Carotid artery stenosis with a high-intensity signal plaque on time-of-flight magnetic resonance angiography and association with evidence of intraplaque hypoxia

Atsushi Ogata, MD, PhD,1 Masatou Kawashima, MD, PhD,1 Tomihiro Wakamiya, MD, PhD,1 Masashi Nishihara, MD,2 Jun Masuoka, MD, PhD,1 Yukiko Nakahara, MD, PhD,1 Ryo Ebashi, MD,1 Kohei Inoue, MD, PhD,1 Yukinori Takase, MD, PhD,1 Hiroyuki Irie, MD, PhD,2 and Tatsuya Abe, MD, PhD1

Departments of 1Neurosurgery and 2Radiology, School of Medicine, Saga University, Saga, Japan

OBJECTIVE Hypoxia induces angiogenesis and plays a major role in the progression of carotid plaques. During carotid intervention, plaques with high-intensity signals on time-of-flight (TOF) magnetic resonance angiography (MRA) often cause ischemic stroke and embolic complications. However, the role of intraplaque hypoxia before carotid endarterectomy (CEA) and carotid artery stenting is not presently understood. In this study the authors aimed to investigate the relationship between intraplaque hypoxia and MRA findings.

METHODS Nineteen consecutive patients with 20 carotid artery stenoses who underwent CEA at Saga University Hospital between August 2008 and December 2014 were enrolled in the study. The expressions of hypoxia-inducible transcription factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) were analyzed by immunohistochemical analysis. In addition, the relationship between the findings on TOF MRA and pathology for the carotid plaques was analyzed.

RESULTS High-intensity plaques on TOF MRA showed higher expression levels of HIF-1α (p = 0.015) and VEGF (p = 0.007) compared with isointensity plaques. The rate of intraplaque hemorrhage (IPH) on TOF MRA was also significantly higher in the high-intensity plaques than in the isointensity plaques (p = 0.024). Finally, the mean number of neovessels was significantly higher in those without plaque hemorrhage than in those with plaque hemorrhage (p = 0.010).

CONCLUSIONS Plaques with high-intensity signals on TOF MRA were associated with IPH and evidence of intraplaque hypoxia. This fact may represent an opportunity to establish novel therapeutic agents targeting intraplaque hypoxia.

https://thejns.org/doi/abs/10.3171/2016.4.JNS16349

KEY WORDS carotid artery; carotid stenosis; hypoxia; vascular disorders

Carotid artery stenosis represents a risk factor for cerebral infarction, and when associated with an unstable plaque, it can progress to atheroembolism.13,14 Unstable plaques are characterized by either a thin fibrous cap with a lipid-rich necrotic core or by intraplaque hemorrhage (IPH).8 Magnetic resonance imaging of human carotid plaques has confirmed histological observations identifying IPH as the main determinant of plaque progression toward instability.19 In addition, high-intensity plaque signals on time-of-flight (TOF) magnetic resonance angiography (MRA) have been shown to be associated with IPH.22

Recently, hypoxic conditions have been reported to play a major role in the progression of carotid plaques.6 As plaques grow, the thickness of the intima and media increases with the proliferation of smooth muscle cells. In turn, this promotes hypoxia deep within the plaque because oxygen diffusion from the lumen decreases with increasing plaque thickness, while oxygen demand increases because of inflammation.2 The hypoxic response is known to be mediated by hypoxia-inducible transcription factor (HIF) 1 and 2. Of these, HIF-1α is only active as a heterodimer and comprises 2 subunits: HIF-1α and HIF-1β. Although the latter is constitutively expressed, the expres-
tion of HIF-1α is strictly regulated by oxygen conditions. HIF-1α is translated and rapidly degraded by proteasomes under normal oxygen conditions; however, its degradation is blocked in hypoxia, causing its levels to rapidly increase. In the nucleus, HIF-1α and HIF-1β form the HIF-1 heterodimer, which binds to hypoxic response elements that cause gene transcription. Among the resulting gene products, vascular endothelial growth factor (VEGF) is among the most relevant to angiogenesis.

Current research suggests that hypoxia promotes plaque growth by stimulating angiogenesis, monocyte infiltration, and oxidized low-density lipoprotein uptake into macrophages, and we also know that HIF-1α and VEGF accumulate in symptomatic or histologically unstable lesions. However, we do not presently understand the role of plaque hypoxia before carotid endarterectomy (CEA) and carotid artery stenting (CAS). In the present study, we aimed to investigate the relationship between intraplaque hypoxia and MRA findings.

Methods

Study approval was obtained from the regional ethics committee of Saga University Hospital, and all patients gave their informed consent to participate. We enrolled consecutive patients with carotid artery stenoses who underwent CEA at the Saga University Hospital between August 2008 and December 2014. CEA was indicated for symptomatic or asymptomatic patients with carotid artery stenosis of >50% or >80%, respectively, using the North American Symptomatic Carotid Endarterectomy Trial criteria. A carotid stenosis was defined as symptomatic if there had been a previous ischemic event, including stroke, transient ischemic attack, or amaurosis fugax, in the 6 months before surgery; patients classified as asymptomatic had 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 point of the maximum stenotic lesion. Approximately 3-mm-thick tissue blocks were fixed in 10% buffered formalin and embedded in paraffin. Serial 4-μm transverse sections were then stained with H & E to evaluate microscopic IPH, and the adjacent sections were used for immunohistochemical analysis. Two researchers histologically assessed all transverse plaque sections independent of the clinical data. IPH was defined as the deposition of red blood cells and was not necessarily associated with plaque rupture.

