Microemboli Composed of Cholesterol Crystals Disrupt the Blood-Brain Barrier and Reduce Cognition

Joseph H. Rapp, MD; Xian Mang Pan, MD; Melanie Neumann, PhD; Michelle Hong, BS; Kelsy Hollenbeck, BS; Jialing Liu, PhD

Background and Purpose—Microemboli occur frequently in patients with asymptomatic carotid atherosclerosis. In other vascular beds, microemboli are known to initiate an inflammatory response, causing organ dysfunction. In the current study, we investigated whether emboli composed of cholesterol crystals, a component of human atherosclerotic plaque, could also cause inflammation and brain dysfunction demonstrated by cognitive impairment.

Methods—Cholesterol crystals of 60 to 100 μm were injected via the rat internal carotid artery. T2-weighted magnetic resonance imaging was conducted after 3 days to estimate infarct volume. Brains were examined for matrix metalloproteinase activation at 24 hours and for albumin leakage and microglia and astrocyte activation at 4 days and 1, 2, and 4 weeks after embolization. To determine changes in cognition, behavioral tests including open field, motor learning, and Barnes Maze tests were conducted on young adult and middle-aged rats 4 weeks after either a single injection or after repeated, bilateral injections given at an interval of 2 weeks.

Results—Matrix metalloproteinase activation was detected in 50% of the animals examined. Perivascular albumin staining was found at 4 days but rarely persisted beyond 1 week. Activation of microglia and astrocytes occurred in all animals and persisted for up to 8 weeks. Cognitive impairment was observed in middle-aged rats after repeated, bilateral injections but not after single injections. In these animals, areas of inflammation were small and scattered but often involved the striatum and hippocampus.

Conclusions—Cholesterol embolization caused an inflammatory response in the brain with persistent activation of microglia and astrocytes and led to cognitive impairment after repeated injections in middle-aged animals with only small foci of neural injury. These data indicate that microembolization causes inflammation and that minimal neuronal injury can cause cognitive impairment in older animals. (Stroke. 2008;39:2354-2361.)

Key Words: cognitive impairment ■ embolic stroke, experimental ■ hippocampus ■ inflammation

Approximately 10% of patients with asymptomatic carotid stenosis have had microemboli detected with 1 hour of transcranial Doppler monitoring.1,2 This incidence increased to 27% with 8 hours of monitoring and to 46% with 16 hours of monitoring.3 Although these patients’ risk of stroke may be low,4 reduced cognitive function5 and silent infarctions6 are more frequent in patients with asymptomatic carotid stenosis compared with controls. The data linking vascular disease and dementia are suggestive of cause and effect, but a mechanism explaining the association has not been identified.7 Atheroemboli in other vascular beds create an inflammatory response with eventual organ dysfunction.8,9 The goal of this study was to determine whether microemboli to the brain also cause inflammation and organ dysfunction, shown by impaired cognition, in a rodent model of microembolization.

In previous work, we examined microembolization in rodents with the use of human atheroma fragments.10 However, the rat immune response to human antigens precludes examination of the inflammatory reaction induced by microemboli as well as any subsequent functional outcome. For the current study, we used USP nonesterified cholesterol crystals, size 60 to 100 μm. These slender crystals are similar to those seen as cholesterol clefts in the plaque necrotic core, where they can account for up to one third of the plaque by weight.11 The extent of brain injury caused by cholesterol embolization was examined in young adult rats. Both young adult and middle-aged rats underwent behavioral testing to determine whether the cognitive decline in rats manifested in advanced age12 could be produced prematurely by microembolization.

Materials and Methods

This study was conducted in accordance with animal care guidelines issued by the National Institutes of Health and the institutional animal care and use committee of the San Francisco Department of Veterans Affairs Medical Center.
Microembolization and Experimental Groups

USP-grade free cholesterol crystals (Sigma) were sized with filters of pore size 60 or 100 μm and counted under 100× magnification with a 100-μm background grid (Figure 1A). To investigate temporal progression of injury and inflammatory response caused by cholesterol crystals in the brain, 300 crystals 60 to 100 μm in diameter were injected into the right internal carotid artery of 3-month-old male Sprague-Dawley rats (n = 31, Charles River, Calif) under isoflurane/O2/N2O (1.5%:30%:68.5%, vol/vol/vol) anesthesia as previously described.10 After cholesterol or sham embolization, 35 rats were randomly assigned to 6 subgroups, including a sham-operated control group. Animals were examined by magnetic resonance imaging after 3 days and then euthanized at 4 days, 1 week, 2 weeks, or 4 weeks after injection. A sixth group was euthanized 24 hours after injection to measure matrix metalloproteinase (MMP) activity by in situ zymography. To investigate the relation between brain injury and the number of 60- to 100-μm crystals injected, 24 animals were injected with 100, 300, or 500 cholesterol crystals. These animals were then euthanized at 4 days.

