Evidence for a Proinflammatory and Proteolytic Environment in Plaques From Endarterectomy Segments of Human Carotid Arteries

Marilena Formato*, Miriam Farina*, Rita Spirito, Marco Maggioni, Anna Guarino, Gian Mario Cherchi, Paolo Biglioli, Celina Edelstein, Angelo M. Scanu

Objectives—Based on previous observations on apolipoprotein(a), apo(a), in human unstable carotid plaques, we explored whether in the inflammatory environment of human atheroma, proteolytic events affect other hepatic and topically generated proteins in relation to the issue of plaque stability.

Methods and Results—Forty unstable and 24 stable plaques from endarterectomy segments of affected human carotid arteries were extracted with buffered saline (PBS) and then 6 mol/L guanidine-hydrochloride (GdHCl) to identify loosely and tightly bound products, respectively. The extracts were studied before and after ultracentrifugation at d 1.21 g/mL. In the extracts, the concentrations of interleukin (IL)-6, -8, and -18 were significantly higher in the unstable plaques and correlated to those of MMP-2 and MMP-9. By Western blots, both apoB and apo(a) were highly fragmented and mostly present in the d 1.21 bottom that also contained fragments of apoE (10 and 22 kDa), decorin, biglycan, and versican. Fragmentation was higher in the unstable plaques. In baseline plasmas, concentrations of lipids, lipoproteins, and ILs did not differ between patients with unstable and stable plaques.

Conclusions—In unstable and to a lesser extent in stable plaques, there is a proinflammatory and proteolytic microenvironment with the generation of fragments with potential pathobiological significance that requires investigation. (Arterioscler Thromb Vasc Biol. 2004;24:129-135.)

Key Words: stable and unstable carotid plaques ■ apolipoproteins ■ interleukins ■ metalloproteinases ■ proteoglycans

Methods

Chemicals and Reagents

Materials purchased from Sigma Chemical were (4-amidinophenyl)-methane-sulfonyl fluoride (APMSF) and leupeptin; kallikrein inactivator (KI) was from Calbiochem, immobilon-P membranes from Millipore, and an enhanced chemiluminescent kit (ECL Western Blotting Detection kit) from Amersham. Human MMP-2, MMP-9, IL-6, IL-8, and IL-18 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. All other chemicals were reagent grade.

For the source of the antibodies, please see online text, available at http://atvb.ahajournals.org.

Patient Population

Sixty-four white subjects underwent carotid endarterectomy for occlusive artery disease in the Cardiovascular Unit of the Istituto Monzino of the University of Milan. Their characteristics are

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summarized in the Table. Carotid atherosclerosis was assessed by duplex ultrasound sonography. The patients selected for surgery had either a high-grade stenosis (>70%) or an ulcerated lesion of a medium grade based on echo-Doppler analysis. Of the total number of subjects who underwent surgery, 17 were symptomatic regarding transient ischemic attacks, 30 were hypertensive, 53 were dyslipidemic, and 30 were smokers. The mean age of the subjects was 70.6 years, and the sex ratio was 42/22.

### Methods

#### Plasma Analyses

Before surgery, blood was collected in Vacutainer tubes containing EDTA. After centrifugation at 1000g at 4°C for 15 minutes, the plasma was separated; supplemented with 100 μmol/L APMSF, 2 μg/mL KI, and 50 μmol/L leupeptin; and stored at −80°C. Total cholesterol, triglycerides, and HDL cholesterol were determined in plasma by the College of American Pathologists. Total LDL cholesterol was calculated by the Friedewald formula. In high-lipoprotein a (Lp[a]) subjects, this value was corrected for the contribution by Lp(a) apoB assuming an average molecular weight for apo(a) of 500 000. ApoB was determined by ELISA, and the value was corrected for the contribution by Lp(a) apoB assuming an average molecular weight for apo(a) of 500 000. The Lp(a) protein assay was insensitive to apo(a) size polymorphism. Apo(a) phenotyping was performed by 4% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) blotted by using a specific polyclonal anti-apo(a) antibody. The number of kringles was estimated by using a standard mixture of apo(a) recombinant of known krigle IV repeats, a gift from Dr E. Angles-Cano (INSERM, Paris, France).

