Complement activation in the brain after experimental intracerebral hemorrhage

YA HUA, M.D., GUOHUA XI, M.D., RICHARD F. KEEP, PH.D., AND JULIAN T. HOFF, M.D.

Department of Surgery (Neurosurgery), University of Michigan, Ann Arbor, Michigan

Object. Brain edema formation following intracerebral hemorrhage (ICH) appears to be partly related to erythrocyte lysis and hemoglobin release. Erythrocyte lysis may be mediated by the complement cascade, which then triggers parenchymal injury. In this study the authors examine whether the complement cascade is activated after ICH and whether inhibition of complement ameliorates brain edema around the hematoma.

Methods. This study was divided into three parts. In the first part, 100 μl of autologous blood was infused into the rats’ right basal ganglia, and the animals were killed at 24 and 72 hours after intracerebral infusion. Their brains were tested for complement factors C9, C3d, and clusterin (a naturally occurring complement inhibitor) by using immunohistochemical analysis. In the second part of the study, the rats were killed at 24 or 72 hours after injection of 100 μl of blood. The C9 and clusterin proteins were quantitated using Western blot analysis. In the third part, the rats received either 100 μl of blood or 100 μl of blood plus 10 μg of N-acetylheparin (a complement activation inhibitor). Then they were killed 24 or 72 hours later for measurement of brain water and ion contents. It was demonstrated on Western blot analysis that there had been a sixfold increase in C9 around the hematoma 24 hours after the infusion of 100 μl of autologous blood. Marked perihematomal C9 immunoreactivity was detected at 72 hours. Clusterin also increased after ICH and was expressed in neurons 72 hours later. The addition of N-acetylheparin significantly reduced brain edema formation in the ipsilateral basal ganglia at 24 hours (78.5 ± 0.5% compared with 81.6 ± 0.8% in control animals, p < 0.001) and at 72 hours (80.9 ± 2.2% compared with 83.6 ± 0.9% in control animals, p < 0.05) after ICH.

Conclusions. It was found that ICH causes complement activation in the brain. Activation of complement and the formation of membrane attack complex contributes to brain edema formation after ICH. Blocking the complement cascade could be an important step in the therapy for ICH.

Key Words • cerebral hemorrhage • clusterin • membrane attack complex • complement factor C3d • N-acetylheparin • rat

Brain edema formation after experimental ICH appears to have several different causes. Delayed edema formation may result from erythrocyte lysis and the release of hemoglobin.29 One potential mediator of erythrocyte lysis is the complement system, which consists of at least 30 proteins and involves in many immune reactions, including cell lysis and inflammatory response. Complement is normally excluded from the brain by the BBB, but it could enter at the time of the initial ICH or later when the BBB is disrupted. Complement activation occurs in several central nervous system disorders, including trauma, cerebral ischemia, subarachnoid hemorrhage, multiple sclerosis, and Alzheimer’s disease.1,17,19,20,32 Complement depletion produced by cobra venom factor reduces cerebral vasospasm after subarachnoid hemorrhage,10 improves somatosensory evoked potentials and neurological function during cerebral ischemia,26 reduces inflammation in experimental allergic neuritis,7 and reduces macrophage infiltration during wallerian degeneration.5 Recently, Huang, et al.,13 found that sialyl Lewis x glycosylated soluble complement receptor-1, a complement activation inhibitory protein, reduced cerebral infarct volume in mice after middle cerebral artery occlusion. Another inhibitor of complement cascade activation, N-acetylheparin, a heparin derivative that lacks anticoagulant activity,28 protects the myocardium from ischemic injury.1,22

Complement-mediated brain injury may be caused by the formation of MAC and the inflammatory response that ensues. The MAC consists of C5b-9 assembled after complement activation. Insertion of MAC into the cell membrane forms a pore, which eventually kills the cell. The formation of MAC in ICH may induce erythrocyte lysis that causes delayed brain edema formation.29 Recent investigations have demonstrated that MAC not only causes cell lysis, but also modulates cellular functions such as the release of cytokines, eicosanoids, oxygen radicals, and matrix proteins.13 Clusterin, which is also called sulfated glycoprotein-2, complement lysis inhibitor, or apolipo-
protein, is a putative complement inhibitor that impedes MAC-induced cell lysis by forming a clusterin–C5b-9 complex. Clusterin has also been shown to reduce amyloid-β–induced neurotoxicity in vitro. Giannakopoulos et al. suggested that clusterin may have a neuroprotective effect in Alzheimer’s disease as well. Complement factor C3d, an indicator of complement activation, is a fragment of complement C3.

