Induction of Nerve Injury-Induced Protein 1 (Ninjurin 1) in Myeloid Cells in Rat Brain after Transient Focal Cerebral Ischemia

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Nerve injury-induced protein-1 (Ninjurin-1, Ninj1) was initially identified as a novel adhesion molecule in rat sciatic nerve and to be up-regulated in neurons and Schwann cells of distal nerve segments after nerve transection or crush injury. Recently, Ninj1 was found to act as a modulator of cell migration, angiogenesis, and apoptosis. Accumulating evidence indicates that innate immune response plays beneficial and deleterious roles in brain ischemia, and the trans-endothelial migration of blood-derived immune cells is key initiator of this response. In the present study, we examined the expression profile and cellular distribution of Ninj1 in rat brain after transient focal cerebral ischemia. Ninj1 expression was found to be significantly induced in cortical penumbras 1 day after 60 min of middle cerebral artery occlusion (MCAO) and to increase gradually for 8 days and then declined. In infarction cores of cortices, patterns of Ninj1 expression were similar to those observed in cortical penumbras, except induction was maintained for 10 days. At 1 day post-MCAO, Ninj1 inductions were detected mainly in neutrophils and endothelial cells in both infarction cores and penumbras, but reactive macrophages were the major cellular expressers of Ninj1 at 4 days post-MCAO. Expressional induction in reactive macrophages was maintained in infarction cores after 12 days post-MCAO but not in penumbras. These dynamic expressions of Ninj1 in different immune cells at different times suggest that this protein performs various, critical roles in the modulation of acute and delayed immune responses in the postischemic brain.

Key words: Ninjurin 1, MCAO, myeloid cells, induction

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shown that Ninj1 enhanced macrophage motility and consequent extravasation by modulating protrusive membrane dynamics [6]. In the ischemic brain, damaging processes are progressed through a complex series of pathophysiological events, such as, energy failure, excitotoxicity, oxidative stress, inflammation, and apoptosis [7]. Whereas excitotoxicity and Zn\(^{2+}\) toxicity contribute to acute and massive neuronal death in the ischemic core [8], apoptosis and inflammation are main causes of delayed neuronal death in the penumbra [9]. Since in penumbra, blood flow reduction is less severe than in the ischemic core, brain cells in penumbra are allowed to respond to damage and transduce signals and synthesize new proteins, which have beneficial or detrimental effects [9]. Therefore, brain damage can be amplified by inflammatory responses in penumbra [7] or be repaired by accelerated re-constructional activities [10].

Since, Ninj1 has been reported to participate in cell migration, angiogenesis, and apoptosis regulation in various diseases of the central nervous system (CNS), this protein might play an important role in delayed damaging process and/or in subsequent repair and regeneration process in the postischemic brain by modulating leukocyte infiltration, inflammation, and angiogenesis. As a first step to investigate the function of Ninj1 in brain ischemia, we examined the expression of Ninj1 using a middle cerebral artery occlusion (MCAO) murine model of stroke. Immunoblotting and triple fluorescent immunohistochemistry were performed to elucidate the spatiotemporal expression of Ninj1 in the postischemic brain with focus on differential expressions of Ninj1 in ischemic cores and peri-infarct regions (penumbras) of ipsilateral (ischemic) hemispheres.

