Progression of vasogenic edema induced by activated microglia under permanent middle cerebral artery occlusion

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Abstract
Brain edema is a severe complication that accompanies ischemic stroke. Increasing evidence shows that inflammatory cytokines impair tight junctions of the blood-brain barrier, suggesting the involvement of microglia in brain edema. In this study, we examined the role of microglia in the progression of ischemic brain edema using mice with permanent middle cerebral artery occlusion. The intensity of T2-weighted imaging (T2WI) in the cerebral cortex and the striatum was elevated 3 h after occlusion and spread to peripheral regions of the ischemic hemisphere. Merged images of 2,3,5-triphenyl tetrazolium chloride staining and T2WI revealed the exact vasogenic edema region, which spread from the ischemic core to outside the ischemic region. Microglia were strongly activated in the ischemic region 3 h after occlusion and, notably, activated microglia were observed in the non-ischemic region 24 h after occlusion. Pre-treatment with minocycline, an inhibitor of microglial activation clearly suppressed not only vasogenic edema but also infarct formation. We demonstrated in this study that vasogenic edema spreads from the ischemic core to the peripheral region, which can be elicited, at least in part, by microglial activation induced by ischemia.

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1. Introduction
Stroke is the third leading cause of death and the most frequent cause of permanent disability worldwide [1]. Edema appears to be critical in the pathogenesis of ischemic stroke, and severe edema can induce exencephaly, leading to a significant aftereffect or occasionally lethality. Brain edema is mainly classified into cytotoxic edema and vasogenic edema [2]. Cytotoxic edema is characterized by intracellular accumulation of fluid and Na⁺ resulting in cell swelling, while vasogenic edema involves the extravasation and extracellular accumulation of fluid into the cerebral parenchyma caused by disruption of the blood-brain barrier (BBB).

The BBB is formed by cerebral endothelial cells interconnected by a continuous line of tight junctions [3]. In addition, pericytes and astrocyte endfeet also contribute to forming the BBB. Several vascular permeability factors such as matrix metalloproteinases (MMP) and vascular endothelial growth factors (VEGF) have been reported to be involved in the formation of vasogenic edema via potentiated permeability of cerebral endothelial cells [4]. Pericyte loss or dysfunction in numerous diseases was recently revealed to promote a leaky BBB [5]. Astrocytes regulate water flux into and out of the brain parenchyma via aquaporin 4 (AQP4), a major water channel of the central nervous system. AQP4-null mice were observed to have more severe brain edema than wild-type mice in cortical cold injury [6] and intracerebral hemorrhage [7]. Together, BBB integrity can be regulated by multiple mechanisms owing to

Abbreviations: BBB, Blood-brain barrier; DWI, Diffusion-weighted images; MCAO, Middle cerebral artery occlusion; MRI, Magnetic resonance imaging; T2WI, T2-weighted images; TTC, 2,3,5-triphenyl tetrazolium chloride.

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Microglia constitute the primary immune cells of the central nervous system (CNS). Microglial activation, which causes neuroinflammation, occurs in the brain during ischemic stroke in human patients as well as in experimental animals [8]. Activated microglia secrete pro-inflammatory cytokines and chemokines, which exacerbate neuronal injury [9]. However, little is known how microglia induce cerebral infarct in the ischemic brain. Of note, inflammatory cytokines can enhance vascular permeability in the brain. Interleukin (IL)-6 was shown to increase the permeability of human umbilical vein endothelial cell confluent monolayers via mechanisms associated with redistribution of the tight junctional protein ZO-1, redistribution of cytoskeletal actin, increased cell contraction, and disorganization of the intercellular borders [10]. IL-6 also decreased the expression of tight junctional proteins, occludin and claudin-5, in the endothelium of microvessels [11]. Confluent human brain microvascular endothelial cells treated with tumor necrosis factor (TNF) α consistently demonstrated significant reductions in both mRNA and protein expression levels of VE-cadherin, occludin and claudin-5 [12]. Therefore, activated microglia might affect BBB integrity followed by vasogenic edema formation/progression. In this study, we examined a role of microglia in ischemic edema in mice using a novel vasogenic edema imaging method based on overlaying magnetic resonance imaging (MRI) with 2,3,5-triphenyl tetrazolium chloride (TTC) staining.