In separate experiments, animals were each injected with 300 cholesterol crystals and underwent behavioral testing to determine changes in cognition. Three-month-old male Sprague-Dawley rats (n = 24) and 14-month-old male Fischer 344 rats (n = 20; National Institute on Aging, Bethesda, Md) were injected as noted earlier to investigate age-related susceptibility to cognitive dysfunction.13,14 To examine the effects of age and repeated exposure to microemboli, 14-month-old Fischer 344 rats (n = 14) were injected with 150 crystals into the left and right internal carotid artery with a 2-week interval between injections. Control animals (sham embolized) received injections of saline in the same manner and volume. Rats that underwent behavioral testing were analyzed for the extent of brain injury at the end of the testing period or 2 months after the initial injection. The volume of brain regions showing loss of microtubule-associated protein immunoreactivity or CD11b marker infiltration was estimated by tracing the contour of the regions of interest multiplied by section thickness.

Tissue Preparation, Immunohistochemistry, and Immunofluorescence

Forty-micron-thick coronal free-floating sections were collected serially after transcardiac perfusion as described previously.15 Immunohistochemistry or immunofluorescence was performed in every 12th section (480 μm apart) according to previous methods15,16 with the following reagents: mouse anti-NeuN (1 μg/mL; Chemicon, Temecula, Calif); mouse anti-rat CD11b (1 μg/mL, Chemicon); mouse anti–glial fibrillary acidic protein (1 μg/mL, Chemicon); rabbit anti-albumin (Accurate Chemical and Scientific Corp, New York, NY); mouse anti–microtubule-associated protein a and b (1 μg/mL, Chemicon); biotinylated sheep anti-mouse, anti-rabbit, and anti-rat secondary antibodies (5 μg/mL; Amersham, Cleveland, Ohio); ABC solution (Vector Laboratories, Burlingame, Calif); 0.05% diaminobenzine tetrachloride (DAB Fast; Sigma, St. Louis, Mo) with 0.01% H2O2 and 0.04% NiCl2 (Sigma); and streptavidin Alexa Fluor 488 (Molecular Probes, Eugene, Ore).

In Situ Zymography

After transcardiac perfusion with ice-cold phosphate-buffered saline, brains were removed without fixation and frozen in acetone with

Figure 1. Microembolization disrupted the blood-brain barrier, activated MMPs, and induced microglial and astroglia response. A, Cholesterol crystals ranging from 30 to 60 μm in diameter. B, Perivascular albumin staining in the hippocampus after a single injection of 60- to 100-μm cholesterol crystals. C, MMP staining by in situ zymography showing focal activation of MMPs after injection of cholesterol crystals. D, Representative T2-weighted magnetic resonance imaging image showing apparent infarct in the hippocampus. E and F, CD11b and glial fibrillary acidic protein staining, respectively, suggesting activation of microglia and astrocytes in the same region where MMPs were activated. Scale bar for A and B = 100 μm; for C, E, and F, 20 μm.
Table 1. Effect of Cholesterol-Containing Microemboli on Albumin Leakage and Microglia and Astroglia Activation Over Time

<table>
<thead>
<tr>
<th>ID</th>
<th>Infarct Volume* mm³</th>
<th>Time After Embolization</th>
<th>CD11b Positive</th>
<th>ED-1 Positive</th>
<th>Albumin Positive</th>
<th>GFAP Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>16.77</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>4.69</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A6</td>
<td>0</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A7</td>
<td>0</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A8</td>
<td>0</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A9</td>
<td>0</td>
<td>4 days</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Positive ratio</td>
<td>2/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

Table 2. Dose-Response Study Showing the Ratio of Animals That Demonstrated Inflammation or Neuronal Injury After Microembolization of 60- to 100-μm Cholesterol Crystals in Various Numbers

<table>
<thead>
<tr>
<th>No. of Emboli</th>
<th>Positive Ratio on Immunohistology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED-1 MAP2</td>
</tr>
<tr>
<td>150</td>
<td>2/7 1/7</td>
</tr>
<tr>
<td>300</td>
<td>7/12 3/12</td>
</tr>
<tr>
<td>500</td>
<td>7/8 4/8</td>
</tr>
</tbody>
</table>

MAP indicates microtubule-associated protein.

