Vasospasm in Monkeys Resolves Because of Loss of and Encasement of Subarachnoid Blood Clot

Zhen-Du Zhang, MD, PhD; Baktair Yamini, MD; Taro Komuro, MD, PhD; Shigeki Ono, MD; Lydia Johns; Linda S. Marton, PhD; Bryce Weir, MD; R. Loch Macdonald, MD, PhD

Background and Purpose—We studied in monkeys why vasospasm resolves after subarachnoid hemorrhage (SAH).

Methods—Monkeys underwent angiography and right (n=17) or bilateral (n=8) SAH. Animals with bilateral SAH underwent angiography 1, 3, 5, and 7 days later. Animals with right SAH underwent angiography 7 days later. The clot was then not removed (n=5), removed and replaced with fresh clot (n=7), or removed and not replaced (n=5). At the same time on day 7, the removed clot (n=12) or fresh clot (n=5) was placed on the left side. Angiography was repeated every 2 days until day 14.

Results—SAH caused significant vasospasm on day 7 that resolved by day 14. Removal of clot on day 7 resulted in more rapid resolution of vasospasm. Placement of fresh clot onto arteries that had already been exposed to clot for 7 days produced vasospasm that persisted without resolving for an additional 7 days. Placement of 7-day-old clot from the right onto previously unexposed left arteries or of clot from blood removed from an animal 7 days after SAH caused significantly more rapid onset of vasospasm compared with de novo vasospasm. Microscopic examination of the clots showed they were surrounded by macrophages 7 days after SAH. Arterial compliance and contractility were reduced in relation to duration of the exposure of arteries to clot.

Conclusions—Vasospasm resolves because of loss of subarachnoid blood clot. We hypothesize that reduced spasmogen release from the clot contributes to resolution of vasospasm. There was no response in the cerebral arteries that rendered them less responsive to the subarachnoid clot. (Stroke. 2001;32:1868-1874.)

Key Words: hemoglobin ■ subarachnoid hemorrhage ■ vasospasm

We previously studied the time course of the dependence of vasospasm on the presence of subarachnoid clot in a nonhuman primate model.1 It was found that the longer the clot remained in contact with the cerebral arteries, the more slowly the arteries returned to normal diameter when the clot was removed. Vasospasm resolved 5 to 7 days after subarachnoid hemorrhage (SAH). This was interesting because the clots that were removed at these times weighed about a third as much as when they were initially placed and still contained substantial amounts of hemoglobin, which has been postulated to be an important mediator of vasospasm.2 The release of hemoglobin from the subarachnoid clot is regarded as a major cause of the narrowing. This raised the question of why vasospasm was resolving in the continuing presence of the substance that was postulated to cause it in the first place. Could subarachnoid blood induce a response in the cerebral arteries that altered their responsiveness to a subsequent application of subarachnoid blood? In support of this, we found that levels of heme oxygenase-1, which was suggested to aid in the resolution of vasospasm,3 were increased 7 days after SAH during resolution of vasospasm.4 In these experiments, we tested the hypothesis that subarachnoid blood clot induces a positive adaptive response in cerebral arteries that renders them less responsive to a subsequent challenge with subarachnoid blood.

Materials and Methods

Animals were randomly assigned to experimental manipulations. All data analysis was blinded. Seventeen cynomolgus monkeys (Macaca fascicularis) underwent cerebral angiography and right subarachnoid blood clot placement (SAH) on day 0 (Figure 1). Angiography, craniectomy, and creation of SAH were carried out as described previously.1 All survival procedures were carried out with the animals under general anesthesia using sterile technique. On day 0, animals were sedated with 10 mg/kg ketamine IM, weighed, intubated, and ventilated on 1% to 2% isofluorane. Body temperature, PaCO₂, PaO₂, blood pressure, and heart rate were maintained in the physiological range. Blood hemoglobin was measured. Cerebral angiography was performed with constant magnification and exposure factors. SAH was created by placing the preweighed clot from 12 mL of fresh, autologous arterial blood into the subarachnoid space next to the right internal carotid, middle (MCA), and anterior cerebral arteries. A clot of this size was used because it filled the intradural space created after craniectomy and arachnoid dissection. Angiography was repeated on day 7. The clot was then not removed (n=5), removed and replaced with fresh clot (n=7), or removed and not replaced (n=5). After this procedure and with the animals under...
Henseleit buffer composed of (in mmol/L) Na\(^+\) 139, K\(^+\) 5.9, Ca\(^{2+}\) 2.5, Mg\(^{2+}\) 1.2, Cl\(^-\) 124, HCO\(_3\)^{-} 25, SO\(_4\)^{2-} 1.2, H\(_2\)PO\(_4\)^{-} 1.2, and glucose 11, bubbled with 95% O\(_2\), and 5% CO\(_2\) at 4°C. Arterial rings 3 mm long were suspended between stainless steel wires in tissue baths filled with this buffer. After equilibration and adjustment to optimal tension, contractility and compliance were compared between experimental groups and in the normal basilar artery, which was shown in preliminary experiments to be of similar diameter and to have identical contractility and compliance as the MCA.\(^6\) Contractility was measured by constructing concentration-tension curves to cumulative half-log additions of KCl.\(^6\) Compliance was measured by progressive stretch of rings under inhibition of the myogenic tone with papaverine (100 \(\mu\)mol/L) and nicardipine (10 \(\mu\)mol/L).\(^6\)