2. Materials and methods

2.1. Animals

All procedures on animals were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Animal Care and Use Committee of Hiroshima University, Hiroshima, Japan. Male ICR mice were obtained from SLC (Shizuoka, Japan) and were maintained in a temperature-controlled animal facility with a 12-h light-dark cycle.

2.2. Permanent middle cerebral artery occlusion (pMCAO)

pMCAO was performed according to the method reported by Clark et al. [13]. Mice were anesthetized with isoflurane (Escain, MERCK, Kenilworth, NJ, USA). A midline incision was made in the neck to access the left carotid bifurcation and the external and internal carotid arteries. After ligation of the vessels, a small incision was made in the external carotid artery, and a round-tip monofilament (filament size 6–0) was inserted and gently advanced through the internal carotid artery until the tip occluded the origin of the middle cerebral artery. The monofilament was secured in place with a ligature, and the skin incision was closed by surgical clips.

Fig. 1. Time-dependent changes in MR signals in the brains of MCAO-elicited mice. The middle cerebral artery was occluded in mice, and MR images were obtained at 0 (Sham), 3, 6, 12 and 24 h after occlusion. A. Representative images of DWI, T2WI and TTC staining in slices at the bregma. Regions of interest (ROIs) for quantitative analysis of DWI and T2WI (Right panel). B and C. MR images obtained of the striatum and the cerebral cortex in bregma slices were quantified using Realia and Imagej software and are represented as values relative to the contralateral signal intensity, which was set to 1. The values represent the mean ± S.E. (n = 4 animals in each group). D. Infarct size was calculated from TTC staining images by Imagej software. The values represent the mean ± S.E. (n = 5–6 animals in each group).
2.3. MRI

MRI was performed according to our previous report [14]. Briefly, mice were anesthetized with isoflurane and body temperature was maintained at a constant 37 ± 0.2 °C. The MRI data were acquired using an MRmini-SA (DS Pharma Biomedical, Osaka, Japan), consisting of a 1.5-Tesla permanent magnet, a compact computer-controlled console, and a solenoid MRI coil with a 30-mm inner diameter. T2-weighted images (T2WI) and diffusion-weighted images (DWI) were obtained with the following parameters: the T2WI parameters were TR (ms)/TE (ms) = 2500/69 and NEX = 4, and the DWI parameters were TR (ms)/TE (ms) = 2500/69, b value = 850 sec/mm², and NEX = 4. To measure the signal intensity from T2WI and DWI, the mean signal intensity was determined using the INTAGE Realia Professional software program (Cybernet Systems Co. Ltd., Tokyo, Japan) and Image J software (National Institutes of Health, Bethesda, MD, USA).

2.4. Measurement of infarct size

Infarct size was assessed by TTC staining. Brains were sliced into 1-mm-thick coronal sections based on the bregma at 0.0 mm and stained with 1% TTC solution in PBS at 37 °C for 10 min. TTC-stained brain sections were analyzed by Image J software. To correct for tissue swelling, the following formula was used to calculate infarct volume: correct infarct volume = (infarct volume / contralateral hemisphere volume)/ipsilateral hemisphere volume [15].

2.5. Brain water content

The brain was rapidly removed and dissected out into two regions, the cerebral cortex and the striatum. The wet weight of each brain region was measured, and the samples were then placed in an oven (100 °C) for 24 h and then reweighed (= dry weight). The percentage of brain water content was calculated as [(wet weight – dry weight)/wet weight of brain tissue] × 100 (%) [14].

2.6. Immunofluorescent histochemistry and image analysis

Iba1/CD68 staining was performed according to our previous report [16]. The images were processed using the accompanying Zen image acquisition software package (Carl Zeiss, Oberkochen, Germany). Microglia were counted by overlaying the Iba1 and DAPI-stained images. Areas for microglial soma were evaluated by Iba1 staining images processed by Imagej software and used as an index of amoeboid microglia formation. The Iba1 and CD68 co-stained area was calculated with ImageJ software and used as an index of microglial phagocytic activity.

