Autocrine Activation of Cerebral Microglia by Allograft Inflammatory Factor-1

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Research

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Abstract

Allograft inflammatory factor-1 (AIF-1) is a marker for activated microglia. Unilateral common carotid artery occlusion (UCCAO) was conducted to elucidate mechanisms that regulate AIF-1 expression in C57BL/6 male mice. Immunohistochemical reactivity of microglia against anti-AIF-1 antibody was increased significantly in the brain of this model. The increased AIF-1 production was further confirmed by ELISA using brain homogenate. Real-time PCR demonstrated that the increased AIF-1 production was regulated at the transcriptional level. Serum AIF-1 levels were further examined by ELISA and marked increase was observed on Day 1 of UCCAO. To examine the influence of AIF-1, immunohistochemical staining was performed and revealed that the immunoreactivity against anti-Iba-1 antibody was significantly increased in various organs. Among them, the accumulation of Iba-1$^+$ cells were observed prominently in the spleen. Intraperitoneal injection of minocycline, a potent microglia inhibitor, reduced the number of Iba-1$^+$ cells suggesting microglia activation-dependent accumulation. Based on these results, AIF-1 expression was further examined in the murine microglia cell line MG6. AIF-1 mRNA expression and secretion were up-regulated when the cells were cultured under hypoxic condition. Importantly, stimulation of the cells with recombinant AIF-1 induced the expression of AIF-1 mRNA. These results suggest that increased AIF-1 production by microglia in cerebral ischemia regulate the AIF-1 mRNA expression at least in part by an autocrine manner.

Introduction

Allograft inflammatory factor-1 (AIF-1) is a calcium binding protein containing a partially conserved EF-hand helix-loop-helix domain [1]. AIF-1 was originally cloned from activated macrophages accumulating in the arterial wall during chronic rejection of atherosclerotic allogenic heart grafts in rats [1]. Since then, three other proteins have been identified that share identical amino acid sequence with AIF-1. These are ionized calcium binding adaptor molecule-1 (Iba-1), microglia response facor-1 (MRF-1), and daintain [2]. In addition, considerable sequence similarity exists for interferon-responsive transcript-1 (IRT-1), balloon angioplasty-responsive transcript-1 (BART-1), and others [2]. These molecules are designated as AIF-1 family proteins.

In an attempt to identify interleukin-2 (IL-2) inducible genes in rat brain, Imai et al. cloned the Iba-1 gene by differential display screening [3]. Using the polyclonal anti-Iba-1 antibody (Ab), microglia, but not other cell populations in brain primary culture, was demonstrated to express Iba-1 antigen [4]. Although the expression level is low in resting microglia, it is strongly enhanced in cases of brain infarction [5]. Moreover, enhanced expression is accompanied by morphological changes in the microglia, a hallmark of microglia activation [6]. Based on these observations, Iba-1 has been used as a marker for activated microglia.

AIF-1 is highly homologous among some species and contains the -KR-KK-GKR- motif, a characteristic sequence for peptide hormone precursors [7]. Glucose-induced insulin secretion has been demonstrated to be affected by the intraperitoneal injection of AIF-1 [7], which suggests that AIF-1 as a secretory

protein. Serum AIF-1 concentration is increased in some experimental and disease conditions such as dextran sulfate sodium-induced colitis [8], diabetic nephropathy [9], ischemia reperfusion injury after liver transplantation [10], and experimental autoimmune neuritis [11]. Moreover, AIF-1 serum concentration is correlated with clinical and biochemical metabolic parameters in humans [12]. In spite of these observations, serum AIF-1 level after brain ischemia have never been investigated. Enhanced immunoreactivity against anti-Iba-1 Ab is a hallmark of microglia activation, however, whether this phenomenon is accompanied with the increased mRNA expression has never been investigated.

In the present study, we examined AIF-1 mRNA expression, serum AIF-1 levels, and Iba-1^+ cell distribution in the organs of mice after brain ischemia induced by permanent unilateral common carotid artery occlusion (UCCAO) and in a mouse microglia cell line.

Materials And Methods

Animal

Mice (6-week-old male C57/BL6JJcl) used in this study were obtained from CLEA (Tokyo,Japan). The experimental protocols used in this study were approved by the Nihon University School of Dentistry Animal Ethical Committee and conducted according to the legal requirement (AP19DEN033-1).

Unilateral common carotid artery occlusion (UCCAO)

The mice were intraperitoneally (i.p.) injected with a mixture of 0.25 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. The right CCA was exposed and ligated with a silk suture, and the surgical wound was closed.

For minocycline experiments, minocycline (MilliporeSigma, St. Louis, MO, USA) was suspended in PBS and injected (i.p.; 50 mg/kg body weight) at 2 h before and right after UCCAO.

Cell culture and treatment

The mice microglia cell line MG6 was obtained from the RIKEN BRC cell bank [13, 14]. The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum, 10 μg/ml insulin (MilliporeSigma, St. Louis, MO, USA) 0.1 mM 2-mercaptoethanol in a 5% CO_2 incubator. The cells (2×10^5/12 well plate) were incubated in hypoxic condition (5% O_2, 5% CO_2, Sugiyamagiken, Tokyo, Japan) for 6, 12, and 24 h. Culture supernatants and total RNA were harvested and subjected to ELISA and real-time polymerase chain reaction (PCR), respectively. For recombinant AIF-1 (enQuire BioReagents, Littleton, CO, USA) stimulation, the cells were cultured with or without 100 ng/ml of recombinant AIF-1 for 6 h [15]. Total RNA was subjected to real-time PCR.

