Contrast Carotid Ultrasound for the Detection of Unstable Plaques with Neoangiogenesis: A Pilot Study


Department of Paride Stefanini, Vascular Surgery Unit, Sapienza University of Rome, Viale del Policlinico 155, 00100 Rome, Italy
Department of Neurological Sciences, Sapienza University of Rome, Rome, Italy
Department of Radiology, University of Rome, S. Andrea, Rome, Italy
Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy
Department of Biopathology and diagnostic Imaging, University of Rome, Tor Vergata, Rome, Italy

Submitted 29 August 2008; accepted 12 December 2008
Available online 27 March 2009

Abstract

Objectives: To evaluate whether contrast ultrasonography can be used to distinguish asymptomatic from symptomatic carotid plaques and provide insight into underlying pathophysiological differences.

Design: Contrast Carotid ultrasound was performed in both symptomatic and asymptomatic patients referred for carotid endarterectomy.

Materials and methods: Of 77 consecutive patients referred for carotid artery evaluation, 64 underwent carotid endarterectomy for asymptomatic cerebrovascular disease and 9 underwent urgent surgery for acute neurological deficits with hemiparesis. The endarterectomy specimens were assessed immunohistologically.

Results: In all 9 patients undergoing urgent surgery, contrast ultrasonography showed the accumulation of diffuse microbubble contrast at the base of the carotid plaque. This pattern was observed only in 1/64 of the patients undergoing surgery for asymptomatic carotid disease. Immunohistologically staining of the endarterectomy specimens showed that the area of microbubble contrast at the base of the symptomatic plaques was associated with an...
Introduction

Ultrasonographic evaluation of carotid plaque morphology represents, apart from the quantification of degree of stenosis, a fundamental tool to evaluate the risk of stroke related to carotid disease. About 1/3 of cerebrovascular accidents are indeed due to a hemodynamic or thromboembolic event arising from a complicated carotid plaque. Many studies have indeed confirmed that the “unstable” plaque is heterogeneous, echolucent and with surface ulcerations and that these features may be responsible for both lesion progression and clinical symptoms. Nevertheless, the pathophysiological mechanisms underlying the progression towards carotid plaque instability require detailed elucidation.

Recent histological studies have demonstrated the importance of inflammation and plaque angiogenesis in the development and progression of the atheroma, and the clinical association between inflammation, atherosclerosis progression and cardio/cerebrovascular events has been investigated. Neoangiogenesis occurs indeed regularly within atherosclerotic plaques and symptomatic carotid disease have been associated with an increased microvessels’ density that, being immature and fragile, could be more permeable to noxious inflammatory components accumulating in the extracellular matrix of the media and intimal layers, increasing the progression of vessel wall disease, plaque functional activity and vulnerability.

Carotid ultrasonography has been proven as a reliable technique to image carotid atherosclerosis, providing better definition of the arterial lumen, plaque morphology and surface. More interestingly, contrast carotid ultrasound may have the potential to visualize plaque microvessels, thus allowing the study of pathophysiology of the plaque “in vivo”. The focus of this study was to evaluate the characteristics of the carotid plaque with contrast carotid ultrasonography and compare these findings with post-operative plaque histology.

Materials and Methods

Standard and contrast carotid ultrasound investigations were performed in patients with documented severe degree of internal carotid artery stenosis, referred to our department for elective or emergency/urgent carotid endarterectomy (CEA), in order to better define plaque morphology and plaque neovascularization.

Patients

Of 77 consecutive patients referred for consideration of carotid endarterectomy: (51 male and 26 female, mean age 67 ± 6), 64 underwent elective carotid endarterectomy for asymptomatic cerebrovascular disease (no symptoms in previous 6 months). Nine patients were symptomatic for recent or acute neurological hemiparesis due to contralateral internal carotid artery stenosis: these patients underwent urgent surgery within 1 week from symptom onset. Two further patients with internal carotid artery occlusion were not treated surgically and the remaining 2 patients were treated endovascularly. The study was approved by the local ethical committee and all patients gave written informed consent.

Ultrasound imaging protocol

Carotid duplex scanning was performed with an Acuson/Siemens Sequoia 512, with the software “Cadence Contrast Pulse Sequencing” (Cadence CPS). Linear phased array probes (6, 8 and 15 Mhz) were used to assess carotid plaques, with same presetting for all patients.

The best visualization of carotid plaques documented in B-Mode, Color and Power modes on both longitudinal and transversal scans were digitally stored. Angle corrected blood flow velocities were obtained with pulsed wave Doppler at the maximum site of stenosis. Plaque echographic morphology has been described according to criteria already well-established in literature: plaques were considered as hyperechoic with acoustic shadow, hyperechoic, isoechoic, hypoechoic, and consequently as calcific, fibro-calcific fibrous, fibro-fatty, haemorrhagic. Plaque surface was classified as regular, irregular and ulcerated, when a surface irregularity of >2 mm was detected. Quantification of total plaque echogenicity with computerized analysis of Gray Scale Median (GSM) analysis was also performed. The degree of stenosis was evaluated according to European Carotid Surgery Trial (ECST) criteria, as percentage of the difference between the original and the residual lumen at the maximum site of stenosis and to the relative increase of blood flow velocities.

