Increased Expression of Interleukin-1β in Mouse Hippocampus after Global Cerebral Ischemia

Yoichi Imaizumi¹, Hidekatsu Mizushima¹, Hiroaki Matsumoto¹, Kenji Dohi¹, Kiyoshi Matsumoto¹, Hirokazu Ohtaki², Hisayuki Funahashi², Seiji Matsunaga², Reiko Horai³, Masahide Asano³, Yoichiro Iwakura³ and Seiji Shioda²,⁴

¹Department of Neurosurgery and ²Department of Anatomy, Showa University School of Medicine, 1–5–8 Hatanodai Shinagawa-ku, Tokyo 142–8555, ³The University of Tokyo, The Institute of Medical Science, Laboratory of Animal Research Center, Minato-ku, Tokyo 108–8639 and ⁴The Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology (JST), Japan

Received May 7, 2001; accepted August 14, 2001

We examined the in vivo expression of IL-1β and its transcript after cerebral ischemia produced in mice cardiac arrest model. The IL-1β mRNA in the hippocampal region reached a detectable level at 1 hr after ischemia and had a peak at 3 hr after ischemia recirculation. But it was markedly decreased at 1 day and reached the control levels at 4 day after ischemia recirculation. The IL-1β-like immunoreactivity was observed at 1, 2, 4 day after ischemia recirculation and its immunoreactivity was detected at 2 day. The IL-1β-like immunoreactivity was observed in both microglia and astrocytes after brain ischemia by double immunostaining. These results provide the direct evidence for the localization and induction of IL-1β expression in vivo in mice after ischemia. It is suggested that IL-1β, produced in both astrocytes and microglia cells after ischemia, directly affect on neurons as well as glial cells to induce delayed neuronal cell death.

Key words: Interleukin-1β, Cytokines, Cerebral ischemia, Cardiac arrest, Glial cells

I. Introduction

Interleukin (IL)-1β is a polypeptide of approximately 17,500 Da [2]. In the central nervous system, IL-1 has been suggested to participate in neuronal death resulting from experimental ischemia [20, 25], while it has been reported to stimulate astroglial proliferation after brain injury, and is protective at low concentrations in vitro [7]. However, the proposal that IL-1 mediates toxicity in brain remains controversial and the mechanisms of neurotoxic effects of IL-1 and its involvement in ischemia and excitotoxic damage are largely unknown. Several studies have described the exacerbation of ischemic brain damage after application of exogenous IL-1β [13, 25]. Early and marked induction of IL-1β mRNA was detected in rat brain after transient forebrain ischemia [23]. Recent physiological and morphological data suggest that IL-1β may be synthesized and secreted by several central nervous system cell types including microglia, astrocytes, and neurons [3, 17, 27]. These studies suggest that endogenous IL-1β plays an important role in controlling the apoptosis. Though the overall studies concerning apoptosis in the brain have been done in rats, few of these studies have been studied in mice. Nearly soon, with the development of gene-deficiency mice, we must prove a direct evidence whether IL-1β is directly involved in neuronal cell apoptosis or not. The cardiac arrest model has recently been developed to improve the defects of the four-vessel occlusion model and is a sure way of hemostasis and less invasive [5, 9, 10]. The purpose of the present study is to determine the expression of IL-1β in brain and to identify cells that produce IL-1β in mice brain after ischemia-recirculation.