Real time-PCR

Total RNA was purified from excised brain or cultured MG6 cells using RNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized with Superscript III™ Reverse Transcriptase (Invitrogen, San Diego, CA, USA) and subjected to real-time PCR. Real-time PCR was performed using Thermal Cycler Dice ® Real Time System TP800 (TaKaRa, Tokyo, Japan) with TB Green ® Premix Ex Taq™II (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan). The primers used are as follows.For AIF-1 5'-GTCCTTGAAGCGAATGCTGG-3' (forward) and 5'-CATTCTCAAGATGGCAGATC-3' (reverse). For β-actin 5'-GGTCAGAAGGACTCCTATGTGG-3' (forward) and 5'-TGTCGTCCCAGTTGGTAACA-3' (reverse).

AIF-1 measurement

On Day 0, 1, 3, and 5 after UCCAO, peripheral blood was collected and incubated for 30 min at 37℃ and then at 4℃ for 18 h. The samples were centrifuged at 1,200 x g for 20 min and the supernatants were preserved at -80℃. The brain tissue was minced with scissors in a cell lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% TritonX-100) and then homogenized using a Dounce homogenizer (Wheaton Industries, Millville, NJ, USA). The samples were centrifuged (12,000 x g, 5 min) and the supernatants were harvested. MG6 cell culture supernatants were harvested after culture under hypoxic condition. The samples were centrifuged (12,000 x g for 3 min) and the supernatants were harvested. AIF-1 concentration was measured using ELISA kit (MyBioSource, San Diego, CA, USA).

Histopathological experiments

The mice were transcardially perfused with 4% paraformaldehyde and the organs (spleen, lung, liver, kidney, and brain) were excised and further fixed with the same fixative for 18 h. The organs were sliced and embedded in paraffin. Four-micrometer-thick sections were prepared, deparaffinized in xylene, rehydrated with 100% ethanol, and subjected to HE staining. For immunohistochemical staining, the sections were incubated with Dako Proteinase K (Agilent Technologies, Santa Clara, CA, USA) for 20 min at room temperature (RT) and washed with PBS. Endogenous peroxidase activity was inactivated with 0.3% hydrogen peroxide in methanol for 20 min at RT. Non-specific binding was blocked by incubating the sections with 1% BSA-PBS for 1 h at RT. The blocking solution was removed, and the rabbit anti-mouse Iba-1 antibody (Ab) (1:100 diluted with 1% BSA-PBS, FUJIFILM Wako, Osaka, Japan) was applied and incubated for 18 h at RT. Negative controls were incubated with 1% BSA-PBS instead of the primary Ab. The sections were then incubated with Dako EnVision™+ Dual Link System-HRP (ready to use, Agilent Technologies, Santa Clara, CA, USA) for 1 h at RT. The sections were washed and were developed with freshly prepared 3,3'-Diaminobenzidine chromogen (DAB, MilliporeSigma, St. Louis, MO, USA) solution for 7 min, counterstained with hematoxylin for 30 sec, dehydrated in a series of ethanol dilutions, cleared in xylene, and mounted on glass coverslips. The images (both HE and immunohistochemical stainings) were viewed and photographed using the all-in-one microscope BZ X-810 (Keyence, Osaka, Japan). Iba-1^+ cell numbers in the spleen were counted under microscope. The area were divided into red pulp, white pulp and perivascular area of the central vein. Five different area (200 μm x 200 μm) were randomly picked up and Iba-1^+ cells were counted. The count was repeated three times by different individuals per specimen. Total of three specimens were counted.

Statistical analysis.

Male mice were used in all experiments. All data were analyzed statistically using IBM SPSS® Statistics 20 software (International Business Machines, Armonk, NY, USA). The data are expressed as the mean ± SD. The error bars represent the SD. For ELISA measurement, One-way ANOVA with Dunnett's test and One-way ANOVA with Tukey's test were used. For real-time PCR, student's t-test and One-way ANOVA with Dunnett's test were used to analyze statistical significance. Differences were considered significant at $P < 0.05$ ($*p < 0.05$, $**p < 0.01$).Sample sizes are shown in the figure legends.

Results

Microglia activation by UCCAO

To confirm the activation of microglia by UCCAO, brain sections were subjected to immunohistochemical staining using anti-Iba-1 Ab, and the corpus callosum of the right cerebral hemisphere was the main focus. In the control brain, microglia did not show strong immunoreactivity against anti-Iba-1 Ab (Fig. 1A). In contrast, significantly strong immunoreactivity was observed in the ischemic brain. Immunoreactivity peaked on Day 1 after UCCAO (Fig. 1A) and remained strong until Day 5. The spines of the immunoreactive microglia were elongated and the cell body was thickened (Fig. 1A), indicating the activation of microglia by UCCAO.

Microglial activation may be accompanied with increased AIF-1 expression. To test this hypothesis, total RNA was extracted from the right hemisphere of the brain and subjected to real-time PCR. With AIF-1 mRNA expression levels in control mice set as 1, AIF-1 mRNA expression was increased significantly in UCCAO mice (7.8 ± 2.5-fold, Fig. 1B). To further confirm the increased production of AIF-1, AIF-1 concentration in the brain extracts was measured by ELISA. As shown in Fig. 1C, AIF-1 concentration increased to 4.1 ± 0.7 ng/ml in the ischemic brain on Day 1 (control, 2.3 ± 0.3 ng/ml). Relatively high AIF-1 levels were maintained until Day 5 of UCCAO (3.0 ± 0.4 ng/ml) (Fig. 1C).

AIF-1 concentration in the serum

To examine whether increased AIF-1 expression in microglia is reflected in peripheral blood, serum was collected before and on Day 1, 3, and 5 after UCCAO and subjected to ELISA. The AIF-1 concentration was 559 ± 120 pg/ml in resting state (Fig. 2). On Day 1, AIF-1 increased to 1,253 ± 174 pg/ml and this level was maintained through Day 3 (1,093 ± 208 pg/ml) and Day 5 (862 ± 214 pg/ml) (Fig. 2). These results indicate that the AIF-1 concentration in peripheral blood increases after UCCAO.

