Evidence of the role of hemolysis in experimental cerebral vasospasm

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The short-term (≤ 72-hour) reaction to subarachnoid injections of various blood components was determined in a canine model of cerebral vasospasm. Platelet-rich plasma (PRP) formed durable clots in the basal cistern surrounding the basilar artery and provoked no vascular reaction in 72 hours or more. Freshly isolated autologous erythrocytes resuspended in PRP likewise provoked no vasoconstriction in 72 hours, although a second injection of fresh erythrocytes in PRP induced significant reaction, as in the conventional “double subarachnoid hemorrhage (SAH)” canine model. Hemolysate of fresh erythrocytes led to a severe immediate vascular reaction after introduction into the basal cistern using PRP as the carrier/clotting medium, as did the injection of intact erythrocytes incubated ex vivo for 72 hours. Resolution of the initial reaction was rapid for hemolysate, but slow and (depending on hematocrit) incomplete for intact “aged” erythrocytes. In vitro measurements of erythrocyte lysis in these media and histological examination indicate that the production of erythrocyte lysate was responsible for the vascular reaction observed, suggesting that the rate of lysis of erythrocytes in the subarachnoid clot is a major factor in the genesis of vasospasm after SAH.

Key Words • vasospasm • basilar artery • erythrocyte • hemolysis • inflammation • dog

Based on the vasoactivity of erythrocyte lysate in vitro,2,9,23,25 the acute vascular reaction to subarachnoid injections of erythrocyte breakdown products4,10,17,24 or incubated blood,6 and the in vitro vasoactivity of cerebrospinal fluid (CSF) from subarachnoid hemorrhage (SAH) patients,25 it has been long proposed that hemolysis of erythrocytes in the subarachnoid blood clot plays an important role in the development of cerebral vasospasm after SAH. While the identity of vasoactive factor(s) in erythrocyte lysate is not certain, most attention has focused on oxyhemoglobin due to its in vivo20 and in vitro7,35,37,39,40 vasoactivity in cerebral vessels. However, a direct role for oxyhemoglobin in cerebral vasospasm is still debated,1,41 and the question of whether the vasoconstrictive action of erythrocyte lysate or hemoglobin is direct or indirect is yet unresolved. Proposed indirect mechanisms include: generation of free radicals,29,31 stimulation of prostaglandin14,21,22 and leukotriene28 synthesis, potentiation of reactivity to other vasoactive substances,34 neurogenic sensitization,35 and the inhibition of prostaglandin38 or endothelium-dependent18,19 mechanisms of relaxation.

Since the investigations cited above focused on short-term (< 24-hour) cerebrovascular reaction the role of the erythrocyte in delayed chronic vasospasm remains undemonstrated. Recent work by Duff, et al.,5 has shown that cat cerebral arteries surrounded by cell-free plasma clot for 7 to 10 days show no significant development of vasoconstriction or vasculopathy, while subarachnoid blood clot leads to vasospasm within 7 days. Mayberg, et al.,16 have shown that application of washed erythrocytes to porcine cerebral vessels induces a chronic vascular reaction comparable to that of whole blood. These studies emphasize the role of the erythrocyte. Similar work in the canine model has shown that moderate-to-severe persistent vasospasm can result solely from inflammatory reactions in the subarachnoid space, even in the complete absence of erythrocytes.28 These observations are not mutually exclusive, but rather emphasize that cerebral vasospasm after SAH is probably multifactorial in origin. A model connecting the roles of inflammation and the erythrocyte has recently been advanced.29

The experiments presented here investigated quantitatively the role that various cellular components of
whole blood might play in the mechanisms of cerebral vasospasm in the canine model. In particular, they examine the extent to which the rate of release of hemolysate from subarachnoid erythrocytes can account for the severity of delayed vascular reaction to subarachnoid blood clot.

Materials and Methods

Subarachnoid injections of various autologous blood components, cerebral angiography, and data analysis were performed exactly as described elsewhere. 28

Preparation of Subarachnoid Injectates

Plasma and Platelet-Rich Plasma. Cell-free plasma was prepared from Ca++-chelated whole blood (with approximately 4.5 mM ethyleneglycol-bis-(β-aminooxyethylether)-N, N’-teta-acetic acid, or EGTA), as described earlier. 28 Clotting was activated by reconstitution of free Ca++ to between 0.70 and 1.0 mM and the addition of trace amounts (0.002 to 0.10 U/ml) of lyophilized bovine thrombin. Plasma was sometimes used as the subarachnoid injection medium; however, earlier studies had shown that plasma clot alone does not remain long in the basal cistern unless other matrix materials are added, such as dextran or latex beads. 28 Inclusion of such foreign materials in that study, led to severe inflammation and consequent vasoconstriction, however. To avoid this problem, plasma-containing platelets at or in excess of the whole-blood platelet count was also used.

