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Original Article

Correlation of Macrophage Inflammatory Protein-2 Expression and Brain Edema in Rats after Intracerebral Hemorrhage

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Abstract: Brain edema is one of the most frequent and serious complications of intracerebral hemorrhage (ICH), but its underlying mechanism remains largely unknown. In order to understand whether inflammatory mediators released from the blood after cerebral hemorrhage plays a role in brain edema, we investigated the dynamic change of the inflammatory mediator macrophage inflammatory protein-2 (MIP-2) in rat brain after ICH. Our results indicate that the expression of MIP-2 increases 2 hours and peaks 2 days after ICH. The expression of MIP-2 correlates with NF-κB activation and brain water content. These results suggest that MIP-2 expression may play an important role in the development of brain edema after ICH in rats.
Key Words: MIP-2, NF-κB, brain edema, intracerebral hemorrhage

Introduction

Stroke is the third leading cause of death and the leading cause of long-term disability in the world. Intracerebral hemorrhage (ICH) is a common and one of the most devastating types of stroke. ICH accounts for 10-15% of all strokes, but it is responsible for approximately 50% of stroke-related deaths. It imparts some form of disability to 88% of its survivors [1, 2]. Secondary brain edema and brain damage may result in further deterioration in neurological functions after ICH. But the cause of secondary brain injury and edema formation is not completely understood. Previous reports showed that ICH was accompanied by inflammation. Whether the inflammation contributes to brain edema and neurological injury or plays a role in repairing the brain tissue is not fully known.

Macrophage inflammatory protein-2 (MIP-2) was first isolated from mouse macrophages in 1988 by Wolpe. MIP-2 is a chemoattractant [3] and an activating factor for neutrophils [4]. In neuropathological processes, MIP-2 is thought to play a pivotal role in the induction and perpetuation of inflammation in the central nervous system (CNS). The origin of MIP-2 in brain has not been fully defined. Recent studies have demonstrated that reactive glial cells also produced MIP-2 [5, 6]. In addition to astrocytes, microglial cells, endothelial cells, macrophages and some neuron cells may be potential sources of MIP-2 production in pathological states of the brain [7]. The release of MIP-2 into the tissue of the CNS may be caused by both in situ and infiltrated cells.

In order to understand the role of inflammatory mediators released from the blood after cerebral hemorrhage in the development of brain edema, we carried out a systemic investigation on the dynamic change of many inflammatory mediators including MIP-2. In this report, we described the expression of MIP-2 in rat brain after ICH and correlated the expression of MIP-2 with brain edema.

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Materials and Methods

Animal Model of Intracerebral Hemorrhage

Ninety male Sprague-Dawley rats (200–250 g) were purchased from the Center for Experimental Animals, Harbin Medical University. NIH Guidelines for the Care and Use of Laboratory Animals were followed in all animal procedures.

We chose to inflict ICH by autologous blood to better mimic the sudden deposition of blood in the brain parenchyma after the rupture of vessels in humans [15]. Rats were anesthetized with chloral hydrate (350 mg/kg intraperitoneally). Animals were placed in a stereotaxic frame using modified ear-bars fitted with blunt rubber ends designed for rats. Procedures for induction of ICH by autologous blood were modified from the previous studies in rats [16] and mice [17]. A midline scalp incision was made, and a hole was drilled in the left skull (3mm lateral to midline, 0.2 mm anterior to bregma). Autologous blood (50 μL) from arteria caudilis was collected into a needleless sterile insulin syringe without any anti-coagulant. A 26-gauge needle was quickly attached to the syringe and stereotaxically introduced into the left caudate nucleus 5.5 mm below the surface of the hole in the skull. The 50 μL blood was injected over 5min, and the needle was left in place for another 5 min to minimize backflow. The syringe was then removed slowly. The burr hole in the skull was sealed with bone wax, the scalp was sutured, and the animals were placed in a cage with *ad libitum* access to food and water. Body temperature was maintained at 37°C with the use of a feedback-controlled heating pad. Blood pressure, rectal temperature and body weight were monitored periodically after ICH. Control animals were subjected to the same manipulations as ICH rats, in which no autologous blood but the same volume of saline was injected. Animals were sacrificed at 2h, 3h, 6h, 10h, 12h, 24h, 2d, or 5d after the injury for analyses. Control animals were killed at 2h after the injection. All experimental procedures were done in accordance with guidelines of the Chinese Council on Animal Care.

