Cerebral Ischemia and Infarction From Atheroemboli <100 μm in Size

Joseph H. Rapp, MD; Xian Mang Pan, MD; Bo Yu, MD; Raymond A. Swanson, MD; Randall T. Higashida, MD; Paul Simpson, MD, PhD; David Saloner, PhD

Background and Purpose—To determine the importance of emboli not trapped by carotid angioplasty filtration devices, we examined fragments <100 μm released with ex vivo angioplasty and asked if fragment composition and size correlated with brain injury.

Methods—Human carotid plaques (21) were excised en bloc, and ex vivo carotid angioplasty was performed. Eight plaques were selected as either highly calcified (4) or highly fibrotic (4) by high-resolution MRI (200 μm³). Fragments were counted by a Coulter counter. Before injection into male Sprague-Dawley rats, fragments from calcified and fibrotic plaques were sized with 60-, 100-, and 200-μm filters. Brain ischemia and infarction were assessed by MRI scans (7-T small-bore magnet) and by immunohistologic staining for HSP70 and NueN.

Results—All 5 animals injected with 100- to 200-μm calcified fragments had infarctions. One was lethal. After injection of 60- to 100-μm calcified fragments, 7 of 12 animals had cerebral infarctions, whereas only 1 of 11 had infarctions with fibrous fragments (P<0.02). HSP70 staining showed that ischemia was more common and more extensive than infarction. Ischemia was found in 10 of 12 animals after injection of calcified fragments and in 9 of 11 after injection of fibrous fragments. The mean number of 60- to 100-μm fragments released was 375±510; the mean number of 20- to 60-μm fragments was 34 196 (range, 2230 to 186 927).

Conclusions—Hundreds of thousands of microemboli can be shed during carotid angioplasty. Fragments from calcified plaques cause greater levels of infarction than fragments from fibrous plaques, although ischemia is common with both fragment types. (Stroke. 2003;34:1976-1980.)

Key Words: atheroembolism ■ cerebral ischemia ■ rats

Hundreds of microembolic signals can be detected in the middle cerebral artery by transcranial doppler (TCD) during carotid angioplasty and other cardiovascular interventions.1-4 Spontaneous microemboli also occur in the carotid artery and can be detected during random TCD monitoring in 21% to 60% of patients with documented carotid stenosis.5-9 The number of spontaneous microemboli shed may be greatest among symptomatic patients. Droste and colleagues5 found up to 142 embolic signals per hour in patients with carotid stenosis and a recent transient ischemic attack. Symptoms may develop as a result of these embolic showers, but at a surprisingly low rate. In evaluating the risk of neurological injury from individual microemboli, these data may be reassuring, but are microemboli truly benign?

Particulates ~100 μm in size may be large enough to occlude the penetrating arterioles of the cerebral cortex10,11 and cause small “silent” areas of ischemia or infarction. For example, Jaeger et al12 reported a series of cerebrovascular angioplasty cases done without a protection device. There was only 1 clinically evident stroke, but diffusion-weighted MR was positive in 29% of cases. These subclinical events may have subtle effects. When >1000 signals were recorded by TCD during cardiac surgery, Pugsley et al13 found measurable neuropsychiatric deterioration in >40% of patients. In a separate study, Padayachee et al14 also found a correlation between higher rates of emboli and subtle alterations in intellectual function after cardiac surgery. Unlike the expected clinical improvement after ischemic stroke, these cognitive deficits became more pronounced when the patients were tested again months after surgery.4 Recently, a 4-year follow-up of coronary artery bypass graft patients reported that patients with cognitive deterioration at discharge may have an accelerated decline in cognitive function compared with age-matched controls.14

In response to concern regarding microembolic events during cardiovascular interventions, including carotid angioplasty, several devices and strategies have been developed to provide cerebral protection. Filtration devices clearly reduce the number of emboli to the brain. However, these devices do not trap...
fragments <80 to 120 μm in size. In previous work, we suggested that there may be a size threshold for acute ischemic cerebral injury. That report did not consider a variation in effect caused by embolus composition. In this report, we address the effect of both microemboli size and composition on the potential for infarction with atherosclerotic fragments 60 to 100 μm in size. Because our protocol involves human-to-rat xenotransplantation, we have limited our observations to 24 hours to minimize the confounding variable of thrombosis or tissue damage resulting from local immune response.