In general, whole blood was drawn into sterile 5-ml glass tubes containing 0.05 ml of 15% K, ethylenediaminetetra-acetic acid (EDTA). Blood sedimented passively at 37°C for 45 to 90 minutes, and supernatant plasma with platelets and white blood cells (WBC’s) was harvested and pooled in sterile siliconized glass tubes. If initial sedimentation rate was too slow, then whole blood was centrifuged for 2 minutes at 100 to 150 G and supernatants were harvested and pooled as above. Plasma containing platelets, WBC’s, and trace erythrocytes was then centrifuged for 2 minutes at 125 G and the supernatant was again harvested. Centrifugation was repeated to yield the final platelet-rich plasma (PRP), the cellular composition of which is given in comparison to whole blood values in Table 1.

After surgical preparation and control cerebral angiography of the animals, CSF equal to the planned injectate volume was withdrawn by needle puncture of the cisterna magna. During this time, ionized free Ca++ in PRP was reconstituted by adding typically 0.035 ml of sterile 100 mM CaCl2/ml PRP. The presence of platelets obviated the need for exogenous thrombin to activate clotting, as further evidenced by the vigorous retraction in vitro of clotted PRP 10 to 30 minutes after Ca++ addition. In vitro trials correlating added Ca++ with clotting time showed that free Ca++ of less than 0.1 mM gave a clotting time of about 2 to 3 minutes, as needed to allow subarachnoid injection (1 minute) and pooling in the basal cistern before completion of clotting. This dependence was found to be remarkably sensitive, so that PRP in excess of injectate volume was routinely prepared and appropriate Ca++ addition was determined in vitro for each experiment.

Erythrocytes and Hemolysate. Whole blood was drawn in sterile 10-ml siliconized glass tubes containing EGTA, EDTA, or heparin (20 U/ml) as anticoagulant. Blood was centrifuged for 8 to 12 minutes at 500 G and plasma/buffy coat was aspirated, including the upper 2 to 4 mm of the erythrocyte pellet. Erythrocytes were then similarly washed twice in warm sterile saline and resuspended in an artificial CSF solution. 29

In various experiments, isolated erythrocytes were then used immediately (“fresh”) or allowed to incubate for 72 hours at 37°C (“aged”). After incubation, “aged” erythrocytes were washed two or three times by resuspension and centrifugation in warm sterile saline, then one final wash was given in freshly prepared Ca++-chelated autologous cell-free plasma before use. “Fresh” erythrocytes also received one final plasma wash before use in subarachnoid injectates.

In some experiments, freshly isolated erythrocytes were lysed by subjecting the washed erythrocyte pellet, resuspended to approximately 45% hematocrit in sterile saline, to between 10 and 12 0.5-second bursts of ultrasound on a cell disruptor.* After high-speed centrifugation at 15,000 G for 5 minutes to remove membranes and insoluble cell debris, 1.4 ml soluble hemolysate supernatant was added to 5.0 ml freshly prepared Ca++-chelated PRP to simulate 25% hemolysis in whole blood. In some experiments, hemolysis was determined

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* Cell disruptor manufactured by Branson Ultrasonics Corp., Danbury, Connecticut.

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<table>
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<tr>
<th>Factor</th>
<th>Erythrocytes</th>
<th>WBC</th>
<th>Platelets</th>
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<tr>
<td>PRP (8 animals)</td>
<td>4700 ± 1300</td>
<td>54 ± 27</td>
<td>367,000 ± 33,000</td>
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<td>control whole blood (17 animals)</td>
<td>5,790,000 ± 240,000</td>
<td>9300 ± 700</td>
<td>276,000 ± 18,000</td>
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<td>% PRP of control</td>
<td>0.08%</td>
<td>0.58%</td>
<td>133%</td>
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* Values are means ± standard error of the means, and are expressed per cubic millimeter of whole blood. WBC = white blood cells.
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FIG. 1. Photograph of the ventral surface of the brain stem of an animal 48 hours after the subarachnoid injection of platelet-rich plasma. The coating is relatively translucent, and is most noted in black and white as a loss of underlying detail.

quantitatively by spectrophotometric analysis of the hemoglobin spectrum using the 575 nm peak in diluted samples of the hemolysate supernatant.

Results

Vascular Reaction to Subarachnoid PRP and Reconstituted Blood

Subarachnoid injections of 3.0 ml “activated” PRP formed a long-lasting clot in the basal cistern surrounding the basilar artery (Fig. 1) and produced no significant change in basilar artery diameter from control value over 72 hours (Fig. 2, open circles). A second subarachnoid injection of PRP 72 hours after the first still produced no significant vascular reaction over an additional 48 to 72 hours.

