The Phenotype of Infiltrating Macrophages Influences Arteriosclerotic Plaque Vulnerability in the Carotid Artery

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Background: Proinflammatory (M1) macrophages and anti-inflammatory (M2) macrophages have been identified in atherosclerotic plaques. While these macrophages have been speculated to be related to plaque vulnerability, there are limited studies investigating this relationship. Therefore, we examined the association between macrophage phenotype (M1 versus M2) and plaque vulnerability and clinical events.

Methods: Patients undergoing carotid endarterectomy received an ultrasound of the carotid artery before surgery. Plaques were processed for analysis by immunohistochemistry, Western blotting, and real-time polymerase chain reaction studies. Medical history and clinical data were obtained from medical records.

Results: Patients were divided into 2 groups: those suffering from acute ischemic attack (symptomatic, n = 31) and those that did not present with symptoms (asymptomatic, n = 34). Ultrasound analysis revealed that plaque vulnerability was greater in the symptomatic group (P = .033; Chi-square test). Immunohistochemistry revealed that plaques from the symptomatic group had a greater concentration of M1 macrophages (CD68-, CD11c-positive) while plaques from the asymptomatic group had more M2 macrophages (CD163-positive). This observation was confirmed by Western blotting. Characterization by real-time polymerase chain reaction studies revealed that plaques from the symptomatic group had increased expression of the M1 markers CD68 and CD11c, as well as monocye chemoattractive protein-1, interleukin-6, and matrix metalloproteinase-9. In addition, more M1 macrophages expressed in unstable plaques were defined by ultrasound analysis, while more M2 macrophages were expressed in stable plaques.

Conclusions: Our data show that M1 macrophage content of atherosclerotic plaques is associated with clinical incidence of ischemic stroke and increased inflammation or fibrinolysis. We also show the benefits of using ultrasound to evaluate vulnerability in the plaques.

Key Words: Atherosclerosis—carotid artery disease—inflammation—macrophage—stroke.

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Atherosclerotic disease in the carotid artery is one important cause of ischemic stroke. Ischemic stroke is estimated to be responsible for >10% of all deaths and is the second cause of mortality worldwide. Atherosclerosis is a progressive and chronic inflammatory disease in which lipids, immune cells, vascular smooth muscle cells, and extracellular matrix accumulate in the subendothelial space to form the growing atherosclerotic lesion. The inflammatory reaction taking place in plaques relies on highly complex processes that are still not completely understood. However, it is known that recruited leukocytes are key facilitators of these events. In mononuclear phagocytes, the so-called M1 and M2 activation pathways are thought to represent the embodiment of the control switches in innate immune response, creating a balance between a proinflammatory environment and an anti-inflammatory environment. The M1 pathway is characterized by the synthesis of proinflammatory cytokines that are potentially harmful in the context of atheroma, while anti-inflammatory/ reparative M2 macrophages are derived from monocytes activated along the alternative pathway.

It is well known that the risk of cardiovascular events is related to the composition and stability of the plaque rather than to the degree of arterial stenosis, and recent studies suggest that inflammation is a critical determinant of plaque stability. Vulnerable plaque imaging techniques currently allow the analysis of plaque morphology and characteristics.

In this study, we aim to investigate the relationship between macrophage polarity (M1 versus M2) and the vulnerability of human atherosclerotic plaques. Patients undergoing carotid endarterectomy (CEA) were divided into 2 groups: those suffering from cerebral infarction (symptomatic group) and those that did not present with symptoms (asymptomatic group). Plaques obtained from both populations were studied to determine the nature of the macrophages associated with the plaque and their association with atherosclerotic factors.

Methods

Patients

Between November 2008 and April 2011, 65 patients underwent CEA at Sapporo Asabu Neurosurgical Hospital, Nakamura Memorial Hospital, Sapporo City General Hospital, or Hokkaido University Hospital. Written informed consent from each patient was obtained before enrollment in the study, which was performed according to Good Clinical Practice and Helsinki Declaration principles. The ethics committees of each hospital and Hokkaido University approved the studies.