Methods

Plaque Specimens
Endarterectomy specimens were removed en bloc to preserve the integrity of the excised lumen. Patients ranged in age from 58 to 73 years. The degree of maximal stenosis ranged from 70% to 99%. All patients gave informed consent in accordance with the Committees on Human Research of the University of California, San Francisco.

Plaque Specimen Imaging
Endarterectomy specimens were gently washed and flushed with saline to remove blood and then immersed in saline at surgery. This solution was exchanged with saline doped with a 1:300 parts GD-DTPA (gadolinium) to maximize signal intensity of the medium surrounding the plaque. The specimen was placed in a 2-cm-diameter cylinder and imaged in a transmit-receive radiofrequency coil constructed with a small, sensitive volume matched closely to the volume of the cylinder containing the plaque. Imaging was done with a Siemens Symphony 1.5-T scanner. The principal geometric morphology sequence used was a 3-dimensional gradient-echo sequence (repetition time, 40 ms; echo time, 12 ms; flip angle, 20°), 256×128 matrix size, with 64 partitions. The field of view was 50×25×13 μm in the x, y, and z directions, respectively, with a resulting slice thickness of 200 μm and 200×200-μm in-plane resolution (Figure 1).

Ex Vivo Angioplasty
To prepare the calcified and fibrous carotid plaques for ex vivo angioplasty (Figure 2), the stump of atheroma from the external carotid artery was ligated, and any protruding portion was excised. An 18F latex tubing was inserted into the common carotid atheroma and fixed in place with tissue adhesive. PTFE vascular grafts (8-mm diameter) (W.L. Gore) were cut open longitudinally, and the outer reinforcing wrap was removed to give the graft expansion qualities similar to those of human adventitia. After the graft was carefully tailored to fit the contour of the plaque, the specimen was blotted dry, dotted with adhesive, and placed into the lumen of the tailored segment. The graft was then sutured closed with Gortex C-V 7 suture, and the suture line was coated with adhesive to ensure that the prepared “vessel” was water tight.

Angioplasties were performed at 37°C. Each wrapped specimen was flushed with 10 mL saline, and the effluent was discarded. As per Neurointerventional Radiology protocol, a 0.018-in guidewire was passed through the lesion. These maneuvers were aided by the 3-dimensional plaque imaging and ligation of the external carotid and were easily performed in all cases. Then, angioplasty was performed twice on each specimen. The initial angioplasty was done with a 3.5- or 4.0-mm balloon, depending on lumen size. The second angioplasty was performed with a 5-mm balloon. Each angioplasty included 2 inflations of 30 seconds at 15 atm.

Collection and Counting of Embolic Fragments
After each maneuver in the angioplasty protocol, the specimens were flushed twice with 10 mL saline, and the effluent was collected in
TABLE 1. Number of Fragments Released With Ex Vivo Angioplasty

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>&lt;20 μm</th>
<th>20–30 μm</th>
<th>30–40 μm</th>
<th>40–60 μm</th>
<th>60–100 μm</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guide wire (n=13), n</td>
<td>193.714 ±117 489</td>
<td>5976 ±9062</td>
<td>1629 ±2908</td>
<td>615 ±983</td>
<td>63 ±93</td>
<td>201 999 ±117 887</td>
</tr>
<tr>
<td>First balloon (n=13), n</td>
<td>228.209 ±112 220</td>
<td>7946 ±12 036</td>
<td>2050 ±3473</td>
<td>843 ±1276</td>
<td>138 ±197</td>
<td>235 356 ±111 548</td>
</tr>
<tr>
<td>Second balloon (n=13), n</td>
<td>317.430 ±154 543</td>
<td>10 925 ±15 367</td>
<td>2976 ±3855</td>
<td>1232 ±1591</td>
<td>173 ±240</td>
<td>369 850 ±202 298</td>
</tr>
<tr>
<td>Total, n</td>
<td>741.779 ±346 223</td>
<td>24 849 ±35 703</td>
<td>6656 ±9989</td>
<td>2691 ±3737</td>
<td>375 ±510</td>
<td>776 351 ±347 823</td>
</tr>
</tbody>
</table>

20-mL conical centrifuge tubes. Fragments <100 μm were sized and counted by a Coulter counter. To prepare the effluent for the Coulter counter, Zap ogoline was added to lyse the red cells. Samples were counted with a Beckman model ZM Coulter counter with a 100-μm filtering orifice. For these measurements, the Coulter counter was calibrated with particle standards of known diameter.