Increased immunoreactivity against anti-Iba-1 Ab

The increased AIF-1 may have some influence on the body. To examine this hypothesis, we performed immunohistochemical analysis of various organs (Fig. 3). Compared with organs from control animals (Fig. 3, left raw), immunoreactivity was increased significantly in the ischemic organs on Day5 (Fig. 3,

middle raw). Relatively strong staining was observed in renal tubular epithelial cells in the kidney, Kupffer's cells in the liver, and bronchial and type 2 alveolar epithelial cells in the lung (Fig. 3). Immunoreactivity was strikingly increased in the spleen, thus, we further analyzed the distribution of Iba-1^+ cells in the spleen.

Distribution of Iba-1^+ cells in the spleen

The spleen was excised on Day 1, 3, 5, and 7 of UCCAO and subjected to HE staining (Fig. 4). Spleen size was drastically reduced on Day 1 compared with that of control and gradually recovered thereafter (data not shown). Histologically, the lymphoid follicle encompasses the clear bright center and the interstitial area of the white pulp was filled with the red pulp in normal spleen (Fig. 4A). In contrast, the lymphoid follicle lacked the bright center and the border of the white and red pulp became much clearer on Day 1. The capillaries in the red pulp were enlarged remarkably until Day 3 (Fig. 4B). The overall follicular structure recovered gradually, and by Day 7, the morphology of the lymphoid follicle recovered to normal (Fig. 4A).

We next attempted to examine Iba-1^+ cell staining before and after UCCAO. The Iba-1^+ immunoreactive cells were sparse in the normal spleen (Fig. 5A, left panel) and slight accumulation was observed in the red pulp area. The number of Iba-1^+ cells increased drastically (155.5 ± 7.8 cells vs control 52.7 ± 2.3 cells) on Day 5 of UCCAO and accumulated heavily in perifollicular area (Fig. 5A, right panel). As shown in Fig. 5B, the number of Iba-1^+ cells increased dramatically on Day 5. The number of Iba-1^+ cells decreased slightly over time, but the higher level was maintained through Day 7.

Minocycline significantly reduced Iba-1^+ cells

Minocycline is a potent inhibitor of microglia activity [16]. To examine whether Iba-1^+ cells accumulating in the spleen in response to UCCAO is affected by minocycline, mice were injected i.p. with minocycline and the number of Iba-1^+ cells was counted. Minocycline injection reduced the number of Iba-1^+ cells in the spleen (81.5 ± 18.2 cells) (Fig. 5C), compared to non-injected UCCAO animals (149.1 ± 14.8 cells). These results indicate that the increased production of AIF-1 in the brain was reflected to the peripheral blood and finally resulted in the increase in Iba-1^+ cells in various organs especially in the spleen.

Increased production of AIF-1

To further analyze the mechanisms underlying the increased production of AIF-1 by microglia, the murine microglia cell line MG6 was cultured under hypoxic conditions and subjected to real-time PCR. As shown in Fig. 6A, AIF-1 mRNA expression increased significantly and peaked at 24 h of hypoxic incubation (5.4 ± 1.1-fold). Increased AIF-1 secretion was confirmed at the protein level by ELISA (Fig. 6B). AIF-1 concentration in the culture supernatant increased time-dependently and reached 2.5 ± 0.2 ng/ml at 24 h (control 0.3 ± 0.02 ng/ml)(Fig. 6B).

Autocrine augmentation of AIF-1 secretion

We reasoned that the increased AIF-1 secretion might be attributed to autocrine activation by AIF-1. To confirm this hypothesis, MG6 cells were cultured in normoxic conditions with the recombinant AIF-1. The AIF-1 mRNA expression was increased by AIF-1 stimulation at 6 h (Fig. 6C, 2.4 ± 0.2-fold).

Discussion

The most widely utilized cerebral ischemia model is a middle cerebral artery occlusion (MCAO) [17]. The inflammatory reactions can be easily observed due to the defined infarct area with this model. However, the MCAO has a low survival rate, even when conducted by skilled technicians. Thus, we selected the UCCAO model [18]. The survival rate is much higher in UCCAO compared to MCAO, with few animal deaths observed. The area of the brain most affected by UCCAO is the corpus callosum, and we focused our attention on this area.

Activated microglia/macrophages have been reported to be strongly immunoreactive for anti-Iba-1 Ab [6]. Consistently, with UCCAO, intense immunoreactivity was observed in the corpus callosum, where activated microglia accumulated (Fig. 1A). In spite of the strong immunoreactivity, no reports have examined whether this increased immunoreactivity is controlled at a transcriptional level. Real-time PCR using RNA obtained from cerebral hemisphere of UCCAO mice showed up-regulated expression of AIF-1 mRNA on Day 1 indicating increased mRNA expression.

The regulatory region of the AIF-1 gene has an interferon responsive element [19], and AIF-1 mRNA expression is regulated by interferon-γ (IFN-γ) [19, 20]. Following neuronal necrosis, numerous immunocytes are recruited to the affected area in the brain and these cells secrete various cytokines, including IFN-γ, which is increased after 1 h of MCAO [21]. The influence of IFN-γ on AIF-1 mRNA expression in the present UCCAO model should be examined in future studies.

Increased AIF-1 production by the cerebral microglia results in increased AIF-1 levels in the serum. In various experimental and disease conditions, AIF-1 levels are known to be increased in serum [22]. In an experimental autoimmune encephalomyelitis (EAE) model, autoreactive T lymphocytes, especially CD4 and CD17 cells, are activated [23]. Chinnasamy et al. developed the EAE in AIF-1 knockout mice and found that demyelination and lymphocyte infiltration were significantly reduced compared with control mice [24]. These results suggest that AIF-1 contributes to T lymphocyte activation. In fact, AIF-1 induces the production and secretion of IL-6, TNF-α, and IL-10 in mouse macrophages [25]. These facts suggest that reducing serum AIF-1 levels might be a possible therapeutic intervention.