Inclusion Criteria

Patients who had high-grade (>70%) stenosis and were asymptomatic or intermediate stenosis (50%) for symptomatic patients in the internal carotid artery according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) and Japanese Guidelines for the Management of Stroke confirmed by carotid B-mode ultrasound were collected. The clinical indication for CEA was met after examination by the neurologist based on the guidelines.

Patient Characteristics, History, and Medication

Medical history was recorded from all patients, and the presence of vascular risk factors, features of metabolic syndrome, and medications were noted. Clinical history was assessed for diabetes mellitus, smoking, hypertension, dyslipidemia, previous acute cerebrovascular or myocardial infarction, and peripheral vascular disease. Hypertension was diagnosed according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-7) and the Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2009) criteria (blood pressure ≥140/90 mm Hg or ≥130/80 mm Hg [with diabetes mellitus] or current antihypertensive treatment). Diabetes was diagnosed in patients with dietary treatment, those taking antidiabetic medications, or those with current fasting plasma glucose levels >7.0 mmol/L. Diagnosis of dyslipidemia was made according to the Japan Atherosclerosis Society Guidelines for Prevention of Atherosclerotic Cardiovascular Diseases 2007 when ≥1 of the following lipid abnormalities was present: low-density lipoprotein cholesterol (LDL-C) ≥3.6 mmol/L (LDL-C was determined using the Friedewald equation), high-density lipoprotein cholesterol <1.0 mmol/L, triglycerides ≥1.7 mmol/L, or when a patient was taking a lipid-lowering drug. Fasting blood samples were taken before CEA for analysis (Table 1).

Definition of Symptomatic Carotid Disease

Patients were categorized by their physician as either symptomatic or asymptomatic based on an evaluation of their history and a clinical examination. Patients who had acute onset focal neurologic symptoms were considered symptomatic, and the median number of days between the acute clinical events and the CEA was 21.5 days (range 8–40 days) in the symptomatic group. Patients who experienced acute clinical events more than 6 months earlier were considered asymptomatic.

Carotid B-mode Ultrasound

The carotid arteries were carefully examined before surgery with commercially available equipment (GE Healthcare LOGIQ9 with 9-MHz linear array transducer; Waukesha, WI). Echo imaging and the evaluation of the degree of stenosis was performed by a well-trained operator who was unaware of the clinical profile of the
patients. Images were also evaluated by well-trained physicians to determine the condition of the arterial surface. Consistent with previous research and to ensure uniformity and an unbiased approach in the analysis, these individuals were also unaware of the clinical profile of the patients.11 Plaque morphology in terms of echogenicity was used to assess plaque stability. Plaques with a smooth wall surface and no echolucent material were considered stable; those with an irregular surface, ulceration, wall flap,12,13 or with predominantly echolucent material were classified as unstable.14,15

**Human Atherosclerosis**

CEA was performed on patients under sedation and anesthesia. Obtained specimens were immediately rinsed in saline and cut into pieces including the most prominent region of the plaque. One section was collected into RNA-later solution (Qiagen, Valencia, CA) for subsequent RNA extraction, the second was pulverized for protein analysis, the third was fixed in formaldehyde for morphologic analysis, and the other sections were stored at –80°C.

**Immunohistochemistry**

The specimens were embedded in paraffin. After deparaffination, to remove endogenous peroxidase, the sections were embedded in hydrogen peroxidase and methanol. Sections were then incubated with mouse monoclonal antihuman CD68 (1:100; Dako Cytomation, Glostrup, Denmark), mouse monoclonal anti-CD11c (1:100; Dako), goat polyclonal antihuman Mannose Receptor (MR) (1:100; R&D Systems, Minneapolis, MN), mouse monoclonal antihuman CD163 (1:100; Leica Biosystems, Newcastle Upon Tyne, United Kingdom), mouse monoclonal antihuman matrix metalloproteinase-2 (MMP-2; 1:100; Abcam, Cambridge, MA), and goat polyclonal antihuman MMP-9 (1:100; R&D Systems) overnight at 4°C. Controls without primary antibody were run for each protocol, resulting in consistently negative observations. Sections were incubated with biotin-conjugated antimouse or antigoat immunoglobulin G (IgG), treated with peroxidase-conjugated streptavidin, and stained with a 3, 3’-diaminobenzidine substrate kit (Nichirei Bioscience, Tokyo, Japan).

