Dual Role of Fcγ Receptor in Transient Focal Cerebral Ischemia in Mice

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Background and Purpose—Cerebral ischemia/reperfusion injury is associated with the development of inflammatory response, including pathological contributions by vascular leukocytes and endogenous microglia. Expression of Fc receptors (FcRs) on macrophages and microglia is thought to be involved in the inflammatory cascade. The present study assessed the role of FcγR in ischemia/reperfusion injury, using FcγR knockout (FcγR−/−) mice and bone marrow chimera FcγR−/− mice, which express enhanced green fluorescent protein (EGFP).

Methods—Mice underwent occlusion of the middle cerebral artery for 60 minutes, followed by reperfusion. Infarct volume and mortality were calculated at several time points after ischemia. To clarify the function and distribution of microglia/macrophages, immunohistochemical staining and immunoblotting of ionized calcium-binding adapter molecule 1 (Iba-1), inducible nitric oxide synthase, and nitrotyrosine were performed.

Results—FcγR−/− mice showed significantly reduced mortality (20%) and smaller infarcts (19.7 ± 3.63 versus 33.28 ± 7.98 mm³; P < 0.001) than wild-type (WT) mice at 72 hours after reperfusion. Western blotting revealed that microglial activation (P < 0.002) and induction of inducible nitric oxide synthase (P < 0.005) were reduced in FcγR−/− mice compared with WT mice. At 7 days after reperfusion, sections double-immunostained for EGFP and Iba-1 showed less activation and migration of EGFP-positive bone marrow-derived macrophages in FcγR−/− chimera mice than in WT mice.

Conclusions—Our results demonstrated that the neuroprotective effect of FcγR deficiency in our model may be primarily attributed to the suppression of activation and infiltration of inflammatory cells. (Stroke. 2004;35:958-963.)

Key Words: microglia ■ macrophages ■ Fe-gamma receptor ■ inflammation ■ ischemia/reperfusion injury

The inflammatory response in the central nervous system is considered important in the pathological process after the onset of cerebral ischemia and is a risk factor for the initial development of cerebral ischemia.1 Brain ischemia induces a marked response of resident microglia and hematopoietic cells, including monocytes and macrophages, and elicits a strong intrinsic inflammatory response involving activation of microglia, recruitment of granulocytes, and infiltration of macrophages in the ischemic area.2 However, whether the molecular mechanisms underlying these inflammatory responses are beneficial or detrimental in cerebral infarction is still unclear. Understanding the intracellular signaling mechanism and cell-to-cell interaction in the inflammatory cascade may help in the design of therapeutic strategies for cerebral infarction.

Recent studies have emphasized the critical roles of Fc receptors (FcRs) expressed on macrophages and microglia in the inflammatory cascade.3,4 Although several studies stressed the importance of the FcγR in the inflammatory response in immunological5 and degenerative diseases6 of the central nervous system, to our knowledge no report has described a link between FcγR and cerebral ischemia.

In a previous study we provided direct evidence for the migration and distribution of bone marrow–derived monocytes/macrophages and the relationship between resident microglia and infiltrated hematogenous elements in the ischemic brain of bone marrow chimera mice that expressed enhanced green fluorescent protein (EGFP).7 It is conceivable that microglia/macrophages may serve a dual and paradoxical role after ischemic injury. To assess the role of Fcγ receptor in ischemia/reperfusion injury, middle cerebral artery (MCA) occlusion/reperfusion was performed in the FcγR knockout (FcγR−/−) mice and the bone marrow chimera FcγR−/− mice with the use of a model system established previously.

Materials and Methods

Transient Focal Cerebral Ischemia

The protocol described here received prior approval by the Committee on Animal Experimental Guidelines of Juntendo University School of Medicine. FcγR−/− mice (Jackson Laboratory) were generated by the homogeneous recombination method, as described previously.8 Studies were conducted in 8-week-old FcγR−/− and C57BL/6 (wild-type [WT]) (n = 50 per group) mice of the same
genetic background. Animals were housed under diurnal lighting and provided with food and water ad libitum.