In ischemic stroke, peripheral immune cells infiltrate to the brain [26]. By labelling splenocytes with fluorescent dye, the spleen is demonstrated as the main source of immune cells [27]. Consistently, spleen size reduces significantly following stroke [27, 28] and splenectomy prevents the exacerbation of neural injury [29]. A reduction of spleen size was observed in our model. Histological examination revealed the lack of a bright center in the lymphoid follicle and an enlargement of the capillaries on Day 1 (Fig. 3). The structure of the spleen recovered gradually by Day 7. Recently, the brain was demonstrated to shape the

humoral immunity via splenic innervation [30]. Elucidation of the functional correlation between brain and spleen must be an important objective.

In concert with the increased serum AIF-1, immunohistochemical examination revealed that the number of Iba-1$^+$ cells increased in various organs with UCCAO. Since Iba-1 is a marker of the activated microglia/macrophage-lineage, strongly immunoreactive cells in the lung, liver, and spleen are expected to be alveolar macrophages, Kupffer's cells, and splenic macrophages, respectively. In fact, the number of Iba-1$^+$ cells in the spleen was reduced significantly with minocycline administration, a potent inhibitor of microglial activation [31]. Interestingly, the increase of Iba-1$^+$ cells (Day 3 to 5) was observed shortly after the peak of AIF-1 levels in serum (Day 1).

What is the role of the Iba-1 strongly immunoreactive cells? One possible explanation might be a clearance of AIF-1. As mentioned previously, AIF-1 can induce inflammation. In order to regulate the inflammatory reaction, residual serum AIF-1 must be reduced to normal levels. Increased Iba-1$^+$ cells were also observed in tubular epithelial cells in the kidney. The role of Iba-1$^+$ cells in these organs should be examined in future studies.

Based on the results of the animal experiments, we investigated whether the cultured microglia cell line MG6 increases expression of AIF-1 mRNA under hypoxic conditions. As expected, under hypoxic conditions, AIF-1 mRNA expression and AIF-1 secretion were drastically increased (Fig. 6A, B). Increased AIF-1 expression has been reported to be maintained relatively long after cerebral ischemia [5]. We reasoned that prolonged increased AIF-1 levels might be attributed to the autocrine regulation of AIF-1 mRNA expression. To examine this possibility, MG6 was stimulated with recombinant AIF-1. The expression of AIF-1 mRNA was increased 2.4 ± 0.2-fold compared with that of non-stimulated cells (Fig. 6C). These results suggested that the hypoxic environment accelerates AIF-1 production and, thus, secreted AIF-1 further induces AIF-1 mRNA expression. If this assumption is the case, AIF-1 requires a receptor on the surface of microglia. Although AIF-1 encompasses the prohormonal motif, its cognate receptor has not been identified. Once AIF-1 binds to its virtual receptor, a signaling cascade might be evoked. In this case, the hypoxia-responsible transcription factor, such as hypoxia-inducible factor-1 (HIF-1) might recruited to the regulatory region of AIF-1 gene. However, a correlation between AIF-1 expression and HIF-1 activation has not been investigated. The mechanisms underlying AIF-1 augmentation under hypoxia should be studied in further experiments.

From our results, we propose that AIF-1 expression and production is, at least in part, controlled by AIF-1 itself by an autocrine manner. In addition, increased AIF-1 production results in increased serum AIF-1 and Iba-1$^+$ cells in various organs. Factors released from the damaged cells are called "alarmins" [32], and AIF-1 might be defined as a type of alarmin. With the gradual reduction of serum AIF-1 level according to the reduction of ischemic symptoms in mice, targeting the AIF-1 may be a therapeutic intervention of brain ischemia. Further investigations are needed to elucidate the underlying mechanisms of AIF-1 expression.

Abbreviations

AIF-1
Allograft inflammatory factor-1
Iba-1
Ionized calcium binding adaptor molecule-1
MRF-1
Microglia response facor-1
IRT-1
Interferon-responsive transcript-1
BART-1
balloon angioplasty-responsive transcript-1
IL-2
Interleukin-2
Ab
Antibody
UCCAO
Unilateral common carotid artery occlusion
MCAO
Middle cerebral artery occlusion
IFN-γ
Interferon-γ
EAE
Experimental autoimmune encephalomyelitis
HIF-1
Hypoxia-inducible factor-1

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Nihon University School of Dentistry Animal Ethical Committee and conducted according to the legal requirement (AP19DEN033-1).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MF and KY conducted the animal experiments.

KN and TI performed the in vitro experiments. TI performed the immunohistochemical experiments. MA was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

