Possible involvement of pericytes in intraplaque hemorrhage of carotid artery stenosis

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OBJECTIVE Intraplaque hemorrhage (IPH) is most often caused by the rupture of neovessels; however, the factors of intraplaque neovessel vulnerability remain unclear. In this study, the authors focused on pericytes and aimed to investigate the relationship between IPH and pericytes.

METHODS The authors retrospectively analyzed the medical records of all patients with carotid artery stenoses who had undergone carotid endarterectomy at their hospitals between August 2008 and March 2016. Patients with carotid plaques that could be evaluated histopathologically were eligible for study inclusion. Intraplaque hemorrhage was analyzed using glycophorin A staining, and patients were divided into the following 2 groups based on the extent of granular staining: high IPH (positive staining area > 10%) and low IPH (positive staining area ≤ 10%). In addition, intraplaque neovessels were immunohistochemically evaluated using antibodies to CD34 as an endothelial cell marker or antibodies to NG2 and CD146 as pericyte markers. The relationship between IPH and pathology for intraplaque neovessels was investigated.

RESULTS Seventy of 126 consecutive carotid stenoses were excluded due to the lack of a specimen for histopathological evaluation; therefore, 53 patients with 56 carotid artery stenoses were eligible for study inclusion. Among the 56 stenoses, 37 lesions had high IPH and 19 had low IPH. The number of CD34-positive neovessels was equivalent between the two groups. However, the densities of NG2- and CD146-positive neovessels were significantly lower in the high IPH group than in the low IPH group (5.7 ± 0.5 vs. 17.1 ± 2.4, p < 0.0001; 6.6 ± 0.8 vs. 18.4 ± 2.5, p < 0.0001, respectively).

CONCLUSIONS Plaques with high IPH are associated with fewer pericytes in the intraplaque neovessels. This finding may help in the development of novel therapeutic strategies targeting pericytes.

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KEYWORDS carotid artery; carotid stenosis; pericyte; vascular disorders
from the blood to neurons is strictly regulated and brain homeostasis is maintained.\textsuperscript{3} However, the role of pericytes in IPH is unclear. In the present study, we aimed to investigate the relationship between IPH and pericytes.

**Methods**

**Patient Population**

This study was approved by the regional ethics committee of Saga University Hospital. We retrospectively analyzed the medical records of all patients with carotid artery stenoses who had undergone carotid endarterectomy (CEA) at Saga University Hospital and affiliated hospitals between August 2008 and March 2016. Patients eligible for inclusion in the study were those with carotid plaques that could be evaluated histopathologically. Carotid endarterectomy was indicated for symptomatic and asymptomatic patients with carotid artery stenosis \(>50\%) and > 80\%,\) respectively, using the North American Symptomatic Carotid Endarterectomy Trial criteria. A carotid stenosis was considered symptomatic if there had been a previous ischemic event such as stroke, transient ischemic attack, or amaurosis fugax in the 6 months before surgery; it was considered asymptomatic if there was no such history. We also recorded the incidence of cardiovascular risk factors.

**Immunohistochemical Analysis**

Carotid plaques obtained during CEA were fixed in formalin immediately after surgical removal and divided transversely at the maximum point of the macroscopic stenotic lesion. Approximately 3-mm-thick tissue blocks were fixed in 10\% buffered formalin and embedded in paraffin. Serial 4-\(\mu\)m transverse sections were then stained with H & E, and the adjacent sections were used for immunohistochemical analysis. Immunohistochemical staining was performed for glycophorin A (dilution 1:800, DAKO), CD68 (dilution 1:100, DAKO), CD34 (dilution 1:2000, Abcam), NG2 (dilution 1:400, Merck Millipore), and CD146 (dilution 1:800, Abcam).

Intraplaque neovascularization was evaluated using antibodies to CD34, as endothelial cell markers, or antibodies to NG2 and CD146, which have been reported as pericyte markers.\textsuperscript{5,8} In this analysis, a “plaque neovessel” was defined as a lumen that had a single layer and showed immunohistochemical-positive tube-like formation. “Shoulder lesions” were defined as tissues adjacent to the outer border of the lipid core down to the vessel lumen.\textsuperscript{19} Most of the newly formed vessels in atherosclerotic plaques have been reported to develop in shoulder lesions;\textsuperscript{10} therefore, we evaluated the number of neovessels in these areas using two sections of the thickest plaque. These sections were imaged using a microscope-mounted high-resolution digital camera (Olympus). Two certified neurosurgeons who were blinded to the clinical data counted the neovessels using ImageJ software (http://rsb.info.nih.gov/ij/down load/), and the mean values were analyzed.

