Acute Tissue Damage After Injections of Thrombin and Plasmin into Rat Striatum

Mengzhou Xue, MD; Marc R. Del Bigio, MD, PhD, FRCPC

Background and Purpose—Extravasation of blood is associated with intracerebral hemorrhage and head trauma. The mechanism of brain cell injury associated with hemorrhage differs from that due to pure ischemia. The purpose of this study was to investigate the acute changes after intracerebral injections of proteins that are involved in blood clotting and clot lysis.

Methods—Sixty-eight adult rats were subjected to stereotaxic intrastriatal injections of normal saline (5 μL), low- (2.5 U/5 μL) and high-dose (25 U/5 μL) thrombin, low- (0.1 μg/5 μL) and high-dose (1 μg/5 μL) tissue plasminogen activator, low- (0.05 U/5 μL) and high-dose (0.5 U/5 μL) plasminogen, and low- (0.335 U/5 μL) and high-dose (3.35 U/5 μL) plasmin. Forty-eight hours later rats were perfusion fixed. Brain damage area, eosinophilic neurons, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL)–positive cells, infiltrating neutrophils, CD8a immunoreactive leukocytes, and reactive microglia were quantified.

Results—Damage area in striatum, dying cells, inflammatory cells, and microglial reaction were significantly greater after the high-dose plasminogen, plasmin, and thrombin injections. Tissue plasminogen activator injections were associated with mild inflammation.

Conclusions—These results suggested that thrombin and plasmin are harmful to brain cells in vivo. Although the doses required to cause damage are relatively great in consideration of the plasma content of these proteins, their pathological effect might be enhanced through synergism with other mechanisms. (Stroke. 2001;32:2164-2169.)

Key Words: blood coagulation ■ cerebral hemorrhage ■ leukocytes ■ microglia ■ proteolysis

Intracerebral hemorrhage can be a consequence of hypertension, bleeding into an ischemic infarct, rupture of abnormal blood vessels, or trauma. Hematomas remain a significant management problem because the blood itself seems to have adverse effects beyond its space-occupying effect.1 Some of the adverse effects of blood have been attributed to proteolytic enzymes involved in blood coagulation and lysis. Thrombin, which activates the formation of fibrin, causes morphological changes of cultured astrocytes, neurite retraction in cultured neurons,3 and edema when injected into brain.4 Plasminogen is converted into plasmin by tissue plasminogen activator (tPA), which is produced by brain endothelia.5 Plasmin serves to lyse blood clots through the digestion of fibrin. Plasmin also causes considerable edema when injected into brain.6 tPA can potentiate brain damage caused by thrombin and that which follows middle cerebral artery occlusion in rats.7,8 However, injection of tPA to aid evacuation of intracerebral hematoma is not associated with adverse effects.9 The consequences of thrombin, plasminogen, and tPA injections into brain are not well documented, in particular to what extent they resemble those caused by intracerebral hemorrhage. Nishino and co-workers10,11 infused thrombin or plasmin into rat striatum for 7 days and studied the histopathological changes after that time. Plasmin caused hemorrhage, and therefore they did not study the animals histologically. At sites of thrombin infusions, neutrophils, macrophages, reactive astrocytes, and new blood vessels were observed. The purpose of this study was to investigate the acute inflammation and cell death that follow intracerebral injections of thrombin, tPA, plasmin, and plasminogen. Our previous experiments showed that cell death and inflammatory cell infiltration peak 2 to 3 days after injection of autologous whole blood in to the rat brain; therefore, we chose a survival period of 2 days.12,13