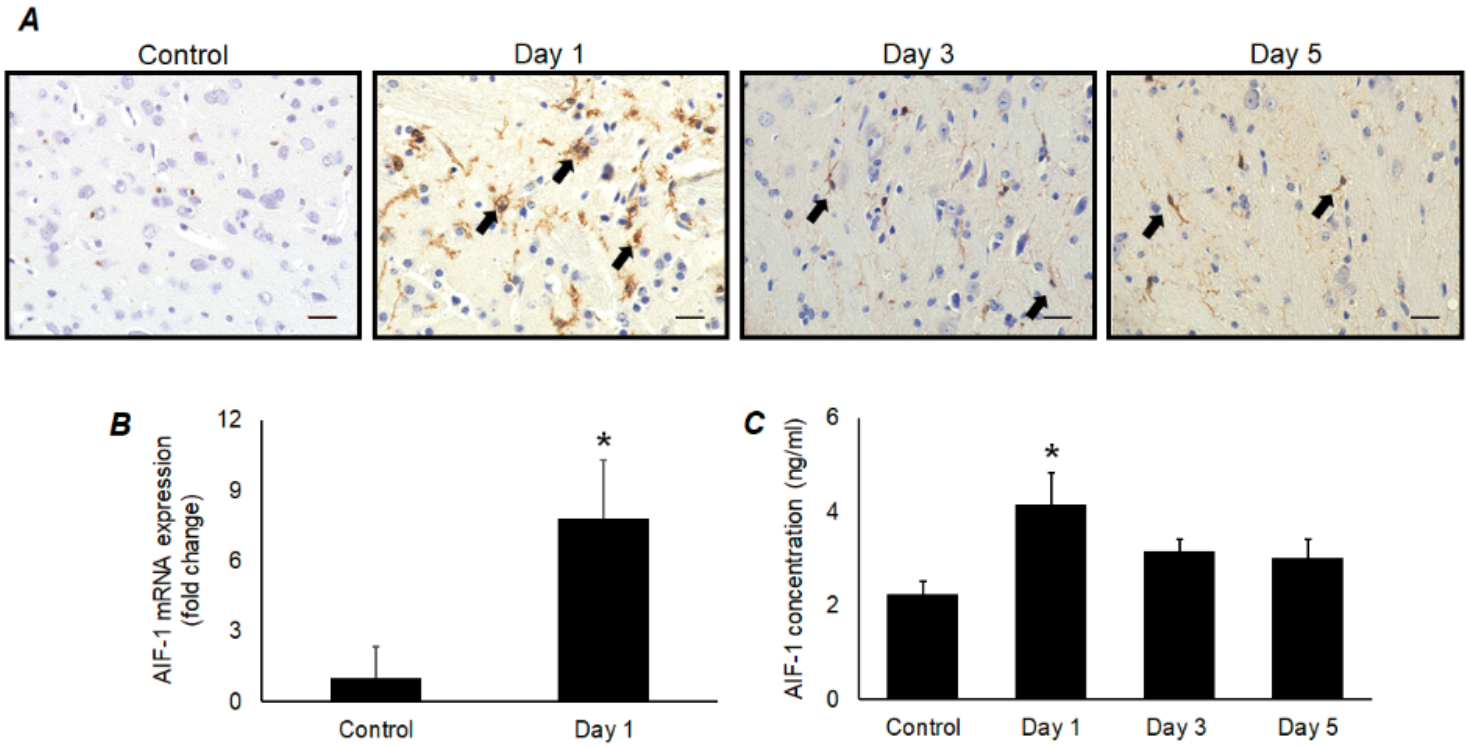

Figure 1

Activation of cerebral microglia by UCCAO. A) The brain was excised on Day 0 (control), 1, 3, and 5 (n=5 for each group) after UCCAO and paraffin sections were prepared and subjected to immunostaining with anti-Iba-1 Ab. Spine elongation and cell swelling and thickening (arrows) are clearly observed on Day 1. These morphological changes are observed until Day 5. The representative of at least 5 different experiments are shown. (Scale bars, 20 μm) B) Total RNA was extracted from the right hemisphere (n=6) on Day 1 after UCCAO and subjected to real-time PCR. The AIF-1 mRNA expression level increased to 7.8 ± 2.5-fold after UCCAO. The mean ± SD of at least 6-independent experiments are shown. *p<0.05 C) The right hemisphere of the brain was excised after UCCAO. The lysates were subjected to AIF-1 measurement. AIF-1 increased to 4.1 ± 0.7 ng/ml on Day 1. The mean ± SD of at least 4-independent experiments are shown. *p<0.05

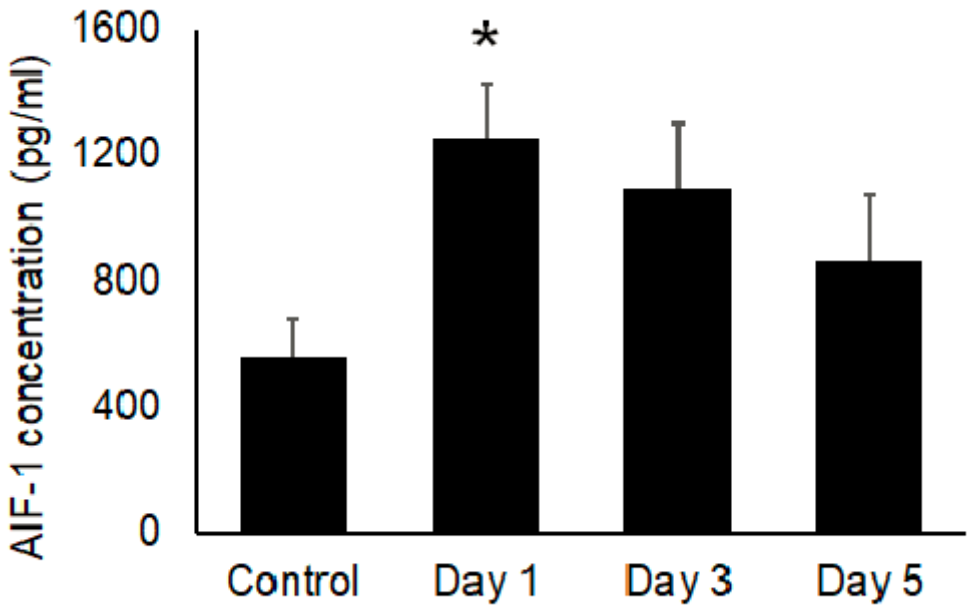

Figure 2

The serum AIF-1 concentration increased after UCCAO. On Day 0 (control), 1, 3, and 5 of UCCAO (n=6 for each group), serum was collected and subjected to analysis using an AIF-1 ELISA kit. The AIF-1 concentration increased significantly with UCCAO and peaked on Day 1. The mean ± SD of 6-independent experiments are shown. *p<0.05