**RNA Extraction and Analysis**

RNA-later-stored plaques (n = 65) were removed from RNAlater and milled with a rotor-stator homogenizer. Total cellular RNA was extracted with a TRI Regent (Qiagen) and purified with the RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturer’s recommendations. cDNA was synthesized from 0.5 μg of total RNA (high capacity RNA-to-cDNA kit; Applied Biosystems, Warrington, United Kingdom). A real-time polymerase chain reaction (PCR) study was performed in triplicate.
on a 7500 Fast Real Time PCR system using SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was determined by the comparative critical threshold method, normalizing expression to 18S.

Protein Extraction and Immunoblot Analysis

Samples (n = 65) were pulverized with a homogenizer in a Tris-ethylenediaminetetra-acetic acid (EDTA) buffer containing 10 mM Tris/hydrochloric acid (pH 7.4) and 1 mM EDTA. Equal amounts (30 μg) of proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane. The primary antibodies used were CD68 (1:1000), CD11c (1:100), CD168 (1:500), and MR (1:100). Actin was used as a loading control. Secondary antibodies were antimouse IgG (CD68, CD11c, and CD168) or antigoat IgG (MR). To ensure equal loading, membranes were stripped and re-probed with the use of goat antihuman actin AB (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Analysis was performed using Amersham ECL Advance Western blotting detection kit (GE Healthcare, Little Chalfont, United Kingdom), and images were obtained using a CCD-camera system LAS-4000 UV mini (Fujifilm, Tokyo, Japan).

Statistical Analysis

Data were summarized using mean and standard deviation or median, minimum, maximum as appropriate for continuous variables and counts as well as percentages for categorical variables. Data distribution was analyzed using the Kolmogorov–Smirnov test. According to these results, appropriate parametric or nonparametric tests were used in further analyses. Unpaired Student t or Mann–Whitney U tests were performed to assess differences among quantitative variables. The Chi-square test was applied to analyze differences in qualitative and categorical variables. Data were analyzed using JMP for Windows software (version 9.0.2; SAS Institute, Cary, NC) and P < .05 was considered statistically significant.

Results

Sixty-five patients (52 males and 13 females; mean age 68.9 years old) were stratified into 2 groups: the asymptomatic group (n = 34) and the symptomatic group (n = 31). The patients’ clinical characteristics and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Asymptomatic (n = 34)</th>
<th>Symptomatic (n = 31)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, × 10^9/L</td>
<td>195 ± 46</td>
<td>203 ± 50</td>
<td>.61</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>125 ± 18</td>
<td>133 ± 12</td>
<td>.18</td>
</tr>
<tr>
<td>WBC, × 10^9/L</td>
<td>6.2 ± 1.6</td>
<td>6.0 ± 1.1</td>
<td>.62</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>80.9 ± 47.5</td>
<td>84.6 ± 20.7</td>
<td>.75</td>
</tr>
<tr>
<td>Uric acid, mmol/L</td>
<td>0.80 ± 0.40</td>
<td>0.80 ± 0.39</td>
<td>.88</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.3 ± 1.7</td>
<td>4.1 ± 0.6</td>
<td>.51</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.1 ± 0.6</td>
<td>1.0 ± 0.5</td>
<td>.52</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.3 ± 1.5</td>
<td>2.1 ± 1.0</td>
<td>.51</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/L</td>
<td>3.1 ± 2.1</td>
<td>3.7 ± 1.7</td>
<td>.31</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>7.6 ± 2.8</td>
<td>7.2 ± 3.2</td>
<td>.68</td>
</tr>
<tr>
<td>Hemoglobin A1c, %</td>
<td>6.1 ± 1.0</td>
<td>5.7 ± 8</td>
<td>.33</td>
</tr>
<tr>
<td>CRP, × 10^4 μg/L</td>
<td>1.7 (0.0-4.3)</td>
<td>1.5 (0.0-6.8)</td>
<td>.78</td>
</tr>
</tbody>
</table>

Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cell count. P values determined by the unpaired Student t or Mann–Whitney U test.

