Increased Endothelial Expression of Intercellular Adhesion Molecule-1 in Symptomatic Versus Asymptomatic Human Carotid Atherosclerotic Plaque

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Background and Purpose—The mechanisms that cause carotid atherosclerotic plaque to become symptomatic remain unclear. Evidence suggests that mediators of inflammation are not only instrumental in the formation of plaque but may also be involved in the rapid progression of atheromatous lesions leading to plaque fissuring, endothelial injury, and intraluminal thrombosis. Our goal is to determine whether intercellular adhesion molecule-1 (ICAM-1), a known component of the inflammatory pathway, is preferentially expressed on symptomatic versus asymptomatic carotid plaques.

Methods—Carotid plaques from symptomatic (n = 25) and asymptomatic (n = 17) patients undergoing carotid endarterectomy with lesions involving >60% stenosis were snap-frozen at the time of surgery. Immunofluorescence studies were performed to measure the percentage of luminal endothelial surface that expressed ICAM-1. The relationships of stroke risk factors, white blood cell count, percent stenosis, and soluble ICAM-1 (sICAM-1) plasma levels to endothelial ICAM-1 expression were investigated.

Results—An increased expression of ICAM-1 was found in the high-grade regions of symptomatic (29.5% ± 2.4%, mean ± SEM) versus asymptomatic (15.7% ± 2.7%, mean ± SEM) plaques (P = 0.002) and in the high-grade versus the low-grade region of symptomatic plaques (29.5 ± 2.4, mean ± SEM, versus 8.9 ± 1.6; P < 0.001). Plasma sICAM-1 levels were not predictive of symptomatic disease, and no significant correlation between risk factor exposure and endothelial ICAM-1 expression was found.

Conclusions—An elevation in ICAM-1 expression in symptomatic versus asymptomatic plaque suggests that mediators of inflammation are involved in the conversion of carotid plaque to a symptomatic state. The data also suggest a differential expression of ICAM-1, with a greater expression found in the high-grade region than in the low-grade region of the plaque specimen. (Stroke. 1998;29:1405-1410.)

Key Words: atherosclerosis • carotid endarterectomy • endothelium • intercellular adhesion molecule-1 • inflammation

The mechanisms involved in the initiation, progression, and maturation of atherosclerotic plaques have been well described previously, and are characterized by endothelial injury, followed by lipid deposition, macrophage migration, vascular smooth muscle proliferation, and deposition of extracellular matrix proteins in the intimal region between the endothelial lining and the media. Processes within the plaque lead to further disruption of the endothelial lining, predisposing the vessel to intraluminal thrombogenesis that culminates in myocardial infarction, stroke, and peripheral vascular disease. In an attempt to isolate key factors that characterize the pathophysiological mechanism that ultimately results in an ischemic event, numerous studies have carefully reported the cellular composition and radiographic patterns found in symptomatic and asymptomatic carotid plaques. Lipid content, calcification, fibrous cap thickness, and intraplaque hemorrhage have been examined as possible harbingers of plaque activation. To date, however, overall morphological differences have been inconsistent indices for predicting plaque conversion to a symptomatic or prothrombotic state.

Recent studies have focused on the role of immune mediators and inflammation in the destabilization of atherosclerotic plaque. Adhesion molecule expression, found preferentially on the endothelium and smooth muscle of atherosclerotic plaque, is an essential step for initiation, maturation, and destabilization of plaques and is mediated by inflammatory cytokines such as IL-1, TNF-α, and interferon-γ. The expression of ICAM-1, vascular cell adhe-
sion molecule-1, and E-selectin is thought to modulate leukocyte/endothelial interaction, resulting in the emigration of leukocytes and enhancement of the potential for plaque rupture. However, plaque rupture and intraplaque hemorrhage, although likely to be strong contributing factors, are not essential for the formation of intraluminal thrombosis, and their presence does not guarantee symptoms. In an effort to find a common mechanism for both plaque rupture and intraluminal thrombosis without fissuring or intraplaque hemorrhage, we hypothesized that the increased expression of adhesion molecules on the vessel luminal endothelial surface and the subsequent intensified interaction between perivascular monocytes/macrophages and the local endothelium are pivotal to multiple processes that predispose the atherosclerotic plaque to convert from an asymptomatic to a symptomatic state. In addition to a strong association with intraplaque hemorrhage, inflammatory cells lead to the increased local production of platelet-activating factor, IL-1, and TNF-α, which transforms the endothelium from an anticoagulant to a prothrombotic state.

