Inflammation within Carotid Atherosclerotic Plaque: Assessment with Late-Phase Contrast-enhanced US

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Original Research

Purpose: To determine if the number of nontargeted microbubbles retained in human carotid plaque is sufficient to be detected with ultrasonography (US).

Materials and Methods: The study protocol was approved by the local research ethics committee. Informed consent was obtained. A total of 37 subjects with carotid atherosclerosis (mean age, 69.9 years; age range, 49–86 years), of whom 27 (73%) were men (mean age, 69.7 years; age range, 58–86 years) and 10 (27%) were women (mean age, 70.3 years; age range, 49–86 years), were studied between December 2008 and May 2009 with late-phase (LP) contrast material–enhanced US by using flash imaging with a nonlinear mode at an intermediate mechanical index of 0.34 6 minutes after bolus contrast agent injection. Plaques were defined as symptomatic if symptoms consistent with stroke, transient ischemic attack, or amaurosis fugax had occurred in the neurovascular territory of the plaque studied within 12 months prior to entry into the study. Plaques were defined as asymptomatic if no such events had ever occurred within the neurovascular territory. Raw linear data were used to quantify echogenicity of the plaque, which was normalized to lumen echogenicity. Gray-scale median score was also calculated.

Results: Of the 37 subjects, 16 (43%) had symptomatic plaques and 21 (57%) had asymptomatic plaques. All examinations yielded evaluable LP contrast-enhanced US data. Normalized LP plaque echogenicity was greater in the symptomatic group (0.39; 95% confidence interval: −0.11, 0.89) than in the asymptomatic group (0.69; 95% confidence interval: −1.04, −0.34) (P = .0005). There was a moderate (ρ = −0.44, P = .016) inverse correlation between normalized LP plaque echogenicity and gray-scale median score.

Conclusion: By quantifying microbubble retention within the carotid plaque, LP contrast-enhanced US depicts clear differences between groups of subjects with plaque ipsilateral to symptoms and asymptomatic plaques. This technique has promise as a tissue-specific marker of inflammation and a potential role in the risk stratification of atherosclerotic carotid stenosis.

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Large-artery atherosclerosis accounts for approximately 30% of strokes (1). The mechanism responsible is normally carotid plaque rupture, when the integrity of the fibrous cap is lost and luminal blood communicates with the thrombogenic core of the plaque (2). This leads to the formation of a thrombus, which can subsequently embolize and occlude a distal cerebral artery, resulting in stroke.

It has long been recognized that the plaques at risk for rupture are not necessarily those that impinge most substantially on the lumen (3). Nevertheless, current clinical imaging investigations still focus on quantification of the degree of luminal stenosis; hence, their performance is relatively poor in the prediction of which previously asymptomatic patients will experience a stroke.

A consensus has emerged that the plaques that are most vulnerable to rupture are those with an abundance of macrophages and a large inflammatory infiltrate (2,4–7). Hence, there has been considerable interest in imaging inflammation within atherosclerotic plaques; this has been achieved with fluorodeoxyglucose positron emission tomography (8) and magnetic resonance (MR) imaging enhanced with either iron oxide (9,10) or gadolinium (11) particles. However, given the prevalence of carotid atherosclerosis, costs prohibit these techniques from being translated into routine clinical practice.

Late-phase (LP) contrast material–enhanced ultrasonography (US) may represent a low-cost modality with which to detect plaque inflammation. US contrast agents (microbubbles) are phagocytosed by monocytes in vitro and remain acoustically active for up to 30 minutes (12,13). Preclinical studies have shown that microbubbles can be detected within monocytes that are attached to the endothelium of inflamed tissue (14). It has also been shown that microbubbles adhere directly to the surface of damaged endothelium (15).

The purpose of this study was to determine if the number of nontargeted microbubbles retained in human carotid plaque is sufficient to be detected with US.

Materials and Methods

Both the US scanner and the software required to analyze the data were provided by Philips (Bothell, Wash); however, the authors had control of the data and information submitted for publication. One author (T.G.) is an employee of Philips; however, this author had no control over the inclusion of any data or information that might have presented a conflict of interest.