Mutant and WT mice, weighing 20 to 25 g, were initially anesthetized with 4.0% isoflurane and maintained on 1.0% to 1.5% isoflurane in 70% \( \text{N}_2 \text{O} \) and 30% \( \text{O}_2 \) with the use of a small-animal anesthesia system. The tip of the laser-Doppler probe was placed on the area selected for regional cerebral blood flow monitoring, which corresponded to the territory of the occluded MCA. The left MCA was occluded for 60 minutes and then reperfused as described previously. In another group of mice (n=10), reperfusion was not performed (permanent MCA occlusion group). Body temperature was kept at 37°C during the experiment with a heating pad. We generated the Fc\( \gamma \)R\(^-/-\)/EGFP transgenic model by bone marrow transplantation of EGFP into Fc\( \gamma \)R\(^-/-\) mice, using the method reported previously, and induced transient cerebral ischemia in these animals 6 weeks later.

**Estimation of Infarct Volume**

At 6, 12, 24, or 72 hours or 14 days after reperfusion, the mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (n=5 per group) and decapitated. The brains were coronally sectioned into six 1-mm-thick slices. The slices were incubated for 20 minutes in 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C and immersion-fixed in 4% paraformaldehyde in PBS at 4°C, until the animals were killed. The observer was blinded to the study protocol and scored the postural reactivity with a modified neurological scoring system described previously. In this system, score 0 represents no observable neurological deficits; 1, failure to extend the left forepaw on lifting the whole body by the tail; 2, circling to the contralateral side; and 3, loss of walking or righting reflex.

**Neurological Evaluation**

Neurological examination was performed daily after reperfusion until the animals were killed. The observer was blinded to the study protocol and scored the postural reactivity with a modified neurological scoring system described previously. In this system, score 0 represents no observable neurological deficits; 1, failure to extend the left forepaw on lifting the whole body by the tail; 2, circling to the contralateral side; and 3, loss of walking or righting reflex.

**Immunohistochemistry**

Five animals of each group were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (n=5 per group) and decapitated. The brains were coronally sectioned into six 1-mm-thick slices. The slices were incubated for 20 minutes in 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C and immersion-fixed in 4% buffered formalin solution. To compensate for brain edema, the correct infarct volume was calculated as described in detail previously.

**Double-Immunofluorescence Staining**

Free-floating sections of EGFP bone marrow chimera mouse were washed with PBS and incubated in a blocking solution, 3% Block-Ace (Yukijirushi) in T-PBS (0.5% Triton X-100), for 30 minutes at room temperature. Double-immunofluorescence staining was performed by simultaneous incubation of sections with anti-Iba-1 antibody, anti-iNOS antibody, or anti-nitrotyrosine antibody (1:50; Upstate Biotechnology) overnight 4°C. For double labeling, the primary antibodies were detected with Texas red–conjugated secondary antibody (1:500; Vector Laboratories) afterward for 2 hours at room temperature. The sections were washed with PBS and mounted on microslide glass with Vectorshield Mounting Medium (Vector Laboratories).

**Western Blots**

For quantitative evaluation, the immunoreactive bands were visualized in the linear range with enhanced chemiluminescence (ECL Western blotting system, Amersham). For double labeling, the primary antibodies were detected with Texas red–conjugated secondary antibody (1:500; Amersham) for 1 hour, the membranes were incubated overnight at 4°C with anti-Iba-1 antibody (1:5000); anti-iNOS antibody (1:1000); anti– nitrotyrosine antibody (1:1000; Santa Cruz Biotechnology Inc). After incubation with the appropriate horseradish peroxidase–conjugated secondary antibody (1:25 000; Amersham) for 1 hour at room temperature, immunoreactive bands were visualized in the linear range with enhanced chemiluminescence (ECL Western blotting system, Amersham). For quantitative evaluation, the immunoreactive bands were subjected to densitometric analysis.

**Cell Count and Statistical Analysis**

In each coronal section of Iba-1 staining, the numbers of Iba-1-positive cells at the transition area were counted independently by 2 investigators. Values presented in this study are expressed as mean±SD. After acquisition of all data, the randomization code was broken, and the data were assigned to the respective group. One-way ANOVA and subsequent post hoc Fisher protected least significant difference test were used to determine the statistical significance of differences in physiological variables, neurological score, and volume of infarction between the 2 groups.

