Contrast-Enhanced Ultrasound for the Evaluation of Neovascularization in Atherosclerotic Carotid Artery Plaques

Kozue Saito, MD; Kazuyuki Nagatsuka, MD; Hatsue Ishibashi-Ueda, MD; Akihiro Watanabe, MD; Hideaki Kannki, MD; Koji Iihara, MD

Background and Purpose—Neovascularization associated with plaque vulnerability, particularly in the plaque shoulder, is susceptible to rupture, causing ischemic events. We aimed to use contrast-enhanced ultrasound to evaluate neovessels in carotid plaques quantitatively, focusing on plaque shoulders.

Methods—Using contrast-enhanced ultrasound with perflubutane, we analyzed 50 consecutive patients who underwent carotid endarterectomy. We measured enhanced intensity and assessed the correlation between contrast effect and histopathology, comparing symptomatic and asymptomatic plaques.

Results—Enhanced intensity of the plaque shoulder was associated with neovessel density (P<0.01; ρ=0.43). Enhanced intensity of the plaque shoulder was higher in plaques with rupture than in those without (P<0.05), and in symptomatic plaques (n=31) than in asymptomatic ones (n=19; P<0.01).

Conclusions—Quantitative evaluation of the contrast effect using contrast-enhanced ultrasound enabled the assessment of neovascularization of plaque shoulders in vivo real time, which may help stratify plaque vulnerability. (Stroke. 2014;45:3073-3075.)

Key Words: carotid stenosis ■ contrast agents ■ neovascularization ■ ultrasonography

Carotid stenosis with atherosclerotic plaques is a risk factor for artery-to-artery embolism. Although risks of ischemic stroke are assessed according to degree of stenosis, even low-grade stenosis causes recurrent embolism and may be refractory to aggressive treatment. Qualitative characterization of plaques, in addition to quantification of the grading of stenosis, should be performed to detect vulnerable plaques. Neovascularization predicts plaque vulnerability, and neovessels in plaque shoulders tend to collapse, inducing intraplaque hemorrhage and plaque rupture.¹

Carotid ultrasound allows less-invasive, serial bedside evaluation of carotid morphology. Second-generation contrast agents containing less soluble gases, such as Sonovue (Bracco, Milan, Italy) and Sonazoid (GE Healthcare, Oslo, Norway), are stable in vivo and provide stable contrast because they are highly compressible and facilitate detection of small and low-flow vessels, such as neovessels in carotid plaques.

We aimed to use contrast-enhanced ultrasound (CEUS) to evaluate intraplaque neovessels quantitatively, focusing on plaque shoulders, for comparing the results with histopathology.

Methods
We enrolled 50 consecutive patients with internal carotid artery stenosis who underwent carotid endarterectomy from July 2011.

Results
We enrolled 60 consecutive patients and excluded 10 because of severe calcification and large ulcers (Table). The contrast

Data collected included vascular risks, stenosis severity, and symptoms associated with previous ischemic events on the ipsilateral side. Carotid ultrasound was performed using an LOGIQ E9 (GE Yokogawa Medical Systems, Hino, Japan) with a linear probe. Plaques were classified as echolucent or echogenic. CEUS examinations were performed after a bolus injection of Sonazoid, a lipiodialized suspension of perflubutane gas microbubbles (0.01 mL/kg body weight), using the amplitude modulation mode for further offline analysis and the phase inversion mode to delineate neovessels. We recorded images using the amplitude modulation mode of the short axis of the most stenotic lesion before and after injection. Regions of interest were set, and a time–intensity curve was generated. Enhanced intensity (EI) was calculated by subtracting baseline from peak intensities in the core (EIₘ), plaque shoulder (EIₛ), and vessel lumen (EIᵢ). We used larger EIᵢ of the 2 shoulders for further analyses (Figure 1).