Materials and Methods

Animal Preparation
All experimental procedures were done in accordance with guidelines of the Canadian Council on Animal Care. Protocols were approved by the local experimental ethics committee. Sixty-eight young adult male Sprague-Dawley rats weighing 175 to 250 g were used. Rats were anesthetized with pentobarbital (50 mg/kg IP) and placed in a stereotaxic frame. The animals were draped, but core temperature was not monitored or regulated during the 15-minute procedure. After midline scalp incision, a hole was drilled through the skull, and a 27-gauge needle attached to a 10-μL Hamilton microsyringe was inserted into the striatum (3 mm lateral to midline, at a 10-μL Hamilton microsyringe was inserted into the striatum (3 mm lateral to midline, 3.5 mm anterior to bregma, 5.5 mm below dura, and 3.5 mm deep to bone). Rats were monitored for signs of postoperative distress, and those that showed signs of distress were sacrificed immediately. On the day of sacrifice, rats were perfusion fixed with 10% buffered formalin. All experimental procedures were done in accordance with guidelines of the Canadian Council on Animal Care. Protocols were approved by the local experimental ethics committee. Sixty-eight young adult male Sprague-Dawley rats weighing 175 to 250 g were used. Rats were anesthetized with pentobarbital (50 mg/kg IP) and placed in a stereotaxic frame. The animals were draped, but core temperature was not monitored or regulated during the 15-minute procedure. After midline scalp incision, a hole was drilled through the skull, and a 27-gauge needle attached to a 10-μL Hamilton microsyringe was inserted into the striatum (3 mm lateral to midline, 3.5 mm anterior to bregma, 5.5 mm below dura, and 3.5 mm deep to bone). Rats were monitored for signs of postoperative distress, and those that showed signs of distress were sacrificed immediately. On the day of sacrifice, rats were perfusion fixed with 10% buffered formalin.

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Histological Examination

Forty-eight hours after injections, rats were reanesthetized and perfused through the heart with 300 mL ice-cold 4% paraformaldehyde in 0.1 mol/L PBS. The brain was removed and stored in the same fixative for 1 to 7 days. Fixed brains were cut coronally approximately 2 mm on either side of the needle entry site, which was identifiable on the brain surface. Brain slices were dehydrated and embedded in paraffin. Sections (6 "m) were cut, and each 30th section from the rostral to the caudal portion of the damage area was stained with hematoxylin and eosin. At the coronal level of the needle entry site, where the brain damage was maximal, a variety of histological and immunohistochemical stains were performed.

To demonstrate mononuclear leukocyte infiltrate, sections were dewaxed and rehydrated, washed with distilled water, quenched with 0.3% H2O2, blocked with 10% normal goat serum, and incubated with anti-CD68 monoclonal antibody (diluted 1/400, PharMingen International) at 4°C overnight. This detects cytoxic T cells and natural killer (NK) cells.14 Slides were then washed with Trition PBS, incubated in biotinylated goat anti-mouse IgG (1/300) for 1 hour at room temperature, washed, incubated with streptavidin-peroxidase (1/400) for 30 minutes at room temperature, colored with diaminobenzidine-H2O2 solution, washed, and coverslipped. Control sections were processed with omission of the biotinylated lectin.

Terminal deoxynucleotidyl transferase (TdT)—mediated deoxyuridine triphosphatase (dUTP)—biotin nick end labeling (TUNEL) was used to identify cells with damaged DNA, most of which are dying cells. Paraffin-embedded sections were dewaxed and rehydrated, then incubated in 20 µL/mL proteinase K for 15 minutes. TUNEL was accomplished with the use of the Apoptag in situ kit (Intergen). After immersion in equilibration buffer for 10 minutes, sections were incubated with TdT and dUTP-digoxigenin in a humidified chamber at 37°C for 1 hour and then incubated in the stop/wash buffer at 37°C for 30 minutes. Sections were washed with PBS before incubation in anti–digoxigenin-peroxidase solution (1/500 in PBS) for 30 minutes at room temperature and colored with diaminobenzidine-H2O2 solution. Sections were counterstained with methyl green. Negative control sections were treated similarly but incubated in the absence of TdT enzyme or dUTP-digoxigenin. TUNEL–positive nuclei with chromatin condensation and fragmented nuclei were considered probable apoptotic cells. TUNEL–positive cells with diffuse light brown labeling of nucleus and cytoplasm were considered probable necrotic cells.16 Together they were considered dying cells.