MATERIALS AND METHODS

Surgical procedure

Male Sprague-Dawley rats were housed under diurnal lighting conditions and allowed food and tap water ad libitum. All animal studies were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health and complied with ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). The animal protocol used in this study was reviewed and approved by the INHA University-Institutional Animal Care and Use Committee (INHA-IACUC) with respect to ethicality (Approval Number INHA-120410-137). MCAO conducted as previously described [11]. In brief, male Sprague-Dawley rats (250–300 g) were anesthetized with 5% isoflurane in a 30% oxygen/70% nitrous oxide mixture and anesthesia was maintained during procedures using 0.5% isoflurane in the same gas mixture. Occlusion of the right middle carotid artery was induced for 1 h by advancing a nylon suture (4-0; AILEE, Busan, South Korea) with a heat-induced bulb at its tip (~0.3 mm in diameter) along the internal carotid artery for 20–22 mm from the bifurcation of the external carotid artery and this was followed by reperfusion for up to 14 days. The left femoral artery was cannulated for blood sampling to determine pH, PaO\(_2\), PaCO\(_2\), and blood glucose concentrations (I-STAT; Sensor Devices, Waukesha, WI). Regional cerebral blood flow (rCBF) was monitored using a laser Doppler flowmeter (Periflux System 5000; Perimed, Jarfalla, Sweden). A thermoregulated heating pad and a heating lamp were used to maintain a rectal temperature of 37.0±0.5°C during MCAO. Animals were randomly allocated to two groups, that is MCAO group (n=27) or a sham control group, animals in sham group were operated upon in an identical manner but the right MCA was not occluded (n=3). In general, mortality was not observed during surgery, but mortality after surgery was 11.1% (3/27).

TTC staining

Rats were decapitated at 2 days post-MCAO, and whole brains were dissected coronally into 2-mm slices using a metallic brain matrix (RBM-40000, ASI, Springville, UT). Slices were immediately stained by immersing them in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 15 min and then fixed in 4% paraformaldehyde. The TTC-stained slices were visualized using a Nikon D40 DSLR (Nikon, Tokyo, Japan).

Immunofluorescence staining

Animals were sacrificed at prescribed times after MCAO and brains were fixed in 4% paraformaldehyde by transcardiac perfusion and then post-fixed in the same solution overnight at 4°C. Brain sections of 1 and 4 days post-MCAO (20 μm) were obtained using a vibratome. Brain of 12 days post-MCAO were embedded in paraffine, and 10 μm-thick sections were prepared. The sections were deparaffinized in zylene and rehydrated through graded series of ethanol. Immunological staining was performed as previously described [12]. Primary antibodies were diluted 1:300 for anti-ionized calcium binding adaptor molecule-1 (Iba-1) (Wako Pure Chemicals, Osaka, Japan), 1:200 for anti-myeloperoxidase-1 (MPO-1) (Abcam, Cambridge, UK), 1:200 for anti-neuronal nuclei (NeuN) (BD Transduction Laboratories, San Jose, CA), 1:200 for anti-glial fibrillary acidic protein (GFAP) (BD Transduction Laboratories, San Jose, CA), 1:200 for anti-endothelial Cell (RECA) (AbDSerotec, Oxford, UK), 1:200 for anti-CD45 (BD Transduction Laboratories, San Jose, CA), 1:200 for mouse monoclonal anti-nerve injury-induced protein 1 (Ninjurin-1) (Merck Millipore Corporation, Darmstadt,
Germany), and 1:200 for rabbit polyclonal anti-Ninjurin1 (Abcam, Cambridge, UK) antibodies. After washing with PBS three times, sections were incubated with 1:200 of FITC-conjugated anti-mouse or -rabbit IgG (Jackson ImmunoRes Lab, West Grove, PA) for anti–ninjurin-1, 1:200 of rhodamine-conjugated anti-rabbit IgG (Jackson ImmunoRes Lab, West Grove, PA) for anti-Ilba-1, MPO-1, NeuN, and 1:200 of rhodamine-conjugated anti-mouse IgG (Jackson ImmunoRes Lab, West Grove, PA) for anti-GFAP, RECA, and CD45 in PBS for 30 min at room temperature and examined under Zeiss LSM 510 META microscope (Carl-Zeiss-Strasse, Oberkochen, Germany). Experiments were repeated at least three times and representative images are presented.

**DAPI staining**

Brain sections were prepared as described above for immunohistochemistry. Following incubation with 0.2% Triton X-100 in PBS for 5 min, sections were incubated with DAPI (1 μg/mL; 4',6-diamidino-2-phenylindole) for 15 min at room temperature, and viewed under the microscope mentioned above.