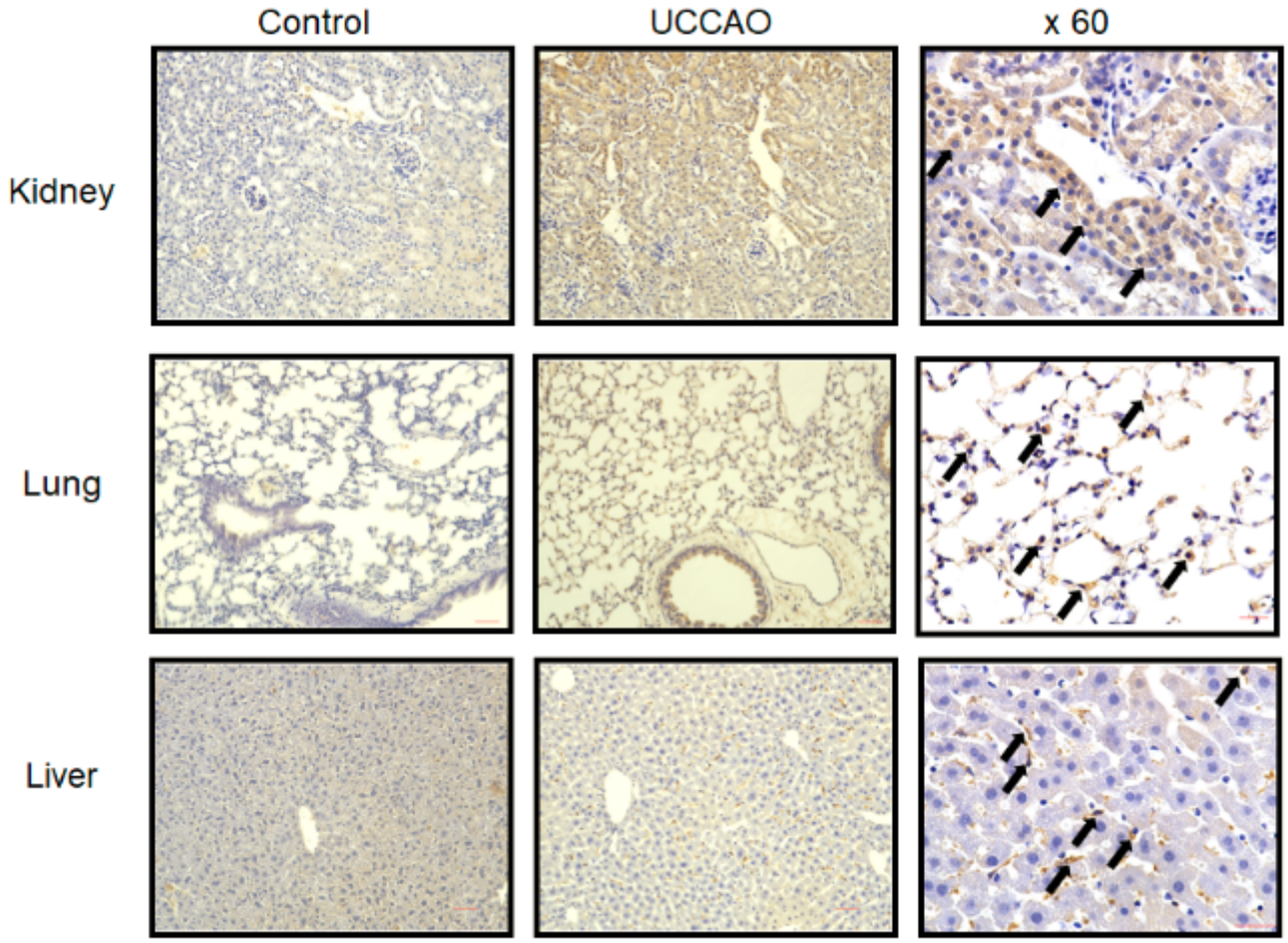

Figure 3

Enhanced immunoreactivity after UCCAO. The kidney (A-C), liver (D-F) and lung (G-I) were obtained after transcardial perfusion with 4% paraformaldehyde (n=5) and subjected to immunohistochemical staining with anti- Iba-1 Ab. The strong positive reactions were observed in renal epithelial cells (B) in the kidney, alveolar macrophages and type II epithelial cells (E) in the lung and Kuppfer's cells (H) in the liver. These cells are shown in larger magnification (arrows) (C, F, I)(x60 objective lens) in the most right panels. The representative of five different experiments are shown. (Scale bar: A, B, D, E, G, H: 50 μm and C, F, I: 20 μm)