Cell Counts and Determination of Damage Area

A camera lucida drawing was used to assess the overall brain morphology on the coronal slice with maximal striatum damage, which was defined by the presence of blood, tissue rarefaction, or necrosis at the injection sites. Computerized planimetry was used to measure the traced areas. With the use of an ocular graticule and ×250 ocular magnification (objective magnification ×20), eosinophilic dying neurons, TUNEL–positive dying cells, neutrophils, CD8a immunoreactive cells, and RCA-1 binding cells were counted in 4 fields (each area 250×250 µm) immediately adjacent to the needle injection/damage site, which was defined by the presence of erythrocytes or necrosis (Figure 1). Areas with large blood vessels were avoided. In brains with large areas of necrosis, counts were made near the edge of the lesion because the necrotic cores were devoid of viable cells. All data are expressed as mean±SEM. Data were analyzed to ensure normal distribution, and then intergroup comparisons were made by ANOVA followed by Fisher’s protected least significant difference post hoc test with the use of StatView 5.01 software (SAS Institute). The differences were considered significantly different when P<0.05. Additional power calculations were made manually with the use of published tables.17

Results

All rats tolerated the surgical procedure well, and there was no surgical mortality. Microscopically, brains with saline injection, all low-dose injections, and tPA high-dose injections exhibited small collections of blood and negligible edema extending up to 50 µm on either side of the needle tract (Figure 2). High-dose injections of thrombin, plasmin,

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### Table: Treatment Groups

<table>
<thead>
<tr>
<th>Injection (in 5 μL)</th>
<th>No. of Rats</th>
<th>Blood Volume Equivalent, μL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>tPA, low (0.1 μg)</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td>tPA, high (1.0 μg)</td>
<td>6</td>
<td>350</td>
</tr>
<tr>
<td>Plasminogen, low (0.05 U)</td>
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<td>110</td>
</tr>
<tr>
<td>Plasminogen, high (0.5 U)</td>
<td>8</td>
<td>1100</td>
</tr>
<tr>
<td>Plasmin, low (0.335 U)</td>
<td>8</td>
<td>750</td>
</tr>
<tr>
<td>Plasmin, high (3.35 U)</td>
<td>8</td>
<td>7500</td>
</tr>
<tr>
<td>Thrombin, low (2.5 U)</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Thrombin, high (25 U)</td>
<td>8</td>
<td>150</td>
</tr>
</tbody>
</table>

*Crude approximation,58,59
and plasminogen resulted in columns of necrosis characterized by lysis of all cell types and absence of nuclear staining extending up to 2 mm on either side of the needle tract (Figure 2). In a minority of these the necrotic core was hemorrhagic up to 200 μm from the needle tract. Scattered karyorrhectic nuclei, eosinophilic or pyknotic neurons, and TUNEL-positive cells were identifiable up to 2 mm from the edge of necrotic lesion. Neutrophils were adherent to vessel walls or passing through the capillaries and venules. Neutrophils and CD8α immunoreactive leukocytes were rarely present within the necrotic tissue except at the periphery. They were found in the surrounding intact edematous striatum as well as occasionally in nearby white matter (Figure 3). Reactive microglia with ramified processes and swollen bodies were present in the parenchyma and around blood vessels.

Quantitative data are shown in Figure 4. Damage area in striatum, dying cells, and inflammation in adjacent nonnecrotic tissue were significantly greater after high-dose thrombin, plasminogen, and plasmin injections compared with other groups. These destructive and reactive changes were roughly proportionate to the total area of injury, although despite the absence of significant necrosis after the low-dose thrombin injection, there were more dying neurons and neutrophils in the penumbra. Neutrophil infiltration and microglial reaction were mildly but significantly elevated after injection of all substances.