We analyzed for the presence of macrophage infiltration in the shoulder lesions using CD68. The proportion of the CD68-positive area was measured by a certified neurosurgeon (A.O.) using ImageJ software. Next, we analyzed IPH using glycophorin A as a protein specific to the erythrocyte membrane.\textsuperscript{11} Glycophorin A grades have been described as follows in coronary arteries: 1, focal granular staining in less than 5\% of the plaque; 2, mild granular staining in 5\%–10\% of the plaque; 3, moderate granular staining in 11\%–25\% of the plaque; and 4, marked granular staining in more than 25\% of the plaque.\textsuperscript{11} Using these grades, we divided the patients into the following 2 groups: low IPH group (grades 1 and 2) and high IPH group (grades 3 and 4). A certified neurosurgeon (A.O.) measured the proportion of the glycophorin A–positive area using ImageJ software.

Finally, neovessel caliber was assessed by a certified neurosurgeon (A.O.). The shortest diameter was defined as the neovessel caliber. We compared the caliber of NG2- and CD146-positive neovessels with that of NG2- and CD146-negative neovessels.

**Statistical Analysis**

Nominal data were analyzed using Fisher’s exact test. Parametric and nonparametric numerical data were analyzed using the \(t\)-test and Mann-Whitney \(U\)-test, respectively. Correlations were examined using Spearman’s rank correlation test. Interobserver variability in the number of neovessels was examined using a paired \(t\)-test. We used JMP Pro 11 software (SAS Institute Inc.) for the statistical analyses. A \(p\) value \(<0.05\) was considered statistically significant.

**Results**

Seventy of 126 consecutive carotid stenoses were excluded given the lack of a specimen for histopathological evaluation. Thus, 53 patients with 56 carotid artery stenoses were eligible for analysis (Fig. 1). There were 50 men and 3 women, and the mean patient age was 70 years (range 38–87 years). Among the 56 lesions, 43 (77\%) were symptomatic and 13 (23\%) were asymptomatic. The patients had a high incidence of cardiovascular risk factors, including hypertension (41 lesions [77\%]) and dyslipidemia (29 lesions [52\%]; Table 1).

On glycophorin A staining, 37 lesions had high IPH and

| TABLE 1. Summary of characteristics among 53 patients with 56 carotid artery stenoses |
|-------------------------------------------|----------------|----------------|----------------|
| Variable                                  | High IPH       | Low IPH        | \(p\) Value    |
| No. of lesions                            | 37             | 19             | 0.035          |
| Age in yrs                                | 72.5 ± 7.5     | 66.4 ± 13.8    |                |
| Male sex                                  | 35 (95)        | 18 (95)        | NS             |
| Rate of stenosis                          | 70.5 ± 14.5    | 80.4 ± 14.9    | 0.02           |
| Symptomatic lesions                       | 29 (78)        | 14 (74)        | NS             |
| Hypertension                              | 25 (68)        | 16 (84)        | NS             |
| Dyslipidemia                              | 21 (57)        | 8 (42)         | NS             |
| Diabetes mellitus                         | 17 (46)        | 9 (47)         | NS             |
| Coronary artery disease                   | 15 (41)        | 6 (32)         | NS             |
| Smoking                                   | 20 (54)        | 13 (68)        | NS             |

NS = not significant. Data are presented as the mean ± standard deviation or number (%), unless indicated otherwise. \(p < 0.05\) considered statistically significant.
Tanaka et al.

19 had low IPH, and these cases were classified into the high and low IPH groups, respectively. The mean number of CD34-positive neovessels was similar in the high and low IPH groups (10.3 ± 0.8 vs. 10.8 ± 1.4, p = 0.75; Fig. 2A). The mean number of NG2-positive neovessels was significantly lower in the high IPH group than in the low IPH group (5.7 ± 0.5 vs. 17.1 ± 2.4, p < 0.0001; Fig. 2B).

Additionally, the mean number of CD146-positive neovessels was significantly lower in the high IPH group than in the low IPH group (6.6 ± 0.8 vs. 18.4 ± 2.5, p < 0.0001; Fig. 2C). Similar results were obtained when two observers analyzed the results separately; interobserver variability was low (Table 2). The mean number of NG2- and CD146-positive neovessels was significantly correlated (Fig. 2D).

The CD68-positive area was significantly larger in lesions with high IPH than in those with low IPH (Fig.

FIG. 1. Flow diagram of patients enrolled and analyzed.