Clots fixed in 10% buffered formalin were cut in half along an axis perpendicular to the horizontal portion of the Sylvian fissure along which the clot had resided in vivo. They were embedded in paraffin, and 10-\(\mu\)m sections were prepared and stained with hematoxylin-eosin. Immunohistochemistry of adjacent sections was performed with mouse primary monoclonal antibodies to human CD68 (DAKO) using techniques previously reported.\(^4\) Sections were deparaffinized and subjected to antigen retrieval by boiling in citrate buffer (7 mmol/L, pH 6). They were exposed to the primary antibody, 1:15, for 10 minutes followed by anti-mouse secondary antibodies conjugated to alkaline phosphatase polymer (Envision system; DAKO). The chromogen was fast red. Control sections were processed with no primary antibody.

Vasospasm was assessed by measurement of angiograms.\(^1\) Results are described and analyzed separately for 3 manipulations of the right cerebral arteries (no removal of clot on day 7 [5 arteries], removal of clot on day 7 and replacement with fresh, autologous clot [7 arteries], and removal of clot on day 7 without replacement [5 arteries]) and 2 manipulations of the left cerebral arteries (placement of 7-day-old clot [12 arteries], placement of new clot with blood from a monkey that was 7 days post-SAH [5 arteries]), plus 1 control group with bilateral SAH (placement of new clot with blood from a previously unoperated monkey [16 arteries]). Percent changes in angiographic diameters from baseline were used for comparisons within groups over time or between groups at corresponding times. Comparisons of physiological variables and angiographic arterial diameters were made with ANOVA followed by Tukey’s test if significant variance was found. Linear regression of arterial diameter versus various physiological variables was conducted to determine whether statistically significant differences within groups over time would significantly alter arterial diameters in some of the physiological variables.

Linear regression of clot weight and percent change in clot weight versus degree of vasospasm also were determined. Comparisons of arterial compliance and contractility between groups also were made with ANOVA followed by Tukey’s test if significant variance was found. Clot histology and immunohistochemistry were interpreted by 2 blinded investigators who provided qualitative descriptions. Significance was taken at \(P<0.05\). Values are given as mean±SD.

**Results**

**Physiological Variables and Clinical Condition**

There were no changes in physiological variables within groups over time or between groups at each time that were of sufficient magnitude to have any effect on angiographic arterial diameters. There were no differences between groups in hemoglobin concentration on day 0 (\(P=0.66,\) ANOVA) (Table 1). Because clots placed intracranially were derived from blood obtained on day 7 in some groups, the hemoglobin concentration in the blood of these groups on day 7 was compared with that on day 0 in the group undergoing bilateral SAH. Hemoglobin concentration was significantly higher in the day 0 group than in the other groups (\(P<0.01,\) ANOVA). There were no differences between groups at any other time. Four monkeys did not survive until day 14. All underwent removal of the clot on the right followed by the placement of fresh clot on the right and the old clot on the left. By day 8, they were lethargic with severe bilateral vasospasm and, in 1 case, right hemiparesis. They were killed on days 8 (\(n=2\)), 9 (\(n=1\)), and 12 (\(n=1\)). Three additional animals undergoing the same procedures were lethargic and hemiparetic beginning on day 8 and improved until they were killed on day 14.

