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Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia

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ABSTRACT

Background: Neuroinflammation is a key cascade after cerebral ischemia. Excessive production of proinflammatory mediators in ischemia exacerbates brain injury. Cold-inducible RNA-binding protein (CIRP) is a newly discovered proinflammatory mediator that can be released into the circulation during hemorrhage or septic shock. Here, we examine the involvement of CIRP in brain injury during ischemic stroke.
Methods: Stroke was induced by middle cerebral artery occlusion (MCAO). *In vitro* hypoxia was conducted in a hypoxia chamber containing 1% oxygen. CIRP and tumor necrosis factor-α (TNF-α) levels were assessed by RT-PCR and Western blot analysis.
Results: CIRP is elevated along with an upregulation of TNF-α expression in mouse brain after MCAO. In CIRP-deficient mice, the brain infarct volume, induction of TNF-α, and activation of microglia are markedly reduced after MCAO. Using microglial BV2 cells, we demonstrate that hypoxia induces the expression, translocation, and release of CIRP, which is associated with an increase of TNF-α levels. Addition of recombinant murine (rm) CIRP directly induces TNF-α release from BV2 cells and such induction is inhibited by neutralizing antisera to CIRP. Moreover, rmCIRP activates the NF-κB signaling pathway in BV2 cells. The conditioned medium from BV2 cells exposed to hypoxia triggers the apoptotic cascade by increasing caspase activity and decreasing Bcl-2 expression in neural SH-SY5Y cells, which is inhibited by antisera to CIRP.
Conclusion: Extracellular CIRP is a detrimental factor in stimulating inflammation to cause neuronal damage in cerebral ischemia.
General significance: Development of an anti-CIRP therapy may benefit patients with brain ischemia.

1. Introduction

Stroke is the third leading cause of death in the Western world and the most frequent cause of permanent disability in adults worldwide [1]. Currently, therapeutic options for treating patients with ischemic stroke are extremely limited. Recombinant tissue-plasminogen activator for thrombolysis is the only approved specific treatment for this devastating illness. It is limited however, by its short therapeutic window (within 3 h) and side effects [1]. In order to develop new neuroprotectant therapies, understanding the complex pathogenesis of brain ischemia and finding new drug targets are imperative.

The mammalian brain is vulnerable to various environmental and pathophysiological insults. When a stroke occurs, the brain is susceptible to hypoxic or ischemic insults. Many studies have shown that inflammation contributes to the pathogenesis of brain injury in stroke by triggering numerous cellular and molecular events that cause neuronal damage [1,2]. In the periphery, immune cells such as leucocytes (neutrophils, monocytes, and macrophages), B cells, and T cells can access most organs in response to inflammation [3,4]. However, due to the blood–brain barrier, most systemic immune cells cannot reach the brain. Microglia are resident macrophages within the central nervous system and are responsible for the majority of inflammatory activity in the brain [4]. When microglia detect invading pathogens or tissue injury, they become 'activated', and start proliferating, migrating, phagocytizing, and producing proinflammatory cytokines and oxidants, leading to neuronal damage [2,3,5].

Cold-inducible RNA-binding protein (CIRP) is the first identified cold shock protein in mammalian cells. The protein sequences of CIRP in mouse and rat are identical, while human CIRP has 95% homology to them [6]. CIRP in both murine and human is a 172-amino-acid nuclear protein consisting of one amino-terminal consensus sequence RNA-binding domain and one carboxyl-terminal glycine-rich domain [6,7]. CIRP is thought to modulate gene expression during mild hypothermia by functioning as an RNA chaperone to facilitate translation [8]. CIRP is constitutively but weakly expressed in various tissues [9,10]. In addition to cold stress, CIRP expression is increased in response to UV irradiation

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[11] and hypoxia [12]. It has been reported that CIRP can be detected in the brain and its expression increases under ischemic conditions [13]. Our recent study also shows that CIRP increases significantly after hemorrhagic and septic shock in animals, and administration of recombinant murine CIRP (rmCIRP) in healthy animals causes organ injury [14].

Based on the information above, we hypothesized that CIRP would contribute to brain inflammation and lead to neuronal damage in cerebral ischemia. In this study, we first determined the expression of CIRP and proinflammatory cytokine tumor necrosis factor-α (TNF-α) in mice after cerebral ischemia induced by middle cerebral artery occlusion (MCAO). Then, we compared brain infarct size and activation of microglia between wild type and CIRP-deficient mice after MCAO. We further applied an *in vitro* cell culture system to elucidate the role of CIRP in regulating inflammation and the signaling pathway in mediating CIRP activity in microglia after exposure to hypoxia. Finally, we assessed CIRP's effects on neural cell death.

2. Materials and methods

2.1. Experimental animals and model of cerebral ischemia

CIRP-deficient (*Cirbp*$^{-/-}$) mice with C57BL/6 background were provided by Kumamoto University in Japan. Male age matched C57BL/6 mice (20–25 g) were purchased from Taconic (Albany, NY). All mice were housed in a temperature controlled room on a 12 h light-to-dark cycle and fed a standard laboratory diet. The animals were allowed to acclimate for at least 5 days under these conditions before being used for experiments. All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the use of experimental animals. The project was approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research.