FIG. 2. Box plots showing the relationship between the number of neovessels and IPH. No significant difference was found in the number of CD34-positive neovessels between the high and low IPH groups (A). The number of NG2-positive neovessels was significantly lower in the high IPH group than in the low IPH group (B). The number of CD146-positive neovessels was significantly lower in the high IPH group than in the low IPH group (C). Horizontal lines within the bars represent medians. The number of NG2- and CD146-positive neovessels was significantly correlated (D).
The correlation between the mean number of NG2-positive neovessels and the CD68-positive area was not significant (Fig. 3B). The correlation between the mean number of CD146-positive neovessels and the CD68-positive area was slightly significant (Fig. 3C). The number of neovessels with NG2- and CD146-positive staining was significantly higher and the CD68-positive area was significantly smaller in the low IPH group than in the high IPH group (Fig. 4).

The NG2- and CD146-negative stained neovessels were frequently enlarged compared with the neovessels that stained positive with NG2 (8.6 ± 0.6 μm vs. 25.7 ± 2.1 μm, \(p < 0.0001\)) and CD146 (9.0 ± 0.4 μm vs. 31.2 ± 1.4 μm, \(p < 0.0001\); Fig. 5).

**FIG. 3.** Box plots showing the relationship between the CD68-positive area and the amount of IPH. The CD68-positive area was significantly larger in lesions with high IPH than in those with low IPH (A). The correlation between the number of NG2-positive neovessels and the CD68-positive area was not significant (B). The correlation between the number of CD146-positive neovessels and the CD68-positive area was slightly significant (C).

**FIG. 4.** Representative cases in the high IPH group (A) and the low IPH group (B). Photomicrographs of glycophorin A and CD68 staining show many positive cells in the high IPH group. The number of CD34-positive neovessels was similar in the two groups. The neovessel densities with NG2- and CD146-positive staining were significantly higher in the low IPH group than in the high IPH group. Square outlines in the panels stained using H & E show plaque shoulder lesions. Original magnification \(\times 40\). GPA = glycophorin A. Figure is available in color online only.
The area with positive glycophorin A staining tended to be greater in symptomatic than in asymptomatic patients (22.7% ± 2.7% vs. 14.4% ± 3.0%, p = 0.12). No differences were found between symptomatic and asymptomatic patients in terms of the CD68-positive area (3.6 ± 0.6% vs. 3.0 ± 0.8%, p = 0.54) and the mean number of CD34-positive vessels (10.5 ± 0.9 vs. 10.4 ± 1.2, p = 0.98), NG2-positive vessels (9.7 ± 1.4 vs. 9.1 ± 2.0, p = 0.79), and CD146-positive vessels (10.6 ± 1.2 vs. 10.7 ± 3.5, p = 0.95).

**Discussion**

In this study, we showed higher expressions of NG2 and CD146 in the intraplaque neovessels with low IPH than in those with high IPH and no significant difference in the expression of CD34 between low and high IPH groups. The causative factor of IPH may not be fewer endothelial cells but may instead involve pericytes in the intraplaque neovessels. Arterial pericytes in advanced and complicated atherosclerotic lesions may be involved in vascular remodeling, calcification, and intraplaque neovascularization. The role of pericytes in carotid plaques has been mentioned in only one report, which showed that pericytes were associated with asymptomatic carotid stenosis and calcified plaque. Additionally, the authors showed that human vascular pericytes secreted osteoprotegerin, which underwent osteoblastic differentiation in vitro, and they suggested that pericytes may contribute to the stabilization of carotid plaques. The present study showed the possible involvement of pericytes in IPH of carotid stenosis.

Recently, several authors have reported on the role of pericytes in experimental ischemic stroke models. Studies have reported an enlargement in the infarct volume due to insufficient blood-brain barrier formation with increasing vascular permeability after ischemic stroke in pericyte-deficient mice compared with that in wild-type mice. Pericyte loss in diabetic retinopathy can advance endothelial cell loss, resulting in the formation of microaneurysms, which cause retinal hemorrhage. In the present study, we showed the enlargement of intraplaque neovessels without the expression of pericytes compared to that with the expression of pericytes. Therefore, pericyte loss in intraplaque neovessels may cause IPH.

