of the plaques. Intra- and interobserver variability was <95%. Cell counting was performed at magnification of 40X using a test grid with an area of 0.22 mm². An average of 10 fields per section or a number of microscopic fields until the SEM was less than 5% were counted. The PAPP-A positivity was semiquantitatively scored as maximal (3+), moderate (2+), low (1+), very low (0.5), or absent (0).

Histologic definitions and atherosclerotic plaque types. Plaques were classified according to the modified American Heart Association atherosclerosis classification (25) and to the recent consensus document of the American Heart Association (26) into three categories: ruptured plaques, characterized by the presence of an acute thrombus associated with plaque rupture; and vulnerable plaques, including thin fibrous cap atheroma, characterized by a lesion composed of a lipid-rich core covered by a <65 μm-thick fibrous cap containing many lipid-laden macrophage foam cells (>25 per high-power magnification) (25). The remaining plaques were classified as stable plaques.

Based on histologic characteristics, carotid plaques were thus divided into three groups (Table 1): Group 1 comprised 38 stable atherosclerotic plaques (5 of whom showed organized thrombosis) and were considered as control group, Group 2 comprised 13 “vulnerable but not ruptured plaques”, and Group 3 comprised 14 “ruptured plaques” showing cap rupture and thrombosis at the histologic examination.

Confocal immunohistochemistry microscopy. To characterize the cells’ subpopulations expressing PAPP-A, double immunostaining for confocal microscopy was performed in selected cases. Briefly, 5-μm paraffin sections of carotid plaques were first incubated with monochlonal antibodies to PAPP-A (clone 234-5, dilution 1:100, 1 h at room temperature), rinsed and then incubated with biotinilated mouse IgG, then rinsed again and incubated with antibodies conjugated to streptavidin peroxidase. Fluorescence was obtained by incubating the sections with a streptavidin-fluorescin conjugate (FITC). After this first reaction, a second reaction with primary antibodies respectively to SMA, CD68, CD3 (dilution 1:100, 1 h at room temperature in the dark) was performed as described above, and fluorescence was obtained with a streptavidin-Texas Red fluorescent conjugate. Control sections were incubated with a mixture of irrelevant monoclonal reagents with a similar isotype. Specimens were observed under an Olympus fluorescence microscope. Images were acquired by means of Noran confocal microscope at 60X/1.4 NA immersion oil lens. Three-dimensional stacks were acquired at resolution of 0.1 μm in X, Y, Z axis.

Quantitative real-time polymerase chain reaction (RT-PCR) assay. To demonstrate local production of PAPP-A within the carotid tissue, a RT-PCR assay was performed on seven carotid plaques (one from asymptomatic patient, four from TIA, and two from stroke) by personnel who had no knowledge of the subjects’ clinical data (C.A.C). Briefly, after removal from the patient of the carotid plaque, a tissue ring was immediately obtained by cutting the central segment of the anatomic tissue sample, submerged in plastic vials containing 3 cc of RNA later ribonuclease acid stabilization reagent (Qiagen, Valencia, California) and immediately stored at −20°C. Total ribonuclease acid was extracted from plaques using the RNeasy Mini Kit (Qiagen) and treated with deoxiribonuclease (DNA-free, Ambion, Inc., Austin, Texas). Four hundred nanograms of ribonuclease acid were reversed transcribed using TaqMan RT reagents (PE Biosystems, Foster City, California) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction analyses were performed using the ABI PRISM 7700 Sequence Detection System and software (PE Applied Biosystems, Foster City, California). Primer and probe sequences for specific detection and amplification of PAPP-A and 28S have been described previously (27). Relative messenger ribonuclease acid abundance was calculated using the 2-TΔΔC method (28).