#### Plaque Analyses

The surgically excised plaques were rinsed in ice-cold saline, containing the same antiproteolytic agents as those used for plasma, and immediately delivered on ice to the histopathology laboratory, where they were sectioned into 2 segments: one for biochemical studies and the other for pathology and plaque classification. For histological examination, the plaques were formalin-fixed, paraffin-embedded, and stained with hematoxylin-eosin, Masson’s trichrome, and orcein. The criteria for plaque instability were presence of a thin fibrous cap, evidence of inflammation, and a clear ulceration over the necrotic core. Moreover, by Masson’s and orcein stain, there was evidence for collagen and elastic fibers fragmentation. The diagnosis was established on the analysis of several samples and was blinded.

### Clinical Characteristics and Lipid and Lipoprotein Levels of Study Subjects

<table>
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<th>Parameters</th>
<th>All (n=64)</th>
<th>Unstable (n=40)</th>
<th>Stable (n=24)</th>
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<tr>
<td>Age, y</td>
<td>70.6 ± 7.8</td>
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<td>Sex ratio, m/f</td>
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<td>29/11</td>
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<td>Body mass index, kg/m²</td>
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<td>Symptomatic, %</td>
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<td>10</td>
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<tr>
<td>Diabetes, %</td>
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<td>Dyslipidemic, %</td>
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<tr>
<td>Smokers, %</td>
<td>30</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>125 ± 57.6</td>
<td>118.2 ± 55.5</td>
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<td>Total cholesterol, mg/dL</td>
<td>177.7 ± 29.7</td>
<td>179.2 ± 31.3</td>
<td>173.8 ± 30.7</td>
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<td>LDL cholesterol (total), mg/dL</td>
<td>116 ± 28</td>
<td>121 ± 25.5</td>
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<td>Lp(a)-protein, mg/dL</td>
<td>2.4 (1–37.7)</td>
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<td>4.3 (1–37.7)</td>
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<td>Lp(a)-cholesterol, mg/dL</td>
<td>3.2 (1.3–49)</td>
<td>1.6 (1.3–28.6)</td>
<td>5.6 (1.3–49)</td>
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<td>LDL cholesterol (corr)*, mg/dL</td>
<td>107.8 ± 30.3</td>
<td>111.5 ± 27.6</td>
<td>95 ± 28</td>
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<td>ApoB, mg/dL</td>
<td>86.8 ± 24.6</td>
<td>90.1 ± 20.4</td>
<td>84.7 ± 24.2</td>
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<td>HDL cholesterol, mg/dL</td>
<td>35.5 ± 10.4</td>
<td>35.7 ± 11.9</td>
<td>35.6 ± 9</td>
</tr>
</tbody>
</table>

Values are mean ± SD or median with ranges in parenthesis.

*See Methods.*
concentration in the extracts was determined by the Lowry method, using bovine serum albumin as a standard. The concentration of MMP-2, MMP-9, IL-6, IL-8, and IL-18 was determined by ELISA using commercial kits. We also quantified apo(a) by a sandwich ELISA, using anti-Lp(a) for coating and alkaline phosphatase-conjugated anti-apo(a) for detection and apoB by the same ELISA procedure as for plasma.

In both PBS and GdHCl extracts, as well as top and bottom fractions, Western blot analyses were performed by using specific antibodies against apo(a), F1, F2, apoB, apoE, decorin, biglycan, and versican. The electrophoretic analyses were conducted under nonreducing and reducing conditions (in the presence of 3% β-mercaptoethanol) on both 4% and 4% to 20% SDS-PAGE. In all cases we used a Novex system for 1.5 hours at constant voltage (130 V) at 22°C. After electroblotting, the Immobilon blots were blocked in PBS containing 5% fat-free dry milk and 0.3% Tween 20, followed by incubation with the given antibody. The blots were visualized with the ECL Western Detection Reagent. The immuno-stained bands were scanned by using a GDS-8000 System (UVP BioImaging Systems).

Statistical Analyses
Statistical analyses were performed according to the method of Glantz.10 The difference between unstable and stable plaques regarding the parameters studied in the plasma and in the extracts was carried out by using the paired Student t test. The correlation between MMP and IL concentrations in the extracts was performed by the nonparametric Spearman rank test that was also used to correlate parameters studied both in plaques and plasma. Value of P<0.05 were considered to be significant.