2.7. Statistical analyses

All of the data are expressed as the mean ± standard error (S.E.). The statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by Student’s t-test or Dunnett’s test. P values of <0.05 were considered to indicate statistical significance.

3. Results

3.1. Time-dependent changes in edema formation after MCAO

Brain edema accompanied by ischemia was monitored time-
dependently using DWI and T2WI after pMCAO. DWI utilizes proton diffusion within tissues as a reporter for evolving neuropathology that reflects cytotoxic edema, while T2WI has been used for evaluation of vasogenic edema because small molecules such as water have long T2 relaxation times compared to solid and large molecules [2]. The signal intensity of DWI in the striatum clearly increased 3 h after MCAO, and then the signal time-dependently elevated 24 h after MCAO (Fig. 1A and B). The DWI signal changes observed in the cerebral cortex were similar to those in the striatum. The T2 signal in the striatum rose at 3 h and continuously increased thereafter, and a similar trend was detected in the cerebral cortex. Greater changes were found in the DWI signal than in the T2WI signal 3 h after ischemia (striatum DWI, 1.49 ± 0.04; striatum T2, 1.31 ± 0.06; cortex DWI, 1.40 ± 0.02; cortex T2WI, 1.27 ± 0.05), suggesting that brain ischemia primarily induces cytotoxic edema followed by vasogenic edema.

The infarct area was detected at the ischemic core 6 h after ischemia and thereafter increased in a time-dependent manner. Because almost no infarct area was observed 3 h after ischemia, infarct formation is preceded by ischemic edema. We next sought to show the exact regions of vasogenic edema because T2WI can be used to visualize both the infarct and edema regions. When T2WI was merged with TTC-stained images 6, 12 and 24 h after MCAO, a hyperintense region in T2WI appeared surrounding the infarct area. Interestingly, vasogenic edema time-dependently moved from the ischemic core to the periphery of the ischemic regions (Fig. 2A). The T2 high-intensity area and the TTC-unstained region increased in time-dependent manners, while the vasogenic edema area exhibited rapidly the same size at all time points examined (Fig. 2B). These data suggest that vasogenic edema is generated at the ischemic core and then spreads to peripheral regions in the ischemic hemisphere. The water content in the ipsilateral cortex and the striatum significantly increased compared with the water content in the contralateral hemisphere 24 h after MCAO (Fig. 2C), confirming that vasogenic edema is elicited by brain ischemia.

3.2. Involvement of microglia in vasogenic edema progression induced by MCAO

Microglial activity was evaluated in the ischemic and non-ischemic regions 3 and 24 h after MCAO, as microglia were reportedly activated by ischemia and may be involved in edema formation and/or progression [17]. Microglia in the ischemic region exhibited amoeboid shapes and increased expression of CD68 3 h after occlusion, clearly indicating microglial activation at early stage of ischemia (Fig. 3A, C and D). In non-ischemic regions, microglial activation was not detected 3 h after ischemia, while amoeboid like-microglia with high CD68 expression were observed 24 h after

Fig. 3. Delayed activation of microglia in the non-ischemic region of MCAO-treated mice. The middle cerebral artery was occluded for 0, 3 and 24 h. The brains were fixed, and then cryosections were prepared. Slices were double-stained with Iba1 and CD68 to evaluate microglial activity in the ischemic and non-ischemic regions of the ischemic hemisphere. A. Representative images of Iba1- and CD68-stained tissues. Scale bar indicates 50 μm. Schematic of a mouse brain at the bregma with the red box marking the location of the analysis (Upper right panel). B-D. The number of microglia (B), amoeboid score (C) and phagocytic activity (D) were determined from staining images according to the method described in the Materials and Methods section. The values represent the mean ± S.E. (n = 3–4 animals in each group). The data were analyzed using one-way ANOVA, followed by Student’s t-test or Dunnett’s test. *P < .05, **P < .01 vs. 0 h values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
ischemia, suggesting that microglia outside the ischemic region are activated at a relatively late phase of ischemic stroke (Fig. 3A, C and D). The number of microglia did not change during the experiments (Fig. 3B).