Table 1. Clinical Presentation and Plaque Histologic Types Observed in the 65 Carotid Plaques Submitted to Histologic Examination

<table>
<thead>
<tr>
<th>Histologic Definition</th>
<th>Stroke (17 Plaques)</th>
<th>TIA (20 Plaques)</th>
<th>Asymptomatic (28 Plaques)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured plaques</td>
<td>7 (41.2%)</td>
<td>4 (20.0%)</td>
<td>3 (10.7%)</td>
</tr>
<tr>
<td>Vulnerable plaques</td>
<td>5 (29.4%)</td>
<td>4 (20.0%)</td>
<td>4 (14.3%)</td>
</tr>
<tr>
<td>Stable plaques</td>
<td>5 (29.4%)*</td>
<td>12 (60.0%)</td>
<td>21 (75.0%)</td>
</tr>
</tbody>
</table>

*All five stable plaques corresponded to plaques with organized thrombosis. TIA = transient ischemic attack.
Statistical analysis. Statistical analysis of the data was performed using SPSS for Windows (version 11.0, SPSS Inc., Chicago, Illinois). Categorical variables were described in frequencies. Unpaired t test and one-way analysis of variance was utilized to assess intergroup differences for continuous variables. The chi-square test was used to establish associations among categorical variables. Correlation coefficients describing the relationship between PAPP-A positivity, cap thickness, and plaque inflammation were also calculated. A value of p < 0.05 was considered as statistically significant in all analysis.

RESULTS

Clinical data. No differences were found between clinical variables and risk factor profiles among of the three study groups (Table 2). Patients who suffered a stroke showed a mean percentage angiographic stenosis of the ipsilateral internal carotid artery of 80.7 ± 10.5%, TIA patients of 80.3 ± 6.2%, and asymptomatic patients of 80.6 ± 8.0% (p = NS). Mean percentage contralateral angiographic stenosis was similar in the three study groups (64.3 ± 22.4% for stroke vs. 64.4 ± 25.9% for TIA vs. 51.5 ± 23.0% for asymptomatic; p = NS).

Plaque morphology. All plaques examined contained a large lipid-necrotic core with cholesterol clefts, sometimes associated with widespread calcification, covered by a fibrous cap. Plaque cellular composition was quite variable but consisted principally of smooth muscle cells and inflammatory cells. These cells, macrophage-foam cells (CD68-positive cells), and T-lymphocytes (CD3-positive) were found in variable amounts around the lipid-necrotic core (shoulder of the plaque) and in the fibrous cap. No neutrophils were observed within the plaque at the histologic examination.

In Group 1 (stable plaques) five plaques with an organized thrombus were characterized by fibrous tissue, sometimes stratified, associated with typical angiomatosis, with a network of large, thin-walled vascular channels and a variable number of macrophage cells loaded with hemosiderin, visible as scattered brown refractive pigments. These plaques were obtained from patients with lacunar stroke operated at least 18 months after symptoms onset. “Vulnerable” and “ruptured” plaques (Groups 2 and 3) were characterized by a thinner cap and a higher inflammatory infiltrate, principally monocytes/macrophages (CD68-positive) and T lymphocytes (CD3-positive), both in the cap and in shoulder, as compared with stable plaques (Group 1).

A highly significant difference was present in the inflammatory cell infiltrate (CD3 and CD68) between stable and both ruptured and vulnerable plaques (19.1 ± 1.3 cell/mm² in Group 1; 47.0 ± 5.6 cell/mm² in Group 2; 54.7 ± 2.5 cell/mm² in Group 3; Group 1 vs. Group 2 p = 0.001; Group 1 vs. Group 3, p = 0.001; respectively). Conversely, no significant differences were observed in the amount of inflammatory cell infiltrates between Group 2 and Group 3 (p = 0.23).

Immunohistochemical and confocal localization of PAPP-A. Single immunostaining PAPP-A antibody showed a diffuse positive reaction both in Group 2 “vulnerable plaque” and Group 3 “ruptured plaque.” In both groups there was a strong and diffuse reaction for PAPP-A, mainly in the foam cells of the cap and shoulder region of the plaque. Conversely, in Groups 1 and 2 the reaction was faint and focal.