Results

Studies in Plasma
As shown in the Table, the mean values of the parameters studied were in normal limits, except that in both unstable and stable plaques, the HDL cholesterol levels were well below the normal values recommended by the Adult Treatment Panel III guidelines.11 Only the true LDL cholesterol, ie, corrected for the contribution by Lp(a) cholesterol, approached significance (P=0.06). Moreover, the plasma Lp(a) levels, expressed as protein, were within normal limits. The number of the KIV repeats of the apo(a) isoforms varied from 15 to 30; there was no difference in their distribution between unstable and stable plaques. Also no significant difference between the 2 groups was observed for the 3 ILs studied. For IL-6 the median values in the unstable and stable plaques were 3.61 and 3.90 pg/mL; for IL-8, 14.32 and 11.70 pg/mL; and for IL-18, 263.81 and 327.60 pg/mL, respectively.

Correlation Between Echo-Doppler Analyses and Pathology Findings
Patients presenting with medium-grade ulcerated lesions by echo-Doppler were also those having plaques meeting the criteria of instability by morphological criteria. The expression of the lesion varied from subject to subject. All stable plaques had a stenotic lumen and had a fibrocalcific appearance by echo-Doppler.

Studies in Plaque Extracts

ILs and MMPs
As shown in the scatter diagrams in Figure 1 (please see online Figure I, available at http://atvb.ahajournals.org), the median concentrations of IL-6 was significantly higher in the unstable than in the stable plaques both in the PBS and GdHCl extracts. This was also true for IL-8 and for IL-18, but only for the PBS extracts because very little of this IL was recovered in the GdHCl fraction. The concentration of MMP-2 was slightly higher in the unstable than in the stable plaques, both in the PBS and GdHCl extracts. The difference was significantly higher for MMP-9 in the PBS extracts. Of note, <10% of this MMP was found in the GdHCl extract.

Apolipoproteins

Apo B
In both unstable and stable plaques, there was no significant difference between the concentrations of apoB in the PBS and in GdHCl extracts (please see online Figure II, available at http://atvb.ahajournals.org). As exemplified in the 4% gel results in Figure 2A, some intact apoB was present in the PBS extracts of the unstable (16%) and stable plaques (35%), whereas there was no intact protein in the GdHCl extracts. The size of the fragments were much smaller in the GdHCl than in the PBS extracts, a high percentage of them (62% for the unstable and 43% for the stable) accumulating in the front 110-kDa band. To resolve the lower-molecular-weight fragments, we analyzed the samples on 4% to 20% gels (Figure 2B). The results corroborated the relatively higher degrada-

Figure 1. Representative examples of stable (A) and unstable (B) plaques. The sections were stained with hematoxylin-eosin, Masson’s trichrome, and orein. Magnification, ×4.
tion of apoB in the GdHCl extracts. Of note, all fragments, irrespective of plaque derivation, were recovered in the d 1.21 bottom, indicating lack of Lp association. On the other hand, the product that in the gels migrated in the 500-kDa position floated at d 1.21 (data not shown), representing either an intact LDL or a highly lipidated apoB. Although there was some variability in results among subjects, the fragmentation of apoB was consistently higher in the unstable than in the stable plaques.

Apo(a)
As shown by ELISA, the concentration of apo(a) in the GdHCl was significantly higher than in the PBS extracts, both in the unstable and stable plaques (online Figure II). We also found a positive correlation ($P=0.009; r=0.46$) between the concentration of Lp(a) protein in the plasma and the apo(a) concentration in the total GdHCl extracts. As shown in the 4% gel blots (Figure 3A), the fragmentation of apo(a) in both unstable and stable plaques was more marked in the GdHCl extracts than in the PBS extracts. In each case, the small-molecular-weight fragments accumulating in the 110-kDa front was resolved in the 4% to 20% gels (Figure 3B), showing the lowest band in the 56-kDa range. By using antibodies specific for either F1 or F2, we found that subspecies of each of those fragments were present (data not shown). Of interest, when 2 apo(a) size isoforms were present in the plasma, they were also present in small amounts but with the same ratio, in the PBS extracts of the stable plaques (see example in Figure 3A). Moreover, the ultracentrifugation studies showed that both intact and the apo(a) fragments were in the d 1.21 g/mL; top and bottom were separated on 4% SDS-PAGE under reducing conditions and probed with anti-apo(a) antibody. C, Unstable plaque extract as in panel A was spun at d 1.21 g/mL; top and bottom were separated on 4% SDS-PAGE under reducing conditions and probed with anti-apo(a) antibody. D, Unstable extract in panel A run on 4% SDS-PAGE under nonreducing conditions and then blotted against anti-apoB and anti-apo(a) antibodies. The arrows indicate the difference position of migration between apoB and apo(a) represented as 18 K (kringle) #. *F1; **F2.