**Immunoblot analysis**

Brain samples were prepared from cortical core, cortical penumbra, striatal core, striatal penumbra as indicated regions in Fig. 1A from MCAO and sham groups, and proteins (10 μg) were purified, separated in 10% sodium dodecyl sulfate-polyacrylamide gels, and transfer to membranes. After blocking membranes with 5% non-fat milk for 1 hr, they were incubated with primary antibodies for anti-Ninjurin-1 (1:2000, BD Transduction Laboratories, San Jose, CA) and anti-α-tubulin (1:5000, Merck Millipore Corporation, Darmstadt, Germany) overnight at 4°C. The next day, blots were detected using anti-mouse HRP-conjugated secondary antibody (1:2000; Santa Cruz Biotechnology) and a chemiluminescence kit (Roche, Basel, Switzerland).

**RESULTS**

**Ninjurin-1 protein expression increased differentially in penumbra and core of the postischemic rat brain**

To examine the expression pattern of Ninj1 in the postischemic brain, immunoblotting and triple fluorescent immunohistochemistry were conducted. For immunoblot analysis, protein samples were prepared from cortical penumbras, cortical cores, striatal penumbras, and striatal cores (Fig. 1A) at 1, 4, 6, 8, 10, and at 14 days after 60 min of MCAO. In the cortical penumbras, the induction of Ninj1 was detected at 1 day post-MCAO and its level gradually increased until day 8 and then decreased thereafter (Fig. 1B). In cortical cores, Ninj1 protein was induced at 1 day post-MCAO, and this level was maintained until day 10 (Fig. 1C). In striatal cores and penumbras, Ninj1 was inducted in a manner similar to that observed in cortex, although levels of induction were slightly higher (Fig. 1D and E). These results describe the dynamic nature of Ninj1 expression in the postischemic brain, particularly, in the penumbra regions of ipsilateral hemispheres.

**Ninj1 was induced in neutrophils and in endothelial cells in cortical penumbras and cores at 1 day post-MCAO**

The observed dynamic expression of Ninj1 protein, in cortices
and striata prompted us to identify the cell types expressing Ninj1 in different regions at different times. Since cortex and striatum revealed similar temporal expression profiles, an immunohistochemical study was conducted with focus on cortices (Fig. 1A). To identify the cell types expressing Ninj1 at 1 day post-MCAO, triple immunofluorescence staining was performed using anti-Ninj1 antibody and antibodies specific for neurons (NeuN), astrocytes (GFAP), microglia (Iba-1), neutrophils (MPO-1), and endothelial cells (RECA-1), and using DAPI. In sham controls, Ninj1+ cells were rarely detected in any brain region (data not shown). However, at 1 day post-MCAO, Ninj1 immunoreactivity was markedly increased in cortical penumbras (Fig. 2A). Most of Ninj1+ cells were merged with MPO-1+ cells (Fig. 2B) or RECA-1+ cells (Fig. 2C), indicating that Ninj1 protein was induced in neutrophils and endothelial cells in cortical penumbras. The majority of Ninj1+/MPO-1+ cells were observed in brain parenchyma (Fig. 2B), and some were detected within blood vessels attached to vessel walls (data not shown). Ninj1 induction was also detected in Iba-1+ cells, but Ninj-1 levels were low and few merged cells were observed (Fig. 2D). However, they were rarely merged with NeuN+ or GFAP+ cells (Fig. 2E and F). Similar observations were made in cortical cores, but numbers of Ninj1+/Iba1+ were higher (Fig. 2J) and those of Ninj1+/MPO-1+ (Fig. 2H) and Ninj1+/RECA-1+ (Fig. 2I) cells were fewer than in cortical penumbras (Fig. 2B–D). These

Fig. 2. Expression of Ninj1 in cortices of ischemic hemispheres of rat brains at 1 day post-MCAO. Coronal brain sections were prepared at 1 day post-MCAO and immunofluorescent stained with anti-Ninj1 antibody (A, G) or anti-Ninj1- and anti-MPO-1 (B, H), anti-RECA (C, I), anti-Iba-1 (D, J), anti-NeuN (E, K), or anti-GFAP (F, L), and DAPI. The pictures shown are representative of three independent experiments. The insets in A-C, G-J are high magnification photographs of each image. Scale bars in A and G represent 1 mm, those in B-F and H-L represent 20 µm, and those in insets represent 10 µm.
results indicate that at 1 day post-MCAO, Ninj1 was induced mainly in neutrophils and endothelial cells in penumbras and in neutrophils and microglia in cores of ischemic hemispheres.