The specific aim of our study was to determine whether ICAM-1, reportedly a key component of the inflammatory pathway by causing endothelial/leukocyte interaction and subsequent luminal thrombosis, is preferentially expressed on the endothelium of carotid plaques of symptomatic versus asymptomatic patients. The current practice of performing CEA in symptomatic and asymptomatic patients to reduce the risk of stroke allows us a unique opportunity to examine and compare vascular specimens between the 2 clinical states.

**Subjects and Methods**

Forty-two carotid plaques were obtained from 40 consecutive patients (37 men and 5 women) undergoing CEA at the National Naval Medical Center in Bethesda, Maryland, with stenotic atheromatous lesions of ≥60%, as measured by NASCET criteria. All patients gave consent for use of their plaque and blood samples for research analysis as per the hospital’s Institutional Review Board. Twenty-five patients were symptomatic (stroke, transient ischemic attack, amaurosis fugax), and 17 were asymptomatic (Table 1). Extensive history and neurological examination were obtained by a neurologist from the National Institute of Neurological Disorders and Stroke Branch before surgery in all patients to determine symptomatic and asymptomatic group selection. Stroke risk factors of hypertension (blood pressure >140/90 for 1 or more years), past history of smoking (at least a 5 pack-year history), diabetes (oral agent or insulin dependent for >1 year), and hypercholesterolemia (low-density lipoprotein >160 untreated, fasting triglycerides >200, or on cholesterol-lowering medication for >1 year), current medications including use of antplatelet agents, and time from last ischemic event were recorded. Patients with atrial fibrillation were excluded from the symptomatic group to avoid the possible confusion between a cardiac and a carotid source of ischemic events. A screening fasting blood glucose and lipid profile was performed on all patients without known history of diabetes or hypercholesterolemia. A CT scan of the head was obtained for all patients and used as supporting evidence of the history and physical in the asymptomatic population to rule out silent infarction.

Carotid plaque specimens were collected in the operating room (with care to remove the plaque in a single piece if possible). The methods used to divide the plaque into “high-grade” and “low-grade” regions are as follows. The distance from the carotid bifurcation to the point of maximal stenosis was measured on the angiogram. The corresponding region on the plaque specimen was identified, and a 7- to 12-mm block surrounding the estimated point of maximal stenosis was recovered. The lumen was filled with Tissue-Tek optimal cutting temperature compound (Laboratory-Tek Products), and the specimens were snap-frozen in liquid nitrogen and stored at −70°C until sectioning. Cryostat sections of 16-μm thickness were obtained, and multiple slides from the highest region of stenosis by visual inspection were used for analysis. The low-grade region was obtained from the distal edge of the internal carotid artery plaque where the specimen tapered to a near normal—appearing vessel. These regions corresponded to <30% stenosis by angiogram and generally had the visual characteristics of near normal vascular endothelial tissue. ICAM-1 expression was identified in each sample by incubating with a mouse anti-human ICAM-1 monoclonal antibody, subclass IgG1 (R&D Systems, 1:1000 dilution) for 48 hours at 4°C and then washed 3 times (10 minutes each). A FITC–labeled second antibody (goat anti-mouse IgG F(ab’2); Jackson ImmunoResearch Laboratories, Inc, 1:100) to reveal endothelial ICAM-1 expression was identified in each sample.

**TABLE 1. Demographics and Distribution of Risk Factors**

<table>
<thead>
<tr>
<th>Age (range), y</th>
<th>Symptomatic (n=25)</th>
<th>Asymptomatic (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>67.7 (41–82)</td>
<td>69.5 (52–89)</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Stenosis (SD), %</td>
<td>77.9±13.3</td>
<td>78.0±12.7</td>
</tr>
<tr>
<td>Hypertension, (%)</td>
<td>19/25 (76)</td>
<td>12/17 (71)</td>
</tr>
<tr>
<td>Smoking, (%)</td>
<td>16/25 (64)</td>
<td>14/17 (82)</td>
</tr>
<tr>
<td>Cholesterol, (%)</td>
<td>12/25 (48)</td>
<td>10/17 (59)</td>
</tr>
<tr>
<td>Diabetes, (%)</td>
<td>4/25 (16)</td>
<td>2/17 (12)</td>
</tr>
<tr>
<td>White blood cell count (SD)</td>
<td>7.91±1.77</td>
<td>7.10±1.51</td>
</tr>
</tbody>
</table>