Similar single injections of 3.0 ml autologous whole blood produce only a small maintained constrictor effect (approximately 15% narrowing) within 72 hours, while a second experimental SAH (that is, the “double-SAH” canine model) leads to significant vasospasm: 36.2% ± 2.7% narrowing (± standard error of the mean) in 27 dogs. That these observations with whole blood are dependent on erythrocytes in the subarachnoid clot was indicated by experiments in which a 20% hematocrit of freshly isolated erythrocytes was added to PRP shortly before subarachnoid injection.

The angiographic results of such injections show no significant reaction within 72 hours (Fig. 2, filled circles), but there was substantial vasospasm in response to a second injection of “reconstituted” whole blood 72 hours after the first. The small but statistically significant reduction in reaction severity to subarachnoid injections of PRP with added erythrocytes relative to whole blood for both the first and second experimental injections (3% and 28% narrowing vs. 15% and 36%, respectively) is likely due to the reduced hematocrit of erythrocytes in the injectate. While there appears to be no significant role for platelets alone in the mechanisms of delayed cerebral vasospasm after SAH, the addition of erythrocytes induces results virtually identical to those with whole blood.

Vascular Reaction to Subarachnoid Hemolysate

In preliminary trials in two dogs, Ca++-chelated autologous whole blood was lysed by ultrasonication and induced to clot within 3 to 4 minutes from time of subarachnoid injection by reconstruction of free Ca++ or by mixing (1:1 v/v) with whole blood without Ca++-chelator. Both animals showed intense immediate re-
FIG. 3. Angiographic basilar artery diameter (expressed as a percentage of control diameter) is shown for two animals receiving injections of whole blood sonicated at 20% and 40% hematocrits (filled circles) and three animals injected with hemolysate equivalent to a 10% hematocrit of sonicated erythrocytes in platelet-rich plasma (PRP, filled squares). For reference, the mean (± standard error of the mean) vascular reaction to subarachnoid injections of PRP and PRP/fresh erythrocytes is shown (open circles). Symbols without error bars represent individual determinations.

Action to hemolysate with 35% to 40% constriction (Fig. 3, filled circles). One animal died overnight. Angiography at 24 and 48 hours in the surviving animal showed nearly complete resolution of the early acute-phase reaction, although neurological deficits persisted.

To promote viability allowing a longer-term study, lysate from freshly isolated erythrocytes was mixed with PRP in an amount equivalent to complete lysis of a lower (10%) hematocrit. After free Ca++ was reconstituted, 3.0 ml of “activated” erythrocyte lysate/PRP mixture was injected via the cisterna magna. Angiographic data for three animals receiving this injectate are shown in Fig. 3 (filled squares).

Introduction of erythrocyte lysate directly into the basal cistern provokes a severe immediate reaction which fully and rather rapidly resolves, likely due to elution of soluble vasoactive components and serum expression upon clot retraction. This is illustrated in Fig. 4A which shows the absence of significance color on the brain stems of animals 48 to 72 hours after subarachnoid injection of hemolysate/PRP.

Vascular Reaction to Subarachnoid “Aged” Erythrocytes

Isolated erythrocytes were incubated in artificial CSF for 72 hours at 37°C without agitation or mixing, then repeatedly washed with warm sterile saline followed by a wash with freshly prepared Ca++-chelated autologous plasma before resuspension to various hematocrits (range 10% to 40%) in plasma, whole blood, or PRP. After activation of clotting, these erythrocytes “aged” ex vivo were administered to the basal cistern of 10 animals. Immediate and severe vasoconstriction was
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**FIG. 5. Left:** Mean (± standard error of the mean) vascular reaction to subarachnoid injections of various media containing 10% to 40% hematocrits (Hct) of intact “aged” erythrocytes (that is, incubated 72 hours ex vivo) is shown by the filled circles in comparison to the mean response to subarachnoid platelet-rich plasma (PRP) and fresh erythrocytes in PRP. The injection media were: plasma in three animals, whole blood in five, and PRP in two. The injection media did not alter the observed intense reaction, and mortality rate (> 70% after 24 hours) prevented analysis at later times. Symbols without error bars represent individual measurements in the surviving animals.

**Right:** Similar data for four animals receiving subarachnoid injections of 2.5% to 5% hematocrits (Hct) of intact “aged” erythrocytes suspended in PRP. All animals survived without deficit noted in all animals at 1 to 2 and 3 to 5 hours postinjection. The data shown in Fig. 5 left were seriously limited by mortality: seven animals died within 24 hours and only two survived to 48 hours. The clear indication, nonetheless, is that resolution from severe acute reaction to moderate vasospasm took place over 72 hours.