Study Subjects

This prospective study was approved by the local research ethics committee and the Medicines and Healthcare Products Regulatory Agency. The approval of this agency was required because the contrast agent was being used for an off-label indication. Informed consent was obtained from all subjects prior to the examination. Between December 2008 and May 2009, we recruited subjects aged at least 18 years who came to our vascular clinic to undergo carotid duplex US and in whom US revealed an atherosclerotic plaque causing more than 30% stenosis as assessed with velocity criteria. We excluded subjects who experienced myocardial infarction or unstable angina within 14 days before the study, as well as those who experienced cardiac failure (New York Heart Association classification, III or IV) or had prosthetic heart valves, as these are contraindications to contrast agent administration. The patients were not consecutive, since not all patients were approached in time to enroll and some patients declined enrollment. A total of 37 subjects were enrolled (mean age, 69.9 years ± 8.5 [standard deviation]). Of these patients, 27 (73%) were men (mean age, 69.7 years; age range, 58–86 years) and 10 (27%) were women (mean age, 70.3 years; age range, 49–86 years); there was no significant difference between the age of men and the age of women.

Plaques were defined as symptomatic if symptoms consistent with stroke, transient ischemic attack (TIA), or amaurosis fugax had occurred in the neurovascular territory of the plaque studied within the 12 months prior to entry into the study. Plaques were defined as asymptomatic if no such event had ever occurred within the neurovascular territory. Of the 37 subjects, 16 were recruited into the symptomatic group and 21 were recruited into the asymptomatic group (Table 1). In the symptomatic group, the time between the cardiovascular event and LP...
Table 1

Characteristics of Symptomatic and Asymptomatic Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Symptomatic Group (n = 16)</th>
<th>Asymptomatic Group (n = 21)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>71 ± 9.9</td>
<td>69 ± 7.5</td>
<td>.65</td>
</tr>
<tr>
<td>Male sex</td>
<td>11 (69)</td>
<td>16 (76)</td>
<td>.72</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>3 (19)</td>
<td>3 (14)</td>
<td>&gt;.99</td>
</tr>
<tr>
<td>Statin use</td>
<td>15 (94)</td>
<td>16 (76)</td>
<td>.24</td>
</tr>
<tr>
<td>Hypertension</td>
<td>12 (75)</td>
<td>17 (81)</td>
<td>.70</td>
</tr>
<tr>
<td>Smoking history</td>
<td>11 (69)</td>
<td>13 (62)</td>
<td>.74</td>
</tr>
</tbody>
</table>

Note.—Unless otherwise indicated, data are numbers of patients and data in parentheses are percentages.
* Data are means ± standard deviations.

Contrast-enhanced US was less than 30 days in 10 subjects (62%) and less than 50 days in 14 subjects (88%). With regard to cerebrovascular events, six subjects (38%) had experienced stroke, eight (50%) had experienced TIA, and two (12%) had experienced amaurosis fugax. In the asymptomatic group, TIA or stroke had been previously diagnosed in seven (33%) patients; this event affected the hemisphere contralateral to the one in which plaque was studied. In the remaining 14 (67%) patients in the asymptomatic group, TIA, stroke, or amaurosis fugax had never been diagnosed. There was no significant difference between the symptomatic and asymptomatic groups in terms of age; sex; history of diabetes mellitus, hypertension, or smoking; and presence of a 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor (statin) in their prescription (Table 1).

Standard and Contrast-enhanced Carotid US

The US examination was performed with the subject in the supine position and with a US scanner (iU22; Philips) equipped with a high-frequency linear-array L12-5-MHz probe. Luminal stenosis was measured in the sagittal plane by using velocity criteria, which approximated the North American Symptomatic Carotid Endarterectomy Trial criteria (16,17). This measurement was made by a clinical vascular scientist with at least 5 years of experience in carotid US as part of each subject’s routine care. The gray-scale median score was calculated as previously described, with the luminal blood and carotid wall adventitia serving as reference points for normalization (18).

LP contrast-enhanced US was subsequently performed. The contrast agent (SonoVue; Bracco Spa, Milan, Italy) consisted of a phospholipid shell containing the inert gas sulfur hexafluoride. The contrast agent was prepared immediately before the examination by mixing 25 mg of hexafluoropropylsiloxane powder with 3 mL of saline. A 2-mL dose of this preparation was injected as an intravenous bolus into an antecubital vein. Subjects were observed for 30 minutes after administration of the contrast agent and verbally asked about the occurrence of adverse events.