Preparation of Tissue Fragments and Microspheres for Injection

Plaque fragments were selected for injection by filtration through sized filters. Particles 100 to 200 μm in size were isolated by taking those fragments that passed through a 200-μm filter but were retained by a 100-μm filter. Particles 60 to 100 μm in size were filtered through first 100- and then 60-μm filters. In each case, the fragments retained on the second filter were removed, resuspended in saline, counted under ×100 magnification, and then diluted to a concentration of 100 fragments per 300 μL. Plaque fragments were stored in saline at 4 °C for up to 5 days before injection. Some of the calcified fragments were dried at 100 °C and stored for separate experiments.

Microspheres (98 or 50 μm) were suspended in saline, counted under ×100 magnification, and then diluted to a concentration of 100 fragments per 300 μL.

Cerebral Embolization in Rats

Male Sprague-Dawley rats (Simonsen Laboratories) weighing 300 to 400 g were given injections of 300 μL of saline alone or saline containing plaque fragments or microspheres. Rats were anesthetized with 80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine IP. Under the operating microscope, both common carotid arteries (CAs) were exposed via a midline incision in the neck. The CA was carefully isolated from the vagus nerve, and the cervical branch of the vagus nerve was cut and ligated. A temporary microvascular clip was applied on the ECA just above its origin. Once the artery was prepared, MRE-033 Micro-Renathane tubing (Brantree Scientific, Inc) was inserted retrograde into the ECA through a transverse arteriotomy, the microvascular clip was removed, and the tubing tip was positioned near the origin of the ECA. The common carotid artery was temporarily occluded with an encircling 4-0 silk. The saline, with or without atheroemboli, was injected slowly (3 to 5 seconds). After injection, the 4-0 silk was removed, the ECA was ligated, and the wound was closed. The animals were monitored continuously for the first hour, and any abnormality of gait or behavior was recorded. Animals were also assessed at 4 and 24 hours after surgery.

MRI Study

Rats were anesthetized with ketamine 50 mg/kg IP, fixed on an animal holder, and placed into the magnet. MRI was performed on a Surrey Medical Imaging System 7-T, 18.3-mm horizontal-bore Oxford magnet system equipped with Magnex self-shielded gradients. A birdcage (nonquadrature) coil was used for the experiments. Temperature was maintained by directing temperature-controlled air down the bore of the magnet (Figure 3).

An initial set of coronal gradient-echo scout images (1-mm-thick slices, 1-mm separation, 128×128 encodings, 8-m sec echo time) was taken to ensure correct positioning of the animal. A sagittal image with the same parameters was taken for positioning the subsequent coronal spin-echo images. Finally, 18 spin-echo images (1-mm-thick slices, no separation, 128×128 encodings, later zero-filled to 256×256, 64-m sec echo time, 2400-ms repetition time) were obtained to visualize the brain.

Immunohistochemistry

Postinjection brain sectioning and staining were performed as follows. Rats were euthanized 24 hours after ICA injection. Under anesthesia, the abdomen was opened, and the aorta was cannulated. Rats were given heparin (100 U/Kg) and perfused with 100 mL of 0.9% saline, followed by 500 mL of 4.0% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4 (PBS). The brains were then removed from the cranial vault and postfixed in paraformaldehyde and PBS for 2 to 4 hours. Coronal sections (100 μm) were cut on a Vibratome and placed in PBS overnight. Immunohistochemistry was performed with the avidin-biotin/horseradish peroxidase technique. Sections were placed in PBS containing 2% horse serum, 0.2% Triton X-100, and 0.1% bovine serum albumin (HS-PBS) for 2 hours at room temperature. They were incubated for 72 hours at 4 °C in the primary monoclonal antibody to HSP72 (Amersham), which was diluted 1:4000 in HS-PBS. Sections were then washed in PBS, incubated for 2 hours in biotinylated horse anti-mouse secondary antibody, and incubated for 2 hours in the avidin-horseradish peroxidase solution prepared from an Elite ABC Kit (Vectastain, Vector Laboratories). Sections were washed in PBS and reacted for horseradish peroxidase with diaminobenzidine (0.04% in PBS) and 0.3% hydrogen peroxide. Reacted sections were then washed, kept in PBS overnight, and mounted on gelatinized slides. To determine nonspecific binding, representative control sections were processed as described above except the first antibody was deleted.