Histological Examination

Rats were overdosed with chloral hydrate at 2h, 3h, 6h, 10h, 12h, 24 h, 2d or 5d after ICH and were perfused through the heart with 300 mL ice-cold 4% paraformaldehyde in 0.1 mol/L PBS. Fixed brains were cut coronally approximately 2 mm on either side of the needle entry site, which was identifiable on the brain surface. These brain slices were dehydrated and embedded in paraffin. Sections (4 μm) were cut and 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. Damaged brain areas were defined by the presence of blood, tissue rarefaction, or necrosis. These analyses were evaluated blindly by an experienced investigator to minimize observation bias.

Brain Water Content

Brain water content was determined by the dry-wet weight method. After decapitation, rat brain samples were immediately weighed on an electronic analytical balance (Changzhou instruments, Inc., China) to obtain the wet weight. Then brain samples were dried at 100°C in an Electric Blast Drying Oven (Chongqing Sida Apparatus, Inc, China) for 24 h to obtain the dry weight. The formula for water content calculation was as follows: wet weight - dry weight) / wet weight × 100%.

Immunohistochemistry

The procedures were processed according to the protocol recommended for MIP-2 and NF-κB immunohistochemistry kit. The rat brain sections were de-waxed and re-hydrated, rinsed with distilled water and PBS, quenched with 3% H_2O_2, blocked with 10% normal goat serum, and incubated with goat anti-MIP-2 polyclonal antibody (diluted at 1:150, Santa Cruz, USA), and rabbit anti-NF-κB polyclonal antibody (diluted at 1:100, Santa Cruz, USA) at 4°C overnight. Slides were then washed with Triton PBS, incubated in biotinylated goat anti-rabbit IgG and rabbit anti-goat IgG (DAKO,USA) for 1 hour at room temperature, washed, incubated with streptavidin peroxidase for 30 minutes at room temperature, colored with diaminobenzidine-H_2O_2 solution, washed, dehydrated in graded ethanol, immersed in xylene and coverslipped. Control sections were processed with omission of the primary antibody.

Quantitative Real-time RT-PCR of MIP-2 mRNA

After decapitation, the rat cerebrum at 2h, 3h,

6h, 10h, 12h, 24 h, 2d or 5d after ICH were kept immediately in liquid nitrogen for the analysis of MIP2 mRNA by real-time RT-PCR. Two procedures were used for RNA extraction and reverse transcription (RT). Total RNA was isolated from rat brain using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR reactions were performed in 96-well plates with an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) using SYBR Green to monitor amplification. Reactions were done in 25 μL volumes containing 200 nM of each primer, 5 μL cDNA (corresponding to ~3 ng DNA), and 10 μL 2 × SYBR Green Master Mix Reagent (Applied Biosystems). Aliquots from the same cDNA sample were used with all primer sets in each experiment. Reactions were run using the manufacturer's recommended cycling parameters of 93°C for 2 min, 40 cycles of 93°C for 1 min and 55°C for 1 min. Each PCR reaction was completed in triplicate. Optical data obtained by real-time PCR was analyzed with the manufacturer's software. Primers used were 5'-cctgggaaggaagaacatgg-3' (sense) and 5'-ggcacatcaggtacgatcca-3' (antisense).

Western Blot Analysis for NF-κB

Animals were anesthetized before undergoing intracardiac perfusion with saline. The brains were sampled as described for brain water content. Western blot analysis was performed as previously described [19]. Briefly, 50 μg protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane. After blocking, membranes were probed with a 1:200 dilution of the primary antibody (rabbit polyclonal anti- NF-κB, Santa Cruz) and a 1:5000 dilution of the second antibody (peroxidase-conjugated goat anti-rabbit antibodies, Rockland). The antigen-antibody complexes were visualized with a chemiluminescence system (Amersham) and exposed to film (Kodak X-OMAT). The relative densities of bands were analyzed with ODYSSEY infrared imaging system (LI-COR). GAPDH was used as an internal control. The experiments were repeated three times with similar results.