Fixed carotid endarterectomy specimens were stained with hematoxylin and eosin and Masson trichrome. Plaque morphology was evaluated according to the American Heart Association classification of atherosclerotic plaques. Immunohistochemistry was performed using a monoclonal antibody against von Willebrand factor (DAKO, Nikko, Japan) to detect neovessels. Neovessel density (per square millimeter) of the side with the higher density plaque shoulder in the most stenotic lesion was used for further analyses (online-only Data Supplement).
of plaque shoulders was greater than that of cores (EIₕ versus EIₚ, 9.6±3.6 versus 1.1±1.6 dB, respectively; *P*<0.0001), consistent with histological findings that cores contained lipid and hemorrhage with few neovessels, mainly localized in plaque shoulders (Figure 2). EIₖ associated with neovessel density in the plaque shoulder (*P*=0.0017; *p*=0.43; Figure I in the online-only Data Supplement). EIₖ was higher in plaques with rupture than in those without rupture (EIₖ, 10.1±3.5 versus 6.9±3.2 dB, respectively; *P*=0.018). EIₖ tended to be higher in plaques with intraplaque hemorrhage than in those without rupture (EIₖ, 9.9±3.6 versus 7.6±2.5 dB, respectively; *P*=0.095).

The contrast effect of the plaque shoulder of symptomatic plaques was significantly greater than that of asymptomatic plaques (EIₖ, 10.8±3.7 versus 7.7±2.4 dB, respectively; *P*=0.0016). Among symptomatic plaques, EIₖ values of plaques that had a time interval from the onset of the last event to carotid endarterectomy of ≤6 months were higher than those of plaques with an interval of >6 months, but the difference was not statistically significant (EIₖ, 11.3±3.7 versus 9.2±3.7 dB, respectively; *P*=0.34; Figure II in the online-only Data Supplement).

**Discussion**

The histological characteristics of vulnerable plaques, including intraplaque hemorrhage, thrombus, inflammatory cells, thin fibrous caps, and neovascularization, were reviewed.² Neovessels are immature and fragile, particularly in plaque shoulders, because local inflammatory damage and shear stress from the arterial lumen lead to collapse, causing intraplaque hemorrhage and plaque rupture,¹ consistent with the present results.

Although MRI³ and computed tomographic angiography⁴ using contrast agents yield highly reproducible evaluations of carotid artery neovascularization, precise delineation of neovessels remains difficult because enhancement depends on endothelial permeability, and contrast agents gradually penetrate the extravascular space. In contrast, CEUS generates real-time images of microbubbles as intravascular tracers that penetrate the plaque from the vessel lumen or adventitial side through neovessels.

This is the first CEUS study to evaluate neovessels in plaque shoulders quantitatively and compare the results with those of histopathologic specimens. Other studies using CEUS correlate symptomatology and CEUS contrast effects of the entire plaque visually⁵ or quantitatively.⁶ Here, the high contrast

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**Table. Characteristics of Patients and Plaques**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=50)</th>
<th>Symptomatic (n=31)</th>
<th>Asymptomatic (n=19)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>71.5±6.9</td>
<td>72.1±6.5</td>
<td>71.2±7.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Male sex</td>
<td>49 (98)</td>
<td>30 (97)</td>
<td>19 (100)</td>
<td>0.43</td>
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<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>43 (86)</td>
<td>26 (84)</td>
<td>17 (89)</td>
<td>0.58</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>14 (28)</td>
<td>5 (16)</td>
<td>9 (47)</td>
<td>0.017</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>37 (74)</td>
<td>21 (68)</td>
<td>16 (84)</td>
<td>0.2</td>
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<tr>
<td>Smokers (former or current)</td>
<td>34 (68)</td>
<td>21 (68)</td>
<td>13 (68)</td>
<td>0.96</td>
</tr>
<tr>
<td>Plaque characteristics</td>
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<td></td>
<td></td>
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<tr>
<td>Severity</td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>3 (6)</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td></td>
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<tr>
<td>50%–69%</td>
<td>7 (14)</td>
<td>6 (19)</td>
<td>1 (5)</td>
<td></td>
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<tr>
<td>≥70%</td>
<td>40 (80)</td>
<td>22 (71)</td>
<td>18 (95)</td>
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<tr>
<td>Echogenicity</td>
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<td></td>
<td></td>
<td>0.20</td>
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<tr>
<td>Echoluent</td>
<td>39 (78)</td>
<td>26 (84)</td>
<td>13 (68)</td>
<td></td>
</tr>
<tr>
<td>Echogenic</td>
<td>11 (22)</td>
<td>5 (16)</td>
<td>6 (32)</td>
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<td>AHA classification</td>
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<tr>
<td>Type V</td>
<td>4 (8)</td>
<td>1 (3)</td>
<td>3 (16)</td>
<td></td>
</tr>
<tr>
<td>Type VI</td>
<td>46 (92)</td>
<td>30 (97)</td>
<td>16 (84)</td>
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<tr>
<td>Histological findings</td>
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<td></td>
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<tr>
<td>Intraplaque hemorrhage</td>
<td>44 (88)</td>
<td>28 (90)</td>
<td>16 (84)</td>
<td>0.52</td>
</tr>
<tr>
<td>Plaque rupture</td>
<td>43 (86)</td>
<td>29 (94)</td>
<td>14 (74)</td>
<td>0.049</td>
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<tr>
<td>CEUS data</td>
<td></td>
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<td></td>
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<tr>
<td>EIₖ (dB)</td>
<td>24.0±6.0</td>
<td>24.0±6.8</td>
<td>23.9±4.7</td>
<td>0.88</td>
</tr>
<tr>
<td>EIₖ (dB)</td>
<td>1.1±1.6</td>
<td>1.1±1.7</td>
<td>1.1±1.4</td>
<td>0.88</td>
</tr>
<tr>
<td>EIₖ (dB)</td>
<td>9.6±3.6</td>
<td>10.8±3.7</td>
<td>7.7±2.4</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