LP contrast-enhanced US of the carotid bifurcation and internal carotid artery was performed with flash imaging at an intermediate mechanical index of 0.34 with a nonlinear imaging (power modulation) contrast mode 6 minutes after bolus contrast agent injection. In the symptomatic group, the plaque on the symptomatic side was studied. In the asymptomatic group, the plaque that was causing the greatest stenosis was studied. Six flash frames were acquired in less than 1 second in the axial orientation at the level of greatest stenosis. The cine loop of the acquisition was saved on the hard drive. Contrast-enhanced examinations were performed by a radiologist (O.D., 3 years of carotid US experience) who was blinded to the subjects’ clinical information.

QLAB software (Philips) was used to quantify the echogenicity of the plaque from the acquired cine loop and to measure the gray-scale median score. Raw linear data were used for analysis. All regions of interest were drawn by an ultrasonologist (E.L.S.L., 17 years experience) who was blinded to patient medical history. On the fundamental B-mode image, a single region of interest was drawn on the outline of the plaque (Fig 1). The region of interest was automatically mapped to the same position on the contrast-enhanced image, and the aforementioned software was used to determine the echogenicity of each pixel within the region of interest. The mean echogenicity was then automatically calculated. A second region of interest was drawn around the residual lumen and used to determine mean echogenicity in the lumen.

Statistical Analysis

The raw linear data generated with the aforementioned software were found to have log-normal distribution; therefore, these data were log transformed for statistical analysis. The echogenicity of plaque was normalized by dividing plaque echogenicity by lumen echogenicity. (Because the data were log transformed, normalization required subtracting lumen echogenicity from plaque echogenicity.) The normalized echogenicity in the symptomatic group was compared with that in the asymptomatic group with the t test, assuming unequal variances (GraphPad Prism 5.01; GraphPad Software, San Diego, Calif). Within the symptomatic group, plaques in the territory of a stroke were compared with those in the territory of a TIA. Within the asymptomatic group, plaques from subjects who had never experienced a cerebrovascular event were compared with plaques from patients who had a history of such events affecting the contralateral hemisphere. These comparisons were made with the t test. The Pearson correlation coefficient was used to investigate the relationship between gray-scale median score and LP contrast-enhanced US echogenicity, as well as the relationship between LP contrast-enhanced US echogenicity and luminal stenosis. Baseline frequencies between the two groups were compared with the Fisher exact test or t tests, where appropriate. The possible relationship between subject
characteristics and LP contrast-enhanced US was assessed by using analysis of variance with the characteristic as a covariate (SAS, version 9.1.3; SAS Institute, Cary, NC). Sensitivity and specificity of LP contrast-enhanced US in the correct identification of plaques as symptomatic or asymptomatic were derived by using receiver operating characteristic curve analysis. Cutoff values that minimized the difference between sensitivity and specificity were chosen. For all statistical tests, a $P$ value of .05 indicated a significant difference. The number of subjects enrolled in the study yielded 90% power to detect effect sizes (difference divided by standard deviation) of 1.1 with a 5% type I error rate. Sample size calculations were performed with PASS software (NCSS, Kaysville, Utah).

### Results

#### Relationship between LP Contrast-enhanced US Echogenicity and Symptoms

Evaluable LP contrast-enhanced US data were obtained in all 37 subjects enrolled, and none of the subjects reported an adverse event during the observation period. The percentage of luminal stenosis ranged from 30% to 99%, and while the mean stenosis in the symptomatic group was greater than that in the asymptomatic group, the difference between the two groups was not significant ($P = .06$) (Table 2). Given the standard deviation observed in this study and the sample size, a difference in stenosis of 20.5% would have been required to yield 90% power to detect a difference between the two groups. The LP contrast-enhanced US normalized plaque echogenicity was significantly greater in the symptomatic group (0.39; 95% confidence interval: $-0.11, 0.89$) than in the asymptomatic group ($-0.69; 95%$ confidence interval: $-1.04, -0.34$) ($P = .0005$) (Fig 2). However, there was overlap in echogenicity between the two groups. Of note, the lowest echogenicity in the symptomatic group was derived from the subject who had the longest event-to-examination time (nearly 1 year). In this subject, echogenicity fell below the mean echogenicity of the asymptomatic group.