ApoE
The results of the PBS extracts of both unstable and stable plaques on 4% to 20% gels showed a single band in the
position of an apoE standard (Figure 4A). On the other hand, in the GdHCl extracts there were 2 major additional bands, 22 and 10 kDa, corresponding to the N-terminal and C-terminal domains, respectively. On spinning the extracts at d 1.21 g/mL, the top fraction of the PBS extract contained only intact apoE likely bound to phospholipid micelles (Figure 4B). The bottom fraction also contained intact apoE, in a lipid-poor form. In the case of the GdHCl extracts, the top d 1.21 faction contained intact apoE and a small amount of C-terminal fragment, whereas in the bottom fraction there were bands corresponding to intact apoE and N- and C-terminal domains. Overall, the results indicate that in the plaque extracts, apoE was both in a lipidated and in a lipid-free or lipid-poor form, whereas the fragments were in a lipid-poor state except for a small amount of C-terminal fragment known to have lipid binding properties. There were no apparent pattern differences between unstable and stable plaques.

**Proteoglycans**

Based on the results of the 4% to 12% gel blots, decorin, biglycan, and versican were all highly degraded. The degradation was more marked in the GdHCl extracts. A representative gel regarding an unstable plaque is shown in Figure 5. Several bands are seen in each case. Given that the results refer to the protein core of each PGs, we are not in the position to determine the fate of the glycan components after core fragmentation. Overall, however, there appeared to be no major differences between extracts from unstable and stable plaques.

**Discussion**

Our studies have provided evidence that in the morphologically classified unstable carotid plaques, and to a lesser extent in the stable ones, there is a proinflammatory and proteolytic microenvironment conducive to the formation of fragments of apoB, apo(a), apoE, and the protein cores of PGs decorin, biglycan, and versican. From the proinflammatory standpoint, the concentrations of IL-6 and IL-8 were positively correlated with the concentrations of MMP-2 and MMP-9, the chosen representatives of the MMP family. Although we performed no direct measurements, we can reasonably assume that most, if not all, of these MMPs were active under the following considerations: the underlying setting conducive to proenzyme activation above that of the tissue inhibitor of MMPs, previous studies on endarterectomy extracts from vulnerable regions of human atherosclerotic plaques\textsuperscript{12}, and our own results on surgical carotid plaques in which the activities of MMP-2 and MMP-9 were documented by zymography.\textsuperscript{2} In addition, there has been a report of an increased MMP-9 activity in human unstable carotid plaques and the postulate of a potential association between this activity and acute plaque disruption.\textsuperscript{13} Both ILs and MMPs were distributed between the PBS and GdHCl plaque extracts, suggesting an heterogeneity in binding of these molecules to the vascular matrix and a likely heterogeneity in bioactivity. Also of note is that the plasma concentrations of IL-6, IL-8, and IL-18, although above the normal values reported in the literature,\textsuperscript{14–19} did not differentiate between subjects with unstable and stable plaques. We are not in the position to determine whether this
failure may have been because we measured protein content and not activity, the latter known to depend on the function of the respective binding proteins and receptors of these cytokines.20–22

Regarding apolipoproteins, we paid particular attention to apoB and apo(a), both proatherogenic and both with reported fragments in human plaques.2–4 The fragmentation that we observed with apoB was more extensive in the unstable than in the stable plaques and, in turn, more evident in the GdHCl extracts than in the PBS extracts, an observation consistent with the notion of apoB trapping based on the Lp retention hypothesis.23 Our findings on apoB fragments make it apparent that studies of apoB in the atheromatous plaques should make use of antibodies capable of detecting not only intact apoB but also fragments that may differ in antigenicity and function.

