Induction of Autophagic Cell Death in the Rat Brain Caused by Iron

Gao Chen, MD, Chao-hui Jing, MD, Pan-pan Liu, MD, Di Ruan, MD and Lin Wang, MD

Abstract: *Background:* The accumulation of iron in the brain is a hallmark of hemorrhagic stroke and several neurodegenerative diseases. Iron overload has been reported to induce brain injury through necrotic and apoptotic mechanisms. This study was taken to examine whether iron in the brain contributes to autophagic cell death. *Methods:* Sprague-Dawley rats received an intracerebral ventricular injection of either ferrous chloride or saline. The expression levels of autophagic markers were measured by Western blot analysis. Immunofluorescent double labeling was used to identify the cell types expressing Beclin 1. Transmission electron microscopy was performed to examine the ultrastructural changes in neural cells 1 day after ferrous iron injection. *Results:* Western blot analysis showed that the ratios of LC3-II to LC3-I and ATG5 levels were significantly upregulated at 6 hours and 1 day after ferrous iron injection. Beclin 1 expression was markedly elevated as early as 6 hours, reaching a peak at 24 hours and remaining elevated at 3 days after the injection. Beclin 1 immunoreactivity was located in both neurons and astrocytes under confocal microscopy. Induction of autophagic cell death was manifested by accumulation of autophagic vacuoles in the contralateral parietal cortex under transmission electron microscopy. *Conclusions:* Our data showed that increased ferrous iron levels in the brain induced autophagic cell death. These results also suggest that autophagy form of programmed cell death may be a mechanism of brain injury in iron overload disorders.

Key Indexing Terms: Autophagic cell death; Iron; Microtubule-associated protein light chain 3; ATG5; Beclin 1. **[Am J Med Sci 2013;345(5):369–374.]**

Iron operates as an essential cofactor for many proteins and is critical in many vital biological pathways, including the synthesis of DNA, RNA and proteins; the formation of myelin; and the development of the neuronal dendritic tree within the brain.[1] The blood-brain barrier (BBB) plays a critical role in the maintenance of iron homeostasis within brain. Deregulation of BBB integrity and iron overload have been well documented after intracerebral hemorrhage (ICH), subarachnoid hemorrhage (SAH) and some neurodegenerative diseases like Alzheimer's (AD) and Parkinson's diseases (PD).[2,3] There is increasing evidence that iron accumulation within the brain has been implicated in cellular damage in a number of pathological situations ranging from hemorrhagic stroke to neurodegenerative diseases.[4–7]

From the Department of Neurosurgery, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

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The first 2 authors contributed equally to this work.

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Correspondence: Lin Wang, MD, Department of Neurosurgery, the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China (E-mail: wanglin_77@hotmail.com).

There are 3 main types of cell death, including necrotic, apoptotic and autophagic cell death. In contrast to programmed cell death (PCD), necrosis has been traditionally thought to be a passive form of cell death with more similarities to a train wreck than a suicide. Autophagic cell death is morphologically defined as a type of PCD occurring with marked proliferation of autophagic vacuoles, relatively preserved cytoskeletal and nuclear integrity until late in the process, whereas apoptosis is characterized by cellular and nuclear shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and the formation of apoptotic bodies.[8,9] The execution and regulation of the autophagic program rely on several autophagy-related genes, which are highly conservative in eukaryotic cells, ranging from yeast to mammals.

Iron-induced cellular damage is involved in many central nervous system disorders and become a debilitating health problem. The mechanisms of brain injury in iron overload disorders are still being elucidated. Iron overload in the brain has been found to induce iron-catalyzed free radical generation, lipid peroxidation, axonal dystrophy, necrosis and apoptotic cell death.[10,11] It has been proved that cellular iron content plays a key role in the induction of apoptosis and necrosis.[12] However, it is unclear whether iron contributes to the autophagic cell death. In this study, therefore, we sought evidence that iron overload in brain leads to autophagy form of PCD.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 250 to 300 g were obtained from SLAC Laboratory Animal Co. Ltd (Shanghai, China). The animals were maintained on a 12-hour light/dark cycle under controlled temperature conditions (22 ± 2°C) and given standard food and water *ad libitum*. The rats were allowed to acclimatize for 3 days before the experiments. The rats were fasted but allowed free access to water overnight before surgery. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University, and they conform to the guidelines of the "Principles of Laboratory Animal Care" (National Institutes of Health Publications No. 80-23, revised 1996). All feasible measures were taken to reduce animal suffering and to minimize the number of animals used for these experiments.

Study Design

A total of 50 rats were randomly assigned to 2 groups: control group (n = 10) and iron group (n = 40). Tissue samples were taken for analysis at 6 hours, 24 hours, 3 days and 7 days after the intracerebral ventricular injection of ferrous chloride. Animals undergoing sham operations were exterminated 24 hours after surgery. Four animals were required for Western blot analysis. Three animals were examined by electron microscopy and immunofluorescent double labeling.

Intracerebral Ventricular Injection

Rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneal). The right femoral artery was catheterized to monitor arterial blood pressure and blood gases. Blood samples were taken before and 30 minutes after the injection. Rectal temperature was maintained at 37.5°C using a self-adjusting feedback-controlled heating pad. The animals were positioned in a stereotactic frame (Kopf Instruments, Tujunga, CA). Twenty microliters of saline or ferrous chloride (2 mmol/L; Sigma, St. Louis, MO) was infused into the right lateral cerebral ventricle over 5 minutes through a microsyringe attached to a microinfusion pump. The coordinates used were 0.8 mm posterior and 1.4 mm lateral to the bregma, and a depth of 3.7 mm. After the intracerebral ventricular injection, the injection needle was left in the site for 30 seconds before removal, the burr hole filled with bone wax and the skin incision closed with sutures.