Percentages are enclosed in parentheses. AHA indicates American Heart Association; CEUS, contrast-enhanced ultrasound; EIₖ, enhanced intensity in the core; EIₖ, enhanced intensity in the lumen; and EIₖ, enhanced intensity in the plaque shoulder.
effect in plaque shoulders may more reliably predict the risk of plaque rupture and intraplaque hemorrhage.

Histopathology revealed an inverse correlation between neovessel density and time from the ischemic event to carotid endarterectomy, suggesting that significant angiogenesis occurs soon before an episode of plaque instability and regresses thereafter as remodeling.7 Our findings support this hypothesis although the results were not statistically significant (Figure II in the online-only Data Supplement).

CEUS can predict and stratify plaque vulnerability and may enhance evaluation of treatment strategies for atherosclerotic diseases. For example, CEUS detected decreased angiogenesis in a patient treated with statins.8 Although we did not evaluate the effects of antiatherosclerotic drugs or levels of serological markers here, additional studies analyzing serological markers or interventional prospective studies of antiatherosclerotic treatment are required.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

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Supplemental Methods

Patient Enrollment

Between July 2011 and January 2013, we enrolled 50 consecutive patients with internal carotid artery stenosis who underwent CEA at the National Cerebral and Cardiovascular Center. The Ethics Committee of the National Cerebral and Cardiovascular Center approved this study. Written informed consent was obtained from all subjects before enrolment. Exclusion criteria were previous allergic reaction to Sonazoid or to eggs, because the lipid-stabilized suspension of Sonazoid contains egg yolk.

Patient Characteristics

The definitions of vascular risk factors were as follows: hypertension (blood pressure > 140/90 mmHg or antihypertensive drug use), diabetes mellitus confirmed according to guidelines or medication for diabetes mellitus, dyslipidemia (low-density lipoprotein > 3.6 mmol/L, high-density lipoprotein < 1.0 mmol/L, triglycerides > 3.8 mmol/L or statin use), and smoking (current or past smoker). The degree of carotid stenosis was assessed according to findings on CT angiography or conventional cerebral angiography and was calculated according to the definition stated in the Northern American Symptomatic Carotid Trial (NASCET). Symptomatic plaques were determined as those associated with a history of transient ischemic attack, cerebral infarction, or both on the ipsilateral side.