In the plaque extracts, most apo(a) was not Lp-associated and was also fragmented, indicating that major structural changes of Lp(a) had occurred in the transfer process from the plasma into the vessel wall. The presence of non–Lp-associated apo(a) in the extracts also suggests, as proposed recently,24 that an important first step in the Lp disassembly is the cleavage of the disulfide bond between apo(a) and apoB either by the action of a specific thiolase or by the fragmentation in the areas in apoB and/or apo(a) comprising the cysteine residues involved in disulfide bond formation. Concerning the origin of the fragments, we reported previously that apo(a) resists degradation by oxidative events even though it is readily susceptible to the action of MMPs.25 These data and the high concentrations of MMPs that we found in the plaque extracts suggest that these enzymes may have played an important role in the generation of apo(a) fragments. Of note, neither apo(a) nor fragments were observed in sections of adjacent unaffected tissues (C. Maggioni, unpublished observations, 2003).

Regarding apoE, an intact protein was present in the PBS extracts of both unstable and stable plaques, whereas the GdHCl extracts also contained fragments (Figure 4A). Moreover, as shown in Figure 4B, there was intact apoE in both lipidated and lipid-poor forms, contrary to the fragments that were essentially all in a lipid-poor state. We did not determine the nature of these lipids, although we speculate to represent phospholipids bound to apoE produced and secreted by topical activated macrophages. An original finding of the current study was the identification in the plaque extracts of 2 main fragments, 10 and 22 kDa, with gel mobility of the N- and C-terminal domains of apoE, respectively. The generation of these 2 fragments was likely owing to proteolytic degradation because there are no literature data supporting an action by oxidative events and we have shown that, in vitro, enzymes of the MMP family cleave apoE (C. Edelstein and A.M. Scanu, unpublished observations, 2003). The fact that apoE in atheromas is susceptible to cleavage raises the issue about the functional relationships between fragments and parent apoE. In this regard, we reported previously that in vitro the C-terminal domain of apo E interacts preferentially with the protein core of biglycan,26,27 an observation that receives support from our current finding of a 10-kDa fragment in the GdHCl extracts.

The degradation scenario observed with the apolipoproteins also applied to decorin, biglycan, and versican, the 3 PGs studied. These findings raise questions about the functional significance of these fragments. Of interest in this regard is a recent study dealing with the degradation of decorin by several MMPs and the consequences of the enzymatic disruption of this multifunctional PG.28 MMP-2 was particularly effective in causing decorin cleavage in the Leu-rich region, ie, the site of binding by transforming growth factor-β. Thus, as suggested by the investigators, enzymatic disruption of decorin may release this growth factor in an active form, potentially contributing to the progression of the atherosclerotic process. Decorin disruption may also affect other functions such as regulation of collagen fibrillogenesis and maintenance of tissue integrity via binding to fibronectin and thrombospondin.3

Of the various parameters studied in the plasma, none discriminated between stable and unstable plaques and fragmentation, including the levels of Lp(a) and the distribution of apo(a) size isoforms. However, in the case of Lp(a), we were dealing with a population, averaging 70 years, well above the age at which the cardiovascular pathogenicity of Lp(a) has been reported to be preferentially expressed.29 Of note, the potential cardiovascular impact of apo(a) size polymorphism in this age group has also not been established. The whole patient population had low plasma HDL levels that, considering the recognized atheroprotective effects of this Lp,30 might have contributed to the cardiovascular pathogenicity in the 2 groups of subjects.

Overall, our current results raise the awareness that within the proinflammatory environment of advanced atheromatous lesions, important degradative events occur involving both topical and plasma-derived proteins. Regarding the resulting degradation products, emerging evidence points at the bioactivity of some of them and at their potential in modulating prothrombotic events as in the case of apo(a) or vessel remodeling as in the case of PGs. In this respect, it is important to note that fibrin degradation products accumulate in the atherosclerotic plaque31,32 and that some of them have been suggested to be chemotactic to monocyte macrophages, to stimulate smooth muscle cell growth and be potentially involved in restenosis.33 It should be apparent that the area of bioactive fragments in atheromas needs further exploration.

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References


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