To reduce this high mortality rate, erythrocytes were incubated ex vivo and resuspended in PRP to hematocrits ranging from 2.5% to 5%. Four animals received subarachnoid injections of these small titters of “aged” erythrocytes, and all survived 48 to 72 hours without serious deficit. There was a clear reduction in the severity of acute-phase reaction and complete progressive resolution of vasospasm within 72 hours (Fig. 5 right).

**In Vitro Hemolysis of “Aged” Erythrocytes**

A comparison of the brain stems of animals that died shortly after subarachnoid injection of “aged” erythrocytes (Fig. 4B) with those surviving to longer term (Fig. 4C) showed rapid progressive loss of color in the subarachnoid clot, suggesting that erythrocyte lysis played an important role in the vascular reaction observed. In five cases, *in vitro* measurements of hemolysis in the subarachnoid injectates of “aged” erythrocytes in various media were performed in parallel to the *in vivo* experiments. Small aliquots of the material prepared for subarachnoid injection were allowed to clot and were incubated for various times at 37°C. Supernatants were assayed spectrophotometrically and hemolysis measured over 3 to 3.5 hours was expressed as a percentage of total initial “aged” erythrocytes (Fig. 6). While the quantitative results were highly variable, all *in vitro* samples showed significant hemolysis (30% to 60%) occurring within several hours of *in vivo* administration. In contrast, hemolysis in freshly drawn samples of clotted whole blood averaged less than 0.25% of erythrocytes in 3 hours.

Erythrocytes incubated ex vivo and added to the subarachnoid space in PRP provoked, in addition to...
nearly complete hemolysis, a potent inflammatory reaction in the perivascular space as indicated by a dense population of inflammatory and phagocytic cells (Fig. 7).

Discussion

The experiments described above demonstrate three main findings. 1) A long-lasting clot containing a normal or supranormal (133%) population of platelets surrounding the canine basilar artery in situ provokes no vascular reaction in 72 hours or more after exposure, as was previously shown for cell-free plasma clots of brief duration.28 2) A similar clot containing platelets and freshly isolated autologous erythrocytes likewise provokes no significant vascular reaction in 72 hours after exposure. 3) A similar clot containing intact autologous erythrocytes prematurely "aged" by incubation ex vivo for 72 hours or hemolysate from fresh autologous erythrocytes provokes a severe and immediate vascular reaction which resolves more rapidly for hemolysate than for "aged" erythrocytes. These findings clearly support a central role for the "aging" erythrocyte in the etiology of delayed cerebral vasospasm after SAH and are in agreement with the in situ studies by Duff, et al.,3 Mayberg, et al.,16 and earlier authors,17,24 as well as with in vitro studies demonstrating the vasoactivity of erythrocyte lysate.2,9,23,25

The following interpretation is consistent with the above findings. Canine erythrocytes incubated 72 hours ex vivo under conditions analogous to those of the subarachnoid clot apparently undergo changes such that exposure to plasma factors greatly accelerates lysis, as evidenced by the data shown in Fig. 6. When "aged" erythrocytes were introduced into the canine subarachnoid space in a plasma-containing medium, hemolysis likely proceeded rapidly as the clot formed around the basilar artery, thus exposing the vessel to high concentrations of vasoactive hemolysate. However, the intense initial vascular reaction began to resolve as hemolysis was either completed (low hematocrit, Fig. 5 right) or slowed when cytokytic plasma factors were lost during clot retraction and serum expression (high hematocrit, Fig. 5 left). Already-released hemolysate was then likely more slowly eluted by bulk CSF circulation.28 Hemolysis of the remaining erythrocytes proceeded then primarily only as fresh cytokytic plasma protein entered the perivascular clot by extravasation through the vessel blood-brain barrier, since spontaneous hemolysis of such cells has been shown to be quite slow.29 This latter idea is based on a comparison of Figs. 5 left and 3, which suggests that hemolysis of "aged" erythrocytes continued for some time after injection, since resolution of the initial "acute-phase" reaction of similar magnitude was slow and incomplete in the presence of intact "aged" cells. This hypothesis is further supported by the observation that extravasation of plasma protein is greatly increased in cerebral vessels surrounded by subarachnoid blood clot.3,27,33 It is possible, however, that a secondary process, namely inflammation in reaction to the "aged" erythrocytes, further prolonged this process, as suggested by the massive infiltration of phagocytic cells seen in Fig. 7 and by earlier studies of the vascular reaction to inflammation.28

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