II. Materials and Methods

Animals

Male adult BALB/c mice (25–30 g) were housed on a 12–24 hr light-dark cycle and given food and water ad lib. The animals were anesthetized by the gas with 3.5% halothane and maintained with 2% sevoflurane in 70% N₂O and 30% O₂. Body temperature was monitored and maintained at 37°C with a rectal thermometer. A 24-gage polyethylene catheter for arterial-line (A-line) was inserted into the left
femoral artery. Blood pressure and the electrocardiogram were monitored during all of the procedures. Arterial blood samples were analyzed at 37°C for pH, pCO₂, and pO₂ 10 min before cardiac arrest, 10 min after reperfusion. An equal amount of saline was injected after every sampling. Regional cerebral blood flow was measured by laser-Doppler flowmetry with a fiber-optic probe placed 2 mm posterior and 6 mm lateral to Bregma on the ipsilateral hemisphere, the site supplied by the proximal segment of the MCA. Global cerebral ischemia was induced according to the rat cardiac-arrest model [5]. The anesthetized mouse was placed in a supine position and a small incision was made in the midline of the chest. The occluding device was gently inserted in the position and a small incision was made in the midline of the chest. The occluding device was gently inserted in the mediastinum at the level of the 4th intercostal segment. The device was manipulated to the dorsal wall of the thorax. Then the distal end was placed under the bundle of major cardiac blood vessels. A complete interruption of the circulation was accomplished by lifting the occluding device and, at the same time, applying the gentle downward pressure with the fingers on the sternum. After 5 min of transient global ischemia, under the artificial ventilation with a tidal volume of 2.5 ml at 200 times per min (100% transient global ischemia, under the artificial ventilation device and, at the same time, applying the gentle downward pressure with the fingers on the sternum. After a recovery period of 30 min, the animals could be disconnected from the respirator. The animals were housed in cages with free access to water and food pellets.

**Northern blotting**

Following reperfusion periods of 0, 30, 60, 180, and 360 min (n=3 in each group), the animals were deeply anesthetized with pentobarbital, the brains were removed and dissected out the hippocampus and cerebral cortex under operating microscope. Total RNA was isolated from the hippocampal tissues by an acid guanidinium thiocyanate-phenol-extraction method and poly A⁺ RNA was purified using the QuikPrep Micro mRNA purification kit (Pharmacia). Northern blot hybridization was performed as described [8]. Total RNA or poly AN⁺ RNA was electrophoresed on a 1.2% denatured agarose gel and transferred to a nylon membrane. Hybridization was performed at 42°C with 32P-labeled DNA probes and membranes were washed in 2X SSC containing 1% SDS at 65°C. Radioactivities were measured using the BAS-2000 system (Fuji Photo Film Co., Tokyo, Japan).

**In situ hybridization**

Following reperfusion periods of 0, 1, 3, 6 hr and 1, 2, 4 days (n=5 in each group), the animals were deeply anesthetized with pentobarbital and sacrificed by decapitation. The brain was quickly removed and frozen in isopentane that had been cooled in liquid nitrogen. Sections were cut at 10 μm on a cryostat (MICROM, Heiderberg, Germany) and fixed by immersion for 20 min in a freshly prepared solution of 4% paraformaldehyde in phosphate. A hybridization probe was prepared by random-primer labeling of the murine IL-1β cDNA with Krenow enzyme and [α-35S]dCTP. The probe cDNA of 870 bp encoding IL-1β was cloned from a mouse brain cDNA library. The labeled cDNA probe was diluted to a final concentration of 0.4–0.8 pmol/ml in a buffer. Autoradiograms were generated by exposing the slide-mounted tissue sections to Hyperfilm-β-max film (Amersham, Buckinghamshire, U.K.) for 3–5 days at room temperature. Subsequently, the slides were dipped in nuclear track emulsion (NTB2; Kodak, Rochester, NY) and exposed for 10–14 days in a dry chamber. Slides were developed in Kodak D19 developer and fixed in Kodak Rapid Fixer. After development, the sections were lightly counterstained with hematoxylin and eosin, dehydrated in a graded ethanol series and covered with coverslips with DPX (SAF Bulk Chemicals, Buchs, Switzerland). Controls for specificity included sections incubated with the 32S-labeled cDNA probe and sections that were pretreated with RNase (20 μg/ml) for 30 min at 40°C. To determine the statistical analysis of IL-1β mRNA positive cells, we counted each section of 4 days (n=5) 120 (width) ×100 (height) μm² in the CA1 region of the hippocampus. Statistical significance was determined with t-test.