Immunohistochemical staining was then performed. Serial paraffin-embedded sections (4 μm) were deparaffinized and rehydrated. Primary antibodies and dilutions were as follows: 1:50 mouse anti-HIF-1α (Novus Biologicals) and 1:80 mouse anti-VEGF (Abcam). Intraplaque neovascularization was evaluated with antibodies against CD34 (1:2000; Dako) as an endothelial cell marker. To make the CD34 and VEGF epitopes accessible for the antibodies, sections were autoclaved for 20 minutes at 121°C in a sodium citrate buffer (pH 6.0). Immune complexes were visualized using Envision + Dual Link System-HRP (Dako). The signals for HIF-1α, VEGF, and CD34 were visualized using diaminobenzidine. All sections were counterstained with hematoxylin.

Shoulder lesions were defined as the tissues adjacent to the outer border of the lipid core down to the vessel lumen. Most newly formed vessels in atherosclerotic plaques are reported to develop in shoulder lesions, so we measured the proportions of HIF-1α and VEGF in these areas using ImageJ software (http://rsb.info.nih.gov/ij/download/). A plaque neovessel was defined as a lumen that had a single layer of CD34-positive endothelial cells. Using magnification ×100, CD34-positive neovessels were counted manually in 4 randomly selected plaque fields, and the means were compared.

Statistical Analysis

We used JMP Pro 11 software (SAS Institute Inc.) for the statistical analyses. Nominal data were analyzed using the Fisher’s exact test. Parametric and nonparametric numerical data were analyzed by t-test and the Mann-Whitney U-test, respectively. Correlations were examined using the Spearman’s rank correlation test. Values of p < 0.05 were considered significant.

MRI Examination and Image Analysis

MRI examination was performed preoperatively, using either a clinical 3.0-T MRI unit (Magnetom Trio, A Tim System, Siemens AG) or a 1.5-T MRI unit (Magnetom Avanto, A Tim System, Siemens AG) with a 12-channel head coil. For 3D TOF MRA with the 3.0-T MRI unit, the imaging parameters were: TR 19–20 msec, TE 3.1–4.9 msec, flip angle 18°–20°, field of view 150–180 × 200, matrix size 240–304 × 320, and slice thickness 1.0–1.2 mm. For 3D TOF MRA with a 1.5-T MRI unit, the imaging parameters were: TR 22–24 msec, TE 6.5–7.2 msec, flip angle 20°–25°, field of view 150–180 × 200, matrix size 192–288 × 320, and slice thickness 1.0 mm. Our scan range included the carotid bifurcation.

Two researchers (M.N. and A.O.) reviewed the TOF source images of the carotid bifurcation and the proximal internal carotid artery, and final interpretations were reached by consensus. For interpretation of the MRA, the researchers were blinded to the patients’ clinical data. Maximum stenotic lesions of the carotid artery were assessed on axial source images of TOF MRA. All images were reviewed on a picture archiving and communication system. For analysis, MRI signal intensities were measured in a circular region of interest over the carotid plaque, measuring 6–10 mm². A high-intensity TOF image was defined as the presence of a hyperintense signal within the carotid plaque greater than 150% of the signal intensity of the adjacent neck muscle on TOF source images. Patients with high-intensity plaques on TOF were labeled the TOF-high group, and the other patients were labeled the TOF-iso group.
Results

Participants
We enrolled 19 consecutive patients with 20 carotid artery stenoses who underwent CEA (Table 1). There were 18 men and 1 woman, and the mean patient age was 68 years (range 42–82 years). Among the 20 lesions, 16 (80%) were symptomatic and 4 (20%) were asymptomatic. The participants had a high incidence of cardiovascular risk factors, including hypertension (15 patients; 79%) and dyslipidemia (10 patients; 53%), which were extremely common.

Expression of HIF-1α and VEGF
On TOF MRA, 11 plaques had high-intensity signals and 9 had iso-intensity signals, and we classified these as the TOF-high and TOF-iso groups, respectively. The mean area staining positive for HIF-1α was 5.48% ± 3.86% in the TOF-high group compared with 1.77% ± 1.68% in the TOF-iso group (p = 0.015; Fig. 1A). The mean area staining positive for VEGF was 4.48% ± 3.05% in the TOF-high group compared with 1.28% ± 0.75% in the TOF-iso group (p = 0.007; Fig. 1B). The expressions of both HIF-1α and VEGF were significantly higher in the TOF-high group and the expressions of both factors were significantly correlated (Fig. 1C).

