Attenuation of intracerebral hemorrhage and thrombin-induced brain edema by overexpression of interleukin-1 receptor antagonist

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Object. Adenovirus-mediated overexpression of interleukin-1 receptor antagonist (IL-1ra) attenuates the inflammatory reaction and brain injury that follows focal cerebral ischemia. Recently, an inflammatory reaction after intracerebral hemorrhage (ICH) was identified. In this study the authors examine the hypothesis that overexpression of IL-1ra reduces brain injury (specifically edema formation) after ICH.

Methods. Adenoviruses expressing IL-1ra (Ad.RSVIL-1ra) or LacZ, a control protein (Ad.RSVlacZ), or saline were injected into the left lateral cerebral ventricle in rats. On the 5th day after virus injection, 100 μl of autologous blood or 5 U thrombin was infused into the right basal ganglia. Rats with ICH were killed 24 or 72 hours later for measurement of brain water and ion content. Thrombin-treated rats were killed 24 hours later for edema measurements and an assessment of polymorphonuclear leukocyte (PMNL) infiltration by myeloperoxidase (MPO) assay, as well as histological evaluation. Compared with saline-treated and Ad.RSVlacZ-transduced controls, Ad.RSVIL-1ra–transduced rats had significantly attenuated edema in the ipsilateral basal ganglia 3 days after ICH (81.5 ± 0.3% compared with 83.4 ± 0.4% and 83.3 ± 0.5% in control animals). Thrombin-induced brain edema was also reduced in Ad.RSVIL-1ra–treated rats (81.3 ± 0.4% compared with 83.2 ± 0.4% and 82.5 ± 0.4% in control rats). The reduction in thrombin-induced edema was associated with a reduction in PMNL infiltration into the basal ganglia, as assessed by MPO assay (49% reduction) and histological examination.

Conclusions. Overexpression of IL-1ra by using an adenovirus vector attenuated brain edema formation and thrombin-induced intracerebral inflammation following ICH. The reduction in ICH-induced edema with IL-1ra may result from reduction of thrombin-induced brain inflammation.

KEY WORDS • intracerebral hemorrhage • brain edema • thrombin • interleukin-1 receptor antagonist • gene therapy • rat

Intracerebral hemorrhage induces the formation of brain edema, which can elevate intracranial pressure and cause brain herniation. Preventing the accumulation of edema is, therefore, an important aspect of the clinical management of ICH, A variety of mechanisms are involved in brain edema formation. Recent studies indicate that an inflammatory reaction occurs around the hematoma, in ischemic and traumatic brain injury, an inflammatory response exacerbates brain edema formation. Neutrophils, essential components of inflammation, release factors, including oxygen radicals and cytokines, which can enhance brain damage, and neutrophils accumulate in the vicinity of the hematoma.

One edemogenic factor involved in brain edema formation after ICH is thrombin. Therefore, inhibiting the inflammatory reaction might reduce thrombin-induced brain edema after ICH, because thrombin triggers an inflammatory response in many tissues, including brain.

Interleukin-1 is a potent inflammatory mediator. Levels of IL-1 in the brain increase after ischemia, injection of bacterial endotoxin, and local brain injury. The IL-1ra inhibits several actions of IL-1, both in vivo and in vitro, and administration of IL-1ra reduces ischemic and traumatic brain injury. Gene transfer has proven to be a useful tool to study the mechanisms of action of IL-1 in vivo, as shown by studies of adenovirus-mediated overexpression of IL-1ra, which attenuates ischemic brain injury. The effect of IL-1ra on ICH has not been studied, and because ischemia does not appear to be a significant contributor to brain injury following ICH (see, for example, Patel, et al.) the effects of this antagonist on ICH are uncertain.