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of bias into data collection. This investigation reports final data evolving from and using techniques from an initial preliminary study.22

Preoperative blood was drawn from all patients for the measurement of sICAM-1 to determine whether plasma levels were representative of focal or systemic vascular upregulation of adhesion molecule expression. Plasma sICAM-1 levels were also compared with levels obtained from volunteer risk factor–free, age-matched control subjects without carotid arteriosclerosis recruited at the National Naval Medical Center. Levels of sICAM-1 were determined using an ELISA kit (R&D Systems).

Statistical Analysis
Repeated measures ANOVA was used to compare the 2 patient groups, symptomatic and asymptomatic, on ICAM-1 expression in both high- and low-grade regions of the CEA plaque. In the analysis the group (symptomatic/asymptomatic) × grade (high/low) interaction was tested. Paired and independent group t tests with Bonferroni correction were applied to determine the difference between high-grade symptomatic and high-grade asymptomatic regions (interplaque differences) and between the high- and low-grade regions of the same plaques (intraplaque differences). Pearson product moment correlations were used to investigate the relationship of percent high-grade ICAM-1 expression with sICAM-1, white blood cell count, and percent stenosis. The correlations were considered for symptomatic and asymptomatic groups combined and separately.

Point–bi-serial correlations were used to investigate the relationship of high-grade ICAM-1 expression to the dichotomous variables of hypertension, diabetes, hypercholesterolemia, and smoking. A 1-way ANOVA with Welch approximation was used to compare sICAM-1 levels among the symptomatic, asymptomatic, and control groups. A stepwise logistic regression analysis was performed to determine the contribution on the occurrence of patient symptoms of the potential risk variables of percent ICAM expression, age, percent stenosis, and presence of stroke risk factors.

Results
Repeated measures ANOVA on ICAM-1 expression resulted in a highly significant group-grade interaction, demonstrating a propensity for increased percent ICAM-1 expression in the symptomatic patients, \( P=0.0003 \) (Figure 2). Pair-wise comparisons of the individual group means with Bonferroni corrected t tests demonstrated that the percentage of the carotid endothelial surface showing positive ICAM-1 reactivity is significantly \( (P=0.002) \) higher in the high-grade regions of symptomatic patients (29.6%±2.3%, mean±SEM) versus the high-grade regions of asymptomatic patients (15.7%±2.7%, mean±SEM). In addition, intraplaque comparison reveals a significantly \( (P<0.001) \) increased percent-
age of endothelial cell surface expressing ICAM-1 in the high-grade regions of the symptomatic plaques (29.6% ± 2.3%, mean ± SEM) compared with the low-grade regions of symptomatic plaque (8.9% ± 1.6%, mean ± SEM). There was no notable difference between the high- and low-grade regions of the asymptomatic plaque or between the low-grade regions of the symptomatic and asymptomatic plaques.

An investigation performed to measure potential relationships between the risk factors for stroke (as listed in Table 1, including hypertension, diabetes, hypercholesterolemia, smoking, and percent plaque stenosis, and leukocyte count) with the degree of adhesion molecule expression showed no correlation.

In logistic regression analyses, none of the risk factor variables contributed significantly to the logistic model prediction, with or without the inclusion of percent ICAM-1. The only variable that contributed significantly to a logistic model was percent ICAM-1 in high-grade symptomatic plaque, both singularly and in conjunction with the other variables.

Finally, analysis of the plasma sICAM-1 levels showed the symptomatic, asymptomatic, and control groups to be statistically significant, P<0.02 (Table 2). Pair-wise group r tests revealed no difference between the symptomatic (255±81, mean±SD, pg/mL) and asymptomatic patients (224±78, mean±SD, pg/mL). Although the sICAM-1 level was an unreliable marker for distinguishing elevated levels of focal expression of the adhesion molecule on carotid plaques, there was a significant difference between the symptomatic patients and the risk factor-free, age-matched control population (194±41, mean±SD, P=0.05).