*Values are reported as the mean ± standard deviation.

†Value reported as median (range).

Table 3. Ultrasound determination of carotid stenosis and plaque vulnerability in asymptomatic and symptomatic patients

<table>
<thead>
<tr>
<th>Condition of arterial surface, n</th>
<th>Asymptomatic (n = 34)</th>
<th>Symptomatic (n = 31)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>30</td>
<td>18</td>
<td>.78</td>
</tr>
<tr>
<td>Irregular</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Ulceration</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Wall flap</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total occlusion</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Vulnerability, n</td>
<td></td>
<td></td>
<td>.033†</td>
</tr>
<tr>
<td>Stable</td>
<td>21</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Unstable</td>
<td>13</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

*Mann–Whitney U test. Values are reported as mean ± standard deviation.

†P < .05 using the Chi-square test.
medications are shown in Table 1. The 2 groups did not differ significantly according to age, sex, individual risk factors, medication, or coexistence of other vascular diseases (angina pectoris, previous myocardial infarction, and previous cerebrovascular disease). Overall, there was no significant difference in metabolic risk factors (hypertension, diabetes mellitus, or dyslipidemia). Basic biochemical and hematologic parameters and inflammatory parameters (white blood cell count and C-reactive protein) did not differ between the groups (Table 2).

The average carotid artery stenosis detected by ultrasound was not statistically different between the 2 groups; however, the frequency of unstable plaques (plaque with an irregular surface, ulceration, wall flap, or with predominantly echolucent material) was considerably higher in the symptomatic group (Chi-square test; \( P = 0.033 \); Table 3).

Representative immunostaining of plaques from both asymptomatic and symptomatic group are shown in Figure 1. Macrophage-rich areas were identified by CD68 \(^{+} \) immunostaining. This staining revealed that plaques from the symptomatic group had a greater concentration of macrophages and especially M1 macrophages (CD11c \(^{+} \)). In addition, enhanced staining of MMP-2 and MMP-9 was observed in histologic sections from symptomatic patients. Conversely, enhanced expression of markers associated with alternative M2 macrophage differentiation (CD163 and MR) was observed in plaques from the asymptomatic group (Fig 1).

Consistent with the immunostaining results, Western blotting revealed increased protein expression of the M1 marker (CD68 and CD11c) in the symptomatic group plaques and increased expression of the M2 markers (CD 163
Relative mRNA expression of the M1 markers CD68 (3.7-fold) and CD11c (3.3-fold) were significantly higher in the plaques from symptomatic patients compared to the asymptomatic patients. Monocyte chemoattractive protein-1 (MCP-1; 3.4-fold), interleukin-6 (IL-6; 9.6-fold), and MMP-9 (10.6-fold) expression was significantly increased in the symptomatic plaques (Fig 3A). Real-time PCR revealed that expression of the M1 markers (CD68 [4.1-fold] and CD11c [5.7-fold]) were significantly increased in the unstable plaques defined by ultrasound analysis in symptomatic group (Fig 3B), while expression of the M2 markers (CD163 [0.3-fold] and MR [0.1-fold]) was significantly lower in the unstable plaques defined by ultrasound analysis in the asymptomatic group (Fig 3C).

