An experimental model of lacunar infarction: Embolization of microthrombi

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Objectives: Microthrombi are undoubtedly the most common embolic material in the cerebral circulation, originating from even minor irregularities of the arterial wall, fibrillating atria, cardiac valves, and patent foramen ovale. Thrombus fragments are globular and likely to completely obstruct terminal vessels. In contrast, previous work with “atheroemboli” of needle-like cholesterol crystals rarely cause occlusions or infarctions instead creating small foci of inflammation. In this work, we asked if microthrombi would occlude terminal vessels and create lacunar type infarctions in the subcortical tissues of the rat brain where, as in human brain, collateral flow is limited relative to the cortex.

Methods: Three treatment groups of adult male Sprague-Dawley rats were studied. All groups underwent general anesthesia with monitoring of temperature and blood pressure during cannulation of the right internal carotid artery. In the group embolized with thrombus fragments (n = 12), animals had injections of 300 fragments of thrombus size 60 to 100 microns, the cholesterol group (n = 6) had injections of 300 cholesterol crystals of similar size, and the control group (n = 4) had injections of saline. Brains were harvested at 4 days with perfusion fixation and were examined by immunohistochemical staining for breaks in the blood brain barrier (BBB) (albumin), microglial activation (CD11b), astrocyte activation (GFAP), and infarction (loss of NeuN staining). Size and location of the areas of injury and infarction were recorded.

Results: Clot fragments caused discreet infarcts in 10/12 animals that were 0.1-1.7 mm in diameter and coincided with activation of microglia and astrocytes. In some areas, necrosis was already underway at this early time point. Consistent with our previous work, the infarcts caused by cholesterol crystals were smaller (P = .014). Foci of BBB disruption and microglial activation were distributed throughout the brain whereas areas of infarction were found almost exclusively in subcortical tissues (P = .029).

Conclusions: Injecting microthrombri reproducibly caused areas of necrosis resembling lacunar type infarctions. These were primarily located in the striatum and thalamus presumably because these areas lack the branching, collateral network seen in the cortex. In addition, these data give further evidence that the extent of brain injury from emboli depends upon composition and shape as well as size. (J Vasc Surg 2008;48:196-200.)

Clinical Relevance: This model gives experimental support that microemboli are an etiologic agent of lacunar infarction.

Lacunar infarcts are defined as small subcortical areas of tissue loss resulting from occlusion of a single penetrating artery.1 Current clinical/pathologic correlations are credited to C. M. Fisher who in a series of articles reported findings from his own patients and refined concepts originally set forth in the late 19th and early 20th century.2 Fisher carefully examined human brain sections to trace single vessels leading to areas of infarction that he believed correlated with recorded clinical events. While there were classic atheromas in a few vessels and some infarcts were attributed to emboli, the majority, 40 of 50, were attributed to highly focal arterial lesions occurring at branch points characterized by lipid deposition, scarring, and modest vessel enlargement.3 Fisher called these lesions “segmental arterial disorganization”, now termed lipohyalinization. As 90% of the patients studied were hypertensive,4 a causal effect was assumed and the “Lacunar Hypothesis” proposed.

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Fisher’s concepts on lacunar infarcts have been challenged over the ensuing decades. Diffuse thickening of small cerebral vessels occurs with hypertension but segmental occlusions as Fisher described are rare,5,6 not found in any other human organs and do not occur in hypertensive animal models.7 Population studies of stroke have shown that there is no greater prevalence or severity of hypertension among patients with lacunar infarction compared with those with cortical stroke.8 In spite of these observations, the Lacunar Hypothesis remains sacrosanct for many,9,10 seemingly because there is no clearly proven alternative etiology.11,12 Authors looking for other etiologic agents have suggested that emboli could be an important, if not primary, cause of lacunar infarctions.7,8

Arguments against embolization causing lacunes include a presumed inability of emboli to access the penetrating arteries and the lack of a recognized embolic source in many patients presenting with lacunar infarction. Neither argument is particularly persuasive. Large emboli are uncommon in the small penetrating arteries, but emboli less than 98 microns have equal access to both penetrating and circumferential arteries of the primate brain.13 The lack of an identified source does not rule out embolization. Even stroke patients with multiple acute lesions on diffusion weighted-magnetic resonance imaging (DW-MRI), a pattern typical of embolization, may present without a clearly identifiable embolic source.14,15 But the hypothesis that
emboli sized less than 100 microns could cause lacunar type infarctions has not been tested. To this end we first compared the effects from embolization of 60 to 100 micron emboli formed from thrombus with those from cholesterol crystals, another common component of atheroemboli; then to determine whether 60 to 100 micron microthrombi could cause small, discreet infarcts in subcortical tissues similar to the lacunar type infarctions in humans.