**Immunohistochemistry**

The animals were anesthetized with pentobarbital and then they were perfused for 30 min with a fixative that consisted of 400–500 ml of 2% paraformaldehyde and 0.1% glutaraldehyde (Electron Microscopy Sciences, Cherry Hill, NJ) in 0.1 M phosphate buffer (pH 7.2) for 20–30 min. Twenty-μm sections from the brains were cut on a cryostat and picked up on gelatin-coated slides. Endogenous peroxidase activity was blocked with 0.01 M phosphate buffer that contained 0.5% hydrogen peroxide and 2% normal goat serum. Sections were rinsed with PBS, preincubated with 10% normal goat serum in PBS, and then incubated with goat anti-IL-1β antibody (R&D Systems, Minneapolis, USA), at a dilution of 1:100–1:1,000. After incubation, tissue sections were immunostained by the avidin-biotin complex method (Vector Laboratories, Burlingame, CA). They were subsequently developed with 3,3′-diaminobenzidine-4HCl (Vector Laboratories) and 0.05 ml of a 5% solution of hydrogen peroxide in Tris-HCl buffer. Sections were dehydrated in a graded ethanol series and coverslipped with DPX. For double immunostaining, coronal sections were incubated with the solution mixed with both anti IL-1β antibody and mouse anti-GFAP antibody (Sigma, St. Louis, USA) or anti-rat Mac-1 (CD11b) antibody (Boehringer Mannheim, Mannheim, Germany) 2 overnight at 4°C. These sections were incubated with Cy3-labeled donkey anti-goat IgG antibody (1:1000, Amersham International Pte, Buckinghamshire, UK) for 2 hr at room temperature and incubated with Alexa 488-labeled goat anti mouse or anti rat IgG antibody (Molecular Probes, Eugene, OR, USA) for 2 hr at room temperature. The sections were mounted and examined with an Olympus AX-70 fluorescence microscope (Olympus Co., Ltd., Tokyo, Japan). To verify the specificity of staining, the sections were (1) incubated with non-immune serum instead of the primary antibody, (2) incubated with the primary antibody without the second and third antibodies, and (3) incubated with the primary antibody that had...
III. Results

The blood pressure was immediately dropped to levels to zero after cardiac arrest. With commencement of resuscitation after 5 minutes’ ischemia, the blood pressure rose promptly. All successfully resuscitated animals showed a quick rise in blood pressure and returned to control levels at 10 min after resuscitation. The physiological data during operation are as follows. No seizures were observed at any time in these animals. Pre-operative blood gas data (n=4) at 10 min before cardiac arrest were pH 7.33±0.02, PaO$_2$ 121.20±7.44 mmHg, PaCO$_2$ 40.12±1.91 mmHg, rectal temperature 36.38±0.18°C, O$_2$ saturation (SAT) 98.06±0.51%. Post-operative data (n=4) at 10 min after cardiac arrest were pH 7.23, PaO$_2$ 164.08±19.13 mmHg, PaCO$_2$ 44.49±2.76 mmHg, rectal temperature 35.88±0.20°C, O$_2$ SAT 98.78±0.25%. Blood pH temporarily decreased after global ischemia.

Northern blot hybridization analysis in IL-1β gene in the hippocampus demonstrated that the induction of IL-1β mRNA in mice was observed as early as 30 min after ischemia-reperfusion and reached a peak at 30 min (Fig. 1). IL-1β mRNA level was comparable, ranging 2.8-fold higher than in sham-operated control mice (Fig. 1). But there could not be seen in the induction of IL-1β mRNA in the cerebral cortex after ischemic insult. In the brains of non-operated and sham-operated mice, gene expression of IL-1β mRNA could be detected but very weak by using in situ hybridization histochemistry (Fig. 2A). In the ischemia-reperfusion mice, IL-1β mRNA induction began as 1 hr after reperfusion (Fig. 2B) in the hippocampal region and peak levels of IL-1β mRNA density were found 3 hr after reperfusion (Fig. 2C)
and gradually declined with time (Fig. 2D). Very few signals could be detected 4 days after reperfusion. From statistical analysis (t-test, \( p < 0.01 \)), the most intense signals of IL-1\( \beta \) mRNA was detected at 3 hr after ischemia-reperfusion (Table 1).