Cell Counting and Statistical Analysis

Two observers, blinded to the experimental surgery, evaluated the morphometric parameters in ten randomly chosen non-overlapping high-power fields (hpf, original magnification×400, Olympus BX51, light microscope) from each section. All positive cells in the ten high-power fields were counted and the number of MIP-2 and NF-κB positive cells per high-power field was calculated. All data were reported as mean ± standard deviation (SD). The statistical significance of the data was analyzed by analysis of variance (ANOVA), post hoc tests and pearson correlation test with SPSS 13.0 for Windows (SPSS Inc). Values of $P<0.05$ were regarded as significant.

Results

Morphological Changes of Rat Brain after ICH

A spherical hematoma was observed in the caudate nucleus area at all time points after ICH. At 3h time point, a few scattered neutrophils were found at the periphery of the hematoma. Little morphological changes of neurons were observed. At 24h, brain edema around the hematoma was visible. The infiltrated inflammatory cells were mainly mononuclear with scattered neutrophils. At 48h, the brain edema around the hematoma was pronounced and the brain tissue was diffluent and necrotic. The hematoma was surrounded by a compact band of cells including viable neutrophils, some cell debris, a few macrophages and rare clusters of intact erythrocytes. At day 5 after ICH, the hematoma started to resolve with glia cell hyperplasia and abundant neovascularization. In contrast, no hematoma or significant changes were observed in the brain tissue of rats from the control group (**Figures 1 A-C**).

Immunocytochemical Analysis of MIP-2 Expression in Rat Brain after ICH

The results of immunohistochemical staining indicated that MIP-2 immunoreactivity was intensive in rat brain after ICH. MIP-2 protein was mainly expressed in cell bodies of neurons, and extended into the major axons in cortical layer and part of punctate substance, which is widespread in cerebral cortex, hippocampus and thalamencephalon (**Figures 2 A-C**). The most intense MIP-2 immunoreactivity was observed in neurons of injured brain. Pyramidal neurons in the cortex showed moderate MIP-2 immunoreactivity, with layers 3 and 5 showing more intense staining than other cortical layers. MIP-2 protein was also moderately expressed in vascular endothelial cells. MIP-2 protein was

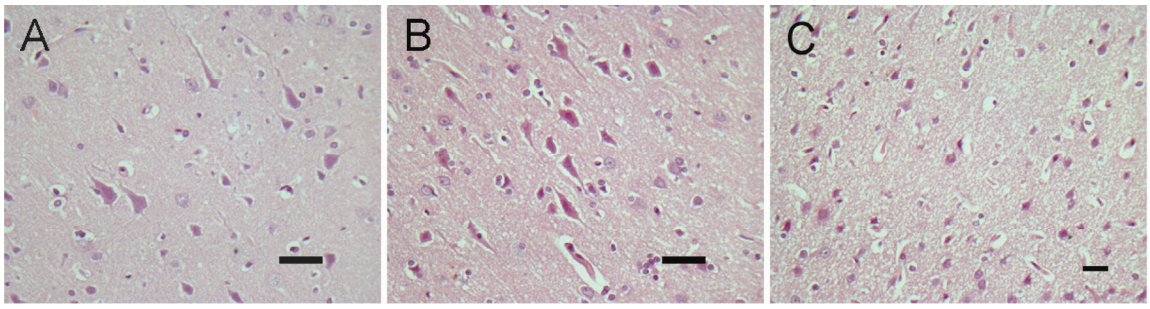

Figure 1 Histopathology of cerebral cortex of rats from control (**A**), 3h after ICH (**B**) and 2d after ICH (**C**) (hematoxylin and eosin stain; scale bar = 100 μm). Mild morphological changes of neurons were observed 3h after ICH while significant changes were present 2d after ICH.

weakly expressed in normal rat brain. Compared to control rat brains, MIP-2 immunoreactivity showed a modest increase 2 hours after ICH, significant increase 3 hours and maximal increase 2 days after ICH (P< 0.01) (**Figure 2D**).