Contrast ultrasound investigation was performed as already described elsewhere. A total amount of a full dose of SonoVue (25 mg) contrast agent injection reconstituted with 5 ml of saline solution (Bracco Altana Pharma, Konstanz, Germany) in an antecubital vein (20 gauge Venflon) was performed in repeated bolus, the first, 1/2 dose (2.5 ml), followed by two other 1/4 doses (1.25 ml). Each bolus was followed by a saline flush.

After identifying the plaque on longitudinal and transverse scans, and after obtaining baseline B-mode, Color and Power images of the plaque, the 15 Mhz linear array probe with a mechanical index varying from 0.4 to 1.4 with Cadence CPS continuous real-time recording software was used to visualize plaque vascularization. This software is based on the processing of nonlinear generated signals in the fundamental frequency band that can improve sensitivity...
and specificity of contrast agents’ detection. The “Contrast Agent only” software feature, in which the image is derived only from the signals of the microbubbles, has been used. The investigations were performed together by two different experienced sonographers (EV, MFG), and digitally stored onto an external hard-disk for off-line analysis. QONTRAST Software by Bracco Imaging was also applied to color-code contrast distribution within the plaque.

Histological sampling and immunohistochemical studies

Carotid endarterectomy specimens, after fixation in 10% buffered formalin and decalcification, if necessary, were sliced transversely every 5 mm, embedded in paraffin and stained with Movat’s pentachrome stains. For each plaque, 3–5 sections were examined according to the extension of the plaque. Plaques were cut from the top edge to the bottom on the longitudinal planes, to obtain a specimen that was directly comparable with the bidimensional images obtained at ultrasound. In the last 6 cases transversal planes were obtained to evaluate the whole section of the plaque. The sonographers (EV, MFG) instructed the pathologist, blinded to clinical data, on how to perform the cut, according to the regions of interest pre-identified at ultrasound. Sections were probed with hematoxylin–eosin coloration, to have a general view of plaque cellularity, and immunostained with antibodies for Vascular Endothelial Growth Factor (VEGF) and Matrix MetalloProteinase 3 (MMP3) (DAKO, Glostrup Denmark). The last 6 cases also were stained for CD31 (1:100 dilution, purchased from Dako, Glostrup, Denmark) and CD34 (1:4000 dilution, Dako) antibodies markers for endothelial cells cocktail with an overnight incubation at 4 °C, with diaminobenzidine as the final chromogen. To control for false positivity, immunostaining was probed with the placenta and without the primary antibody. Intraobserver agreement was 95%. The regions of interest observed at ultrasound images were identified.

Results

Standard ultrasonography

All the 73 patients undergoing carotid endarterectomy presented with complicated plaques characterized by a heterogeneous echographic pattern, predominantly hypoechoic and with irregular surface, determining severe internal carotid stenosis.23 According to the criteria established3,4 for carotid plaque GSM and cerebrovascular risk evaluation, the majority (86.3%, 63 of 73) was classified as major risk plaques, with GSM values <25. The remaining 10/73 (13.7%) had GSM values higher than 25, due to the presence of small hyperechoic calcified spots within the plaque. All 9 symptomatic patients undergoing urgent surgery had very low GSM scores ranging from 5 to 12. Asymptomatic patients had higher GSM values than symptomatic ones (23.4 ± 8 versus 9.1 ± 3; Mann–Whitney p < 0.0001).

Contrast ultrasound imaging

In all 64 asymptomatic patients weak, discrete contrast-enhancement was detected mainly in the hypo-echoic areas, corresponding to fibrous and fibro-fatty tissue (Fig. 1). Isolated microvessels, readily identifiable by high intensity spots of microbubbles running from the adventitial layers towards the vessel lumen, were the main vascularization pattern (type I, Table 1). Vascularization was not detected either in the hyperechoic with acoustic shadow calcified tissue, or in the hypoechocic necrotic and haemorrhagic areas.

In the 9 acute symptomatic patients a completely different pattern of contrast enhancement was detected (Fig. 2). In the first seconds after contrast agent administration, little contrast was identified in the hypoechocic areas (type I pattern observed in 5/9 patients). A few seconds later, a major diffuse area of contrast enhancement appeared at the base of the plaques, due to an agglomerate of many very small microvessels, difficult to differentiate from each other (type II, Table 1). The residual hypoechocic part of the plaque, corresponding to the necrotic or haemorrhagic contents, remained without contrast. A similar pattern of contrast was observed in only 1/64 asymptomatic plaques, in a patient who had presented with a stroke 2 years earlier. Therefore the presence of microbubble contrast at the base of the plaque was associated strongly with the presence of symptomatic plaque (1/64 versus 9/9, Fisher exact test p < .0001).