In nonischemic hippocampal tissue, low levels of IL-1\( \beta \)-like immunoreactivity were detected (Fig. 3A). 1 day after ischemia, IL-1\( \beta \)-like immunoreactivity was increased in glial cells in the hippocampal region (Fig. 3B). At 2 day after ischemia, many glial cells showing IL-1\( \beta \)-like immunoreactivity were increased and the pyramidal neurons began to display condensation and segregation of chromatin toward the nuclear envelop (Fig. 3C). By 4 day after ischemia, amoeboid microglia and astrocytes were abundant near the hippocampal fissure and were highly immunoreactive for IL-1\( \beta \) (Fig. 3D). The increase in IL-1\( \beta \)-like immunoreactivity seemed to be increase in number of astrocytes and microglia in the hippocampal region. During 1–7 days after ischemia-reperfusion, no IL-1\( \beta \)-like immunoreactivity was observed in both the CA1 and CA2 pyramidal neurons and the dentate gyrus granule neurons.

The number of microglial cells and astrocytes were gradually increased during 2–7 days after ischemia-reperfusion. These cells were small, with elongated cell bodies, and had one or more thick and thin processes and some of them had numerous short secondary branches along their length, suggesting glial cells. Therefore we used double-labeling immunohistochemistry to determine the cell type of IL-1\( \beta \) expressing cells in the hippocampal region. As a result both microglial cells (CD11b-positive) and astrocytes (GFAP-positive) showed IL-1\( \beta \) immunoreactivity at 2 and 4 days after ischemia-reperfusion (Fig. 4). No other cells including neurons and endothelial cells in the hippocampus were detected IL-1\( \beta \)-like immunoreactivity during ischemia-reperfusion.

**IV. Discussion**

We have used here the cardiac-arrest model, which was first introduced in rat by Korpatchev et al. [10]. The characteristics and the benefits of this cardiac-arrest model are that it is less invasive, a simpler operation, and produces a more

### Table 1. Number of IL-1\( \beta \) mRNA expressing cells in the hippocampus after ischemia-reperfusion

<table>
<thead>
<tr>
<th>time course</th>
<th>0 hr</th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>1 day</th>
<th>2 day</th>
<th>4 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>21.92</td>
<td>52.04**</td>
<td>160.78*</td>
<td>112.11*</td>
<td>68.67*</td>
<td>54.37*</td>
<td>23.35</td>
</tr>
<tr>
<td>S.D.</td>
<td>5.24</td>
<td>8.00</td>
<td>33.68</td>
<td>21.75</td>
<td>4.92</td>
<td>2.95</td>
<td>2.24</td>
</tr>
</tbody>
</table>

**\( p < 0.01 \)**, *\( p < 0.0001 \)**

Values are presented as mean and SD. 0 hr data was control and was compared with others data. The peak level was 3 hr after reperfusion. The data of 3 hr, 6 hr, 1 day, 2 days was evident difference value (\( p < 0.0001 \)). The same level of control was 4 days after ischemia. Value for each of 5 numbers were averaged for each subject. The standard deviation (SD) values showed lower column.

![Fig. 3](A) Expression of IL-1\( \beta \) like immunoreactivity in glial cells in hippocampal region after global ischemia. Time course of day 0 (A), day 1 (B), day 2 (C), day 4 (D) after ischemia. Day 0 was observed a few IL-1\( \beta \) like immunoreactive cells in the hippocampal region. At 1, 2 and 4 days after reperfusion, IL-1\( \beta \) like immunoreactive cells became more evident. The most counts of IL-1\( \beta \) like immunoreactive cells was at 2 days after reperfusion.
uniform global ischemia than the four-vessel occlusion model. The operative mortality is relatively low (less than 10%), and the resuscitated animals remain physiologically stable and available for extended observations for indefinite periods of time [5, 9, 22]. The morphological features of the ultrastructural apoptotic changes appeared nuclear and cytoplasmic condensation, cell shrinkage, and nuclear fragmentation as observed in the rat and gerbil hippocampal CA1 region [5, 16, 18, 22]. Apoptotic changes were demonstrated not only in CA1 and CA2 of hippocampus but also in dentate gyrus [22].