Ultrasound Investigations

Carotid ultrasound examination was performed using an LOGIQ E9 ultrasound system (GE Yokogawa Medical Systems, Hino, Japan) equipped with a 6–9 MHz phased-array transducer (9L-D linear probe). The examination of both carotid arteries without contrast that included the common and internal carotid arteries was performed using B-mode, color Doppler mode, and pulse Doppler spectral analysis, and the results were stored digitally for further analysis. Each plaque was classified according to echogenicity as follows; uniformly echolucent (class I), predominantly echolucent (class II), predominantly echogenic (class III), predominantly echogenic lesions with small areas of echolucency (class IV), or uniformly echogenic or extensively calcified (class V). We defined classes I and II as the echolucent group, and classes III, IV, and V as the echogenic group. Upon completion of the noncontrast portion of the examination, CEUS examinations were performed using the amplitude modulation (AM) and phase inversion (PI) modes. The mechanical index was 0.2–0.3. Image depth was adjusted to 4–5 cm, and focus position was 3–4 cm. Sonazoid (0.01 ml/kg body weight) was injected as an intravenous bolus, followed by a 10-ml saline flush through an antecubital vein. It was necessary to discriminate the true contrast effect from artifacts appearing as bright echoes. We initiated the observation before the injection of contrast agent and traced the microbubbles moving into the plaques from the vessel lumen or adventitial side to eliminate artifacts. The appearance of microbubbles was observed within 10–20 s following injection. In AM mode, images were recorded on the short-axis at the most stenotic lesion before (as a reference image) and after (perfusion image including first-pass) starting the injection. After the first-pass, we observed the plaques and recorded images in the short- and long-axes in AM and PI modes. Neovessels of the plaques were delineated by accumulation of these cine memory images in PI mode. Evaluable images were acquired for at least 5 min after injecting each bolus. Ultrasound examinations were performed by two investigators (H.K., K.S.) with no prior knowledge of the patients’ clinical
information.

**CEUS Image Analysis**

The cine clip with GE-exclusive raw data format files was saved on the LOGIQ E9. Data were analyzed offline. Regions of interest (ROIs) were set in the vessel lumen (to ensure filling with the contrast agent), the plaque core, and the both sides of the plaque shoulders, which were defined as lateral edges of the plaques on the short-axis image with the highest grade of stenosis (Figure 1A). A time-intensity plot was generated for each area. We measured the baseline intensity (BI) (dB) before injection of contrast agent and the peak intensity (PI) (dB) in the ROIs. The enhanced intensity (EI) was calculated by subtracting BI from PI in the core (EI_c), the plaque shoulder (EI_s), and the lumen (EI_l). We used the greater EI_s of the two sides for further analyses (Figure 1-B).

**Histologic Analysis**

CEA specimens were immediately fixed in Histochoic (Amresco, Solon, OH, USA) for 48 h, decalcified in EDTA for 1 week, and then 3-mm thick transverse tissue slices of the carotid artery were prepared and embedded in paraffin. Serial transverse 3-μm sections were prepared and stained with haematoxylin and eosin and Masson’s trichrome. Histological examination was performed by an experienced pathologist (H.I.U.) who was uninformed of the ultrasonographic findings. The images were used to evaluate plaque morphology according to the American Heart Association (AHA) classification for atherosclerotic plaques as follows: type IV lesions, atheroma containing lipid core; type V lesions containing prominent new fibrous connective tissue; and type VI lesions appearing as complex plaques with disrupted surfaces, hematoma, thrombus, or all of these.

Immunohistochemistry was performed using a monoclonal antibody (diluted 1:50) against the endothelial-cell marker von Willebrand factor (DAKO, Japan) to stain neovessels in the plaque. We defined all the microvessels that reacted with the antibody in the fibrous cap as neovessels, because normal human intima lacks blood vessels. Shoulder regions were defined as the fibrous cap of the lateral edges between intima with and without plaques.

Images of the sections were captured and analyzed using a digital image analysis system (Aperio, CA, USA). Neovessel density was measured on both sides of the plaque shoulder in each section (/mm²). The higher neovessel density between both sides was utilized at the most stenotic lesion for further analyses to choose the same slice and the same side of the plaque shoulder as the ultrasound image.

**Statistical Analysis**

JMP 8.0.2 software (SAS Institute, Cary, NC, USA) was used for statistical analysis. Statistical analysis was performed using the Wilcoxon rank sum test, chi-square test, or Fisher’s exact test. Correlation analysis between enhanced intensity and neovessel density was performed using Spearmann’s rho correlation. p <0.05 was defined as indicating statistical significance.

**References for Methods**


Figure I

Analysis of neovessel density and enhanced intensity in the plaque shoulders.

Spearman’s rho 0.43  
$p = 0.0017$
Box plot of EI in plaque shoulders.
The borders of the boxes show the 75th and 25th percentiles, the whiskers show the range of data, and the lines within the boxes indicate the median.