**Right Angiography**

This group of MCAs were from animals that had undergone clot placement on day 0 followed on day 7 by no removal of clot, removal of the clot and placement of fresh autologous clot, or removal of the clot without replacement. Analysis of angiographic MCA diameter over time showed that all 3 groups had significant vasospasm on day 7. If the clot was not removed, vasospasm also resolved and by day 14 was significantly less than on day 7 (\(P<0.001,\) ANOVA). If the clot was removed, vasospasm also resolved and was significantly less than on day 7 by days 12 and 14 (\(P<0.05,\) ANOVA). If fresh
clot was placed after the clot was removed on day 7, however, there was no reversal of vasospasm.

Comparisons between groups at days 0 and 7 showed no significant differences, and each group developed significant vasospasm at day 7. Analysis between the groups over time showed no differences on days 8 and 10, but by day 12, there was significant variance in percent changes in MCA diameter compared with that on day 7 (P < 0.05, ANOVA, Figure 2).

The group that underwent clot removal without replacement had significantly less vasospasm than the group that underwent clot removal and placement of fresh autologous clot. By day 14, all 3 groups were significantly different, with clot removal resulting in less vasospasm and placement of fresh clot resulting in more vasospasm compared with the group with no removal of the clot. The results for the intracranial internal carotid and anterior cerebral arteries showed similar patterns of changes.

**Left Angiography**

This group of MCAs were from animals that had undergone placement of 7-day-old clot removed from the right side of the head onto the previously unexposed left arteries or placement of new clot with blood from a monkey that was 7 days post-SAH. The third group of MCAs to be analyzed were from animals that had bilateral clot placement followed by serial angiography, which demonstrates the de novo time course of vasospasm during the first 7 days in this model. Analysis of percent change in angiographic MCA diameter over time showed that all 3 groups had significant reductions in MCA diameter starting 1 day after clot placement (day 8 or day 1, depending on the group; P < 0.001, ANOVA) and that vasospasm persisted without significant change until the animals were killed on day 7 or 14. The trend in all 3 groups was for vasospasm to become less severe after day 3, although this was not significant.

Comparisons between groups at day 0 and day 7 before clot placement showed no significant differences in MCA diameters. The only significant difference between the groups was 1 day after clot placement when there was more severe vasospasm in groups that were already 7 days post–right SAH and that had placement of 7-day-old clot or of new blood from a previously unoperated monkey (P < 0.001, ANOVA) (Figure 3). Furthermore, vasospasm was significantly more severe in animals with placement of 7-day-old clot than in the other groups (P < 0.001, ANOVA). The results for the other intracranial arteries showed the same pattern of changes.

### Table 1. Blood Hemoglobin Concentration for Each Group Over Time

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>Hemoglobin, g/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Right angiography</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No removal of clot on day 7 (n=5)</td>
<td>0</td>
<td>10.2±1.5*</td>
</tr>
<tr>
<td>Removal of clot on day 7 and replacement with fresh autologous clot (n=7)</td>
<td>7</td>
<td>8.6±0.9</td>
</tr>
<tr>
<td>Removal of clot on day 7 without replacement (n=5)</td>
<td>0</td>
<td>10.8±1.6*</td>
</tr>
<tr>
<td>Left angiography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placement of 7-day-old clot (n=12)</td>
<td>0</td>
<td>10.7±1.1*</td>
</tr>
<tr>
<td>Placement of new clot from a monkey 7 days after SAH (n=5)</td>
<td>7</td>
<td>9.1±1.9</td>
</tr>
<tr>
<td>Bilateral angiography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placement of new clot from previously unoperated monkey (n=16)</td>
<td>0</td>
<td>11.5±1.4*</td>
</tr>
</tbody>
</table>

All values are mean±SD.

*P < 0.01, ANOVA within and between groups over time, day 0 significantly different from day 7 for each group.

**Figure 2.** Percent change in MCA diameter from day 7 vs the day after clot placement for right-side arteries that had no clot removal on day 7, new clot placed on day 7, or clot removed on day 7. Vasospasm reverses significantly more rapidly in the clot removal group than in the group with new clot placed by day 12 (P < 0.05, ANOVA) and than in the group with new clot placed and the group with no clot removal by day 14 (P < 0.05, ANOVA).
Comparison of the severity of vasospasm 7 days after clot placement was made for all 6 groups. There was a trend for the most severe spasm to occur after placement of fresh clot on the right-side arteries that already had been exposed to clot for 7 days (P = 0.08, ANOVA).

### Clot Weights and Relation to Vasospasm

There were no significant differences in the weights of fresh clots placed on day 0 or day 7 (Table 2). There were no significant differences in the weights of clots removed on day 7, although the weights of these clots were all significantly less than the weights placed on day 0 (P < 0.001, ANOVA). Similarly, there were no differences in the weights of clots that had been present intracranially for 14 days, although they were significantly less than weights present at 7 days (P = 0.05, ANOVA).