Assessment of Neurobehavioral Outcomes

One month after cholesterol crystal or sham embolization, rats with a unilateral injection (3-month-old Sprague-Dawley: n=13 for sham, n=11 for embolized; 14-month-old Fisher 344: n=9 for sham, n=11 for embolized) or bilateral injections (14-month-old Fisher 344: n=10 for sham, n=14 for embolized) were subjected to behavioral tests in the following order.

**Open Field**

Rats were placed in a brightly lit, square Plexiglas enclosure (40×40 in.) surrounded by automated infrared photocells interfaced with a computer (Hamilton & Kinder, San Diego, Calif) to record the data. “Beam breaks” generated by movement were monitored, allowing measurement of the number of movements, active times, path length, and rearing events.15 On each of 3 consecutive days, open field activity was recorded for 10 minutes after an initial 1-minute adaptation period. For analysis of exploratory behavior, the arena was divided on a zone map consisting of a center region (15×15 in.); 4 corner regions of 7.5×7.5 inches each, and a peripheral region (the remaining area).

**Motor Learning (Rota-Rod)**

Rats were tested on accelerating rotating rods (San Diego Instruments, San Diego, Calif) to assess motor coordination and balance for 4 consecutive days. After a 1-minute adaptation period on the rod at rest, the rod was accelerated by 5 rpm every 15 seconds, and the length of time that the rat remained on the rod (fall latency) was recorded and averaged for 3 consecutive trials with 5-minute intervals between trials.18

**Barnes Maze**

The Barnes Maze was used to measure spatial memory acquisition and retention. A black acrylic escape tunnel was placed under 1 of the holes on a circular platform (120-cm diameter) with 18 holes (10-cm diameter per hole) along the platform perimeter (Hamilton Kinder, Poway, Calif). The platform was elevated 60 cm above the floor. Rats from each treatment group were randomly assigned to locate the escape tunnel from 1 of the 3 predetermined locations to rule out spatial preference. Mildly aversive stimuli, blowing fans and 500 lux of bright light, were used to increase the motivation for finding the escape tunnel. A Noldus EthoVision video tracking system (Noldus, Leesburg, Va) was used to record and analyze the data. Rats were trained to locate the escape tunnel in 2 successive daily sessions (3 trials per session, 3 minutes per trial) with 1-hour intersession interval from different counterbalanced starting positions. A 3-minute probe trial was performed 1 hour after the last trial on day 5. The order and number of holes visited were recorded and analyzed by viewing the recorded videotape to calculate primary errors (errors committed before the first encounter with the escape hole), omission errors (encounters with the target hole followed by further exploration of nontarget holes), and search strategies (direct: the first hole visited was the escape hole or a hole directly adjacent to the escape hole; serial: the first visit to the target hole was preceded by visits to at least 2 adjacent holes in a serial fashion; or random: no systematic search pattern was evident).19
Statistical Analysis

Data were expressed as mean±SEM. Behavioral study data with repeated measures were analyzed by a “mixed model regression” with SAS version 9 (SAS Institute, Cary, NC) PROC MIXED. We used the Kenward-Roger method,20 which constructs $F$ statistics to compare groups and uses estimated denominator degrees of freedom to accommodate potentially unequal variances and/or unequal correlations among the repeated measurements on each animal over time. Our models allowed animal-specific slopes and intercepts and fit flexible functions of the session per day. Other data were analyzed by ANOVA with StatView software (SAS Institute, Cary NC). Post hoc tests were conducted when appropriate. Differences between groups were considered significant for a 2-sided $P<0.05$.

Results

Cholesterol Emboli Disrupt the Blood-Brain Barrier and Elicit an Immune Response

Thirty-five animals underwent surgery for cholesterol injection or sham operation. Two animals died as a result of anesthetic complications, and 3 had infarcts that caused neurologic deficits that met end-point criteria and were euthanized. Among the 25 animals examined by magnetic resonance imaging (Figure 1D) after injection with cholesterol crystals, 7 (28%) had infarcts without apparent neurologic deficit. Of those examined 4 days after embolization, all but 1 animal had areas of perivascular albumin staining involving vessels that ranged from 30 to 60 μm (Table 1). This was generally eccentric to the lumen, and there was no evidence of infarction. When albumin staining persisted through 7 days, it appeared to have progressed to circumferential involvement (Figure 1B). Perivascular areas of MMP

activity were found in 3 of 6 (50%) animals examined 24 hours after injection (Figure 1C) but in none of the controls. The most consistent histologic feature after cholesterol crystal embolization was activation of microglia, shown by CD11b and ED-1 staining (Figure 1E, Table 1) seen in all rats after 4 days and persisting in the majority of animals through the duration of the study. The pattern of astrocyte activation shown by glial fibrillary acidic protein expression (Figure 1F, Table 1) mirrored that of microglia and also persisted among the majority of rats, including animals that had no evidence of