To examine whether microglial activation is involved in edema formation and progression, we challenged mice with a potent inhibitor of microglial activation, minocycline [18]. Minocycline administration clearly suppressed microglial activation induced by MCAO in non-ischemic regions (Fig. 4A). Mice treated with minocycline exhibited vasogenic edema after MCAO, but the vasogenic edema was limited to the ischemic region and did not spread to the peripheral region of the ischemic hemisphere (Fig. 4B and C). The infarct area, induced by MCAO, was decreased by minocycline treatment (Fig. 4B and C). These results suggest that microglia activated by ischemia contribute to, at least in part, vasogenic edema progression and subsequent infarct formation.

4. Discussion

The brain edema, especially vasogenic edema is a serious complication that accompanies stroke and is occasionally lethal because it can cause severe brain damage such as exencephaly. T2WI can be used to visualize vasogenic edema, although both vasogenic edema and the infarct have high intensity on T2WI. Therefore, we merged TTC-stained images with T2WI to visualize the exact area of vasogenic edema. By this procedure, vasogenic edema was observed in the regions surrounding the infarct and then spread to peripheral regions of the ischemic hemisphere. Vasogenic edema is characterized by the extravasation and extra-cellular accumulation of fluid in the cerebral parenchyma, which is induced by BBB rupture. The BBB consists of vascular endothelial cells, pericytes and astrocyte endfeet [3]. Damage and/or shrinkage of endothelial cells and modulation of aquaporins expressed in astrocytes have been considered to be involved in BBB dysfunction [4,19]. Interestingly, the vasogenic edema that initially occurred at the ischemic core expanded to non-ischemic regions 24 h after MCAO, suggesting that diffusional molecules are involved in the progression of vasogenic edema.

Increased evidence has shown that activated microglia can induce enhanced permeability of the BBB. TNFα and IL-6, which are released from activated microglia, are reported to potentiate the permeability of endothelial cells by reactive oxygen species generated by NADPH oxidase and to decrease the expression of claudin-5 and occludin, which form tight junctions [12]. Furthermore, treatment with minocycline was reported to suppress the BBB damage induced by ischemia and subsequent reperfusion [20], suggesting that activated microglia can cause a leaky BBB. However, the relationship between activated microglia and vasogenic edema was still unclear. In this study, microglia were strongly activated in the ischemic region at the acute phase, and delayed microglial activation was observed in the non-ischemic region. Interestingly, minocycline did not suppress edema formation, but it limited edema to the ischemic region, preventing expansion of edema to the non-ischemic region. These results suggest that microglia are involved in vasogenic edema progression but not edema formation. BBB components such as endothelial cells and astrocytes were damaged by ischemia at the acute stage due to a shortage of oxygen and nutrients, followed by ATP depletion. Based on this knowledge and our findings, the development of vasogenic edema might be divided into 2 mechanisms. One mechanism is the dysfunction of cells constituting the BBB by damage directly associated with ischemia, such as ATP depletion, which occurs at the early phase of ischemia.
stroke. Another mechanism could be microglial activation. Activated microglia during ischemia can act on vasogenic edema progression likely because inflammatory cytokines released from microglia cause the BBB to become leaky. This microglia-dependent mechanism is considered to enable the spread of vasogenic edema from the ischemic core to the non-ischemic region. Our study showed minocycline-inhabitable spreading of vasogenic edema that accompanies microglial activation, which strongly supports the above hypothesis that microglia are involved in secondary vasogenic edema formation.

Activated microglia are reported to be involved in the progression of infarcts induced by brain ischemia-reperfusion via mechanisms that decrease the production of inflammatory cytokines, suppress neuronal apoptosis and increase neurotrophic factor production by neural stem cells [21–23]. However, few reports have focused on vasogenic edema partly because it is difficult to evaluate vasogenic edema. This study showed by visualizing vasogenic edema with superimpose of T2WI and TTC staining that microglia are involved in the progression of vasogenic edema accompanied by permanent ischemia, which causes severe brain damage. Therefore, the exacerbation of vasogenic edema could be one of the mechanisms by which microglia mediate infarct progression in the ischemic brain. Microglial activation might be suppressed via neuroprotective strategies administered at the acute phase of ischemic stroke that involve inhibition of vasogenic edema progression.

Competing financial interests

The authors declare no conflicts of interest in association with the present study.

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