In this study we examine whether adenovirus-mediated overexpression of IL-1ra can reduce the brain edema that follows ICH or thrombin instillation into the basal ganglia, and whether IL-1ra overexpression reduces the thrombin-induced inflammatory response. We used MPO activity as
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a marker of PMNLs to assess the thrombin-induced inflammatory reaction.1

Materials and Methods

Animal Preparation

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 75 adult male Sprague–Dawley rats weighing 275 to 350 g were used in these experiments. The rats were given free access to food and water.

Adenoviral Gene Transduction

The production of replication-deficient human adenovirus serotype 5–derived adenoviral vectors is described elsewhere.34 Two different recombinant virus vectors were used in this study: one containing the human IL-1Ra cDNA, the other containing the Escherichia coli β-galactosidase gene. For both viruses, the RSV promoter was used to drive gene transcription. The two viruses were designated Ad.RSVIL-1ra and Ad.RSVlacZ. The Ad.RSVlacZ virus was used as a control because it carried a gene that was not expected to affect cerebral injury.

For administration of the virus, each rat was positioned in a stereotactic frame and its scalp was incised along the sagittal midline by using sterile procedures. A burr hole was drilled to the pericranium 1 mm lateral to the sagittal suture and 1 mm posterior to the coroanal suture. A stereotactically-guided needle-tipped 10-μl Hamilton syringe was inserted into the left lateral ventricle approximately 4 mm beneath the cortex. Ten microliters of adenoviral suspension containing 1012 particles/ml was injected into the lateral cerebral ventricle at a rate of 1 μl/minute, and the needle was then withdrawn over the course of 5 minutes. The burr hole was sealed with bone wax, the wound closed with sutures, and the animals were allowed to recover.

Intracerebral Infusion of Blood or Thrombin

Five days after administration of virus or vehicle, the rats were positioned in a stereotactic head frame and the scalp was opened using sterile procedures. A cranial burr hole was drilled near the right coronal suture 3 mm lateral to the midline. A stereotactically-guided 26-gauge needle was inserted into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3 mm lateral to the bregma). Whole blood from the femoral artery or thrombin was infused into the right basal ganglia, after which the rats were killed at 24 hours. The brains were dissected into cortex and basal ganglia. Thus, a total of four samples was obtained from each brain: ipsilateral and contralateral cortex, and ipsilateral and contralateral basal ganglia. The cerebellum was used as a control specimen. Brain samples were immediately weighed on an electronic analytical balance to obtain the wet weight. The samples were then dried in a gravity oven at 100°C for 24 hours to obtain the dry weight and to determine the water content, which was calculated as (wet weight – dry weight)/wet weight. The dehydrated samples were digested in 1 ml of 1 M nitric acid for 1 week before determination of sodium content by flame photometry. Ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue (mEq/kg dry wt).

Myeloperoxidase Assay

The rats were anesthetized with 80 mg/kg pentobarbital and perfused transcardially with cold (4°C) isotonic saline. The brains were removed and cut as described earlier. A total of four samples was obtained from each brain: ipsilateral and contralateral cortex, and ipsilateral and contralateral basal ganglia. The brain samples were immediately weighed and frozen in liquid nitrogen.

Tissue segments were thawed on ice and homogenized in 4 ml of 50 mM Tris-HCl (pH 7.4) at 4°C. Samples were added to 20 ml of 5 mM phosphate buffer (pH 6) at 4°C, homogenized, and centrifuged at 30,000 G for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed again as described earlier. After the supernatant was decanted, the pellet was extracted by suspension for approximately 2 minutes in 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer at 25°C, at an original tissue weight/volume ratio of 1:4. The samples were immediately frozen in liquid nitrogen, and freeze and thaw cycles were then performed three times, with 10-second sonications at 25°C between cycles. After the last sonication, the samples were incubated at 4°C for 20 minutes and centrifuged at 12,500 G for 15 minutes at 4°C. The 100-μl supernatants were mixed with 100 μl of 50 mM phosphate buffer containing 0.001% hydrogen peroxidase (pH 6), and 50 μl of 0.334 mg/ml o-dianisidine dihydrochloride. Absorbance was measured at 460 nm at 15-second intervals over 3 minutes by using a spectrophotometer, and results were expressed as the relative change in absorbance per minute at 460 nm.