Semiquantitative analysis of PAPP-A localization showed a mean score value of 0.62 ± 0.06 for stable plaques, 2.54 ± 0.14 for vulnerable plaques, and 2.71 ± 0.12 for ruptured plaques (Group 1 vs. Group 2, p = 0.001; Group

Table 2. Clinical Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>Asymptomatics (29 Patients)</th>
<th>TIA (24 Patients)</th>
<th>Stroke (19 Patients)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>69.7 ± 6.3</td>
<td>69.8 ± 6.7</td>
<td>66.7 ± 9.3</td>
<td>0.31</td>
</tr>
<tr>
<td>Male gender (F/M)</td>
<td>21 (72.4%)</td>
<td>19 (72.9%)</td>
<td>14 (73.7%)</td>
<td>0.84</td>
</tr>
<tr>
<td>Angiographic stenosis (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ipsilateral carotid</td>
<td>80.6 ± 8.0</td>
<td>80.3 ± 6.2</td>
<td>80.7 ± 10.5</td>
<td>0.99</td>
</tr>
<tr>
<td>Contralateral carotid</td>
<td>51.5 ± 23.0</td>
<td>64.4 ± 25.9</td>
<td>64.3 ± 22.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>19 (65.5%)</td>
<td>10 (38.3%)</td>
<td>16 (84.2%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>2 (6.9%)</td>
<td>1 (4.2%)</td>
<td>0</td>
<td></td>
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<tr>
<td>Type II</td>
<td>4 (13.8%)</td>
<td>3 (12.5%)</td>
<td>6 (31.6%)</td>
<td>0.37</td>
</tr>
<tr>
<td>Cigarette smoking (%)</td>
<td>3 (10.3%)</td>
<td>6 (25.0%)</td>
<td>6 (31.6%)</td>
<td>0.17</td>
</tr>
<tr>
<td>Hypercholesterolemia (%)</td>
<td>19 (65.5%)</td>
<td>15 (62.5%)</td>
<td>16 (84.2%)</td>
<td>0.26</td>
</tr>
<tr>
<td>Hypertrygliceridemia (%)</td>
<td>9 (31.0%)</td>
<td>10 (41.7%)</td>
<td>11 (57.9%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>1 (3.4%)</td>
<td>3 (12.5%)</td>
<td>4 (21.1%)</td>
<td>0.16</td>
</tr>
<tr>
<td>History of coronary artery diseases (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable angina (%)</td>
<td>3 (10.3%)</td>
<td>5 (20.8%)</td>
<td>1 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>Unstable angina (%)</td>
<td>0</td>
<td>1 (4.2%)</td>
<td>1 (5.3%)</td>
<td>0.40</td>
</tr>
<tr>
<td>Medication with statins (%)</td>
<td>12 (41.4%)</td>
<td>9 (37.5%)</td>
<td>10 (52.6%)</td>
<td>0.59</td>
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Values are mean ± SD or n (%).
TIA = transient ischemic attack.
1 vs. Group 3, p = 0.001; Group 2 vs. Group 3, p = 0.37, respectively).

A significant inverse correlation between cap thickness and PAPP-A score was observed in plaque samples ($r = 0.62, p = 0.01$) (Fig. 1A), whereas positive correlation was present between plaque inflammation and PAPP-A score ($r = 0.70, p = 0.01$) (Fig. 1B), suggesting that both vulnerable and ruptured plaques are characterized by high PAPP-A expression.

Double immunostaining for confocal microscopy confirmed strong and diffuse expression of PAPP-A in carotid plaques from Groups 2 and 3 (Figs. 2D to 2L). Double immunostaining showed that almost all monocyte/macrophages, both in the cap and in shoulder of the plaque, strongly expressed PAPP-A.