Quantitative RT-PCR Analyses of MIP-2 mRNA

Using real-time quantitative RT-PCR, we further investigated the expression of MIP-2 at mRNA level. MIP-2 mRNA continued to increase after

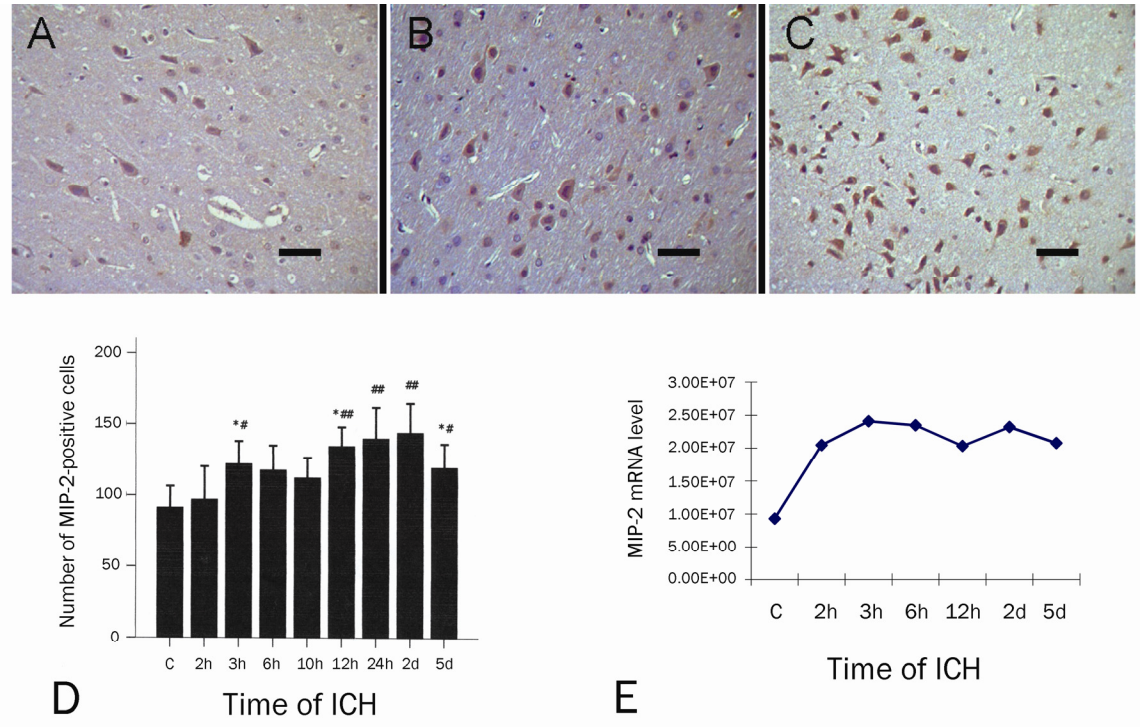

Figure 2 MIP-2 immunoreactivity in cerebral cortex of rats from control (**A**), 3h after ICH (**B**) and 2d after ICH (**C**) (immunohistochemical stain with anti-MIP-2 antibody; scale bar = 100 μm). MIP-2 immunoreactivity is predominantly present in cell bodies of neuron. Graphic presentation of the number of MIP-2 positive cells per high power field after ICH (**D**). Vertical bars indicate mean ± SD. # P＜0.05 compared with control group rats; ## P＜0.01 compared with control group rats; *P＜0.05 compared with rats at a prior time point after ICH. Graphic presentation of MIP-2 mRNA expression by quantitative real-time RT-PCR (**E**). Line indicates the quantity of MIP-2 mRNA expressed in arbitrary unit.