Histological Assessment

The rats underwent either Ad.RSVIL-1ra or Ad.RSVlacZ gene transduction as described earlier. After recovery for 5 days, the rats underwent thrombin injection in the right basal ganglia. Twenty-four hours after thrombin injection, rats were reanesthetized and perfused through the heart with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). The brains were removed, postfixed for 24 hours, and sectioned into 50-μm slices on a vibratome before processing for hematoxylin and eosin staining.

Statistical Analysis

All data in this study are presented as the means ± the standard error. Data were analyzed using analysis of variance with the Scheffe F-test or Student t-test. Statistical significance was accepted at probability values of less than 0.05.

Sources of Supplies and Equipment

The Sprague–Dawley rats were purchased from Charles River Laboratories, Portage, MI. The rat thrombin and the hexadecyltrimethylammonium bromide were provided by Sigma Chemical Co., St. Louis, MO. The electronic scale (model AE 100) was obtained from Mettler Instrument Co., Hightstown, NJ. The flame photometer (model IL943) was acquired from Instrumentation Laboratory, Inc., Lexington, MA.

The first tissue homogenization was performed using a model RZR-2000 homogenizer manufactured by Crafco. Wiarton, ON, Canada, and the second homogenization was performed using an Ultra-Turrax model T25 manufactured by Janke and Kunkel, Staufen, Germany. The Ultrospec 3 spectrophotometer was purchased from Amersham Pharmacia Biotech, Piscataway, NJ.
Results

Physiological Parameters

Neither Ad.RSVIL-1ra nor Ad.RSVlacZ recombinant virus affected body weight during the 5 days after virus injection, and no neurological deficits were observed in either group. There were no differences in blood gas levels, blood pH, blood glucose, hematocrit, and blood pressure measured among the groups at the time of ICH induction. The combined mean physiological variables are shown in Table 1.

Brain Edema Formation

In rats that had previously received a ventricular infusion of saline, injection of 100 μl of blood into the right basal ganglia resulted in edema formation in that location. One day post-ICH, water content in the ipsilateral basal ganglia was 80.8 ± 0.5%, compared with 77.9 ± 0.2% in the contralateral hemisphere (Fig. 1A). By 3 days post-ICH, the water content increased further in the ipsilateral hemisphere, to 83.4 ± 0.4% (Fig. 1B). At both 1 and 3 days post-ICH, prior treatment with Ad.RSVlacZ did not change the amount of edema formation (Fig. 1). Rats that received prior treatment with Ad.RSVIL-1ra, however, had less edema formation in the ipsilateral basal ganglia than did the other two groups at 3 days (Fig. 1B), but not at 1 day (Fig. 1A). The changes in brain sodium content in the three groups mirrored the changes in brain edema. Thus, there were no differences in sodium accumulation among the three groups at 1 day post-ICH (Fig. 2A), but the reduction in edema formation in the Ad.RSVIL-1ra–treated rats at 3 days post-ICH was associated with a reduction in brain sodium accumulation in the ipsilateral basal ganglia (Fig. 2B).

In rats that had previously received a ventricular infusion of saline or Ad.RSVlacZ, injection of 5 U of thrombin into the right basal ganglia resulted in marked edema formation and sodium accumulation in that location (Fig. 3). In the ipsilateral basal ganglia (the site of the thrombin injection) of Ad.RSVlacZ–transduced rats, however, there was a marked increase in MPO activity compared with the contralateral basal ganglia. This increase was inhibited in Ad.RSVIL-1ra–transduced rats.