Figure 1. Graphs showing linear regression between pregnancy-associated protein-A (PAPP-A) score versus plaque thickness (A) and PAPP-A score versus total cells’ plaque cap inflammatory infiltrate (B) for the 65 carotid atherosclerotic plaques submitted to histopathologic examination. A decrease in cap thickness and an increase in inflammatory infiltrate in the cap region of the plaque are both strongly correlated with increase in PAPP-A expression.
Figure 2. Photomicrographs of a carotid atherosclerotic plaque removed by carotid endarterectomy from a patient who suffered a major stroke. A vulnerable plaque characterized by a large lipidic-necrotic core, a thin fibrous cap (A, Movat pentachrome, 4×) associated to a heavy inflammatory infiltrate constituted mainly by macrofagic foam cells, CD68-positive (B, 10×). A strong positive reaction for pregnancy-associated protein-A was observed (C, 20×). (D, 10× and G, 20×) show CD68 antibody revealed by streptavidin-Texas Red fluorescent conjugate. (E, 10× and H, 20×) Show pregnancy-associated protein-A reaction revealed by streptavidin-fluorescin conjugate. Two dimension confocal analysis of the two fluorochromes (F and I) clearly showed a strong and diffuse positive reaction for pregnancy-associated protein-A in macrophagic cells (concordant double positivity appears as a yellow stain). (K) Shows confocal analysis of the two fluorochromes, smooth muscle actin immunostain (red) and pregnancy-associated protein-A stain (green). Smooth muscle cells observed in this plaque gave rise to a strong positive reaction for pregnancy-associated protein-A (yellow stain). (L) Shows confocal analysis of the two fluorochromes, CD3 immunostain (red) and pregnancy-associated protein-A stain (green). Almost all T lymphocytes (red) observed were negative for pregnancy-associated protein-A in plaques where a diffuse positive reaction was observed (green). Only occasional T lymphocytes were pregnancy-associated protein-A-positive (double positivity, yellow stain, arrow).
Moreover, in the same plaques, PAPP-A was less expressed by smooth muscle cells, whereas T-lymphocytes (CD3-positive) did not co-localize with PAPP-A. Stable plaques and plaques with organized thrombosis showed focal PAPP-A expression co-localizing only with few monocytes/macrophages and smooth muscle cells expressing PAPP-A (Figs. 3D to 3H).

**PAPP-A plasma assessment.** Patients with vulnerable and ruptured plaques (Groups 2 and 3) showed significant greater plasmatic expression of PAPP-A compared with patients with stable plaques (Group 1). In particular, PAPP-A plasma values were 4.02 ± 0.18 mIU/l in Group 1, 7.43 ± 0.97 mIU/l in Group 2, and 6.97 ± 0.75 mIU/l in Group 3 (Group 1 vs. Group 3, p = 0.01; Group 1 vs. Group 2, p = 0.004; Group 2 vs. Group 3, p = 0.71, respectively). Pregnancy-associated protein-A plasma levels significantly correlated with PAPP-A score (r = 0.35, p = 0.035), suggesting that circulating levels of this biomarker reflect its expression within the plaque.

**RT-PCR assay.** Quantitative real-time polymerase chain reaction demonstrated local messenger ribonucleic acid expression in carotid plaques from symptomatic patients (two affected by stroke and four affected by TIA) relative to a plaque from an asymptomatic patient (Fig. 4). All samples showed detectable PAPP-A messenger ribonucleic acid with two of the plaques from patients affected by stroke demonstrating PAPP-A messenger ribonucleic acid levels two- to four-fold greater than control.

**DISCUSSION**

The present study shows for the first time a clear association between PAPP-A, carotid plaque morphology, and related complications. First, PAPP-A was highly expressed in complicated and vulnerable carotid plaques (i.e., with cap rupture or with a thin fibrous plaque associated with a high inflammatory infiltrate) and was mainly found in both monocyte/macrophage cells present in the cap and shoulder region of the plaque. Second, carotid plaques classified as stable showed little inflammatory cell infiltration and PAPP-A expression in a small percentage of monocyte/macrophage cells. Third, blood analysis demonstrated that PAPP-A levels are elevated in the plasma of patients bearing vulnerable/rupture carotid plaques compared with stable plaques. Therefore, the study strongly suggests that PAPP-A is a specific marker of carotid plaque vulnerability and that the clinical evaluation of this marker may be of extreme help in identifying not only “the plaque” at risk but, moreover, “the patient” at risk of developing an acute cerebrovascular event.