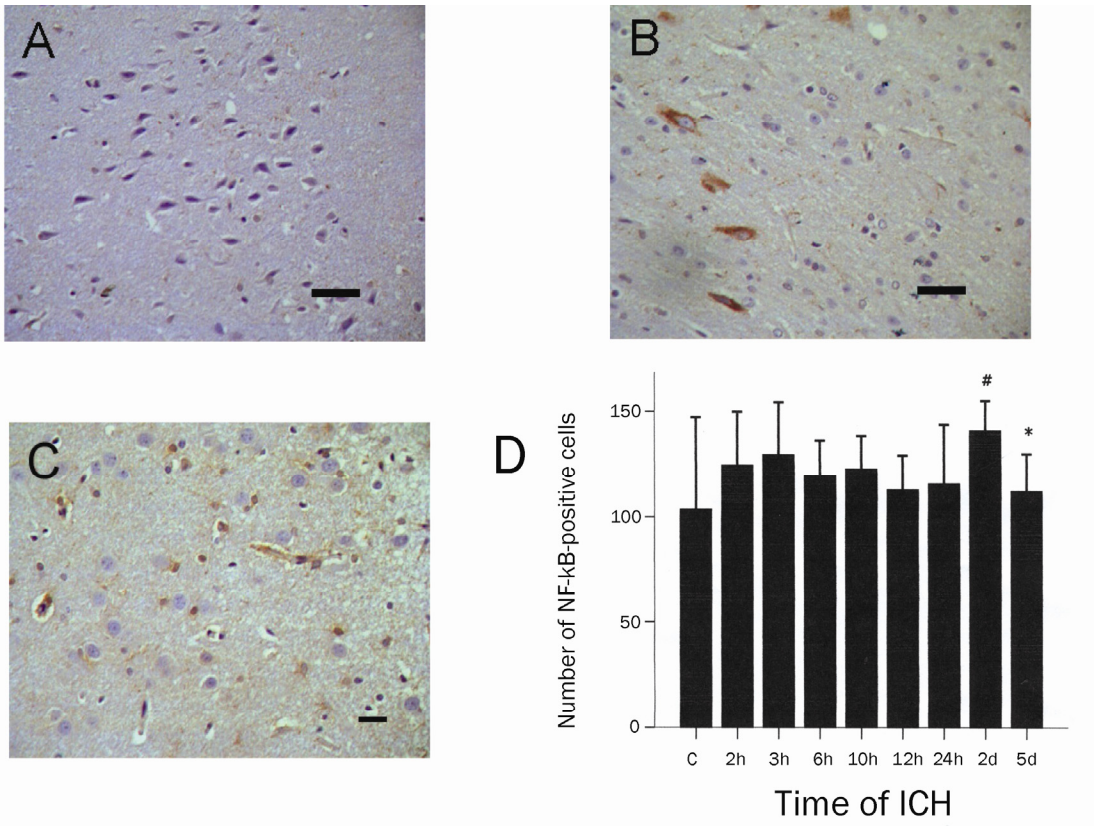

Figure 3 NF-κB immunoreactivity in cerebral cortex of rats from control (**A**), 3h after ICH (**B**) and 2d after ICH (**C**). (immunohistochemical stain with anti NF-kB antibody; scale bar = 100 μm). NF-κB protein was weakly positive in the control, strongly cytoplasmic positive 3h after ICH, and cytoplasmic and nuclear positive 2d after ICH. Graphic presentation of the number of NF-kB positive cells per high power field after ICH (**D**). Vertical bars indicate the mean ± SD. # P＜0.05 compared with control group rats; *P＜0.05 compared with rats at a prior time point after ICH.

ICH and peaked at 3 hours (**Figure 2E**). The kinetics of MIP-2 mRNA expression is similar that of MIP-2 immunoreactivity.

NF-κB Immunocytochemistry in Rat Brain after ICH

NF-κB protein in rat brain was expressed in cell bodies of neurons and astrocytes. NF-κB protein was also expressed in vascular endothelial cells and smooth muscle cells of the cerebral vasculature. NF-κB protein was weakly positive in the cytoplasm of the control group. It was translocated from cytoplasm to nucleus in the cells of the hemorrhage hemisphere (**Figure 3 A-C**). Similar to MIC-2, NF-κB immunoreactivity began to increase at 2 hours after ICH, dramatically increased at 3 hours and reached the maximum at 2 days after ICH (P<0.05 compared with control group) (**Figure 3D**).

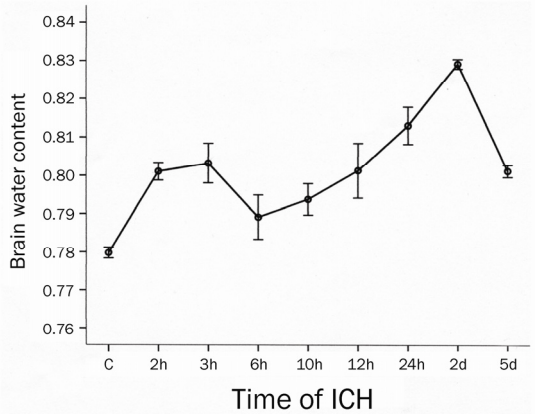

Figure 5 Using the dry-wet weight method, we found that cerebral edema was significant at the acute stage and resolved slightly from 6 to 12 hours after ICH. But 24 hours later, cerebral edema became more severe and reached the maximum at day 2 after ICH. Line indicates mean ± SD.