To determine the dependence of vasospasm on subarachnoid clot, plots were made of percent reductions in angiographic MCA diameter over 7 days after a particular clot was placed compared with the weight of clot placed, the weight cleared over 7 days, and the percent of clot cleared during the same time (Figure 4). Separate analyses were conducted for clots placed on day 0 and for fresh and old clots placed on day 7. No significant relationships could be demonstrated for any of these comparisons. It was noted that the small weights of day 7 clots often elicited as much spasm as did fresh clots of much greater weights (Figure 4).

### Arterial Contractility and Compliance

There was significant variance in tension developed in response to 60 mmol/L KCl, with normal basilar arteries (0.33 ± 0.12 g) significantly greater than MCAs of any experimental group. Furthermore, contractility was lowest in arteries exposed to SAH for 14 days (no clot removal on day 7 and therefore exposure to clot for 14 days, 0.045 ± 0.057 g; removal of clot on day 7 and replacement with fresh autologous clot, 0.035 ± 0.058 g) compared with arteries exposed to SAH for 7 days (removal of clot on day 7 without replacement, 0.10 ± 0.19 g; placement of 7-day-old clot into the previously unexposed left MCA, 0.080 ± 0.014 g; placement of fresh clot in a previously unoperated monkey, 0.091 ± 0.015 g). Measurement of compliance showed the same pattern of progressive decrease with increasing duration of exposure to SAH (Figure 5). There was significant variance in tension at lengths of 0.25, 0.5, 1.5, and 1.75 mm, with lowest tension in normal basilar arteries and MCAs from the group undergoing removal of clot on day 7 without replacement and highest tension in MCAs exposed to SAH for 14 days or to clot for 7 days followed by removal of clot and replacement with fresh clot (P < 0.05, ANOVA).

### Table 2. Weights of Clots Placed and Removed

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0: Fresh Clot Placed</th>
<th>Day 7: Fresh Clot Placed</th>
<th>Day 7: 7-Day-Old Clot</th>
<th>Day 14: Clot Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right angiography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No clot removal on day 7 (n=5)</td>
<td>3.85 ± 0.76</td>
<td>...</td>
<td>...</td>
<td>0.28 ± 0.17</td>
</tr>
<tr>
<td>Removal of clot on day 7 and replacement with fresh clot (n=7)</td>
<td>3.58 ± 0.71</td>
<td>3.18 ± 0.52</td>
<td>...</td>
<td>0.96 ± 0.13</td>
</tr>
<tr>
<td>Removal of clot on day 7 without replacement (n=5)</td>
<td>3.51 ± 0.38</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Placement of 7-day-old clot (n=12)</td>
<td>...</td>
<td>...</td>
<td>0.85 ± 0.47</td>
<td>0.37 ± 0.31</td>
</tr>
<tr>
<td>Placement of new clot from blood from a monkey 7 days after SAH (n=5)</td>
<td>...</td>
<td>3.81 ± 0.45</td>
<td>...</td>
<td>0.74 ± 0.24</td>
</tr>
<tr>
<td>Bilateral angiography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placement of new clot from blood from a previously unoperated monkey (n=16)</td>
<td>3.61 ± 0.32</td>
<td>...</td>
<td>0.79 ± 0.23</td>
<td>...</td>
</tr>
</tbody>
</table>

All values are mean ± SD.
Clot Histopathology and Immunohistochemistry

On day 7, the surface of the clot was covered with a layer of cells that varied from 5 to 15 layers thick (Figure 6); this layer appeared to encase the clot. The clot itself was composed of erythrocytes in varying stages of breakdown. There was minimal inflammatory cell infiltration into the clot, although in some areas, groups of cells were observed that appeared to be migrating into the clot. Some of the cells in the layer covering the clot showed immunoreactivity for CD68. By day 14, there was a similar layer of cells, some with CD68 immunoreactivity, surrounding the clot. The principal change was a decrease in the size of the remaining clot and a more amorphous appearance of the remaining erythrocytes. Cells around the clot that did not contain CD68 immunoreactivity resembled proliferating cells derived from arachnoid cells.