The aims of this study were to determine whether the complement cascade is activated after ICH and to examine whether N-acetylspermin, an inhibitor of complement activation, can reduce ICH-related brain edema.

Materials and Methods

Animal Preparation and Intracerebral Infusion

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 62 male Sprague–Dawley rats, each weighing 300 to 400 g, were used in this study. The animals were anesthetized with 40 mg/kg of intraperitoneally injected pentobarbital; then the right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling, and as the route for intracerebral blood infusion. Blood pH, PaO₂, PaCO₂, hematocrit, and glucose levels were monitored. The animals’ body temperatures were maintained at 37.5°C by using a feedback-controlled heating pad. The rats were placed in a stereotactic head frame and a 1-mm cranial burr hole was drilled on the right coronal suture 4 mm lateral to the midline. One hundred microliters of blood or saline was infused into the right caudate nucleus at a rate of 10 μl per minute through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4 mm lateral to the bregma) by using a microinfusion pump. The needle was removed and the skin incision was closed with sutures following infusion. These animals were then used in our three-part study.

Experimental Groups

Part 1: Immunohistochemical Analysis for Complement C3d, C9, and Clusterin. Four rats in each of four groups were tested, all of which had received an intracerebral infusion of 100 μl of either saline or autologous blood and were killed at 24 or 72 hours post-infusion. Their brains were used for immunohistochemical studies to detect complement C3d, C9, and clusterin.

Part 2: Western Blot Analysis for Complement C9 and Clusterin. Four groups of four rats each were examined. These rats received an intracerebral infusion of 100 μl of either saline or autologous blood and were killed at 24 or 72 hours postinfusion. Their brains were sampled for Western blot analysis.

Part 3: N-Acetylspermin and Brain Edema After ICH. Ten rats each received an intracerebral injection of 100 μl of autologous blood or 100 μl of autologous blood plus 10 μg of N-acetylspermin. A group of 10 sham-operated rats underwent needle insertion without infusion. The rats were killed 24 or 72 hours later and their brains were used for water content measurement.

Measurement of Brain Water and Ion Contents

Animals were reanesthetized with 40 mg/kg of intraperitoneally injected pentobarbital and killed by decapitation. Their brains were removed and a 3-mm-thick coronal brain slice was cut with a blade approximately 4 mm from the frontal pole. The brain slice was separated into ipsilateral and contralateral cortex, and ipsilateral and contralateral basal ganglia. The cerebellum served as a control specimen. Brain samples were immediately weighed on an electronic analytical balance for wet weight, and were dried at 100°C for 24 hours to obtain the dry weight. Water content was determined as (wet weight – dry weight)/wet weight. The dehydrated samples were then digested in 1 ml of 1 M nitric acid for 7 days. Sodium and potassium contents were measured using an automatic flame photometer; ion contents were expressed in milliequivalents per kilogram of dehydrated brain tissue.

Western Blot Analysis

Rats were reanesthetized with 40 mg/kg of intraperitoneally injected pentobarbital and killed at 24 and 72 hours after intracerebral infusion. The brains were perfused with saline, and a coronal brain slice was cut as described for water content measurements. The brain tissue was immersed in 0.5 ml of Western sample buffer and then sonicated and frozen at −80°C for Western blot analysis. Briefly, 2 μg (for C9) or 25 μg (for clusterin) protein was run on 7.5% polyacrylamide gels with a 4% stacking gel after boiling for 5 minutes at 95°C. The protein was transferred to pure nitrocellulose membrane, and membranes were probed with a 1:2500 dilution of the primary antibody (rabbit anti–rat C9 PAb; rabbit anti–rat clusterin PAb) and a 1:2500 dilution of the secondary antibody (peroxidase-conjugated goat anti–rabbit antibody). Finally, the antigen–antibody complexes were visualized with a chemiluminescence system and exposed to photosensitive film. The relative densities of C9 and clusterin protein bands were analyzed using the NIH image software.

