Expression of Tissue Factor in High-Grade Carotid Artery Stenosis
Association With Plaque Destabilization

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Background and Purpose—The procoagulant protein tissue factor (TF) has been implicated in thromboembolic complications associated with advanced atherosclerosis. In this study, we investigated whether TF expression in high-grade stenoses of the internal carotid artery (ICA) is associated with clinical features of plaque destabilization and addressed the relationship between TF expression and plaque inflammation.

Methods—In 36 consecutive patients undergoing surgery for high-grade ICA stenosis, clinical evidence of plaque instability was provided by the recent occurrence of ischemic symptoms attributable to the stenosis and the detection of cerebral microembolism by means of transcranial Doppler ultrasound monitoring of the ipsilateral middle cerebral artery. Endarterectomy specimens were stained immunocytochemically for TF expression as well as macrophage (CD68) and T cell (CD3) infiltration.

Results—Morphologically, TF immunoreactivity was codistributed with plaque inflammation and predominantly localized to CD68+ macrophages. Accordingly, statistical analysis revealed a significant association of TF expression with plaque infiltration by macrophages (P<0.0001) and T cells (P=0.013). Plaques extensively stained for TF (median of TF+ total section area >40% in semiquantitative assessment) were more frequent in symptomatic (12/27) than in asymptomatic patients (1/9). Conversely, plaques exhibiting little TF expression (median of TF+ section area <20%) were more frequent in asymptomatic (3/9) than in symptomatic (1/27) patients (P=0.016). Likewise, we found a highly significant association of TF expression with the occurrence of cerebral microembolism (P=0.008).

Conclusions—Induction of TF at sites of plaque inflammation may play an important role in the destabilization of high-grade ICA stenosis. (Stroke. 2001;32:850-854.)

Key Words: atherosclerosis ■ carotid arteries ■ inflammation ■ leukocytes ■ procoagulant

Arterio-arterial thromboembolism from extracranial stenoses of the internal carotid artery (ICA) is an important pathomechanism of ischemic stroke. However, even high-grade ICA stenoses (>70% luminal narrowing) carry a highly variable annual risk of stroke that can be as high as 13% after the recent occurrence of transient cerebral or retinal ischemia or as low as 1% to 2% in clinically asymptomatic patients. The cellular and molecular mechanisms converting a stable plaque into “unstable ICA disease” are incompletely understood. In many patients with high-grade ICA stenosis, long-term transcranial Doppler ultrasonography (TCD) can reveal clinically silent formed-element microemboli passing through the ipsilateral middle cerebral artery. The rate of microemboli is higher in recently symptomatic than asymptomatic patients, predicts the occurrence of future ischemic symptoms, and declines after carotid endarterectomy. Thus, cerebral microembolism provides reliable paraclinical evidence of plaque destabilization.

Inflammatory mechanisms are considered to play a key role in the pathogenesis of atherosclerosis. Plaque-infiltrating T cells and macrophages are potential sources of matrix-degrading enzymes and thrombogenic substances that have been implicated in the key events of plaque destabilization, ie, rupture of the fibrous cap and subsequent luminal thrombosis, finally leading to the manifestation of acute ischemic syndromes such as stroke or myocardial infarction. In patients with high-grade ICA stenosis, several groups have recently found a significant correlation between the extent of inflammatory pathology and the development of plaque-related ischemic complications (see also...
Golledge et al\textsuperscript{22} for a recent review). Tissue factor (TF) is a glycoprotein that is strongly induced in activated inflammatory macrophages and T cells.\textsuperscript{7,13–25} By its ability to bind factor VIIa, TF directly activates the coagulation cascade. Therefore, TF is a candidate molecule linking plaque inflammation with arterial thromboembolism.\textsuperscript{26,27} In the present study, we performed an immunocytochemical analysis of TF expression in endarterectomy specimens from 36 consecutive patients undergoing surgery for high-grade ICA stenosis and addressed the relationship of TF expression to clinical features of plaque destabilization and inflammatory plaque infiltration by macrophages and T cells.