Brain sections also were processed with the avidin-biotin/horseradish peroxidase technique for staining with a monoclonal antibody to the neuron-specific protein NeuN. Lack of staining with antibody to NeuN indicates nonviability of neuronal cells.

Results

Number of Fragments Released With Ex Vivo Carotid Angioplasty

Fragments were dislodged with guidewire insertion alone and each of the 2 balloon inflations. The second, larger balloon yielded more fragments in each size category (Table 1). The total number of fragments <100 μm ranged from 328 180 to 1 596 200 (mean, 776 351 ±347 823). There was an inverse relationship of size to number of fragments released, with most of the fragments being <20 μm. In the 60- to 100-μm size range, the mean number of fragments was 375 (range, 30 to 1684); in the 20- to 60-μm size range, there were tens of thousands of fragments (range, 2230 to 186 927; mean 34 196). The large range in the number of fragments released from individual plaques underscores the considerable variation between plaques in their propensity to shed emboli when manipulated.
A Comparison of the Cerebral Injury Created With Fragments Dislodged From Calcified Versus Fibrous Plaques 60 to 100 μm in Size

When male Sprague-Dawley rats were injected with 60- to 100-μm fragments from calcified carotid plaques, there were no deaths and no obvious alterations in gait or behavior. However, MRI done 24 hours after injection showed infarction in 7 of the 12 animals. Immunohistological staining with NeuN correlated with the MRI findings, showing infarctions in these 7 only. HSP staining demonstrated that areas of ischemia were more common with positive staining in 10 of the 12 animals injected (Table 2).

After injection of 60- to 100-μm fragments from primarily fibrous carotid plaques, MRI at 24 hours showed infarction in only 1 animal (P<0.02 versus injection of the fragments from calcified plaque). Among these 11 animals (1 animal was excluded because of wound complications), NeuN staining correlated with the MRI findings in the 1 animal and detected small areas of infarction in 3 animals that had negative MRI studies. HSP staining was positive in 9 of the animals, indicating that areas of tissue ischemia were common after the injection of fibrous fragments, although infarction was unusual.

Sham injections of the carotid artery with 300 μL saline alone was done in 5 animals. There were no positive findings on MRI or staining with NeuN or HSP.

Cerebral Injury 24 Hours After Injection of 60- to 100-μm Dried Fragments From Calcified Plaque

We heated and dried calcified fragments to denature any biologically active compounds accompanying these fragments. This was an initial experiment to determine whether cerebral injury from calcified fragments could be reduced by denaturing associated compounds. Some of the fragments from heavily calcified plaques were rinsed with sterile water and dried at 100°C. They were then stored at 4°C for up to 3 months. Before injection, they were rehydrated with saline, sized, and counted, and 100 fragments 60 to 100 μm in size were injected into rat ICAs as noted above. Four of 8 animals developed infarcts noted on MRI, with 7 of 8 developing areas that were positive for HSP staining.

Cerebral Ischemia 24 Hours After Injection of 50- and 98-μm Microspheres

To develop more precise guidelines as to the risk of injecting 100 particles in the 60- to 100-μm size range, we followed our cerebral ischemia protocol using either 50- or 98-μm microspheres. After injection of one hundred 50-μm microspheres in 6 animals, there were no strokes on MRI, but 2 of the animals had areas of ischemia on HSP staining (Table 3). In contrast, 10 of 11 animals injected with 98-μm microspheres had lesions seen on MRI, and all animals had areas of infarction by NeuN staining and areas of ischemia shown with HSP staining.

Cerebral Ischemia 24 Hours After Injection of 100- to 200-μm Fragments From Calcified Plaques

When we increased the size of the calcified fragments injected to 100 to 200 μm, there was a substantial increase in brain injury. Injections of these fragments were highly morbid events. Twenty-four hours after injection of 5 animals with 100- to 200-μm calcified fragments, 1 of 5 animals developed an obvious neurological deficit and died. Each of the remaining 4 animals injected with fragments from calcified plaques had areas of infarction on MRI scans.