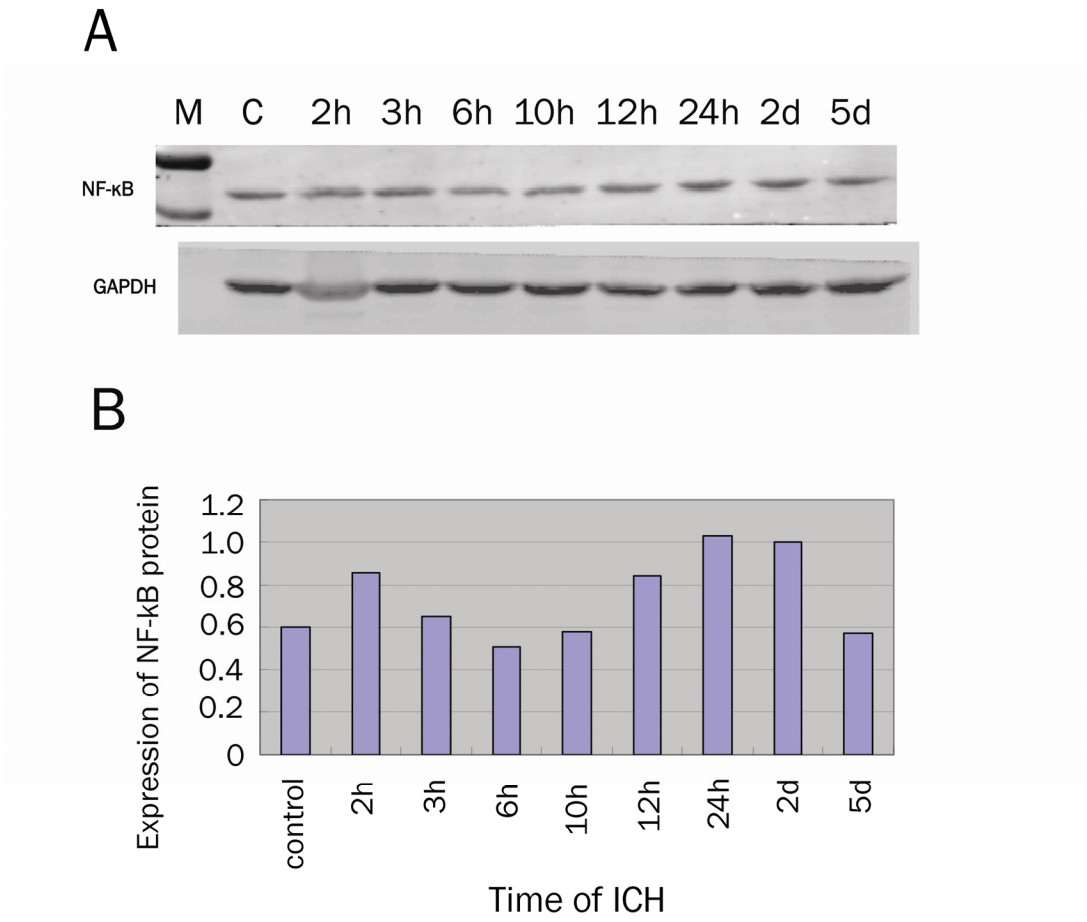

Figure 4 Western blot analysis of NF-κB in rat cerebral cortex. Expression of NF-κB protein increased 2 hours after ICH and reached the maximum at 1-2 days after ICH. GAPDH was used as an internal control. The experiments were repeated three times with similar results.

Western Blot Analysis of NF-κB in Rat Brain after ICH

Western blot analyses showed that NF-κB protein dramatically increased at 2 hours and reached the maximum at 1-2 days after ICH (**Figure 4**).