**Subjects and Methods**

**Patients**

This study prospectively included 36 consecutive surgical inpatients enlisted to undergo carotid endarterectomy for extracranial high-grade ICA stenosis (\geq 70\% luminal narrowing). The study was approved by the local ethics review committee and performed in accordance with institutional guidelines. Informed consent was obtained from all patients before each examination. Baseline characteristics of the study population are provided in Table 1. The degree of luminal narrowing was determined by intra-arterial cerebral angiography, using the criteria of the North American Symptomatic Carotid Endarterectomy Trial (NASCET).\textsuperscript{28} Antiplatelet medication (n=50 patients) or oral anticoagulants (n=1) were routinely stopped at least 6 days before operation. At the day of operation, routine coagulation parameters were normal in all patients. During surgery, no anticoagulation was performed. Routine cardiological and medical assessment performed preoperatively did not provide evidence of cardiac embolism or systemic infectious disease. According to NASCET criteria,\textsuperscript{28} patients were defined as symptomatic if they had recently (<121 days before) experienced transient retinal or cerebral symptoms or minor ischemic strokes attributable to the high-grade ICA stenosis. For the assessment of cerebral microembolism, all patients received long-term transcranial Doppler (TCD) signal recording of the middle cerebral artery ipsilateral to the high-grade ICA stenosis for at least 1 hour, as described in detail elsewhere.\textsuperscript{10} The patients were monitored 1 to 21 days before endarterectomy (median 4.5 days).

**Histological Procedures and Immunocytochemistry**

After longitudinal arteriotomy, the carotid atherosclerotic plaque was excised en bloc (routine endarterectomy), fixed immediately in 4\% paraformaldehyde, decalcified, and transversely sectioned at 2-mm intervals. Each 2-mm tissue block was embedded separately into paraffin. The quantitative analysis was based on all blocks derived from each individual plaque. The number of blocks examined per plaque was 11.1±3.0 (mean±SD). The total number of blocks examined was 403. For immunocytochemistry, 10-µm sections were mounted onto gelatin-coated slides. After deparaffinization, sections were incubated with a monoclonal antibody (mAb) against human tissue factor (No. 4509, American Diagnostica Inc) at 1:100 dilution, followed by biotinylated horse anti-mouse IgG (Vector Laboratories) and the ABC ELITE kit reagents (Vector) with diaminobenzidine as substrate. In parallel experiments, substitution of the TF-specific primary mAb with an irrelevant, isotype-matched control antibody yielded negative results. Furthermore, preadsorption of the TF mAb with an excess of human recombinant TF (American Diagnostica) led to a complete disappearance of staining (Figures 1D and 1E).

**Quantification**

Because TF-immunoreactivity (TF-IR) was distributed both diffusely in the extracellular matrix and in large confluent infiltrates, quantification by cell counting was not possible. In addition, attempts to quantify TF+ section area by computer-aided planimetry were unsuccessful because of the diffuse appearance of the staining that caused large interrater variability. Therefore, TF expression was determined semiquantitatively by 2 independent observers who were blinded as to the identity and clinical status of the patients. For each section, TF expression was rated as either weak (<20\% of section area staining positively for TF), moderate (20\% to 40\% TF+ section area), or abundant (>40\% TF+ section area). Based on these values, the median of TF+ section area was calculated for the entire plaque. For the semiquantitative rating procedure, κ statistics revealed excellent interrater agreement (κ=0.96). T-cell counting (number of cells per mm\textsuperscript{2} total section area) and macrophage planimetry (% total section area occupied by CD68+ cells) were performed as described previously.\textsuperscript{21}

**Statistical Analysis**

To analyze the relationship between the expression of TF and inflammation, we performed Pearson correlation analyses, including TF+ section area, the percentage of macrophage-rich areas, and the number of T cells per mm\textsuperscript{2} section area determined for each section. To analyze the relationship between the extent of TF expression (median TF+ total section area of each individual plaque) and the occurrence of ischemic symptoms and cerebral microembolism, respectively, we used the nonparametric Mann-Whitney U test. Because we performed 2 consecutive statistical tests, values of P<0.025 (0.05/2) were considered indicative of statistically significant findings (α adjustment).

**Results**

**TF Expression in the Fibrous Cap: Codistribution With Macrophages and T Cells**

TF immunoreactivity was found in all plaques, although its extent showed large interindividual variation. The median TF+ section area was <20\% in 4 plaques, 20\% to 40\% in 19 plaques, and >40\% in 13 plaques. TF expression was overall strongly accentuated within the atheromatous core and its immediate surroundings (Figure). In the lesion core, TF immunostaining was predominantly diffuse in the extracellular matrix. In contrast, strong cellular immunostaining was found at the transition between atheromatous core and fibrous cap, where large confluent infiltrates stained positively for TF.
Figure, panel A). In addition, TF was localized in cells immediately lining the luminal surface of the plaque (arrowheads). Staining of serial sections for macrophages (B) and T cells (C) revealed that TF immunoreactivity was overall codistributed with inflammatory infiltration and predominantly localized to CD68+ macrophages (A, B, and D).

To corroborate the relationship between inflammation and TF expression, we quantified the extent of macrophage and T cell infiltration in each plaque section as described previously21 and studied their relationship to TF expression by linear regression analysis. In line with the morphological observations, we found a significant association between TF expression and the percentage of macrophage-rich areas ($r = 0.335$, $P < 0.0001$), and the number of T cells per mm$^2$ section area ($r = 0.124$, $P = 0.013$), respectively.