Clinical trials aimed to demonstrate a benefit of surgical carotid endarterectomy in reducing the risk of cerebrovascular events have focused on carotid stenosis severity as major risk factor for cerebrovascular events. The results of the NASCET study (21), the European Carotid Surgery Trial (ECST) (29), and the Asymptomatic Carotid Atherosclerotic Study (ACAS) (30) suggest that the benefit of surgery is higher in the presence of severe carotid stenosis and that the aggregate risk over five years for ipsilateral stroke and any perioperative stroke is around 11% for asymptomatic patients treated medically. In general, it has been demonstrated that asymptomatic severe carotid stenosis carries a low stroke risk rate (31,32). Therefore, these data demonstrate that stenosis grade is not sufficient to identify patients at high risk of acute cerebrovascular events, and other risk factors are of greater importance.

While it is likely that some strokes associated with carotid artery disease result from hypoperfusion (33), the majority of such strokes appear to result from embolization from a complicated atherosclerotic plaque or acute occlusion of the carotid artery with propagation of thrombus in the distal cerebral vascular bed. Different histopathologic studies have evaluated the morphological aspects of carotid plaques removed from symptomatic and asymptomatic patients to focus on mechanisms underlying plaque destabilization (11,34–37). These studies showed that plaque rupture or ulceration is more common in symptomatic patients, but lumen thrombus and intraplaque hemorrhage is not (38). Moreover, the fibrous cap of symptomatic plaques is thinner with greater inflammation, especially macrophage and T lymphocytes (38–40). In this setting, we have demonstrated that patients affected by hyperfibrinogenemia were at increased risk of carotid thrombosis and plaque rupture related to a greater inflammatory infiltrate and thinner atherosclerotic plaque cap compared with patients with lower fibrinogen levels, independently from other risk factors (24).

Finally, high levels of MMPs have been demonstrated at the site of inflammatory infiltrate in the fibrous cap (41). Because plaque rupture depends on a balance between the tensile strength of the plaque and stress exerted on it, rupture is likely triggered by a sudden increase in stress on the plaque or a sudden reduction in plaque strength. While the former may be caused by sudden increase in blood pressure or pulse rate, the latter may be caused by increased collagen breakdown due to MMPs release at the fibrous cap site. In this setting, increased expression of PAPP-A may induce an increase in MMPs due to macrophage chemotaxis activation and tumor necrosis factor-alpha release (42).

At present, sophisticated imaging techniques such as pixel density analysis and elastography at Duplex examination (43,44), magnetic resonance imaging tissue characterization (45), or local temperature probes (46,47) all hold promise for identifying vulnerable plaques and detecting silent atheroma noninvasively. In addition, inflammatory circulating markers such as hsCRP, and adhesion molecules such as intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 have been correlated with high density of inflammatory cells in the cap and are potential candidates for vulnerable plaque detection (48–52).