**Discussion**

The processes instrumental in the initiation, progression, and activation of atherosclerotic plaques are a complex concert of events involving vessel conformation, hemodynamic forces, multiple risk factor exposure, cellular composition, and physiological mechanisms that include upregulation of inflammatory mediators and plaque destabilization. Attempts to reduce the incidence of strokes that result from carotid atherosclerotic thromboemboli require a clearer understanding of the mediators associated with the conversion of plaques from an asymptomatic to a symptomatic state. Our data show a strong correlation between a major mediator of local inflammation, the expression of the adhesion molecule ICAM-1, with the symptomatic state, which potentially represents the next piece of the puzzle for predicting and understanding plaque activation.

Findings from the NASCET revealed evidence that high-grade stenotic symptomatic lesions (70% to 99%) resulted in a high incidence of recurrent strokes in the medically treated group.21 However, degree of stenosis alone does not explain the stroke risk, since patients on aspirin in the Asymptomatic Carotid Atherosclerosis Study (with 60% to 99% stenosis) had a significantly lower yearly stroke rate.22 Although there are differences between how these 2 studies were performed, it is not inappropriate to conclude that atherosclerotic plaque with a given degree of stenosis imparts a greater risk of recurrent stroke in a patient who has become symptomatic than in one who has not.

Various studies have analyzed the potential role of atherosclerotic plaque components in the development of patient symptoms. Avril et al reported that “soft plaques” containing atheromatous debris or intraplaque hemorrhage were more commonly seen in symptomatic carotid lesions than “hard plaques” composed primarily of collagen or calcium. These findings were echoed by O’Holleran et al who reached similar conclusions via ultrasound analysis of carotid plaques.

Lusby et al2 found acute or recent plaque hemorrhage in 49 of 53 (92.5%) symptomatic patients undergoing CEA compared with only 7 of 26 (27%) in asymptomatic patients. However, more recent studies by Hatsuakuri et al and Bassiony et al revealed no correlation between the preoperative ischemic symptom status and the presence and quantity of fibrous intimal tissue, lipid core/total cholesterol, fibrinogen, necrotic plaque core, calcification, or intraplaque hemorrhage.

Intuitively, intraplaque hemorrhage and the potential for plaque rupture and ulceration would seem to be associated with a luminal surface ripe for focal thrombosis. However, even the data presented by Lusby et al revealed that 90% of asymptomatic patients had evidence of remote intraplaque hemorrhage, suggesting that the severity of hemorrhage represents a spectrum that most likely serves as an interrelated contributing factor to plaque activation but not the single element that institutes focal endothelial thrombosis. In addition, evidence for plaque ulceration was found to be absent on the intimal lining of the symptomatic plaque in greater than 40% of the first 500 patients examined in the

**TABLE 2. Analysis of Plasma sICAM Levels by Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>sICAM-1, pg/mL</th>
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<tbody>
<tr>
<td>Symptomatic, n=25</td>
<td>255±81*</td>
</tr>
<tr>
<td>Asymptomatic, n=17</td>
<td>224±78</td>
</tr>
<tr>
<td>Control, n=15</td>
<td>194±41</td>
</tr>
</tbody>
</table>

One-way ANOVA yielded F_{0.05}=4.71, P=0.0158, indicating statistical significance among the 3 group means for sICAM-1. The 3 groups compared in pairs revealed only control mean vs symptomatic mean was statistically significant (P=0.05).
Accumulation of data from numerous sources has permitted an emerging characterization of the role of inflammatory mediators in the process of atherosclerosis. Proinflammatory cytokines, such as TNF-α, and adhesion molecule expression have been well localized to the atherosclerotic vessel wall, and there is evidence that this profile shifts the endothelium toward a prothrombotic state. A strong association between the presence of angina/myocardial infarction and plaque rupture/ulceration, luminal thrombus, and inflammation has been noted in coronary vessels. Luminal thrombus has been identified in the presence of inflammation alone, although the majority of thrombus formation in coronary arteries has been associated with ulceration. Milei et al have shown a correlation between macrophage infiltration and carotid artery plaque rupture.