Figure 2. Middle-aged rats with repeated embolization showed anxiety-like behavior. There was no overall difference in vertical (A) and horizontal (B, C) activity displayed between groups in the open field. Both groups also spent equal amounts of time resting (D). However, embolized rats showed increased anxiety-like behavior, as they spent less time in the center zone (E) and more time in the corner zone (F) of the open field (comparison between sham and embolized groups; *$P<0.05$, **$P<0.01$).

Figure 3. Middle-aged rats with repeated embolization demonstrated impairment in motor learning on the Rota-Rod. Performance on the accelerating Rota-Rod in both groups of rats improved over time. However, sham rats performed significantly better during 4 days of training ($P<0.05$).
significant infarction. Sham-embolized rats were negative for the aforementioned markers.

Relation Between Brain Injury and the Number of Injected Emboli

When the ratio of inflammation to neuronal infarction was examined after injection of 100, 300, or 500 cholesterol crystals, evidence of inflammation and infarction was found at each dose. However, there was a progressive increase in damage with an increasing number of emboli. For this model, one in which we wished to have a high rate of inflammation with a low rate of infarction, a dose of 300 crystals appeared optimal (Table 2).

Neurobehavioral Testing

When neurobehavioral testing was conducted 4 weeks after a single injection of 300 cholesterol crystals, there was no significant impairment in either young adult or middle-aged animals compared with sham-operated controls (data not shown). However, when 150 crystals were injected into the right internal carotid artery of middle-aged rats on day 1 and then into the left internal carotid artery on day 14, neurobehavioral testing performed 4 weeks later showed modest but significant cognitive impairment, as detailed next.

Altered Exploratory Behavior in Middle-Aged Rats After Repeated Embolization

There was no overall difference in locomotor activity between middle-aged sham and embolized rats during a 3-day open field test (for rearing: treatment effect $F_{1,67}=0.05$, $P=0.82$; day×treatment effect $F_{1,46}=0.18$, $P=0.68$; for total active time: treatment effect $F_{1,67}=0.45$, $P=0.51$; day×treatment effect $F_{1,46}=0.01$, $P=0.95$; for total path length: treatment effect $F_{1,67}=1.17$, $P=0.28$; day×treatment effect $F_{1,46}=0.13$, $P=0.72$; for total rest time: treatment effect $F_{1,67}=0.05$, $P=0.82$; day×treatment effect $F_{1,46}=0.18$, $P=0.68$; Figures 2A–2F). However, during the first day of exploration, embolized rats spent less time exploring the center zone, either being active ($P=0.029$) or resting ($P=0.034$; Figure 3E). Instead, they demonstrated a preference for the corner zones (active time $P=0.0059$, rest time $P=0.035$; Figure 3F), suggesting an anxiety-like behavior.

Reduced Motor Learning in Middle-Aged Rats After Repeated Embolization

Performance on the Rota-Rod task with days of repeated training can be interpreted as a measure of motor learning. Both middle-aged sham and embolized rats improved perfor-
mance, showing motor learning after a 4-day training (day effect $F_{1,70}=63.48$, $P<0.0001$), but the embolized group showed less improvement over time compared with sham-operated rats (day being embolization effect $F_{1,70}=4.04$, $P=0.0484$; Figure 3).

Spatial Learning and Memory Are Impaired in Middle-Aged Rats After Repeated Embolization
Both middle-aged sham and embolized rats learned the spatial task, as evidenced by decreasing path length to reach the escape hole with successive sessions in the Barnes Maze test ($F_{1,9}=10.91$, $P=0.0014$). There was no significant overall difference in performance during acquisition of the Barnes Maze test between groups (treatment effect in path length $F_{1,93}=2.31$, $P=0.13$; day×treatment effect $F_{123}=0.37$, $P=0.548$; Figure 4A). Significant group differences in the path length taken to locate the target were observed only at day 5 ($P=0.0138$). However, the embolized rats made more errors before they encountered the escape hole during acquisition (primary error $F_{1,23}=6.37$, $P=0.019$; Figure 4C). There was also a significant difference between the search strategy used between the sham and embolized rats. Overall, the sham rats used a direct strategy ($P=0.04$), whereas the embolized rats used a random search strategy ($P=0.019$; Figure 4B). The probe trial results showed that both groups exhibited a bias in favor of the zone where the escape tunnel was previously located (sham $F_{17,170}=14.869$, $P<0.0001$; embolized $F_{17,238}=27.425$, $P<0.0001$; Figure 4E).