METHODS

Surgery/emboli injection

Male Sprague Dawley rats weighing approximately 300 grams and fed a normal chow diet were injected with 300 microemboli sized 60 to 100 microns. The injections were done into the right internal carotid artery as described previously.16 Twelve animals were injected with thrombus fragments, six with cholesterol crystals, and four were sham operated controls. The procedure was done under isoflurane anesthesia and was conducted as follows. The carotid bifurcation was isolated from surrounding tissues and the common carotid artery and the external carotid arteries were surrounded with 6-0 silk. After distal ligation, the external carotid was cannulated and emboli injected in saline. The common carotid was temporarily occluded as the emboli were injected. The external carotid was then ligated proximally.

Microemboli preparation

Thrombus fragments. To provide uniformity of the prepared fragments, homologous thrombus was used. Five milliliters of homologous blood was drawn from the tail vein of a donor animal. Blood clot was prepared as described by Kudo. Briefly, blood was allowed to clot at room temperature and the serum removed. After the clot was minced mechanically, it was passed through a series of filters. The fragments that passed through a 100 micron mesh but were filtered by the 60 micron mesh were resuspended in 3 ml of saline and portions counted on a 100 micron grid at 100 magnification. The clot fragments were diluted to a final concentration of 300 fragments per 300 ul and injected within 24 hours after the initial blood draw.

Cholesterol crystals. USP cholesterol crystals (Sigma Chemical Co, St. Louis, Mo) were suspended in saline and filtered as noted above. The crystals were also counted at 100 magnification and diluted to a final concentration of 300 crystals per 300 ul of saline.

Tissue preparation and immunohistochemistry

To perform the brain sectioning and histologic analysis, rats were euthanized and the brain processed as follows. Under anesthesia, the aorta was cannulated. Each rat was given heparin (100 U/Kg) and perfused with 200 ml of 0.9% saline, followed by 200 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PBS). The brain was removed from the cranial vault, and placed in 4% paraformaldehyde for 12 hours, PBS for 12 hours and 30% sucrose for 2 days. Coronal free-floating sections 40 mm thick were cut and collected serially as described by Raber et al.18 Immunohistochemistry was performed on every 12th section (480 mu apart) according to established protocol and examined for activated microglia (CD11b), activated astrocytes (GFAP) albumin, and neuronal cell death (NeuN) using the following reagents: mouse anti-NeuN (1 mu/ml, Chemicon, Temecula, Calif); mouse anti-rat CD11b (1 mu/ml, Chemicon); mouse anti-GFAP (1 mu/ml, Chemicon); rabbit anti-albumin (Accurate Chemical and Scientific Corp, Westbury, New York); biotinylated sheep anti-mouse, anti-rabbit, and anti-rat secondary antibodies (5 mu/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); Streptavidine Alexa Fluor 488 (Molecular Probes, Eugene, Ore).

Scoring of the brain injury

The entire brain was assessed to evaluate the extent of injury or infarction and the location. The scoring criterion used to assess injury caused by embolization was chosen to offer a basis for comparisons between animals with small areas of injury. Scoring was assigned as follows: 0: no abnormality; 1: the diameter of injured or infarcted area was less than 500 microns with no necrotic tissue. 2: the diameter of injured or infarcted area was more than 500 microns or there was necrosis with a diameter of less than 500 microns; 3: the diameter of the areas of necrotic tissue was more than 500 microns. Injury/infarction scores were determined for the striatum, cortex, septum, hippocampus, thalamus and corpus callosum separately with total scores including all six areas. The scoring was done by consensus of two non-blinded readers.