Discussion

Intracerebral hemorrhage causes brain damage through multiple mechanisms. Direct tissue destruction by the hemorrhagic event and dissection of blood along tissue planes occurs immediately. This is followed by development of edema and secondary ischemic damage due to raised intracranial pressure and distortion of the microvasculature. The enzymes involved in blood clotting and clot lysis are potentially toxic in the first day after hemorrhage. Delayed damage also occurs through release of toxins by blood breakdown products. This study demonstrated that injection of thrombin, plasminogen, and plasmin into the striatum of rats is associated with dose-dependent tissue necrosis, cell death, and inflammation at 48 hours. The focal necrosis associated with enzyme injections was rapid and most likely due to a direct effect of these agents on the neuropil or the vasculature. Inflammation, including influx of neutrophils and lymphocytes as well as reaction by microglia, was generally proportionate to the total area of damage and not to the quantity of foreign protein injected. It was also similar in magnitude to that seen after infusions of autologous whole blood. Therefore, with the exception of tPA-associated changes, the inflammation is likely stimulated by the damage and not directly by the infusate (or by small quantities of contaminants in the infusate such as endotoxin).

In blood, thrombin is produced by proteolytic cleavage of the plasma protein prothrombin. Thrombin converts fibrinogen into fibrin, which is ultimately involved in formation of a blood clot. Brain and spinal cord tissues, including neurons and endothelia, have a large number of thrombin receptors (also known as protease-activated receptor [PAR-1]) as well as the related PAR-2. These can be activated by low concentrations of trypsin, thrombin, and plasmin. The brain...
itself appears to be capable of producing small quantities of prothrombin. Through these receptors, whose precise role in normal signaling is unclear, thrombin causes retraction of cell processes of cultured neurons and is toxic to neurons in brain slices in a dose-dependent manner. When injected into the brain, thrombin can cause brain edema. The edema-inducing effect of thrombin can be inhibited by several thrombin inhibitors, thrombin preconditioning, or heparin. Plasminogen is a plasma protein that is converted into plasmin by tPA. They are produced by brain endothelia as well as by some neurons. Plasmin can digest fibrin to allow lysis of blood clots. When injected into brain, plasmin also causes considerable edema, potentially through an effect on the blood-brain barrier.

Brain necrosis and cell death caused by injection of high-dose thrombin and plasmin are likely due to direct proteolytic activity. We suggest this because the tissue necrosis was rapid and involved all cell elements, even those without known thrombin receptors. Thrombin, plasmin, and tPA are all trypsin-like serine proteinases of the tissue kallikrein family. Their active sites have similar substrate specificity, although their affinity varies, being modified by additional binding sites. At lower doses thrombin also causes apoptosis of neurons and astrocytes in culture, apparently through surface receptors that are activated by proteolytic cleavage at a specific site. Because the receptors are proteolytic substrates, experiments with antagonists would not help to determine whether thrombin and plasmin are acting in a selective or indiscriminate manner. More detailed investigation of the dose-response relationship would help to determine this. Although it is conceivable that thrombin or plasmin can induce endothelin synthesis and subsequent vasospasm and ischemia, vasoconstriction after thrombin injection has been previously excluded. Thrombin, tPA, and plasminogen are normally present in brain at low concentrations, especially during development and during reactive changes. In addition to action through the PAR receptors, plasmin is known to degrade a range of extracellular matrix proteins and to activate matrix metalloproteinases, which can also digest matrix proteins. In high doses their proteolytic activity likely exceeds that which can be controlled by endogenous regulatory proteins (eg, a2-macroglobulin, protease nexin-1, plasminogen activator inhibitors), and proteolysis continues unchecked. Plasminogen activator inhibitor-1 (PAI-1), the major regulator of plasminogen activation, exists in brain only in very small quantities, although it can be upregulated after experimental stroke. PAI-1 deficient mice exhibit larger infarcts after middle cerebral artery occlusion. It has also been shown that mice deficient in tPA, in which plasmin is not activated, are less susceptible to neuronal injury after brain ischemia or excitotoxin injection.