Previously, we demonstrated a relationship between risk factor exposure and intensification of an interaction between perivascular monocytes and the cerebral endothelium mediated by inflammatory cytokines. We hypothesize that prolonged exposure to risk factors can prepare segments of the vascular bed for focal thrombosis and hemorrhage on exposure to TNF-α and IL-1, which may be released in response to a local or systemic stimulus according to the localized Shwartzman paradigm. To accumulate the data presented here we used immunostaining techniques to best localize the adhesion molecule expression to the endothelial cell surface in an effort to characterize the interactive surface of the plaque with the blood interface. The findings demonstrate that the greatest percentage of ICAM-1 expression was observed in the high-grade regions of the symptomatic plaque. These findings strongly suggest that components of the inflammatory pathway are directly involved in the conversion of the atherosclerotic plaque to the symptomatic or prothrombotic state. They also reveal that ICAM-1 expression favors the high-grade region and not the entire endothelial surface of the symptomatic plaque, which implies a dynamic interaction at the plaque site. Analysis showed no significant relationship of ICAM-1 expression with the risk factor profile that would explain the difference in adhesion molecule expression. It is of particular interest that asymptomatic patients with very similar degrees of high-grade stenosis had a significantly lower percentage of ICAM-1 expression, supporting the opinion that stenosis alone does not determine the transition to a symptomatic state. Despite the focal elevation of ICAM-1 expression, the soluble form of ICAM-1 in the plasma was not significantly different in symptomatic compared with asymptomatic patients. However, when compared with a risk factor-free, age-matched control group, sICAM-1 was significantly elevated in patients with symptomatic atherosclerosis.

Limitations of this study are related to the quantification techniques by which the expression of ICAM-1 was determined. Immunostaining techniques were specifically used to best localize the adhesion molecule expression to the endothelial cell surface in an effort to characterize the interactive surface of the plaque with the blood interface. However, nonreactive or shielded epitopes of ICAM-1 may lead to an underestimation of protein expression. Analysis was targeted at the endothelial expression of ICAM-1, which represents tissue that has direct contact with blood flow in all patients and does not demonstrate autofluorescence seen in the body of the plaque.

Numerous mechanisms could be invoked to explain our findings. First, it should be recognized that our results are a “snapshot” in time. The findings could represent a periodic event measurable at the times the plaque is most active or a sustained condition that adds to the overall potential of a positive thrombogenic event. One possible explanation for the findings would be the presence of a focal infection that could trigger already “prepared” (susceptible) atheromatous tissue and push it toward a transiently uncompensated prothrombotic state. This may explain why 2 comparably sized bilateral carotid plaques in the same patients exhibit very different symptom profiles. Along the same line, even systemic infection without direct invasion of the vessel wall may trigger a previously upregulated plaque to advance to a more prothrombotic state in accordance with the localized Shwartzman paradigm. Another possibility is that the underlying genetic makeup of the patient may predispose to a more proinflammatory plaque. It is known that shear stress forces and oxidative stress regulate gene expression through transcription-regulating proteins, such as nuclear factor-κB, and shear stress response element, thus affecting genes that regulate adhesion molecule expression. In addition, genes that control the expression of families of cytokines, which regulate inflammation, are known to be polymorphic in nature. Finally, periodicity to the release of mediators of inflammation such as cytokines and chemokines may result in transient or episodic elevations in the overall potential for a plaque to become symptomatic.

It must be considered that adhesion molecule expression is an epiphenomenon of the luminal thrombosis that caused the ischemic event in the symptomatic patient. Though possible, it is felt to be less likely given the preponderance of evidence that inflammatory endothelial changes generally precede thrombogenesis in atherosclerosis and are felt to significantly contribute to the maturation of atherosclerotic plaque. The mechanisms that lead to these findings need further characterization, but our results are consistent with the notion that inflammation is a pivotal factor in carotid plaque symptom status. Inflammatory mediators may participate in thrombus formation either in the hemorrhagic/ulcerative state or in the circumstance of intact but modified endothelial lining.

In summary, the data from this study suggest that a local increase of endothelial inflammatory mediator expression correlates with the symptoms of thromboembolic ischemia and may play a role in the conversion of atheromatous plaque to a prothrombotic state. The data also support the fact that this line of investigation may be useful in identifying new mechanisms in patients at risk for stroke, which may suggest novel strategies for intervention and prevention. This important possibility deserves further exploration.

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ICAM-1 in Symptomatic Human Carotid Plaque

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