Statistical comparisons

The Mann Whitney rank sum test was used to compare the median score of injury after the injection of thrombus fragments with the extent of injury after injection of cholesterol crystals. A comparison of proportions was used to compare the distribution of lesions noted by CD11b staining vs the areas with a lack of NeuN staining.

RESULTS

General

No animals died as a result of the surgery and the carotid injections. There were no limitations in motor function after injection of either clot fragments or cholesterol crystals.

Immunohistochemistry of areas of injury and infarction

Injection of thrombus fragments. After injection with thrombus fragments, 10 of the 12 animals had at least one area of infarction and only one animal had more than three separate infarcts. The infarctions were small with maximal diameters varying from less than 100 microns to 1.7 mm in diameter (Table 1, Fig 1). Using a scoring system to quantitate the accumulated damage from small areas of injury and infarction (Table 1), the average infarction score
Table I. Brain Injury assessed by immunostaining after injection of 300 sized 60 to 100 micron clot fragments or cholesterol crystals in rats

<table>
<thead>
<tr>
<th>Animals Studied</th>
<th>Sham</th>
<th>Thrombus Fragment</th>
<th>Cholesterol crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b staininga</td>
<td>0</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Score (total)b</td>
<td>0</td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>Average scorec</td>
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<td>5.67 ± 2.27</td>
<td>1.83 ± 1.94</td>
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<tr>
<td>NeuN stain lossa</td>
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<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Score (total)b</td>
<td>0</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>Average scorec</td>
<td>0</td>
<td>2.25 ± 1.60</td>
<td>0.5 ± 0.55*</td>
</tr>
<tr>
<td>GFAP staininga</td>
<td>0</td>
<td>9</td>
<td>3</td>
</tr>
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<td>4</td>
</tr>
<tr>
<td>Average scorec</td>
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<td>1.64 ± 1.29</td>
<td>0.67 ± 0.82</td>
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<tr>
<td>Albumin staininga</td>
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<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>5</td>
</tr>
<tr>
<td>Average scorec</td>
<td>0</td>
<td>0.55 ± 0.82</td>
<td>0.83 ± 0.75</td>
</tr>
</tbody>
</table>

*a* = 0.014 comparing area of infarction caused by thrombus fragments versus cholesterol crystals.

*Key: CD11b stain shows activated microglia, NeuN stain loss indicates loss of neurons, GFAP staining shows activated astrocytes.

*b* Scoring criteria: 0: no infarct or injury staining; 1: the diameter of injury/infarction area is less than 500 microns with no necrotic tissue; 2: the diameter of injured/infarcted area is more than 500 microns or the diameter of necrotic tissue less than 500 microns; 3: the diameter of necrotic tissue area more than 500 microns. The score was determined in striatum, cortex, septum, hippocampus, thalamus, and corpus callosum separately. Total scores include all above six areas, the maximum total score in one animal was 18.

*c* Average score was shown with mean ± SD.

Table I: Brain Injury assessed by immunostaining after injection of 300 sized 60 to 100 micron clot fragments or cholesterol crystals in rats

was 2.25 ± 1.60 (range 1-6). In eight of the animals, there were areas of inflammation shown by microglial activation (CD11b staining) in areas that did not have neuronal cell death. The areas of injury were distributed in both the cortical and subcortical regions while the infarctions almost exclusively the striatum or the thalamus (Table II). GFAP staining signaling astrocyte activation occurred less frequently outside of infarct areas than microglial activation. Albumin staining was not seen in areas of infarction (suggesting a complete loss of perfusion), but was seen in areas where there was injury only. In a few specimens after injection of clot fragments, vessels in close proximity to infarcts were observed to be occluded by thrombus (Fig 2).

**Injection of cholesterol crystals.** After the injection of cholesterol crystals, only three of six animals had areas of infarction and these were all smaller than 500 microns. These represented significantly smaller areas of infarction compared with thrombus fragments \( P = .014 \). There were several areas of microglial activation and albumin staining (Fig 3) without associated areas of infarct. No thrombosis was seen after cholesterol crystal embolization. There was no evidence of injury in the saline injected animals.