The plasma proteins we injected, with the exception of tPA, caused dose-dependent brain injury. We must point out several caveats to the experiment. First, expressed in terms of the whole blood volume that would contain that quantity (Table), it seems obvious that the toxic doses could only be delivered in unrealistically large blood volumes relative to the
brain size. Furthermore, plasma infusions alone are not overtly toxic. However, one cannot exclude the possibility that a large hematoma in a large brain would allow diffusion of toxic quantities into the surrounding tissue, at least at the microscopic level. Second, there were considerable differences in the apparent potency of the different proteins. Although the high-dose plasminogen and the low-dose plasmin were roughly equivalent in terms of nominal enzyme activity, the effect of plasminogen was much greater. We speculate that some plasmin activity is lost when it is purified in the postactivated form. In contrast, the plasminogen is activated in situ. Third, the relative potency of thrombin delivered in a hematoma would appear to be greater than that of plasminogen (compare high-dose thrombin with low-dose plasminogen, which are contained in roughly the same amount of blood). However, we must consider for several reasons that this type of comparison is naïve. The true quantity of thrombin delivered is not accurately known because it adsorb to glass and plastic. Furthermore, the actual activity of thrombin at a particular site can be very difficult to predict because it is self-amplifying and because it is rapidly inactivated by binding to fibrin.

Fourth, tPA injections appeared to cause mild inflammation, perhaps a nonspecific effect of foreign protein, but minimal cell death. We thank Mary Cheang for advice concerning the statistical analysis.

In summary, the results demonstrate that injections of thrombin, plasminogen, or plasmin into rat striatum are overtly toxic. However, one cannot exclude the possibility that a large hematoma in a large brain would allow diffusion of toxic quantities into the surrounding tissue, at least at the microscopic level. Second, there were considerable differences in the apparent potency of the different proteins. Although the high-dose plasminogen and the low-dose plasmin were roughly equivalent in terms of nominal enzyme activity, the effect of plasminogen was much greater. We speculate that some plasmin activity is lost when it is purified in the postactivated form. In contrast, the plasminogen is activated in situ. Third, the relative potency of thrombin delivered in a hematoma would appear to be greater than that of plasminogen (compare high-dose thrombin with low-dose plasminogen, which are contained in roughly the same amount of blood). However, we must consider for several reasons that this type of comparison is naïve. The true quantity of thrombin delivered is not accurately known because it adsorb to glass and plastic. Furthermore, the actual activity of thrombin at a particular site can be very difficult to predict because it is self-amplifying and because it is rapidly inactivated by binding to fibrin.

Fifth, with only 2 doses, we cannot know that the maximal adverse effect would not be achievable at a much lower dose. Therefore, we have not determined the dose-response relationship necessary to speculate accurately on the mechanism of injury. Finally, we cannot exclude the possibility that mild hypothermia was protective in the low-dose situation, magnifying the apparent difference between low and high doses. In summary, the results demonstrate that injections of thrombin, plasminogen, or plasmin into rat striatum are associated with necrosis, cell death, and inflammation in a dose-dependent manner. Because of the rapid evolution, the most likely mechanism of action is uncontrolled proteolytic digestion of neurons, glia, and vascular cells. Peripheral to the necrotic core, cell death might be mediated by indiscriminate proteolysis, selective cleavage of protease-activated receptors, or inflammation, which might be induced by general mediators of tissue injury or perhaps directly by thrombin. Although the toxic doses are seemingly high when injected individually, we cannot exclude the likelihood that applied together, as in the case of intracerebral hemorrhage, they can act synergistically along with other plasma proteins not studied here. The plasma enzymes thrombin and plasmin may play an important role in the brain injury that follows intracerebral hemorrhage and therefore represent potential targets for therapeutic intervention.

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