Bayes-Genis et al. (17) demonstrated that PAPP-A, a novel zinc-binding MMP that stimulates insulin-like
Figure 3. Photomicrographs of a stable carotid atherosclerotic plaque removed by carotid endarterectomy from an asymptomatic patient. In the shoulder of the plaque (A, Movat pentachrome, 4×), there is a moderate inflammatory infiltrate, constituted mainly by macrophagic foam cells, CD68-positive (B, a contiguous section of A). The expression of pregnancy-associated protein-A is very low (C, contiguous section of A). Only few cells are pregnancy-associated protein-A-positive (inset). (D) (20×) shows CD68 antibody revealed by streptavidin-Texas Red fluorescent conjugate. (E) (20×) Pregnancy-associated protein-A reaction revealed by streptavidin-fluorescin conjugate. Two dimension confocal analysis of the two fluorochromes reported in F shows that monocytes/macrophages resident in this “stable” plaque were almost negative for pregnancy-associated protein-A. The pregnancy-associated protein-A reaction showed only slight and focal positivity. Smooth muscle cells were almost negative for pregnancy-associated protein-A. The pregnancy-associated protein-A reaction showed only slight and focal positivity (G, concordant double positivity appears as a yellow stain, arrow, 10×). (H) T lymphocytes and pregnancy-associated protein-A double immunostain (10×). Few T lymphocytes (CD3 antibody revealed by streptavidin-Texas Red fluorescent conjugate) were observed in this plaque, and the reaction for pregnancy-associated protein-A was negative (pregnancy-associated protein-A revealed by streptavidin-fluorescin conjugate).
growth factor-1 and acts as a pro-atherosclerotic molecule promoting cell growth responses (42), was associated with acute coronary syndromes in patients affected by coronary artery disease. These authors found that serum levels of circulating PAPP-A were increased in patients with unstable angina and myocardial infarction. At histopathologic examination, PAPP-A was also highly expressed in coronary vulnerable plaques but was only minimally expressed in stable plaques. Moreover, the same study demonstrated that C-reactive protein levels were elevated in 50% of patients with acute coronary syndromes, whereas PAPP-A levels were significantly high in 85% of patients with unstable angina and in 94% of patients with myocardial infarction. Thus, these authors suggested that C-reactive protein is perhaps a sensitive marker of low-grade, diffuse vascular inflammation, whereas PAPP-A seems to be a valuable marker for detecting unstable coronary disease even when the levels of C-reactive protein are not elevated. Pregnancy-associated protein-A plasma levels have been recently demonstrated to correlate with the presence and extent of coronary artery disease in patients affected by stable chronic angina (18). Moreover, an elegant study done by Heeschen et al. (19) demonstrated that PAPP-A reliably identifies high-risk patients affected by coronary artery disease and is a strong independent predictor of cardiovascular events. On the carotid district, Beaudeux et al. (20) demonstrated that in asymptomatic dyslipidemic subjects, elevated serum levels of PAPP-A were associated with more hyperechoic or isoechoic atherosclerotic carotid lesions and with an enhanced inflammatory state compared with patients with hypoechoic lesions, as well as normolipidemic controls.

The present study confirms and extends these previous observations, giving histopathologic rationale to the identification of PAPP-A as a potential marker of vulnerable carotid atherosclerotic lesions. Our results indicate that PAPP-A is expressed in vulnerable and ruptured carotid plaques but not in stable ones. Moreover, the production of PAPP-A by activated cells and its release into the extracellular matrix appear to be strongly linked to the local inflammatory process occurring within the carotid plaque. In this setting, serologic analysis demonstrating that circulating PAPP-A levels are associated with vulnerable and ruptured lesions may help in identify high-risk lesion/patient subsets before the onset of clinical symptoms. In addition, the present study demonstrates by RT-PCR a local production of PAPP-A within the carotid plaque and not a merely absorption of this marker from the blood. All symptomatic plaques showed increased PAPP-A production compared with control plaques, and interplaque variability existed between different samples. This suggests the hypothesis that the latter may be due to different inflammatory cell infiltration related, in turn, to different biological activity. In addition, it can not be excluded that a different time interval between symptom onset and surgical plaque removal may also have influenced the amount of PAPP-A expression.

Conclusions. The present study indicates that PAPP-A is expressed in symptomatic patients bearing complex carotid plaques characterized by increased inflammatory cell infiltration compared with stable plaques from asymptomatic subjects. It is likely that pharmacologic or mechanical treatments aimed both to decrease the inflammatory infiltration occurring in high-risk lesions and stabilize the atherosclerotic plaque will reduce the risk of subsequent cerebrovascular events. Although clinical assessment is fundamental to estimate stroke risk, the future use of PAPP-A as a marker of vulnerable carotid atherosclerotic plaque may help in identifying patients at higher risk among those with carotid stenosis. In this setting, an early determination of which plaque carries a higher risk of rapid destabilization by the means of a biochemical marker detection would contribute to improving the quality of management of ischemic stroke. Furthermore, future studies are necessary to establish if carotid plaque stabilization by the means of mechanical intervention may be also coupled with a reduction in vulnerable markers expression.

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