### Increased TF Expression Is Associated With Plaque Destabilization

To clarify the clinical significance of TF expression in carotid artery plaques, we first studied the relationship between TF immunoreactivity and the occurrence of ischemic symptoms attributable to the stenosis during the last 120 days before inclusion into the study (Table 2). Plaques extensively stained for TF (median of TF+ total section area >40%) were more frequent in symptomatic (12/27 patients; 44%) than in asymptomatic (1/9; 11%) patients. Conversely, plaques exhibiting little TF immunoreactivity (median of TF+ total section area <20%) were more frequent in asymptomatic (3/9; 33%) than in symptomatic (1/27; 4%) patients. Plaques with an intermediate degree of TF+ section area (20% to 40%) displayed a similar frequency in both symptomatic and asymptomatic patients. Statistical testing revealed a signifi-

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<th>TF+ Section Area, median</th>
<th>Ipsilateral Ischemic Symptoms Within Past 120 Days</th>
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<td>n %</td>
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<td>&lt;20%</td>
<td>3 33</td>
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<td>20–40%</td>
<td>5 56</td>
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TF expression was determined semiquantitatively in each plaque section and for each entire plaque the median of TF+ section area was calculated. Mann-Whitney $U$ test revealed a significant association between TF expression and the occurrence of ischemic symptoms ($P = 0.016$).
cavitant association between TF expression and a history of previous ischemic symptoms ($P=0.016$).

To further substantiate the association of TF expression with plaque destabilization, we analyzed the relationship between TF+ section area and the occurrence of cerebral microembolism in long-term TCD monitoring. Table 3 shows that strongly TF+ plaques were more frequent in microemboli-positive patients (48% versus 15% in the microemboli-negative group) whereas plaques with little TF immunoreactivity were more frequent in microemboli-negative patients (31% versus 0% in microemboli-positive patients). For ischemic symptoms, statistical testing showed a highly significant association of TF expression with the occurrence of cerebral microembolism ($P=0.008$).

**Discussion**

In our study we have shown that increased expression of TF in high-grade stenoses of the ICA is associated with plaque destabilization evidenced clinically both by a history of previous ischemic symptoms and the detection of microemboli in long-term TCD monitoring of the ipsilateral middle cerebral artery. Morphologically, TF immunoreactivity was codistributed with plaque inflammation and mostly localized to CD68+ macrophages. In line with this observation, previous studies showed induction of TF by inflammatory cytokines such as interferon-γ and CD40 ligand (CD154). Our data therefore strongly suggest that TF induction at sites of plaque inflammation may play an important role in the destabilization of high-grade ICA stenosis.

TF has the ability to directly activate the coagulation cascade by the interaction with factors VIIa and X. Accordingly, TF has been implicated in the development of thromboembolism due to advanced atherosclerotic lesions. In our present study, most TF immunoreactivity was localized diffusely in the acellular necrotic core and within inflammatory infiltrates in the fibrous cap of the atheroma. However, we also found some TF expression in cells immediately lining the vascular lumen. Thus, exposure of TF activity to coagulation factors may either occur directly at the intimal surface or may result from plaque rupture leading to the release of macrophage-bound or extracellular material from deeper parts of the plaque to the bloodstream. The rupture of complicated plaques has been suggested to be due to the expression of matrix metalloproteinases (MMPs) that degrade extracellular matrix components and thereby weaken the fibrous cap. Interestingly, a recent study by Lofus et al. indeed indicates a correlation of MMP-9 expression with carotid plaque destabilization. Thus, it is an intriguing hypothesis that the concerted action of MMP-9 and TF may be a key mechanism of plaque destabilization in cerebrovascular disease patients at risk of stroke.

A potential limitation of our present study arises from the fact that the sensitive immunohistochemical staining procedure used for the detection of TF antigen does not allow direct conclusions with respect to the actual presence of TF bioactivity. However, in a comparative study, Thiruvikraman et al. used both TF-specific antibody and digoxygenin-labeled factors VIIa and X for the in situ detection of TF and observed essentially identical staining patterns with both types of detection reagents. Similar to our immunohistochemical data, the digoxygenin-labeled factors bound to the acellular lipid core and numerous macrophages. It is therefore likely that the TF immunoreactivity detected in our study indeed reflects TF binding activity for its physiologically relevant ligands. On the other hand, TF expression in atherosclerotic plaques has been shown to be paralleled by the induction of an endogenous TF pathway inhibitor (TFPI) that may interfere with TF bioactivity in a complex manner. Therefore, additional studies using TF as well as TFPI bioassays will be necessary to definitively clarify the role of TF in ICA plaque destabilization.

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**References**


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