Immunohistochemical Studies

Immunohistochemical studies were performed as in our previous report. The rats were reanesthetized with 40 mg/kg of intraperitoneally injected pentobarbital and their brains were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The removed brains were kept in 4% paraformaldehyde for 6 hours, and immersed in 25% sucrose for 3 to 4 days at 4°C. Brains were then placed in embedding compound and sectioned in 18-μm slices on a cryostat. Sections were incubated using the avidin-biotin complex technique. Primary antibodies were rabbit anti–rat C9 PAb, rabbit anti–human C3d PAb, and rabbit anti–rat clusterin PAb. Normal rabbit immunoglobulin G was used as a control.

Statistical Analysis

All data in this study are presented as the mean ± SD. Data were analyzed using Student’s t-test. Significance levels were set at probabilities of less than 0.05.

Sources of Supplies and Equipment

The rats used in this study were acquired from Charles River Laboratories, Portage, MI. The stereotactic head frame was purchased from Kopf Instruments, Tujunga, CA. The microinfusion pump was obtained from Harvard Apparatus, Inc., S. Natick, MA. The electronic analytical balance (model AE 100) was purchased from Mettler Instrument Co., Hightstown, NJ. The flame photometer (model IL943) was obtained from Instrumentation Laboratory, Inc., Lexington, MA. The Hybrid C nitrocellulose membranes, peroxidase-conjugated goat anti–rabbit antibody, and ECL chemiluminescence system were all acquired from Amersham International, Buckinghamshire, England. The rabbit anti–rat C9 PAb and the rabbit anti–rat clusterin PAb were kind gifts from Drs. P. Morgan, University of Wales, and M. Griswold, Washington State University, respectively. The rabbit anti–human C3d PAb was purchased from DAKO, Dakopatts, Denmark. The Kodak X-OMAT film was obtained from Eastman Kodak, Rochester, NY. The imaging software (NIH Image Version 1.61) was acquired from the NIH, Bethesda, MD. The O.C.T. embedding compound was purchased from Sakura Finetek USA, Inc., Torrance, CA.

Results

All physiological variables including mean arterial blood pressure, pH, PaO₂, PaCO₂, hematocrit, and blood glucose were within normal ranges, and this information is presented in Table 1.
Perihematomal Complement C9 Accumulation and MAC Formation

Western blot analysis revealed that complement C9 content was significantly increased (sixfold) in the ipsilateral basal ganglia 24 hours after intracerebral infusion of 100 µl of autologous blood (Fig. 1). The C9 was maintained at a high level for 3 days (fivefold increase compared with controls). Accumulation of C9 was not detected on Western blot analysis in contralateral basal ganglia and control animals at either 1 or 3 days. The C9 had accumulated around the hematoma without deposition 24 hours after ICH. However, 3 days later C9 was observed to be deposited on the neuronal membrane (Fig. 2A), indicating activation of the complement cascade and formation of MAC. Little C9 immunoreactivity was detected in the ipsilateral basal ganglia of saline-injected animals and in the contralateral cortex and basal ganglia after ICH. The C9 immunoreactivity was not found in control brain sections.

Complement C3d Deposition Around the Hematoma and in the Clot

Complement C3d is a fragment of C3, seen as minute particles on immunohistochemical studies. Complement C3d immunoreactivity was found around the clot 24 hours post-ICH (Fig. 3A), but was not detected in the clot itself. However, 3 days later, C3d immunoreactivity was detected not only around the hematoma but also within the clot (Fig. 3B). Few immunoreactive C3d particles were found in the contralateral hemisphere and in the brain of control rats. The immunoreactive C3d particles were not detected in control brain sections.

Clusterin Upregulation

Perihematomal clusterin protein was markedly upregulated after ICH. Western blot analysis showed a twofold increase in clusterin protein levels in the ipsilateral basal ganglia 3 days after ICH (Fig. 4). Clusterin was slightly upregulated in the saline-infused brain as well. Clusterin immunoreactivity was not detected in the first 24 hours; however, it was detected around the hematoma 3 days post-ICH (Fig. 2C). Clusterin immunoreactivity was not detected in the ipsilateral cortex, contralateral cortex, or contralateral basal ganglia. Only a few clusterin-positive cells were found in the ipsilateral basal ganglia of control rats.