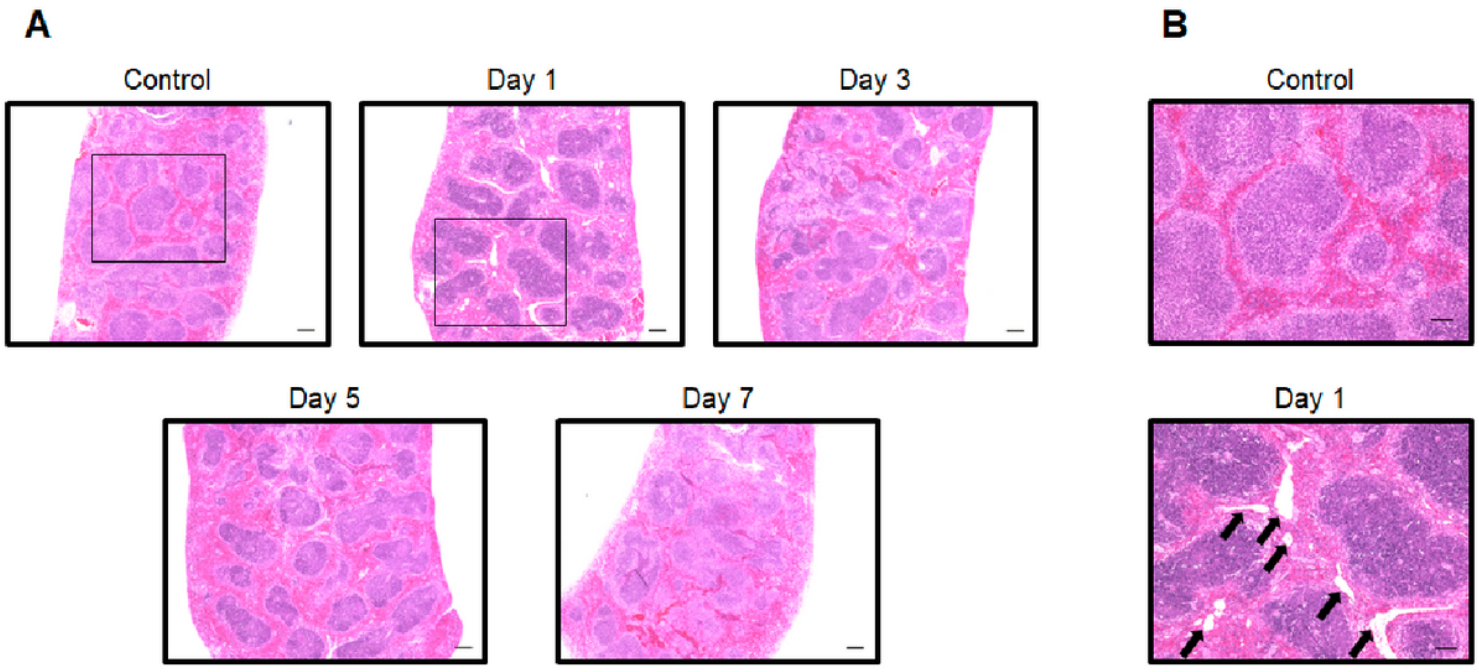

Figure 4

Morphological changes of the spleen. The spleen was excised on Day 0 (control), 1, 3, 5, and 7 of UCCAO (n=10) and subjected to A) HE staining (Scale bars: 200 μm). The rectangle area of control and Day 1 images are enlarged in B) (Scale bars: 100 μm). Clear capillary enlargement is observed in UCCAO (arrows).