#### Relationship between LP Contrast-enhanced US Findings and US Findings

There was a significant difference between the two groups with regard to gray-scale median score. The asymptomatic group (mean, 29.01; 95% confidence interval: 32.844, 25.176) had a higher gray-scale median score than did the symptomatic group (mean, 16.95; 95% confidence interval: 18.544, 15.356) ($P = .0124$). There was a moderate ($r = -0.44, P = .016$) inverse correlation between normalized plaque echogenicity and gray-scale median score, demonstrating the tendency.
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for plaques with greater normalized plaque echogenicity to have a lower gray-scale median score. There was no correlation between normalized LP contrast-enhanced US plaque echogenicity and the percentage of luminal stenosis ($P = .27$).

**Relationship between LP Contrast-enhanced US and Subject Characteristics**

There was no evidence of a relationship between diabetes, smoking history, or statin dose and LP contrast-enhanced US echogenicity, nor was there evidence of differential LP contrast-enhanced US echogenicity between subjects in the symptomatic group with TIA and those with cerebrovascular attack. Within the asymptomatic group, there was no significant difference in LP contrast-enhanced US echogenicity between subjects who had never experienced a cerebrovascular event and those who had a history of such an event affecting the contralateral hemisphere.

**Receiver Operating Characteristic Curve Analysis**

Sensitivity and specificity were 75% and 86%, respectively, for an LP contrast-enhanced US normalized peak echogenicity cutoff of more than 0.

**Discussion**

Our results show that the LP contrast-enhanced US echogenicity of carotid plaques is greater in symptomatic plaques (those within the neurovascular territory of a recent cerebrovascular event) than in asymptomatic plaques. This suggests that plaques responsible for cerebrovascular events are those that tend to have late US enhancement. This finding may merely represent the fact that symptomatic plaques have more intraplaque blood volume than do asymptomatic plaques; thus, they contain more circulating microbubbles in the LP, just as they do with dynamic contrast-enhanced US (19). However, this is unlikely because during the dynamic phase, echogenicity in the lumen is approximately two orders of magnitude greater than echogenicity in the plaque. Were the LP plaque echogenicity purely a result of circulating microbubbles, it should only represent a small fraction of the late-phase lumen echogenicity. The fact that the late-phase echogenicities of plaque and lumen are of similar magnitude implies that microbubbles have accumulated within the plaque. Biologic plausibility of this theory is provided by preclinical work showing that microbubbles are passively targeted to tissue with activated endothelium, inflammation, or both (12,14,15). Therefore, we suggest that by depicting retained untargeted microbubbles, LP contrast-enhanced US has the potential to depict inflammation, endothelial activation, or both within carotid plaque in vivo.

To our knowledge, this is the first study to show that microbubbles passively target atherosclerotic carotid plaque in humans. This finding may have important clinical consequences for patients at high risk of plaque rupture and consequent stroke because ex vivo analysis of atherosclerotic plaques has shown that plaques that cause rupture are characterized by an abundance of macrophages and an inflammatory infiltrate (2,4–7). The fact that the echogenicity derived from the symptomatic group overlaps with that derived from the asymptomatic group implies LP contrast-enhanced US may have the potential to be used to identify asymptomatic patients who are at high risk of cerebrovascular events and who might benefit from intensive medical treatment or surgical intervention. Histologic validation studies will be required to determine whether features of the plaque that pertain to inflammation or endothelial dysfunction correlate with the LP contrast-enhanced US echogenicity. Such studies might also shed light on the mechanism by which nontargeted microbubbles adhere to the plaque. Subsequent prospective studies will be required to investigate the natural history of the LP contrast-enhanced US echogenicity, as well as to

![Figure 2](image-url)

Figure 2: Box plot shows normalized plaque LP contrast-enhanced US echogenicity. Middle lines indicate the mean, vertical bars indicate standard error of mean.
determine if it can be used to predict the clinical outcome and, if so, over how long a time frame it can be used.

As expected, gray-scale median score was greater in asymptomatic plaques than in symptomatic plaques (20). Although the strength of the correlation was only moderate, the LP contrast-enhanced US echogenicity was inversely associated with gray-scale median score. Such a correlation is expected, as plaques with a low gray-scale median score have high lipid, hemorrhage, and macrophage content (21–24) and would therefore be expected to retain more microbubbles.