Effect of N-Acetylheparin on Brain Edema

Intracerebral injection of blood caused marked increases in brain water content. Water content in the ipsilateral basal ganglia was greater (81.6 ± 0.8%) than that in sham-operated rats (77.6 ± 0.5%) after 24 hours. Blood injection also induced more edema in the contralateral basal ganglia (78.7 ± 0.7%) compared with that in sham-operated rats (77.5 ± 0.6%) and in the ipsilateral cortex (80.5 ± 0.7%) compared with that in sham-operated controls (78.7 ± 0.2%).

The intracerebral injection of blood containing N-acetylheparin significantly reduced brain edema at 24 hours post-ICH in the ipsilateral basal ganglia (78.5 ± 0.5% compared with 81.6 ± 0.8% in controls, p < 0.001; Fig. 5A), in the contralateral basal ganglia (77.7 ± 0.4% compared with 78.7 ± 0.7% in controls, p < 0.05), and in the ipsilateral cortex (79.6 ± 0.3% compared with 80.5 ± 0.7% in controls, p < 0.05). The reduction in brain edema formation was associated with a reduction of sodium ion accumulation in the ipsilateral basal ganglia (Fig. 5B). The addition of N-acetylheparin also attenuated brain edema in the ipsilateral cortex (80.7 ± 1.2% compared with 83.3 ± 0.4% in controls; p < 0.01) and in the ipsilateral basal ganglia (80.9 ± 2.2% compared with 83.6 ± 0.9% in controls, p < 0.05; Fig. 6) at 72 hours post-ICH.

Discussion

In this report we provide evidence for activation of the
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Complement cascade after ICH by using C3d and C9 immunohistochemical studies and Western blot analysis. In addition, we suggest that activation of the complement cascade is involved in brain injury and that N-acetylheparin, a complement inhibitor, reduces brain edema formation associated with ICH.

Complement C3d and C9 immunoreactive products were detected either around the hematoma in the ipsilateral basal ganglia or in the hematoma. The presence of C3d, a segment of C3, indicated that the complement cascade had been activated. Depositions of complement C9 on the cell membrane indicated that the whole complement cascade had been activated and MAC had been formed. On Western blot analysis we also found significant C9 accumulation (a sixfold increase) 1 day post-ICH (Fig. 1), indicating that sufficient amounts of complement C9 were...

Fig. 2. Photomicrographs showing complement C9 or clusterin immunoreactivity in ipsilateral basal ganglia 72 hours after intracerebral infusion of 100 μl of either blood or saline. Complement C9 immunoreactivity after blood infusion (A) and saline infusion (B), and clusterin immunoreactivity after blood infusion (C) and saline infusion (D) are shown. Examples of C9- or clusterin-positive cells are indicated by arrows. Bar = 20 μm.

Fig. 3. Photomicrographs showing complement C3d immunoreactivity around the hematoma and within the clot after intracerebral infusion of 100 μl of blood. A: Perihematomal complement C3d immunoreactivity 24 hours after blood infusion. B: Complement C3d immunoreactivity in the clot 72 hours after blood infusion. Examples of C3d immunoreactivity are shown by arrows. Bar = 20 μm.
available for MAC assembly. Maintenance of perihematomal C9 at a high level 3 days post-ICH may have been due to the C9 deposition on cell membranes. Thus, our hypothesis that the complement cascade is activated and MAC formed after ICH was supported by upregulation of clusterin (an MAC inhibitor) in the brain.

After complement cascade activation, complement factors C5b, C6, C7, C8, and C9 are formed into the MAC, which is the final product of the complement cascade and is able to attach to the cell membrane, forming a transmembrane pore that may cause cell lysis. On electron microscopy, MAC can be seen as ringlike structures on erythrocyte membranes. Thus, erythrocyte lysis after ICH may be related to activation of the complement cascade and formation of MAC. Our previous study indicated that erythrocyte lysis and hemoglobin toxicity cause delayed brain edema following ICH. In addition, MAC insertion may occur in neurons, causing neuronal death, and may account for BBB leakage by damaging endothelial cells. Such an effect on nearby cells (the bystander effect) was reported by Park, et al., who demonstrated that complement activation by aged red blood cells can result in MAC insertion into bystander smooth-muscle cell membranes.