Intraplaque Hemorrhage and Neovessel Formation
The rate of IPH was significantly higher in the TOF-high group compared with the TOF-iso group (p = 0.024); it was present in 10 (90.9%) of the 11 lesions in the TOF-high group and 4 (44.4%) of the 9 lesions in the TOF-iso group. We also showed that the mean number of neovessels was lower in the TOF-high group than in the TOF-iso group; however, the difference was not significant (16.8 ± 11.2 compared with 32.5 ± 26.2, p = 0.089; Fig. 2A). However, the mean number of neovessels was significantly higher in plaques without hemorrhage than in those with hemorrhage (40.9 ± 28.7 compared with 16.6 ± 10.1, p = 0.010; Fig. 2B).

Overall, we detected higher HIF-1α and VEGF expression with fewer neovascularizations in the TOF-high group and lower HIF-1α and VEGF expression with an abundance of neovascularization in the TOF-iso group (Fig. 3).

Discussion
In this study, we showed higher expression of HIF-1α and VEGF in high-intensity plaques compared with iso-intensity plaques on TOF MRA for the first time. We also showed that the rate of IPH was significantly elevated in the high-intensity plaques; this is consistent with previously published data. Under normoxic conditions, HIF-1α is usually degraded immediately; in contrast, hypoxic conditions inhibit that process and allow HIF-1α to enter the nucleus and function as a transcription factor, making it a very reliable hypoxic sensor. The angiogenic growth factor VEGF, which is then produced, has a proinflammatory action that causes instability by inducing neovascularization inside the atherosclerotic plaque.

In this study, we showed that high-intensity plaques on TOF MRA correspond to unstable plaques with IPH and hypoxic conditions.

Esposito-Bauer et al. conducted an interesting prospective investigation using MRI, including TOF MRA. They showed that, among patients with asymptomatic carotid stenosis, ipsilateral ischemic cerebrovascular events occurred at a higher rate among those with unstable plaques than among those with stable plaques. High-intensity plaque signals on TOF MRA have also been associated with IPH and ischemic symptoms after CAS. Therefore, finding a way to stabilize high-intensity plaques on TOF MRA may help prevent ischemic cerebrovascular events and reduce embolic complications during CAS. To this end, it has been reported that fluvastatin attenuates HIF-1-dependent ET-1 gene expression through the stimulation of HIF-1α ubiquitin or proteasome-dependent degradation and that pitavastatin treatment for 1 month can decrease serum VEGF levels in patients with unstable carotid plaques. Therefore, it is conceivable that the use of statins may improve intraplaque hypoxic conditions and mitigate some of the adverse sequelae.

In the present study, we showed that fewer plaque neovessels were present in plaques with IPH than in plaques without IPH. A recent study showed that IPH was more frequent in men than in women but that neovessel density was not significantly different. However, the relationship between neovessel density and IPH is not clear. Vink et al. observed that more neovessels developed in plaques without nuclear HIF-1α than in plaques with HIF-1α and concluded that this could have resulted from the presence of a hypoxic environment within plaques, with little or no neovessels to induce HIF-1α expression. We showed that high-intensity plaques on TOF MRA were associated with hypoxic conditions and fewer neovessels. Therefore, the prime causative factor underlying IPH may be hypoxic conditions rather than neovessel density. In that case, VEGF may promote both angiogenesis and vascular permeability, and its expression may induce IPH.

There are a few limitations to the present study. First, TOF MRA is not the most suitable method for detecting hypoxic conditions in plaques, and only a small subset of
patients underwent magnetization-prepared rapid acquisition with gradient echo. Second, because of the cross-sectional study design, we can only infer an association between the factors and not a causative mechanism. Further study is required to clarify the relationship between intraplaque hypoxia and the vulnerability of plaque neovessels.

Conclusions

In this study we showed that plaques with high-intensity signals on TOF MRA were associated with IPH and hypoxic conditions. This may represent an opportunity to establish novel therapeutic agents targeting intraplaque hypoxia.

Acknowledgments

We thank Mrs. Yumiko Oishi and Akiko Soejima of the Department of Neurosurgery, School of Medicine, Saga University, for their assistance.
FIG. 3. Representative cases of the TOF-high group (A) and the TOF-iso group (B). TOF MRA shows a high-intensity plaque (arrowhead) and an iso-intensity plaque (arrow). Photomicrographs of HIF-1α and VEGF staining show many positive cells in the TOF-high group, while CD34 staining shows many neovessels in the TOF-iso group. Square outlines in the panels stained using H & E (HE) show plaque shoulder lesions (original magnification ×40). Figure is available in color online only.

References


**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. Acquisition of data: Ogata. Analysis and interpretation of data: Ogata, Wakamiya, Nishihara. Drafting the article: Ogata. Critically revising the article: Ogata. Reviewed submitted version of manuscript: Ogata. Approved the final version of the manuscript on behalf of all authors: Ogata. Statistical analysis: Ogata. Administrative/technical/material support: Ogata, Masuoka, Nakahara, Ebashi, Inoue, Takase. Study supervision: Ogata, Kawashima, Irie, Abe.

**Correspondence**

Atsushi Ogata, Department of Neurosurgery, School of Medicine, Saga University, 5-1-1 Nabeshima, Saga-shi, Saga 849-8501, Japan. email: ogata.a24@gmail.com.