Discussion

Our data demonstrate that the composition of carotid plaque undergoing manipulation may influence the severity of neurological injury from the resulting microemboli. MRI scanning at 24 hours detected infarction in 7 of 12 animals injected with one hundred 60- to 100-μm fragments from calcified plaques, whereas only 1 infarct was seen after injection of similar-sized fragments from fibrous plaques. Injection of fragments from fibrous plaques caused only 1 infarct on MRI, but these fragments should not be considered

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TABLE 2. Cerebral Injury at 24 Hours After Injection of 60- to 100-μm Fragments From Calcified Versus Fibrous Plaques

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>MRI Positive</th>
<th>HSP70 Staining</th>
<th>Focal Neuronal Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcified Plaques, n</td>
<td>12</td>
<td>7*</td>
<td>10†</td>
<td>7†</td>
</tr>
<tr>
<td>Fibrous Plaques, n</td>
<td>11</td>
<td>1*</td>
<td>9†</td>
<td>4†</td>
</tr>
<tr>
<td>Control, n</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*P=0.041; †P=0.649; ‡P=0.525 (χ²).

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TABLE 3. Cerebral Ischemia at 24 Hours Created With Microspheres

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals</th>
<th>MRI Positive</th>
<th>HSP70 Positive</th>
<th>Focal Neuronal Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsphere (50 μm), n</td>
<td>6</td>
<td>0/3*</td>
<td>2/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Microsphere (98 μm), n</td>
<td>11</td>
<td>10/11</td>
<td>11/11</td>
<td>11/11</td>
</tr>
<tr>
<td>Control, n</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*MRI study was done only in 3 animals.
benign. Nine of these animals had areas producing HSP70, indicating ischemic injury, and 3 had areas of infarction too small to be detected by MRI. The physical properties of these 2 fragment types were quite different when manipulated under the microscope. The calcified fragments were like grains of sand, whereas the fibrous fragments were filamentous and pliable. These physical properties alone may account for the different biological behaviors. This conclusion is supported by the findings that (1) dried and rehydrated calcified fragments caused a rate of stroke similar to calcified fragments from fresh specimens and (2) larger calcified fragments, 100 to 200 μm, caused larger strokes in these animals.

To document the number of fragments released during carotid angioplasty, we sized and counted fragments using a Coulter counter. Our results are similar to those of Coggia and colleagues,17 who performed angioplasty on excised whole ICAs. As we have previously reported with fragments >100 μm,15 there was a large variation in the number of fragments released from individual plaques. In the 60- to 100-μm size range, there were hundreds of fragments (range, 132 to 1684; mean, 375); in the 20- to 60-μm size range, there were tens of thousands of fragments (range, 2230 to 186 927; mean 34 196).

Assuming equivalency of in vivo and ex vivo angioplasty, the 100 fragments injected into the 2-g rat brain represents an approximate 12-fold increase over the maximum number of 60- to 100-μm fragments (1684) released during carotid angioplasty into the 400 g of brain supplied by the middle cerebral artery. Given the extensive collaterals within the microcirculation of the human cortex, a shower of this size of fragment represents a relatively low risk for a clinically apparent stroke even though current carotid filtration devices catch few, if any, fragments of this size. However, our HSP and NeuN data suggest that injection of these microemboli may create small areas of infarction and tissue ischemia. Furthermore, passage of the guidewire is unprotected and releases significant numbers of microemboli and larger fragments.15 Patients with critical carotid stenosis have several factors, including age, hypertension, and/or diabetes, that could affect the microcirculation and increase the vulnerability of the brain to ischemic injury from microemboli. These patients also are likely to have had previous microembolic episodes that would reduce the available microcirculation collaterals5-9 and further increase the potential risk.

We found that injection of one hundred 50-μm microspheres caused little damage, although the injection of one hundred 98-μm microspheres created major infarctions in every animal. One might infer from this that 50 μm could be a reasonable lower limit about which to be concerned. We have reached this conclusion before,15 only to be proven wrong with further experiments. There was a 90-fold increase in the number of 20- to 60-μm fragments released compared with the number of 60- to 100-μm fragments during ex vivo angioplasty. Presuming that some inverse relationship exists between embolus size and the number of emboli required to create ischemic injury, it may be that the increased number of fragments in the 20- to 60-μm range offsets the reduced risk of infarction with these smaller emboli. Clearly, further experiments need to be done to clarify the size threshold for microemboli below which fragments can be considered truly benign.

Finally, there are chronic and acute effects of atheroembolization. Plaque fragments initiate an inflammatory process that eventually leads to cellular infiltration and fibrosis.18-20 This phenomenon is best described for atheroemboli to the kidney, but it has also been observed with atheroemboli to the brain11 and could explain the late deterioration in intellectual function in patients after atheroembolization during coronary artery bypass graft surgery compared with controls.13,14

References
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