Discussion

Multiple mechanisms could be involved in the maintenance and reversal of vasospasm after SAH. Vasospasm may resolve due to loss of the stimulus for the vasospasm, which is subarachnoid blood. There may be decreased release of spasmogens from the clot or decreased access of them to the smooth muscle in the arterial wall. There may be a positive adaptive response that allows the artery to relax even though clot still is present. Spasmogen or spasmogens may become embedded in the arterial wall, or a temporarily irreversible process may occur in the arterial wall such that clot removal does not have any effect on the time course of vasospasm. There may be a negative adaptive response that makes vasospasm more severe in response to a repeated stimulus. Adaptive responses could be local in the artery exposed to blood or general in the entire organism.

In the right-side arteries, clot removal at 7 days resulted in more significant resolution of vasospasm than if the clot was left in place. Therefore, vasospasm resolves in part due to the loss of subarachnoid blood. In prior studies of the effect of clot removal up to 5 days after SAH, vasospasm was always to some extent dependent on the presence of subarachnoid blood clot.1,8,9 The effect of clot removal, however, decreased as the time to clot removal after SAH increased, and it took progressively longer after the clot removal for the effect to become evident.1 Therefore, in addition to resolution through loss of the clot, other mechanisms must be involved. This is supported by reports of the presence of hemoglobin, a prime candidate for the cause of vasospasm, in the arterial wall after experimental SAH.10,11 The results from the right-side arteries do not support the development of a positive adaptive response, because the placement of a new clot after 7 days resulted in even more severe vasospasm than did the initial clot placement.

Regarding the left arteries, there was no general positive adaptive response, because fresh clot placement produced vasospasm similar to that produced by de novo clot placement. Interestingly, placement of 7-day-old clot on the left
resulted in severe spasm, even though when the same clot remained on the right vasospasm was resolving. We suggest that the clot becomes encased in a cellular capsule that impairs spasmogen release. Histopathological examination of the clots supports this theory. Most of the cells around the clot showed immunoreactivity for CD68, a macrophage marker. In rats, macrophages are abundant in the cerebrospinal fluid during the resolution of vasospasm.12 It is unlikely that there is a change in the artery that prevents spasmogens from reaching it, because the placement of fresh clot onto the same artery produces vasospasm.

The right-side arteries suggest there is a negative adaptive response or sensitization of the arteries after SAH, because vasospasm was more severe 7 days after clot replacement on the right than it was in any other group 7 days after a first SAH. On the left, the placement of fresh clot in animals 7 days after SAH produced more vasospasm 1 day later than did the placement of fresh clot de novo. This was not due to hemoconcentration. These findings support a general negative adaptive response. It is also possible that there is some alteration in the blood that makes spasm worse under these circumstances. Another possibility is that the clot is cleared less rapidly after a second application. This was not the case, because the weights of the remaining clot were the same as those 1 week after de novo clot placement. Furthermore, there was no relationship between 2 measures of clot clearance and those 1 week after de novo clot placement. Furthermore, there was no relationship between 2 measures of clot clearance and those 1 week after de novo clot placement.

Weir et al13 injected blood every 7 days into the subarachnoid space of monkeys and noted transient vasospasm after each injection. Repeated injections did not alter the severity of vasospasm. A single injection of blood into the cisterna magna of rats, rabbits, or dogs produces less vasospasm than do 2 injections spaced 2 or 3 days apart.10,14 One mechanism may be that the first injection blocks pathways that clear the subarachnoid blood, making the erythrocytes from the second injection remain in the subarachnoid space until they hemolys and release their spasmogenic contents. The rate of blood clot clearance does not completely explain the results of this study (Table 2 and Figure 4). Zabramski et al15 repeatedly injected blood into the cisterna magna of dogs. In 2 dogs, an injection 3 weeks after the first resulted in the rapid onset of severe vasospasm. This finding is consistent with the present study in that there is no positive adaptive response but there may be sensitization of the arteries to subarachnoid blood.

Vasospastic arteries have decreased contractility and compliance.6,16 These changes become progressively more severe as the time after SAH increases and as vasospasm resolves. They have been suggested to contribute to the persistence of vasospasm.17,18 The importance of such changes can be evaluated in the present study. The removal of the clot even after 7 days was shown to result in more rapid reversal of vasospasm, suggesting that these changes do not prevent vasospasm from resolving, although an examination of the rates of reversal of vasospasm after clot removal does show that it resolves more slowly after clot removal as the time to clot removal after SAH increases.1 The changes in the arterial wall also do not prevent the artery from contracting, because the placement of fresh clot on an artery 7 days after SAH, at a time when decreased contractility and compliance are reduced in this model, results in even more spasm than the initial application of blood.

Acknowledgments

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References


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