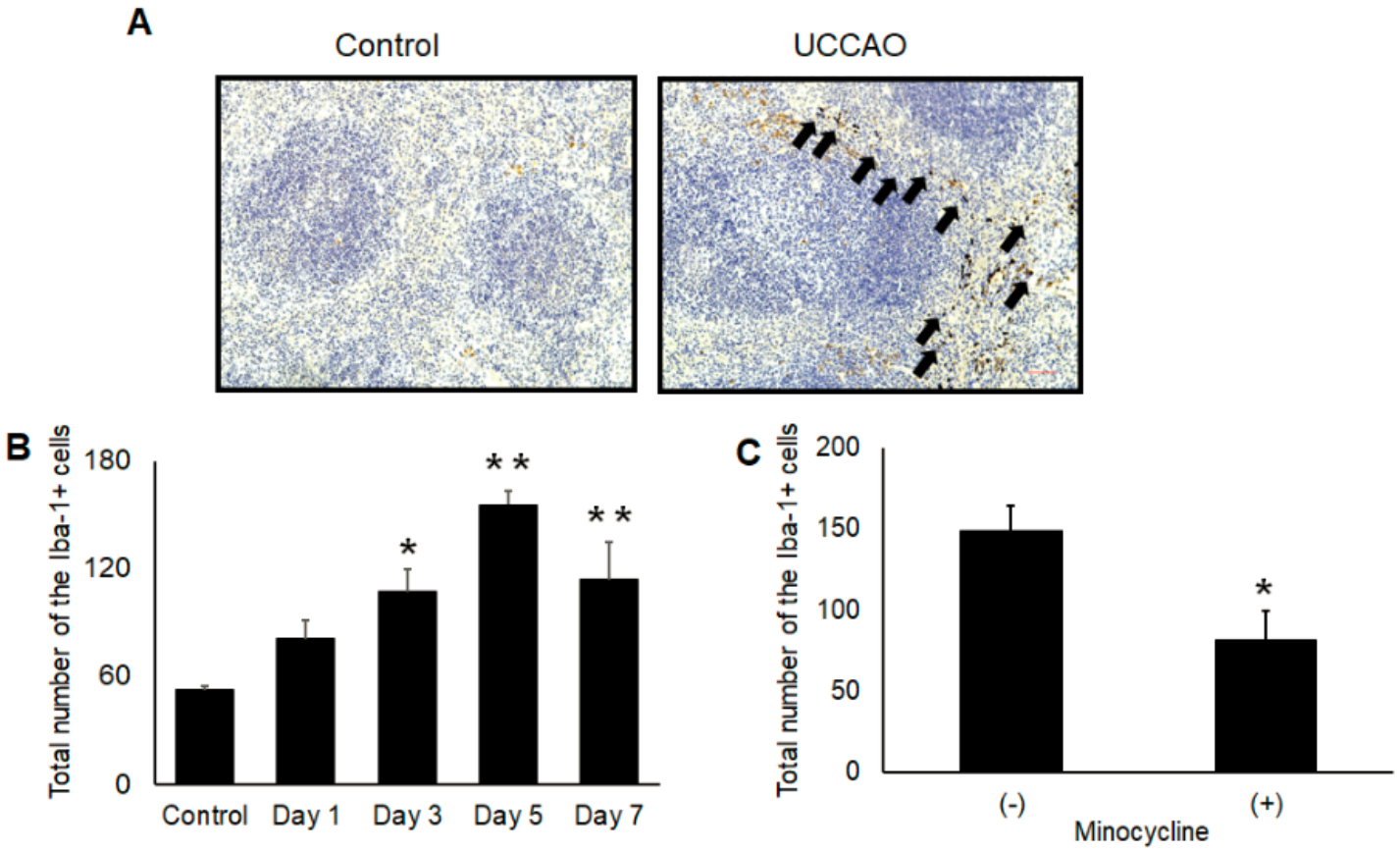

Figure 5

Significant increase of the Iba-1+ cells in the spleen A) Immunohistochemical staining (n=7) revealed a significant increase of Iba-1+ cells in the spleen. On Day 5 (right panel), Iba-1+ cells were accumulated in the red pulp area. (Scale bar: 20 μm) B) The number of Iba-1+ cell accumulated in the spleen was counted under microscopy. Peak accumulation was observed on Day 5 (control: 52.7 ± 2.3 cells vs Day 5: 155.5 ± 7.8 cells). (n=6) *p<0.05, **p<0.01 C) Minocycline was injected to mice (n=5) before and right after UCCAO and the Iba-1+ cell number was counted. Minocycline injection significantly reduced the Iba-1+ cells (81.5 ± 18.2 cells vs minocycline non-injected UCCAO mice: 149 ± 14 cells). *p<0.05

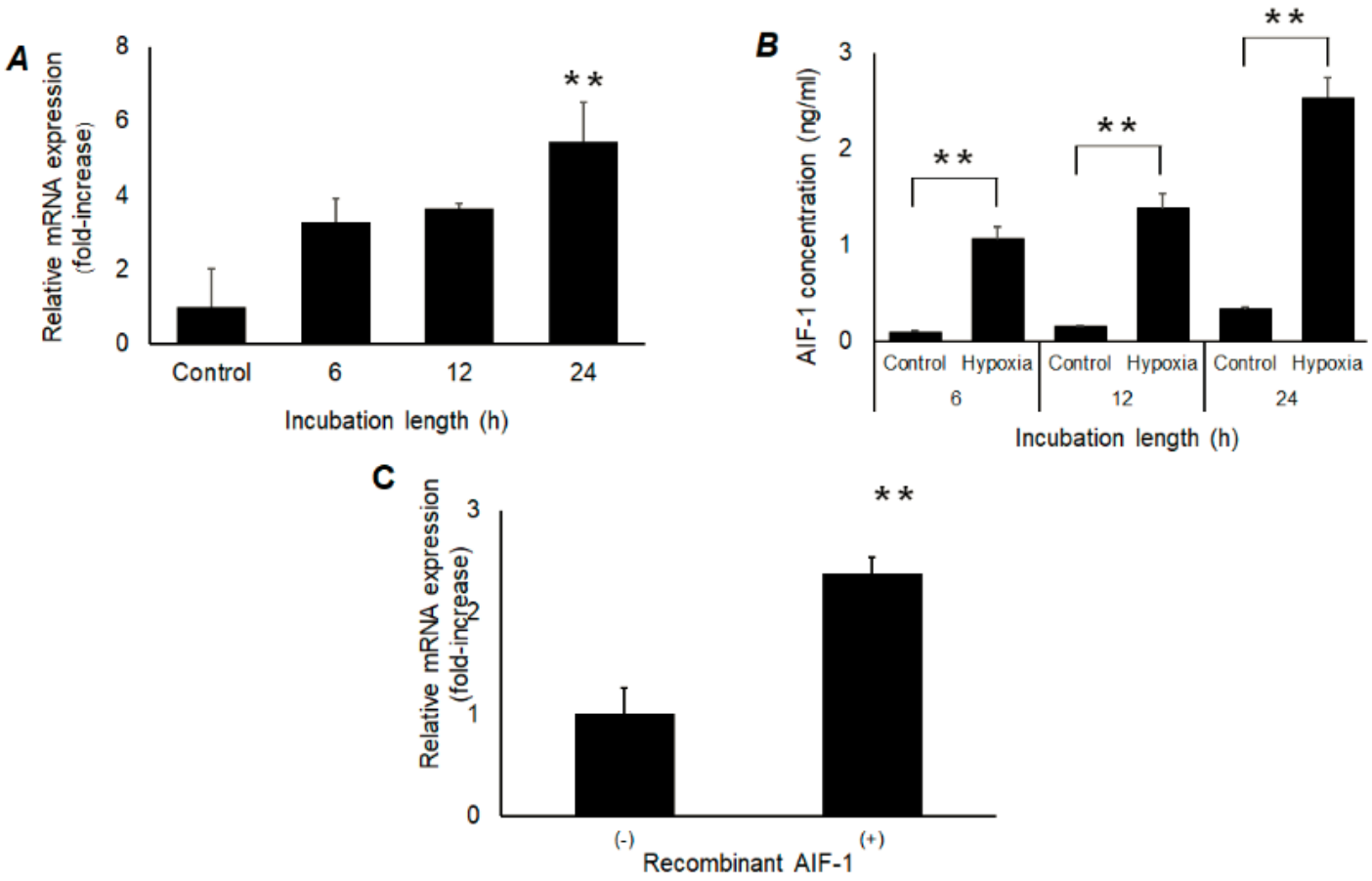

Figure 6

Augmented AIF-1 production A) MG6 cells were cultured under hypoxic condition for 6, 12, and 24 h. Total RNA was extracted and subjected to real-time PCR. The expression of AIF-1 mRNA increased time-dependently and reached 5.4 ± 1.1-fold after 24 h. The mean ± SD of five-independent experiments are shown. **p<0.01 B) After 6, 12, and 24 h of hypoxic culture, MG6 culture supernatants were harvested and the AIF-1 concentration was measured by ELISA. The AIF-1 concentration increased time-dependently and reached 2.5 ± 0.2 ng/ml after 24 h. The mean ± SD of at least five independent experiments were shown. **p<0.01 C) MG6 cells were incubated with recombinant mouse AIF-1 for 6 h. Total RNA was

extracted and subjected to real-time PCR. The AIF-1 mRNA level increased 2.4 ± 0.2-fold. The mean ± SD of seven independent experiments are shown. **p<0.01