The present study revealed that the peak level of IL-1β mRNA expression and IL-1β-like immunoreactivity was at 3 hr and 2 days respectively. It has been reported that the intense gene expression of IL-1β mRNA is at 3 to 4 hr after the rat brain ischemia [1, 23]. Minami et al. [15] have reported that the gene expression of IL-1β mRNA has two peaks at 30 to 240 min after the transient forebrain ischemia by northern blot analysis. Liu et al. [12] have reported that IL-1β mRNA expression appears peak at 12 hr in the ischemic lesion after permanent middle cerebral artery occlusion. As to the IL-1β immunoreactivity in the brain ischemia, Davies et al. [4] have reported that IL-1β immunoreactive microglial cells appear peak at 24 to 48 hr in the ischemic lesion. Legos et al. [11] have reported that the peak of IL-1β protein expression is at 3 to 5 days in the ischemic lesion in the permanent occlusion model. These results and the present study reveal that there may be time lag between the expression of IL-1β mRNA and protein level. The time course of the expression of IL-1β and its transcript has not been completely known in the hippocampal tissue after the brain ischemia. There is a possibility that IL-1β is expressed in different cells during the period of ischemia reperfusion. It has been shown that IL-1β mRNA is expressed in the activated microglia and macrophages artery the brain ischemia [1]. The postischemic induction of glial IL-1β protein is a result of the upregulation of mRNA expression [21]. We found that IL-1β-like immunoreactivity was detected in astrocytes and microglia after brain ischemia reperfusion by double-labeling immunohistochemistry. It seems that IL-1β immunoreactivity appears earlier in microglial cells than astrocytes in the hippocampal tissue after the ischemia, but further studies are needed to clarify in detail.

We found marked increase in IL-1β mRNA expression and its immunoreactivity in the mouse hippocampus after ischemia-reperfusion and IL-1β-like immunoreactivity was detected in astrocytes and microglia by double-labeling immunohistochemistry. Despite the observation that apoptotic cell death occurs in the CA1 and CA2 pyramidal neurons and dentate gyrus granule neurons [22], IL-1β expression was not altered in neurons in these areas. Instead, IL-1β immunoreactivity increased in astrocytes and microglia after ischemia-reperfusion. There have been many studies showing a marked increase in IL-1β mRNA expression in the rat brain following cerebral ischemia [1, 23]. It is suggested that IL-1β mRNA-positive cells are macrophages or fully activated microglia [1]. IL-1β mRNA was also reported to be expressed in the astrocytes when stimulated with lipopolysaccharide [6]. According to Davies et al. [4], the IL-1β protein expression was observed within a few hours after the onset of ischemia. The discrepancy concerning the IL-1β protein expression with our data may be originated from the difference of animal species and degree of ischemia. These findings suggest that the induction of IL-1β by ischemia-reperfusion might be caused in the microglia and astrocytes. These observations are also supported by in vitro study that astrocytes contributed to the cytokines, such as IL-1α, TNF-α, IL-6, released under ischemia [26].

Potential cellular targets for IL-1β in the early post-injury period include microglia, astrocytes, endothelial cells, macrophages, and neurons. IL-1β released by microglia and astrocytes after ischemia could recruit additional microglia locally, thereby contributing to increased cell killing of healthy and/or compromised neurons. It is reported that intra-cerebral ventricle infusion of IL-1β receptor antagonist significantly reduces neuronal damage that occurs in focal models of ischemia as well as after excitotoxic lesions [19]. Treatment with the functional IL-1 antagonist zinc protoporphyrin resulted in acute reduction of ischemia-induced cerebral edema [24]. In spite of these findings demonstrating the
adverse influence of IL-1β on neuronal survival, IL-1β has been considered to be neuroprotective [14]. Thus, IL-1β released by microglia, astrocytes, or macrophages could stimulate to proliferate additional microglia and astrocytes locally, thereby contributing to increase neuronal cell death. Further studies are needed to clarify the functional significance of IL-1β in brain ischemia.

In summary, we have provided evidence for an increase in IL-1β and its transcript in the hippocampus after ischemia-reperfusion. Furthermore, we have found that increase in IL-1β-like immunoreactivity was localized selectively in microglia and astrocytes. IL-1β, secreted by microglia and astrocytes, may play very important role in neuronal damage directly or indirectly through mediation of inflammation in neuronal tissue.

V. Acknowledgments

We thank Dr. Jerome Maderdrut at Tulane University Medical Center for his many valuable suggestions and critical review of this manuscript. We also thank Dr. Takiko Oguro at Showa University School of Pharmacy for the kind gift of the IL-1β cDNA for probe. This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan.

VI. References