In several studies, researchers used contrast-enhanced US to assess carotid plaque; however, in these studies, the researchers used the contrast agent as a blood pool agent and acquired data during the dynamic phase to detect intraplaque angiogenesis (19,25–27). In the majority of these studies, visual assessment of the postprocessed data was used (25–27). In only one study, echogenicity was quantified with image analysis software, and the results were used to perform receiver operating characteristic curve analysis (19). The receiver operating characteristic curve analysis in the present study compares favorably with that generated from the aforementioned US contrast-enhanced study (sensitivity and specificity were 75% and 86%, respectively, in our study and 74% and 75%, respectively, in the dynamic contrast-enhanced study). Such comparisons between the two techniques are premature at this stage, as both techniques are in their infancy. However, LP contrast-enhanced US offers the advantages of a shorter time frame for acquisition, making the examination technically easier to perform. Also, because image acquisition lasts less than 1 second, motion artifacts do not cause a problem.

This study had several limitations. Plaques were labeled as symptomatic or asymptomatic depending on whether they were within the neurovascular territory of a recent cerebrovascular event. It was assumed that symptomatic plaques were the culprit lesions responsible for these events and therefore would exhibit a high degree of inflammation compared with asymptomatic plaques. However, this assumption is unlikely to be correct in all cases for two reasons. First, some of the subjects in the symptomatic group are likely to have experienced a cerebrovascular event that had a noncarotid cause, such as intracranial atherosclerosis, and therefore would not necessarily be expected to have inflamed plaque. Second, some plaques from the asymptomatic group may have been grossly inflamed, but they may have caused only subclinical disease. MR imaging of the brain performed to detect silent infarcts and histologic examination of surgical specimens would have reduced these limitations.

The symptomatic and asymptomatic groups were both heterogeneous. The symptomatic group was composed of subjects with TIA, stroke, or amarousia fugax. Recent evidence suggests that patients with plaques deemed responsible for causing a stroke heal differently than those with plaques deemed responsible for causing a TIA in the postevent period (28). The asymptomatic group comprised subjects who had never experienced a cerebrovascular event and those who had experienced an event that affected the contralateral hemisphere. MR imaging studies with iron oxide particles show that plaques that are contralateral to symptomatic plaques have greater inflammation than do plaques in subjects who have never experienced a cerebrovascular event (29), reflecting the systemic nature of atherosclerosis. Although no evidence of a difference in LP contrast-enhanced US echogenicity was detected between these subgroups, the study was not powered for this purpose and such differences cannot be excluded. A larger study would be required to definitively address the relationship between LP contrast-enhanced US and these characteristics.

The symptomatic group was also heterogeneous with regard to the time between the cerebrovascular event and LP contrast-enhanced US assessment. Because the plaque heals after stroke (28), the receiver operating characteristic curve analysis may have yielded improved sensitivity and specificity had the inclusion criteria for the symptomatic group been more stringent and an event-to-examination time of less than 2 weeks been stipulated.

The nonlinear pulse sequence used was not designed for this indication and represents a crude means with which to detect the low concentration of microbubbles expected to be present in the plaque in the LP. To accurately quantify the degree of microbubble retention and correlate the echogenicity with histologic features of the plaque, improved nonlinear pulse sequences developed specifically for this purpose are required.

Because the late-phase technique destroys retained bubbles, we were able to assess only one slice of the plaque. To assume that this one slice of plaque was representative of the entire plaque would have been undoubtedly incorrect. Contrast modes for three-dimensional US are now available and will help to address this problem in future studies.

As with the majority of modalities that are used to measure echogenicity, the late-phase echogenicity of plaque requires normalization to avoid variation from irrelevant other factors that affect echogenicity, such as focal zone depth, plasma volume, and microbubble concentration. To mitigate these sources of variation, we divided the plaque echogenicity by the lumen echogenicity. However, this quantification method is sensitive to factors that affect luminal echogenicity. Furthermore, the kinetics of those microbubbles flowing within the vascular space probably differ from those of microbubbles retained in the plaque; therefore, this method will also be sensitive to the time point at which LP contrast-enhanced US is performed. Further studies are required to investigate the most appropriate method with which to normalize the echogenicity.

In conclusion, by quantifying microbubble retention within the carotid plaque, LP contrast-enhanced US shows clear differences between groups of plaques within the neurovascular territory of recent cerebrovascular events and asymptomatic plaques; thus, it has promise as a tissue-specific marker of inflammation. This technique may be useful in the identification of asymptomatic patients who might benefit from intensive medical or surgical therapy.
or as a biomarker with which to investigate pharmacodynamic effects of experimental molecules.

References


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