**Ninj1 protein was induced in reactive macrophages in both penumbras and cores at 4 days post-MCAO**

At 4 days post-MCAO, Ninj1 immunoreactivity was markedly increased in infarct penumbras (Fig. 3A) and cores (Fig. 3G). At this time, the numbers of both CD45+ (pan-leukocyte marker) and Iba-1+ (a marker of cells of myeloid origin) cells were remarkably increased and these cells were accumulated in infarction cores and penumbras. Iba-1+ cells were larger than those observed at 1 day post-MCAO (Fig. 2D and J) and were amoeboid in shape, indicating an activated state (Fig. 3C and I). Ninj1 immunoreactivity was found to co-localize with these Iba-1+ and CD45+ cells in both cortical penumbras (Fig. 3B and C) and cores (Fig. 3H and I), indicating that Ninj1 was mainly induced in activated microglia/macrophages at 4 days post-MCAO. The Ninj1+ cells were also merged with RECA-1+ cells (Fig. 3D) but Ninj1+/MPO-1+ cells were rarely detected (data not shown). Similarly, Ninj1 immunoreactivity was hardly detected in NeuN+ and in GFAP+ cells (Fig. 3E and F). Similar results were obtained at 8 days post-MCAO (data not shown).

**Ninj1 remained in reactive macrophages in cortical cores at 12 days post-MCAO**

Ninj1 levels were gradually decreased in cortical penumbras from 8 days to 14 days post-MCAO, but the levels were maintained in cortical cores at 10 days post-MCAO and decreased thereafter (Fig. 1B and C). To determine whether there are changes in cellular distribution of Ninj1 in cortex at 12 days post-MCAO, we conveyed immunohistochemical studies. In cortical penumbras, Ninj1 immunoreactivity was mainly detected in a few CD45+ cells but hardly detected in Iba-1+ cells at 12 days post-MCAO (Fig. 4B and C). Since, it has been known that in contrast to macrophages, microglia expresses low or intermediate levels of the leukocytes.

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**Fig. 3.** Expression of Ninj1 protein in cortices of the ischemic hemispheres of the rat brain at 4 day post-MCAO. Coronal brain sections were prepared at 4 days post-MCAO and immunostained with anti-Ninj1 antibody (A, G) or anti-Ninj1- and anti-CD45 (B, H), anti-Iba-1 (C, I), anti-anti-RECA (D), anti-NeuN (E), or anti-GFAP (F) and DAPI. The pictures shown are representative of three independent experiments. The insets in A-D, G-I are high magnification photographs of each image. Scale bars in A and G represent 1 mm, those in B-F and H-I represent 20 µm, and those in insets represent 10 µm.
marker CD45. Ninj1 was expressed in reactive macrophages but not in resident microglia in cortical penumbras (Fig. 4B and C). On the contrary, in cortical cores, Ninj1 immunoreactivity was detected both in CD45+ and Iba-1+ cells, which might be reactive macrophages, and the numbers of merged cells were significantly higher than in cortical penumbras (Fig. 4H and I). Importantly, Ninj1/Iba-1+ cells displayed ramified morphology (Fig. 4C), indicating that they were in resting state. Ninj1 expression was detected in endothelial cells, but hardly detected in neurons and astrocytes (Fig. 4D-F).

**DISCUSSION**

The present study described the temporal profiles and cellular distributions of Ninj1 in rat brain after transient focal ischemia. As it was reported by Araki and Milbrandt (1996) [1], we found relatively low levels of Ninj1 expression in normal and sham-operated animals. However, the expression pattern of Ninj1 was found to change rapidly in the peri-infarct region (penumbra) of the posts ischemic brain, which is viewed as a physiologically and functionally active area, and thus, a salvageable region in the postischemic brain [13]. Ninj1 protein expression was up-regulated from 1 day post-MCAO, and then gradually increased until 8 days in both infarction cores and penumbras. In penumbras, its levels decreased from day 8, but in infarction cores, its levels were maintained until day 10 (Fig. 1). In addition, Ninj1 was detected in specific cell types at different times, suggesting that it performs different critical roles at different times in the postischemic brain.