Mice were fasted overnight but had access to water *ad libitum* before induction of cerebral ischemia. Permanent focal cerebral ischemia was induced by MCAO as previously described by Belayev et al. [15], with some modifications. Briefly, anesthesia was induced by isoflurane inhalation and subsequently maintained by intravenous boluses of pentobarbital (15 mg/kg BW). Body temperature was maintained at 37 °C using a heating pad with a temperature monitor (Harvard Apparatus, Holliston, MA). The right common carotid artery (CCA) was exposed through a ventral midline neck incision and was carefully dissected free from the vagus nerve and fascia, from its bifurcation to the base of the skull. The distal branches of the external carotid artery (ECA) were then dissected, ligated and divided to create an ECA stump. The internal carotid artery (ICA) was isolated and separated from the adjacent vagus nerve and the pterygopalatine artery was dissected and ligated close to its origin. Then, a 1-cm length of 7-0 poly-L-lysine coated monofilament nylon suture was inserted through the proximal ECA into the ICA and advanced to the middle cerebral artery (MCA) origin to occlude it. The silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding. Occlusion of the MCA was ascertained by inserting the suture to a pre-determined length of 8–10 mm from the carotid bifurcation and feeling for resistance as the suture tip approached the proximal anterior cerebral artery. The cervical wound was then closed in layers and mice were allowed to recover from anesthesia. The intraluminal suture was left *in-situ* and mice were allowed unrestricted access to food and water. The sham-operated animals had the same procedures except for the MCA occlusion. The 48 h post sham-operation group was used as a sham control. At 30 and 48 h post-operation, the animals were sacrificed and brain tissue from the infarcted hemisphere was collected for various analyses. The induction of stroke by the MCAO procedure was confirmed by staining the brain with 1.5% triphenyl tetrazolium chloride (TTC) at 37 °C for 30 min and then immersing it in 10% formalin overnight.

2.2. Immunohistochemistry analysis of the brain sections

Paraffin sections of brain tissue were de-waxed and rehydrated. Slides were soaked in 20% citric acid pH 6.0 buffer (Vector Labs, Burlingame, CA) and heated in the microwave oven and maintained at 95 °C for 15 min for antigen retrieval. Endogenous peroxidase was blocked by 2% H_2O_2 in 60% methanol for 20 min. Normal goat serum (3%) was used to block the nonspecific binding sites. The sections were then incubated with rabbit anti-allograft inflammatory factor 1 (AIF1) primary antibodies (1:100, Proteintech Group, Chicago, IL) overnight at 4 °C, followed by biotinylated anti-rabbit IgG (1:200, Vector Labs) for 1 h. Vectastain ABC reagent and DAB kit (Vector Labs) were used to reveal the immunohistochemical reaction and counterstained with hematoxylin. The primary antibody was substituted with normal rabbit IgG as the negative control. The immunostaining was examined under a Nikon Eclipse E600 microscope.

2.3. BV2 cell culture and exposure to hypoxia

Murine microglial BV2 cells were obtained from Dr. Philippe Marambaud at The Feinstein Institute for Medical Research and are a reliable model for studying the biology of primary microglia [16,17]. BV2 cells have been used to study the cellular responses of microglia to hypoxic stress [18,19]. BV2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 37 °C incubator with 5% CO_2. To simulate the *in vivo* hypoxic condition, BV2 cells were cultured in a sealed chamber containing 1% O_2, 5% CO_2 and 94% N_2 for 20 h or 30 h.

2.4. Determination of gene expression by real-time polymerase chain reaction (PCR)

Total RNA was extracted from brain tissues and BV2 cells using the TRLzol reagent (Invitrogen). Real-time PCR was carried out on cDNA samples which were reversely transcribed from 2 μg RNA by using murine leukemia virus reverse-transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was carried out in 24 μl of the final volume containing 0.08 μmol of each forward and reverse primer, 2 μl cDNA, 9.2 μl H_2O and 12 μl Power SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50 °C for 2 min, 95 °C for 10 min and followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The level of mouse β-actin mRNA was used for normalization and each specific mRNA was conducted in duplicate. Relative expression of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and results were expressed as fold change in comparison with the control group. The sequences of primers used in PCR are the following: CIRP (NM_007705) forward: 5′-AGC TCG GGA GGG TCC TAC AG-3′ and reverse: 5′-GAG GGC TTT TAC TCG TTG TGT GT-3′; TNF-α (X02611) forward: 5′-AGA CCC TCA CAC TCA GAT CAT CTT C-3′ and reverse: 5′-TTG CTA CGA CGT GGG CTA CA-3′; β-actin (NM_007393) forward: 5′-CGT GAA AAG ATG ACC CAG ATC A-3′ and reverse: 5′-TGG TAC GAC CAG AGG CAT ACA G-3′. A dissociation curve was performed for each analysis to confirm the specificity of the PCR product.

2.5. Western blot analysis of protein expression

Brain tissues and BV2 cells were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche Applied Science; Indianapolis, IN). The protein concentration of the lysate was determined by the DC protein assay kit (Bio-Rad, Hercules, CA). 30 μg of protein was subjected to a 4–12% Bis–Tris gel electrophoresis in MES-SDS running buffer (Invitrogen). After electrophoresis, the gel was transferred onto a 0.2-μm nitrocellulose membrane and then blocked with 0.1% casein in 10 mM phosphate buffer, pH 7.5. Then, the membrane was incubated