Western Blot Analysis

The rats were anesthetized and intracardially perfused with 0.1 mmol phosphate-buffered saline (PBS). The contralateral cortex (B: −1.0 to −4.0 mm) was separated and frozen using liquid nitrogen and stored at −80°C until analysis. The frozen brain samples were homogenized in extract buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 0.1% sodium dodecyl sulfate, 1% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 nmol/L leupeptin, 10 nmol/L pepstatin and 10 nmol/L aprotinin, pH 7.4) and centrifuged at 1000*g* for 10 minutes at 4°C to remove the cellular debris. The resultant samples were further centrifuged at 13,000*g* for 30 minutes at 4°C to obtain the supernatants. Protein concentration was determined with the bicinchoninic acid kit. A 50-μg portion of protein from each sample was resuspended in loading buffer, denatured for 5 minutes at 95°C, separated by 10% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked by nonfat milk for 2 hours and then incubated overnight at 4°C with primary antibodies against ATG5 (1: 500; Abcam, Cambridge, UK), LC3 (1: 1000; Cell Signaling Technology, Beverly, MA), Beclin 1 (1: 1000; Abcam) or β-actin (1: 5000; Cell Signaling Technology). After incubation, the membranes were exposed to horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. The membranes were washed again with Tris-buffered saline containing 0.05% Tween-20. The antigen-antibody complexes were visualized using the ECL Plus chemiluminescence reagent kit (Millipore, Billerica, MA). The optical densities of the image signals were quantitatively analyzed using the Quantity One software (Bio-Rad, Hercules, CA).

Immunofluorescence and Laser Scanning Confocal Microscopy

The rats were anesthetized and transcardially perfused with 0.1 mmol PBS, followed by 4% paraformaldehyde. The fixed brain was immersed in 4% formaldehyde for 6 to 8 hours and then immersed in 30% sucrose solution until the tissue sank. Serial frozen sections (about 20 μm thick) were collected onto alum gelatin-coated slides. The sections were incubated in 5% bovine serum albumin in PBS with 0.25% Triton X-100 at room temperature for 30 minutes to block nonspecific binding, followed by incubation at 4°C overnight in the primary antibody. The primary antibodies (and concentrations) used were as follows: rabbit anti-beclin1 (1: 200; Abcam), mouse anti–microtubule-associated protein 2 monoclonal antibody (1:400; Abcam) and mouse anti–glial fibrillary acidic protein monoclonal antibody (1:400; Abcam). The sections were then incubated with goat anti-mouse fluorescein-isothiocyanate–conjugated secondary antibody (1:200; JacksonImmuno, West Grove, PA) and goat anti-rabbit rhodamine-conjugated secondary antibody (1:200; JacksonImmuno) for 2 hours at room temperature. The nucleus was stained with 4′, 6-diamidino-2-phenylindole (1 ng/mL). The sections were covered with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and examined under a laser scanning confocal microscope (LSM-710; Zeiss, Göttingen, Germany). Mouse immunoglobulin IgG was used instead of primary antibody as a negative control.

Transmission Electron Microscopy

The rats were anesthetized with a lethal dose of pentobarbital and intracardially perfused with 0.1 mmol PBS, followed by 4% paraformaldehyde. The brains were exposed, and samples about 1 mm^3 were taken from the contralateral parietal cortex (B: −2.0 to −3.0 mm). The samples were immersed in 2.5% glutaraldehyde overnight at 4°C. The tissue was rinsed in buffer and postfixed with 1% osmium tetroxide for 1 hour. Then, the tissue was rinsed with distilled water before undergoing dehydration in a graded ethanol series. The tissue was then infiltrated using a mixture of one-half propylene oxide and one-half resin overnight. Twenty-four hours later, the tissue was embedded in resin. The sections (approximately 100 nm thick) were cut and stained with 4% uranyl acetate for 20 minutes and 0.5% lead citrate for 5 minutes. Ultrastructures were observed under a transmission electron microscope (Tecnai 10; Philips, Eindhoven, The Netherlands).

Statistical Analysis

All data are expressed as mean ± standard deviation. One-way analysis of variance, followed by a Dunnett's test, was used for statistical analysis. Significant differences were accepted when a value of $P < 0.05$ was attained.

RESULTS

Physiological Variables

All physiological parameters among rats during the experimental period were maintained within normal ranges for mean arterial blood pressure (80–120 mm Hg), arterial pH (7.35–7.45), pO_2 blood gas level (80–95 mm Hg), pCO_2 blood gas level (35–45 mm Hg) and blood glucose level (90–120 mg/dL). These parameters were not significantly different between the groups.

Ferrous Iron-Induced Conversion of LC3-I to LC3-II

LC3 is the mammalian homologue of yeast ATG8 and exists in 2 forms: LC3-I and LC3-II (18 and 16 kDa, respectively). LC3-I is a polypeptide normally found in the cytosol, whereas LC-3 II, the product of its proteolytic maturation, resides in the autophagosomal membranes. Increased autophagic activity can be reflected by the enhanced conversion of LC3-I (cytosolic) to LC-3 II (lipidated) before they are destroyed through fusion with lysosomes.[13] Western blots for LC3 showed that both LC3-I and LC3-II were present in normal control brains. However, the ferrous iron caused a remarkable increase in LC-3 II in the contralateral cortex. The Western blot analysis demonstrated that the ratio of LC3-II to LC3-I was significantly increased at 6 (1.21 ± 0.21) and 24 (1.46 ± 0.31) hours after the ferrous iron injection compared with the saline controls (0.63 ± 0.20; Figure 1A). A time course of the LC3