Histology and microvessel diameter

Histological examination confirmed the presence of vascularization at the base of all 9 symptomatic plaques, consistent with the presence of ultrasound contrast-enhanced areas. All symptomatic carotid plaques contained an increased number of small (diameter 20–30 μm), immature microvessels at their base consistent in with strong neoangiogenetic activity (black arrows in Fig. 2 E). Microvessels and angiogenesis were less evident in asymptomatic plaques, with microvessel diameter being of larger diameter (>50 μm) (Black arrows in Fig. 1E).

Immunohistochemical staining

Immunostaining for VEGF and MMP3 revealed different patterns for asymptomatic and symptomatic plaques. In asymptomatic plaques immunostaining was slight and localized mainly along the course of the scant microvessels. In symptomatic plaques high intensity immunostaining was observed in the adventitial aspect of the plaque, associated with the presence of small diameter microvessels. In the single asymptomatic patient with type II contrast enhancement in the plaque, numerous microvessels were detected under the fibrous cap in the shoulder of the atheroma, clearly identified after CD31 and CD34 immunostaining.

Discussion

The NASCET24 and the ECST22 trials clearly demonstrated that carotid endarterectomy significantly reduces the risk of neurological events in patients with severe internal carotid stenosis. Nevertheless, the risk of thromboembolic events is related more to the instability of the atheroma than to the extent of disease.6,7 Further, the identification
of plaque echogenicity, supported by the quantitative computerized assessment, has been identified as an accurate objective tool for the evaluation of unstable plaques.\textsuperscript{3,4} Data collected from literature have indeed demonstrated that low GSM plaques, composed of hemorrhage and lipidic necrotic core, are associated with an increased risk of embolic events.\textsuperscript{5,20,21} However, the pathophysiological mechanisms responsible of the progression towards carotid plaque instability still remain to be better elucidated.

Recent studies have observed that plaque instability is a dynamic process in which inflammation and neoangiogenesis are linked to atherosclerotic progression and development of neurological events.\textsuperscript{25,26} Moreover, recent histological studies have observed that stable plaques are characterized by a low grade chronic inflammatory infiltrate, whereas unstable and symptomatic plaques are characterized by an active inflammation and “plaque activity” processes, involved in the thinning of the fibrous cap and thus predisposing to the plaque rupture.\textsuperscript{6–8} A high

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & Asymptomatic plaques ($n = 64$) & Symptomatic plaques ($n = 9$) \\
\hline
GSM & $23.4 \pm 8$ & $9.1 \pm 3$ \\
\hline
Type I & 64/64 & 5/9 \\
\hline
(rare, discrete contrast enhancement with microvessels of large diameter 50–60 µm) & & \\
\hline
Type II & 1/64 & 9/9 \\
\hline
(diffuse contrast enhancement, with microvessels of small diameter, 20–30 µm, with VEGF staining, at the base of the plaque) & & \\
\hline
\end{tabular}
\caption{Assessment of plaques by echogenicity by Gray Scale Median (GSM), contrast enhancement and microvessel size and distribution.}
\end{table}
density of vasa vasorum is correlated strongly with the number of infiltrating mononuclear cells, suggesting that neovessels are an important route for the entry for leukocytes and inflammatory cells into advanced lesions, to correlate with development of clinical symptoms. These processes have not been subject to “in vivo” imaging techniques, which are considered as the “gold standard” for the demonstration of the direct temporal correlation between inflammation, angiogenesis and developing of clinical symptoms in carotid atherosclerosis.

Our pilot study indicates that contrast ultrasonography has considerable potential as an “in vivo” imaging technique of plaque vascularization and neovascularization. Patients with acute symptomatic disease showed predominantly a specific pattern of diffuse contrast enhancement at the base of the plaques, close to the adventitial layer, corresponding to a high density of small diameter microvessels. The histological specimens of these pre-selected areas showed the presence of small microvessels with high density, to confirm the presence of plaque neoangiogenesis in the acute symptomatic patients. On the other hand, the vascularization pattern was very different in patients with asymptomatic disease, with more mature larger diameter microvessels, diffusely distributed. Further prospective data is necessary to confirm and extend our findings and to assess the role of the detection of plaque vascularization in the work-up of patients presenting with cerebrovascular disease.

Study limitations

The major limitation of this approach is the subjective characterisation of the patterns of contrast enhancement. An objective quantitative method of assessing contrast enhancement remains to be developed. Additional immunohistology, including CD31 staining with image analysis, to quantify microvessels also is required.

Conclusion

Contrast carotid ultrasound investigation is a reliable non-invasive technique, with the potential for investigating the severity and pathophysiology of carotid artery plaques “in vivo” and in “real time”.

Conflict of Interest/Funding

None.

Acknowledgments

We acknowledge Dr Francesca Magri (MSc, Bracco Imaging SPA, Milan, Italy) for her precious support in computerized image analysis with QONTRAST software.
References