Repeated Exposure to Microemboli Led to Mild Hippocampal and Striatal Injury
After completion of behavioral testing, the middle-aged animals with repeated (bilateral) injections of cholesterol crystals were euthanized 8 weeks after their initial injection. Immunohistochemistry of the brains showed no areas of Neun staining loss, but persistent evidence of focal microglial activation was observed in more than half the animals that often occurred in the hippocampus and/or striatum (Figures 5A–5I). The largest volume of injury was $2.0 \text{ mm}^3$ after repeated embolization with 150 crystals (Table 3).

Discussion
Cholesterol crystals, a surrogate for atheroemboli, initiated an inflammatory response in the brain and, after repeated bilat-
crystals, Steiner et al examined cholesterol crystal emboli—report of experimental cerebral embolization with cholesterol number of emboli injected (300 vs 100). In the only other fibrous fragments but required a 3-fold increase in the caused an inflammation to infarct ratio similar to that of atheroembolic disease. The number and size of crystals plaque necrotic core, cholesterol crystals are the sine qua non embolic material. Forming the cholesterol clefts of the carotid to avoid this issue, we used USP cholesterol crystals as our clouded by the potential of a cross-species immune response. To occur only in humans, and any long-term results in experimental models of embolization with human atheroma are clouded by the potential of a cross-species immune response. To avoid this issue, we used USP cholesterol crystals as our embolic material. Forming the cholesterol clefts of the carotid plaque necrotic core, cholesterol crystals are the sine qua non of atheroembolic disease. The number and size of crystals injected were based on our dose-response study and our previous work, which showed that 24 hours after injection of 60- to 100-µm fibrous fragments from human plaque, 90% of animals had evidence of brain inflammation, whereas only 9% had areas of infarction. Microemboli with greater mass composed of calcified plaque fragments or thrombus appear to cause higher rates of infarction. Cholesterol crystals caused an inflammation to infarct ratio similar to that of fibrous fragments but required a 3-fold increase in the number of emboli injected (300 vs 100). In the only other report of experimental cerebral embolization with cholesterol crystals, Steiner et al examined cholesterol crystal embolization in a living craniotomy model. They found that only localized and temporary hemodynamic disturbances occurred, even with large crystals, and there were no vascular thromboses, ie, occlusions. Intravascular thrombus also was absent in our model, presumably explaining the low incidence of infarction.

Although we tried to mimic atheroembolism, there are obvious limitations to this work. USP cholesterol crystals are chemically similar to those in human atherosclerotic plaque, but in our model, the size and number injected were titrated to create injury with rare neuronal infarction. Cholesterol crystals in human plaque exist in a range of sizes that include lengths both less and greater than 100 µm. The emboli injected create a “shower” of crystals, whereas the emboli in humans occur at a low rate and accumulate over time. The importance of this is unknown, but our attempt at repeated embolic episodes caused cognitive impairment whereas single injections of the same total number of crystals did not. Although cholesterol emboli can occur as individual crystals, they more commonly include amorphous material from the necrotic core. These complex emboli would be larger than isolated crystals and probably more likely to cause vascular occlusions, although the frequency of “silent” microembolic episodes in asymptomatic patients suggests that true atheroemboli also must be quite small and are more likely to create inflammation without apparent neuronal infarction. Unfortunately, for technical reasons, repeated embolization in this model required bilateral injections, adding another variable. Thus, further research will be required to determine whether age, repeated challenge, the bilaterality of injury, or some combination of these factors was responsible for the impaired cognition caused by microemboli.

The most consistent histologic finding after cholesterol embolization was the prolonged activation of microglia and astrocytes. Chronic inflammation with microglial activation has been implicated as a causative factor in several neurodegenerative diseases, including Alzheimer’s, and has been associated with cognitive decline in both humans and animals. Activated microglia elaborate interleukin-1β and other cytokines that appear to precipitate cognitive impairment by interference with synaptic plasticity. In addition, blocking microglial activation has been shown to preserve spatial memory. Our data linking microembolization and chronic inflammation suggest a mechanism for the reported clinical association of microembolic events and cognitive decline.

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**Disclosures**

None.

**References**


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