**Location of injury and infarction after the injection of thrombus fragments (Table II)**

After the injection of thrombus fragments, areas of microglial activation, signifying either injury or infarction were distribution throughout the brain. In contrast, areas lacking staining with NeuN, signifying infarction, were found exclusively in the subcortex particularly the striatum and thalamus \( P = .029 \) (Table II). No such trend occurred after the injection of cholesterol crystals.
DISCUSSION

We compared the ischemic injury caused by embolization of thrombus fragments sized 60 to 100 microns with that caused by similarly sized cholesterol crystals in the rat. Areas of infarction were more frequent, larger, and more likely to include frank necrosis after injection of thrombus fragments (injury score 2.25 ± 0.60 vs 0.50 ± 0.55, \( P = .014 \)). Vessel occlusions were seen with thrombus injection, but not after the injection of cholesterol emboli, which may reduce flow by transient vessel constriction around the crystals.20 Both embolic materials caused foci of injury more frequently than infarction. These were characterized by albumin leakage and focal activation of microglia.

In these experiments, the size of emboli injected was limited to those that would be equally distributed throughout the primate brain. The number was chosen to compare thrombus fragments with current and previous work on embolization of cholesterol crystals.21 Homologous blood from a single animal was used to ensure a uniform preparation of thrombus fragments. While this could have caused an immune response even at this early time point, the injury caused by the globular thrombus fragments is consistent with our previous results after the injection of microspheres,16 suggesting that the use of homologous blood did not substantively changed our results. We are not the first to inject homologous thrombus fragments into the rat brain. Kudo and colleagues developed the model but did not count or limit fragment size, which was reported to have had a mean of 200 to 300 microns.17,22,23 Their preparation created large areas of infarction and reduced total blood flow to the brain. Futrell et al created platelet thrombi by producing a photochemical injury to the rat internal carotid artery. Like the Kudo clot fragment model, the number and size of emboli created was not determined,24 but this model did create multiple small lacunar type infarctions.

In the current study, we injected 300 thrombus fragments into the two gram rat brain and created an average of two to three lesions per animal. This resembles the clinical situation, where, over time, hundreds, if not thousands, of microemboli may enter the cerebral circulation25 without causing apparent injury. We suspect that particulates that did not create infarctions either marginated and became adherent to the vessel wall, or lodged in arteries where the distal tissue had adequate collateral flow. However, the fate of these silent emboli is unknown. Perhaps they underwent thrombolysis or in the case of cholesterol crystals, mobilization or absorption.

The infarctions after injection of thrombus fragments occurred exclusively in the subcortex (\( P = .001 \)). Lacunar type infarctions seen in humans are also predominantly limited to the subcortical tissues but account for one-quarter to one-third of all ischemic strokes.26,27 The rat striatum’s known susceptibility to ischemic injury compared with the cortex is thought to reflect greater branching and collateralization of the vessels within the cortex.28,29 Data from examinations of human brain microvascular architecture reflect a similar disparity of potential collateralization between the cortex and other areas.
and subcortex. Fisher, in his extensive dissections of human brains, noted reduced branching in the subcortical penetrating arteries compared with cortical arteries. This finding has been confirmed by microscopic examinations of human brain vascular casts. Furthermore, with aging, arteries of the subcortex are susceptible to coiling of the lumen within the adventitial space. Vessels of the cortex are not affected. Coiling appears to begin in middle age and while initial studies have not shown a correlation of coiling with white matter disease, these abnormal vessels may be more susceptible to occlusion by microemboli.

Our work using homologous thrombus fragments in rats does not prove that lacunar infarcts in humans are due to microemboli. However, it does add to the evidence indicating that microemboli could have an etiologic role. The vulnerability of the rat subcortex to ischemic injury from microemboli make this an excellent model for further study of the role of emboli in lacunar infarction and other manifestations of what has been thought to be intrinsic small vessel disease of the brain.

AUTHOR CONTRIBUTIONS

Conception and design: JR, XP
Analysis and interpretation: JR, KH, XP
Data collection: KH, XP
Writing the article: JR
Critical revision of the article: JR
Final approval of the article: JR
Statistical analysis: JR, XP
Obtained funding: JR
Overall responsibility: JR

REFERENCES