Histological Evaluation

The accumulation of neutrophils around the site of thrombin injection was examined using hematoxylin and eosin staining. The PMNLs in the ipsilateral cortex and basal ganglia of Ad.RSVlacZ–transduced rats were increased, particularly around the site of injection and the needle

<table>
<thead>
<tr>
<th>Variable</th>
<th>Saline (17 rats)</th>
<th>Ad.RSVlacZ (18 rats)</th>
<th>Ad.RSVIL-1ra (18 rats)</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.45 ± 0.01</td>
<td>7.44 ± 0.01</td>
<td>7.42 ± 0.01</td>
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<td>PaO₂ (mm Hg)</td>
<td>72.4 ± 1.3</td>
<td>74.1 ± 2.0</td>
<td>75.5 ± 2.2</td>
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<td>PaCO₂ (mm Hg)</td>
<td>49.2 ± 1.4</td>
<td>49.0 ± 1.7</td>
<td>49.1 ± 1.5</td>
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<tr>
<td>glucose (mg/dl)</td>
<td>145.0 ± 6.0</td>
<td>147.0 ± 6.0</td>
<td>146.0 ± 4.0</td>
</tr>
<tr>
<td>hematocrit (%)</td>
<td>43.0 ± 1.0</td>
<td>43.0 ± 1.0</td>
<td>43.0 ± 1.0</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>104.0 ± 4.0</td>
<td>102.0 ± 2.0</td>
<td>98.0 ± 2.0</td>
</tr>
</tbody>
</table>

* There were no significant differences among the three experimental groups. Abbreviation: MABP = mean arterial blood pressure.
track. Compared with the Ad.RSVlacZ group, Ad.RSVIL-1ra–transduced rats had reduced accumulation of PMNLs in the ipsilateral basal ganglia (Fig. 5).

Discussion

In this study we demonstrated that adenovirus-mediated overexpression of IL-1ra attenuates brain edema formation following ICH and after thrombin injection into the brain parenchyma. This effect of IL-1ra overexpression may be mediated, at least in part, by a reduction in thrombin-induced inflammation.

A protective effect of Ad.RSVIL-1ra on ICH-induced brain edema was found 3 days after ICH but not after 1 day. In previous studies it has been suggested that neutrophil infiltration peaks 48 hours after ICH and that the brain inflammatory reaction is at its maximum level 48 to 72 hours after ICH.\(^{42,43}\) The lack of effect of IL-1ra overexpression at 1 day is probably related to the time needed for the inflammatory process to reach a peak.

In these experiments we used an adenovirus to mediate IL-1ra overexpression, raising the question whether the adenovirus rather than the gene product might be affecting edema formation after ICH. Two factors lead us to suggest that this is not the case: the control adenovirus expressing LacZ did not affect edema formation at 1 or 3 days, and pretreatment with RSVIL-1ra only affected edema formation at 3 days post-ICH and not at 1 day, which coincides with the time course of brain inflammation following ICH.\(^{14,42,43}\)

The adenovirus was injected into the lateral ventricle. As indicated by LacZ staining (data not shown), choroid plex-
us epithelial cells and ependymal cells are the main types transduced by this route of administration. This confirmed our earlier observations in rat and mouse studies.\(^3,46\) Those earlier results demonstrated that production of IL-1ra in the brain begins on the 1st day after transduction, peaks at 5 to 7 days, and is sustained for 13 days.\(^3,46\) Regardless of the location of transduction, the increase in IL-1ra is found in brain tissue as well as cerebrospinal fluid, probably because of the lack of a cerebrospinal fluid–brain barrier in those cell types.\(^3\)

One of the advantages of gene therapy is the potential for continuous production of a protective protein in the brain. Because of its size and short half-life, IL-1ra can be administered repetitively in the lateral ventricle to produce a protective effect.\(^37\) Although not directly addressed in this study, the fact that a protective effect of IL-1ra overexpression was not found until 3 days after ICH indicates that it may be possible to use gene therapy to induce IL-1ra overexpression after ICH and still produce protective effects. This may be the case in particular if a different vector, such as herpes simplex virus,\(^18\) is used, which can result in an earlier upregulation of the gene product.