**Discussion**

We characterized the phenotype of macrophages present in symptomatic and asymptomatic plaques. Analysis of plaque immunostaining and Western blotting revealed a greater concentration of macrophages present in the plaques isolated from the symptomatic group. In addition, the majority of these macrophages were stained by CD11c, which is indicative of a M1 macrophage population. In the asymptomatic plaque, small numbers of macrophages are observed, and they predominantly stained positive for M2 macrophage markers. Previous studies have reported that increased macrophage volume and the presence of inflammatory cytokines or chemokines in atherosclerotic carotid plaques were associated with plaque vulnerability.16–18

During the preparation of this work, a study commented on the relationship between plaque vulnerability and macrophage polarity. However, these authors compared the plaque to an adjacent nonatherosclerotic zone in the carotid artery in identical patients, and found that both M1 and M2 macrophages were more abundantly expressed in the plaque area.3 We are the first authors to compare the polarity of macrophages in plaques from symptomatic or asymptomatic patients. Our data show that M1 macrophage expression is exclusive to symptomatic plaques while M2 macrophages are present in both symptomatic and asymptomatic plaques.

Gene expression of proinflammatory cytokines and chemokines, such as IL-617 or MCP-1,19 is increased in the plaques of the symptomatic group compared with the asymptomatic group. These data are in agreement with previous reports showing that vulnerable plaques contain large numbers of macrophages and lymphocytes. This is also consistent with our observation that expression of proinflammatory M1 macrophages is elevated in the symptomatic group plaques. The ratio of the increase in IL-6 compared with that of IL-10 suggests that both M1 and M2 macrophages increase in the symptomatic group compared with asymptomatic group, and that the M1 phenotype is much more predominant in the symptomatic group. Enhancement of MMP-2 and MMP-9 contributes to plaque rupture by inducing tissue fragility.20–23 MMP-2 and MMP-9 were found to be more greatly expressed by proinflammatory M1 macrophages in symptomatic patients. These data suggest that a series of events linked to M1 macrophage infiltration are occurring.
in the symptomatic group plaques that are influencing plaque stability and predisposing them to collapse.

It is clear that diabetes mellitus,24,25 dyslipidemia,26,27 hypertension,28 and metabolic syndrome29 are associated with the development and progression of atherosclerosis. A number of large clinical trials with an endpoint of atherosclerotic disease show that rigorous risk management suppresses the progression of arteriosclerosis.30-33 However, the association between plaque stability and clinical measures is more complex. Several studies have shown an association between carotid plaque stability and differences in background or clinical parameters.34,35 In contrast, our study and others have reported no significant differences in background or clinical parameters between symptomatic and asymptomatic patients.21,22,36 This lack of consistent correlation between clinical measures and plaque stability may be explained by the duration a patient has had atherosclerotic disease and/or when a medical treatment for the disease was initiated. Specifically in our study, most patients in the symptomatic and asymptomatic groups had been routinely receiving several antiatherosclerotic agents, and it is feasible that clinical laboratory values were normalized in advance of changes in plaque stability. This hypothesis is supported by separate studies where MMP22 and cholesterol uptake genes37 remained up-regulated in the arteriosclerotic plaques of symptomatic patients even when their peripheral blood parameters had been normalized. Additional longitudinal studies will be required to clarify the relationship between plaque stability and traditional clinical diagnostic measures. Although histologic analysis after clinical events would be the best way to determine when macrophage polarity switches or is altered in plaques, this is not feasible for human studies.

Another important finding of our study was that plaque morphology and not stenosis could be used as a marker for symptomatic events. The contents of the plaque, including the polarity of macrophages, could be estimated by ultrasound before surgery. Our data show that the frequency of unstable plaques defined by ultrasound.
was significantly higher in the symptomatic group, and that expression of M1 macrophage markers was also significantly elevated in plaques from the symptomatic group. However, unstable plaques were observed in both symptomatic and asymptomatic patient populations. In order to examine the association between macrophage polarity and plaque vulnerability, we further investigated the gene expression profile of stable and unstable plaques within these 2 separate populations. Consistent with our comparison of plaques from symptomatic and asymptomatic patients, the unstable plaques within the symptomatic population had increased expression of M1 markers and reduced expression of the M2 marker. This relationship became less apparent in the asymptomatic patient plaques, because only the M2 marker was reduced. In this study, we investigated the relationship between macrophage polarity and the vulnerability of human atherosclerotic plaques and clinical events. Macrophage polarity of atherosclerotic plaques was associated with clinical incidence of ischemic stroke and unstable plaques defined by ultrasound.

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References


