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

Sebastian Jander, MD; Matthias Sitzer, MD; Angélique Wendt, MS; Michael Schroeter, MD; Martin Buchkremer, MD; Mario Siebler, MD; Wolfram Müller, MD; Wilhelm Sandmann, MD; Guido Stoll, MD

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.1,2 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.3,5 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.6 The rate of microemboli is higher in recently symptomatic than asymptomatic patients,7 predicts the occurrence of future ischemic symp-
Golledge et al22 for a recent review). Tissue factor (TF) is a glycoprotein that is strongly induced in activated inflammatory macrophages and T cells.23–25 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.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 (>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).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,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.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.16 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, or rabbit polyclonal IgG against human CD31 (1:100) as a T cell marker (both primary antibodies from DAKO). For antigen retrieval, sections were microwaved in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 minutes before staining.

Statistical Analysis

To analyze the relationship between the expression of TF and inflammation, we performed Pearson correlation analyses, including the number of T cells per mm² total section area and macrophage planimetry (% total section area occupied by CD68+ cells) were performed as described previously.21

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.

<table>
<thead>
<tr>
<th>Age (mean±SD), y</th>
<th>Luminal Narrowing, mean (%)</th>
<th>Microemboli Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic (n=9)</td>
<td>63±9</td>
<td>70–95 (84.6)</td>
</tr>
<tr>
<td>Symptomatic (n=27)</td>
<td>62±11</td>
<td>70–95 (84.3)</td>
</tr>
</tbody>
</table>

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TABLE 1. Clinical Features of Symptomatic and Asymptomatic Patients With High-Grade ICA Stenosis

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Summary:

1. TF is a candidate molecule linking plaque inflammation with arterial thromboembolism.
2. The study found TF expression in endarterectomy specimens from 36 consecutive patients undergoing surgery for high-grade ICA stenosis.
3. TF expression was related to clinical features of plaque destabilization and inflammatory plaque infiltration by macrophages and T cells.
4. TF immunocytochemistry was performed on 403 blocks from 36 patients.
5. TF expression was quantified and correlated with the number of T cells and macrophages.
6. TF expression was highest in the lesion core and decreased towards the atheromatous core.
7. TF expression was more pronounced in symptomatic patients.

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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 previously 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 mm² 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 significant association between TF expression and the occurrence of ischemic symptoms (P = 0.016).

**TABLE 2. Relationship Between TF Expression and Ischemic Symptoms**

<table>
<thead>
<tr>
<th>TF+ Section Area, median</th>
<th>Ipsilateral Ischemic Symptoms Within Past 120 Days</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
<td></td>
</tr>
<tr>
<td>&lt;20%</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>20–40%</td>
<td>5</td>
<td>14</td>
<td>52</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>1</td>
<td>12</td>
<td>44</td>
</tr>
</tbody>
</table>

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).

Immunocytochemical localization of TF (A, D), macrophages (CD68, B), and T cells (CD3, C) in a carotid plaque from a symptomatic patient. TF is expressed both diffusely in the necrotic lesion core (asterisk) and in dense cellular infiltrates at the transition between lesion core and fibrous cap. In addition, some TF+ cells are localized at the luminal surface of the plaque (arrowheads). Note the similar distribution and morphology of TF+ and CD68+ cells. D, High-power view of TF immunoreactivity. E, TF immunostaining is completely abolished after preadsorption of TF-specific mAb with recombinant TF. Scale bars: 100 μm in A–C, 50 μm in D and E.
TABLE 3. Relationship Between TF Expression and Preoperative Microemboli Count

<table>
<thead>
<tr>
<th>TF+ Section Area, Median</th>
<th>Microemboli Count</th>
<th>0 h⁻¹</th>
<th>&gt;0 h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20%</td>
<td>4</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>20–40%</td>
<td>7</td>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>&gt;40%</td>
<td>2</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

See Table 2 for details concerning TF quantification. Overall, a significant association between TF expression and the occurrence of cerebral microembolism was found ($P=0.008$).

A significant 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). As 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. 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). 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. According to the interaction between TF and X.

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 be an important role in the destabilization of high-grade ICA stenosis.

fibrous cap. Interestingly, a recent study by Loefus 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.

Acknowledgment

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References

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