To explore a potential mechanism for the protective effect of IL-1ra overexpression on ICH-induced edema, we also examined the effects of IL-1ra on thrombin-induced injury. Thrombin is one component of the hematoma that is responsible for edema formation,\(^19,20\) and thrombin-induced brain edema formation is also reduced by administration Ad.RSVIL-1ra.

In previous studies in a number of tissues, other investigators have demonstrated that thrombin upregulates inflammatory cytokines such as IL-1 and tumor necrosis factor-\(\alpha\)\(^16,29\) and the adhesion molecules ICAM-1, E-selectin, and P-selectin,\(^16,17,36\) which are involved in the migration of white blood cells from blood to tissue. Infiltration of inflammatory cells in the brain and release of cytokines, such as IL-1\(\beta\) and tumor necrosis factor-\(\alpha\) induce tissue injury.\(^11,12\) Therefore, we examined whether reduction in thrombin-induced edema in Ad.RSVIL-1ra–transduced rats is associated with a reduction in inflammation and PMNL infiltration. As observed both on MPO assay and on histological examination, thrombin induced infiltration of PMNLs, which was lower in Ad.RSVIL-1ra–treated rats. The effect of IL-1ra overexpression on MPO activity after ICH was not examined because hemoglobin interferes with the assay,\(^41\) and because the source of neutrophils in the clot might be either the clot itself or the surrounding tissue vasculature.

One difference between the thrombin- and ICH-induced injuries, and the protective effects of Ad.RSVIL-1ra in those injuries, is timing. After administration of thrombin in the acute phase, there is a marked increase in PMNLs around the injection site within 24 hours (Fig. 4), whereas the infiltration of PMNLs seems to be delayed after

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**Fig. 4.** Bar graph showing MPO activity 1 day after injection with 5 U of thrombin. The rats received intraventricular injections of either Ad.RSVIL-1ra or Ad.RSVlacZ 5 days before thrombin injection. The MPO activity was measured in the ipsilateral and contralateral cortex and basal ganglia. *p < 0.05 compared with LacZ. OD = optical density.

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**Fig. 5.** Photomicrographs of brain sections adjacent to the thrombin injection site obtained 1 day after intracerebral injection of 5 U of rat thrombin. The photomicrographs are of tissue obtained in an Ad.RSVlacZ-transduced rat (A) and an Ad.RSVIL-1ra–transduced rat (B). A massive infiltration of PMNLs was observed in the Ad.RSVlacZ-transduced rat, but this was markedly reduced in the Ad.RSVIL-1ra–transduced rat. Bar = 5 \(\mu\)m. N = needle track.
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ICH.

Similarly, a protective effect of Ad.RSVIL-1ra on edema formation was found 24 hours after thrombin injection, but not until 72 hours after ICH. The reason for this difference is unknown, although it could reflect the thrombin dose or the timing of its administration. After an ICH, some of the thrombin generated from the clotting cascade remains within the clot, bound to fibrin, and may be gradually released into the surrounding tissue.

The contribution of IL-1 to ICH-induced injury has not been previously examined. The expression of IL-1 increases markedly following endotoxin infection, local brain injury, and ischemia. This phenomenon is also observed in patients with brain injury, bacterial infection, and Alzheimer disease. The source of IL-1 after brain injury is unclear, although there is evidence that it can be produced by a number of cell types, including microglia, astrocytes, brain endothelial cells, and invading macrophages and neutrophils. Interleukin-1 has multiple potentially harmful effects on the brain, including neurotoxicity, opening of the blood–brain barrier, induction of apoptosis, neutrophil infiltration, and activation of microglia. All these events are also observed after ICH, but whether IL-1 is the main cause of these events remains to be determined.

Conclusions

Adenovirus-mediated overexpression of IL-1ra attenuates brain edema formation induced by ICH, perhaps by reduction of thrombin-induced brain inflammation. This may provide a new therapeutic target after ICH. This study also contains the first demonstration of a successful use of gene therapy for this condition.

References


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