Brain Water Content

Our results indicated that cerebral edema was severe at the acute stage and relieved from 6 hours to 12 hours after ICH. However, after 24 hours, cerebral edema was dramatically severe ($P<0.05$ compared with control group) and reached the maximum at 2 days (**Figure 5**). There was a positive correlation between brain water content and MIP-2 as well as NF-κB expression. Cerebral edema and the expression of MIP-2 was positively correlated at the 0.01 level (correlation coefficient is 0.548) on the whole. The expression of NF-κB was also positively correlated with cerebral edema at the 0.05 level (correlation coefficient is 0.314).

Discussion

The pathological consequences of primary intracerebral hemorrhage (ICH) include the immediate primary injury and more delayed secondary processes [8]. Ischemia, inflammation, excitotoxicity and apoptosis have all been observed in animal models of ICH [9]. Clinical and experimental evidence indicates a very limited if any role for ICH alone, while inflammation increasingly appears to be a significant factor in the development of secondary changes.

A major side effect of inflammation is an increased permeability of the cerebral microvessels, resulting in vasogenic brain edema and a rise in intracranial pressure [10]. Cytokine increases the permeability of blood brain barrier, induces leukocytosis in cerebrospinal fluid and brain edema [11]. Experimentally, recruitment of neutrophils to the CNS is followed by a breeching of the blood-brain barrier. Administration of MIP-2 exacerbates damage of the blood-brain barrier [12] that may further contribute to inflammation by causing indiscriminate entry of leukocytes into the brain. The expression of MIP-2 mRNA correlates with injury to the blood-brain barrier as demonstrated by the appearance of serum proteins and leukocytes in cerebrospinal fluid and by the increase in brain water content [13].

To further study the role of MIP-2 in brain edema, we quantified mRNA and protein levels for MIP-2 in rat brain after ICH. We found that in addition to previously described inflammatory changes in the brain, stroke induced a complex, but organ specific, pattern of inflammatory factors in the brain as early as 2h after ICH. Pyramidal neurons in layers 3 and 5 of cortex showed more prominent MIP-2 immunoreactivity than those in the other cortical layers. The result of immunohistochemistry correlates with the mRNA levels by real-time quantitative RT-PCR.

Our results also demonstrate that the drastic inflammatory changes occurring in the damaged brain are dynamically reflected with the time course of ICH. The brain water content after ICH appears to follow the expression pattern of MIP-2. Based on the recent reports in the literature and our own findings, we speculate that MIP-2, as an important chemotactic cytokine, may increase blood brain barrier permeability and cause brain edema after ICH.

However, although MIP-2 is present in the brain parenchyma, neutrophils are unable to respond, and hence the subsequent activation-dependent step of leukocyte emigration cannot occur. The up-regulation of some chemokines has been attributed to constitutive activation of nuclear factor-kappaB (NF-κB) [14]. NF-κB is a transcription factor present in cytoplasm as a heterodimer composed of p50 and p65 subunits. After activation, NF-κB translocates from the cytoplasm to the nucleus of the cells, binds to specific DNA sequences, and initiates transcription. Several reports have indicated the involvement of NF-κB in a large number of cellular processes such as inflammatory and immune response. Our study revealed that NF-κB protein in rat brain was mildly expressed in cytoplasm in control group, and translocated from cytoplasm to nucleus of the cells in the hemorrhage hemisphere after ICH. NF-κB binding sites are present in the promoter region of MIP-2 gene. When bound to MIP-2 gene, NF-κB activates transcription of MIP-2 [15]. Studies have indicated that expression of MIP-2 gene in rat lung epithelial cells is dependent on the transcription factor NF-κB [16]. NF-κB also plays a key role in secondary impairments of the tissues around the hematoma [17, 18] by inducing the expression of various genes related to cell injury and apoptosis. Here we demonstrated that both MIP-2 and NF-κB proteins began to increase 2 hours after ICH and reached the maximum 1-2 days after ICH. The expression of NF-κB follows the same pattern of MIP-2 during the entire course of ICH, which also correlated with the developmental course of brain edema. We speculate that NF-κB may induce MIP-2 expression, which subsequently increases the permeability of the blood-brain barrier and results in formation of brain edema after ICH. These findings may provide information for developing new therapeutic strategies and therapeutic targets for cerebral edema after ICH.

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