Clusterin, an MAC inhibitor, interacts with MAC, thus forming a soluble, inactive SC5b-9 compound. Clusterin messenger RNA is expressed in most neurons and astrocytes in normal adult rat brain, but clusterin immunoreactivity has not been found in most clusterin messenger RNA–positive cells. In this study, we found that clusterin in the brain was markedly upregulated after ICH. Clusterin immunoreactivity was detected around the hematoma 3 days post-ICH (Figs. 2C and 4). Perihematomal upregulation of clusterin may be a selfprotection mechanism against both complement activation and MAC insertion after brain contusion. As in the present study, they found clusterin upregulation began 4 days postinjury. Clusterin was present in both neurons and astrocytes.

Although MAC formation may cause erythrocyte cell lysis and bystander brain injury, other factors in the complement cascade may also be involved in brain damage after ICH. For example, complement C5a is an anaphylatoxin and also a potent chemoattractant for polymorphonuclear leukocytes. Inflammatory cells react to nanomolar concentrations of C5a with chemotaxis, upregulation of adhesion molecules, and release of oxygen radicals. Polymorphonuclear leukocytes migrate into the brain after...
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ICH. Whether complement is involved in polymorphonuclear leukocyte migration remains to be investigated.

Our study indicates that N-acetylheparin attenuates brain edema formation after ICH, leading us to suggest that complement activation may be one of the major factors that exacerbates brain edema post-ICH. Both N-acetylheparin and heparin block complement activation by inhibiting several steps in the classic and alternative pathways. The ability of heparin to inhibit complement activation was first reported when Ecker and Gross found that heparin protects sheep erythrocytes from guinea pig plasma–induced cell lysis. Heparin increases C1 esterase inhibitor activity and inhibits C3 convertase formation. Furthermore, both heparin and N-acetylheparin inhibit zymosan and cobra venom factor–induced complement activation. Friedrichs et al. found that heparin and N-acetylheparin reduced ischemic and reperfusion-induced heart injury. Recently it has been reported that N-acetylheparin pretreatment reduces myocardial infarct size and reduces MAC content and interleukin-8 levels after myocardial ischemia/reperfusion in rabbits. In our study, N-acetylheparin reduced brain edema at both 24 and 72 hours post-ICH. Our previous studies have indicated that erythrocyte lysis may not be involved in edema formation at the earlier time point, an observation supported by the finding that MAC insertion into membranes may not occur at 24 hours. If N-acetylheparin causes thorough inhibition of complement activation, the reductions in edema at 24 hours may reflect an antiinflammatory response caused by inhibition of C3a and C5a production. Further studies are required to clarify the mechanisms by which N-acetylheparin reduces perihematomal brain edema.

Our previous studies have indicated that heparin reduces ICH-induced brain edema formation, probably by the anticoagulant effects of heparin. Our previous results have indicated that intracerebral injections of thrombin can induce brain edema formation and that the injection of thrombin inhibitors can reduce ICH-induced edema formation. However, N-acetylheparin, unlike heparin, is not an anticoagulant. This indicates that some of the protective effects of heparin may also not be related to inhibition of coagulation.

Recent studies indicate that complement inhibition can reduce ischemic brain damage. Huang et al. have shown that sialyl Lewis x glycosylated soluble complement receptor-1, a complement inhibitory protein, inhibits leukocyte and platelet accumulation and reduces infarct volume in a cerebral ischemia/reperfusion model. Also, Vashare et al. found that complement depletion with cobra venom factor improves blood flow and neurological outcome after cerebral ischemia/reperfusion. Whether the underlying mechanisms of protection are similar to those involved in ischemia is uncertain, although it should be noted that ICH is not associated with reductions in blood flow to levels that induce ischemic brain damage. Consequently, the protective effects of N-acetylheparin in ICH are unlikely to be mediated by blood flow. The fact that different agents used to inhibit complement activation (sialyl Lewis x glycosylated soluble complement receptor-1, cobra venom factor, and N-acetylheparin) all inhibit brain injury lends credence to the hypothesis that the effects are complement-mediated.

Conclusions

The complement cascade is activated in the brain after ICH, contributing to perihematomal edema formation, and complement inhibition attenuates that same process of edema formation. Complement inhibition is a new direction for potential therapeutic intervention in ICH.

References


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