We showed the possible involvement of pericytes in IPH of carotid stenosis. The relationships between

### TABLE 2. Interobserver variability in measuring the number of neovessels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean No. of Neovessels</th>
<th>p Value for Interobserver Variability*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High IPH</td>
<td>Low IPH</td>
</tr>
<tr>
<td>CD34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observer 1</td>
<td>10.4 ± 1.1</td>
<td>12.0 ± 1.6</td>
</tr>
<tr>
<td>Observer 2</td>
<td>10.2 ± 1.1</td>
<td>9.6 ± 1.9</td>
</tr>
<tr>
<td>NG2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observer 1</td>
<td>5.1 ± 0.5</td>
<td>21.4 ± 3.0</td>
</tr>
<tr>
<td>Observer 2</td>
<td>6.5 ± 0.7</td>
<td>12.8 ± 2.0</td>
</tr>
<tr>
<td>CD146</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observer 1</td>
<td>5.8 ± 0.9</td>
<td>18.4 ± 3.9</td>
</tr>
<tr>
<td>Observer 2</td>
<td>7.4 ± 1.1</td>
<td>18.4 ± 2.8</td>
</tr>
</tbody>
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* Paired t-test.

![FIG. 5. Photomicrographs of NG2 (A) and CD146 (B) staining show enlargement of negatively stained neovessels (black arrows) compared with the size of positively stained neovessels (white arrowheads). Box plot (C) showing the relationship of vessel caliber with the results of NG2 staining. The caliber of NG2-negative vessels was significantly larger than that of NG2-positive vessels. Box plot (D) showing the relationship of vessel caliber with the results of CD146 staining. The caliber of CD146-negative vessels was significantly larger than that of CD146-positive vessels. Figure is available in color online only.](image-url)
pericytes and drugs have been described by several authors.\textsuperscript{20,24} In an experimental ischemic stroke animal study, only cilostazol, as compared to other antiplatelet drugs such as aspirin and clopidogrel, promoted angiogenesis with the proliferation of pericytes in the peri-infarct area.\textsuperscript{20} Takagi et al. showed that cilostazol induced the proliferation of pericytes in vitro and contributed to the reduction in cerebral hematoma volume in experimental cerebral hemorrhage models.\textsuperscript{24} Therefore, it is conceivable that the use of cilostazol may enhance pericyte function and reduce the occurrence of IPH. Medical therapy for carotid stenosis, particularly in asymptomatic patients, has improved.\textsuperscript{1} Nevertheless, among patients with asymptomatic carotid stenosis, ipsilateral ischemic cerebrovascular events occurred at a higher rate in those with IPH than in those with stable plaques.\textsuperscript{6} In addition, cerebrovascular events occurred at a higher rate in those with CD146 expression on macrophages infiltrating human plaques.\textsuperscript{3} A study reported that plaque vulnerability was associated with the occurrence of IPH in carotid stenosis patients.\textsuperscript{11} These authors showed that the previous use of antiplatelet agents was significantly associated with the occurrence of IPH and that aspirin was used in 70\% of the patients who had used antiplatelet agents.\textsuperscript{13} The appropriate medical therapy for carotid stenosis patients with IPH remains unclear. To establish treatments targeting pericytes, it may be possible to tailor treatments according to the plaque characteristics assessed by non-invasive imaging modalities such as MRI.

The present study has a few limitations. First, because of the cross-sectional study design, we can only infer an association between the factors and not a causative mechanism. Second, the sample size is small, and larger patient numbers are required. Third, findings of the present study may have implications for the healing process of IPH since pericytes are multipotent cells and differentiate not only into mural cells but also into fibroblasts.\textsuperscript{14} The present study also showed that the relationship between macrophage infiltration and the expression of pericytes in the intraplaque neovessels was weak. In contrast, one study reported that plaque vulnerability was associated with CD146 expression on macrophages infiltrating human atherosclerotic plaques.\textsuperscript{22} Therefore, the relationship between macrophage infiltration and pericytes of intraplaque neovessels is unclear. Further studies are required to clarify the relationship between pericytes and the vulnerability of plaque neovessels.

Conclusions

Plaques with high IPH are associated with fewer pericytes in the intraplaque neovessels. This finding may help in the development of novel therapeutic strategies targeting pericytes.

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Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Ogata, Wakamiya. Acquisition of data: Ogata, Tanaka, Mizokami, Wakamiya, Nakahara, Inoue, Shimokawa, Yoshioka, Momozaki. Analysis and interpretation of data: Ogata, Tanaka. Drafting the article: Ogata. Critically revising the article: Ogata, Masuoka. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Ogata. Statistical analysis: Ogata. Administrative/technical/material support: Tanaka, Masuoka, Mizokami, Nakahara, Inoue, Shimokawa, Yoshioka, Momozaki, Sakata. Study supervision: Ogata, Masuoka, Momozaki, Sakata, Abe.

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