In the normal rat brain, Ninj1 is detected in meninges, choroid plexus, and the parenchymal perivascular region [5]. However, in the present study, at 1 day post-MCAO, Ninj1 was detected mainly in MPO-1+ cells (neutrophils) and RECA+ cells (endothelial cells). Neutrophils are the first blood-derived immune cells to accumulate around injured brain tissue after ischemic stroke, and contribute to BBB disruption, cerebral edema, and aggravation...
of tissue injury [14]. Rolling and adhesion of neutrophils begin at 2 hrs post-ischemia, and their accumulations within vessels near injured brain regions occurs 6 to 8 hrs later, and infiltration of neutrophils into brain tissue peaks at 24 to 48 hrs post-ischemia [15]. Considering that neutrophil infiltration into the ischemic brain is associated with increases in adhesion molecule levels, Ninj1 induction in neutrophils and in endothelial cells at 1 day post-MCAO coincided well with neutrophil infiltration kinetics. Therefore, these observations suggest that Ninj1 might contribute to neutrophil infiltration and to the aggravation of brain injury in the postischemic brain. In the present study, we carried out triple fluorescent staining, and DAPI staining revealed that Ninj1+ cells were mainly neutrophils and not monocytes or macrophages because of the multi-lobulated nuclear morphologies. However, since Ninj1 has been known to promote the migration of other myeloid and T cells [4, 5, 6, 16], it is possible that Ninj1 mediates the migrations of other immune cells at 1 day post-MCAO. Furthermore, the induction of Ninj1 in Iba-1+ cells, although at low levels, implies that Ninj1 might promote the infiltration of monocyte from blood and/or the pro-inflammatory activation of these cells. This topic needs further study.

At four days after MCAO, Ninj1 was induced in reactive macrophages in infarct cores and in penumbras. Macrophages are viewed as a deleterious component that aggravates ischemic injury and exacerbates secondary progression of ischemic lesions. It has been reported that reactive macrophages in the ischemic brain consist of two populations of cells; one originating from the proliferation of resident microglia and another derived from circulation, and those derived from circulation have been shown to be cytotoxic [17]. It has also been shown that they are recruited from 2 days post-MCAO and remain abundant in brain tissue to day 7 [18]. Although the CCL2/CCR2 axis is a well-known mediator of monocyte/macrophage recruitment [19], it is possible that Ninj1 also participates in the infiltration of blood-borne monocytes into injured brain tissues. In addition to its well-known function in the trans-endothelial migration of immune cells [4, 5, 6, 16], we hypothesize that Ninj1 might function as a proinflammatory mediator and activate macrophages. Further studies are required to determine whether this function conveys in a cell autonomous manner and to identify molecular mechanisms responsible.

It is interesting to note that at 12 days post-MCAO, Iba-1+ cells in the cortical cores were amoeboid shape, indicating activated state, in contrast, they were ramified shape in penumbras and Ninj1 expression was detected only in the cores. Based on these observations, we speculate that reduced Ninj1 immunoreactivity in reactive macrophages from 8 days post-MCAO, in particular, in penumbra might reflect the resolution of delayed inflammation. Although Ninj1 was found to be up-regulated in neurons and Schwann cells after an experimental peripheral nerve injury and contribute to Schwann cell-mediated neurite outgrowth and repair [20], we did not find Ninj1 expression in neurons or oligodendrocytes in the normal or MCAO rat brain. Further studies are required to investigate whether it is due to the differences between peripheral and central nervous systems or to the differences in specific pathological conditions.

In the present study, we describe the dynamic expression of Ninj1 in the postischemic brain, in particular, in cortical penumbras. This time-dependent expression of Ninj1 in various immune cells at different time points suggests Ninj1 plays differential and critical roles in the modulations of acute and delayed immune responses in the postischemic brain.

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