**Results**

**Effects of Fc\( \gamma \)R Deficiency on Transient Focal Cerebral Ischemia**

Figure 1A shows that the survival rate of Fc\( \gamma \)R\(^-/-\) mice (80%) was significantly increased 14 days after reperfusion compared with WT mice. In the permanent MCA occlusion group, 10% of WT mice and 50% of Fc\( \gamma \)R\(^-/-\) mice died with signs of brain swelling and herniation within 24 hours (data not shown). At 24...
hours after reperfusion, the infarct size (10.89±1.26 mm³) in FcγR⁻/⁻ mice was significantly smaller (P<0.001) than that in WT littermates (26.59±1.26 mm³) (Figure 1B). After 72 hours of reperfusion, the infarct size was 19.7±3.63 mm³ in FcγR⁻/⁻ mice and 33.28±7.98 mm³ in WT littermates. The neurological deficit scores are shown in Figure 1C. The scores of FcγR⁻/⁻ mice recorded at several time points after reperfusion were significantly lower (P<0.005) than those of WT mice.

Microglial Activation in FcγR⁻/⁻ Mice After Ischemia/Reperfusion

FcγRI immunostaining was detected in glial cells, which morphologically resembled microglia (Figure 2a). However, no specific FcγRI immunostaining was noted in the brain of FcγR⁻/⁻ mice (Figure 2b).

The distribution of infarct area was analyzed with the use of cresyl violet-stained sections. We defined each ischemic lesion by location in 3 areas (ischemic core, transition area, and peri-infarct area), as shown schematically in Figure 3A as areas C, B, and A, respectively. In WT mice, activation of microglia was identified by Iba-1 antibody in the peri-infarct and transition areas. Ramified Iba-1–positive microglia were detected in the ischemic core at 12 hours after reperfusion (Figure 3Aa). Such microglial activation was widely distributed and gradually increased in the peri-infarct and transition areas until 7 days after MCA reperfusion and then tended to decrease (Figure 3Aa to 3Af). On the other hand, the microglial response was less evident in the ischemic core in a time-dependent manner. In FcγR⁻/⁻ mice, microglial activity in the transition area was weak (Figure 3Ag to 3Al).

Figure 2. Photographs show FcγRI (CD64) immunostaining in untreated WT and FcγR⁻/⁻ mice. a, FcγR immunostaining was observed in many glial cells with microglial morphology in the cortex of WT mice. b, However, there was no specific FcγRI immunostaining in FcγR⁻/⁻ mice. Magnification ×200.

Figure 3. A, Schematic representation of distribution of neuronal damage in mouse brain after reperfusion. Shaded area represents the infarct zone (a). Three areas subjected to immunohistochemical analysis are illustrated: A, peri-infarct area; B, transition area; C, ischemic core area. Photographs show Iba-1 immunostaining in the transition area of representative WT (a to f) and FcγR⁻/⁻ (g to l) mice. Shown are 12 hours (a, g), 24 hours (b, h), 48 hours (c, i), 72 hours (d, j), 7 days (e, k), and 14 days (f, l) after reperfusion. Dots on the brain schema represent the distribution of Iba-1–immunoreactive cells. Magnification ×200. B, Numbers of Iba-1–positive microglia at different time points. C, Western blot analysis. Samples were prepared from the brain at 24 hours (FcγR⁻/⁻ mice: lanes 1 and 2; WT mice: lanes 3 and 4) and 72 hours (FcγR⁻/⁻ mice: lanes 5 and 6; WT mice: 7 and 8) after reperfusion. A 17-kDa band corresponding to Iba-1 protein was clearly detected in the ischemic lesion, and the intensity of the band increased in the stroke side in a time-dependent manner. A weaker band was noted in FcγR⁻/⁻ mice than in WT littermates. D, Densitometric analysis. Values are expressed as percentage of control. C indicates contralateral lesion; S, stroke side.
compared with WT mice (Figure 3Aa to 3Af, 3B). Immunoblots of Iba-1 were clearly detected in the ischemic lesion as a protein band at 17 kDa. In WT mice, the intensity of the band increased in the stroke side \((P < 0.002)\) in a time-dependent manner compared with FcγR−/− mice (Figure 3C, 3D).

**Induction of iNOS in FcγR−/− Mice**

In WT mice, induction of iNOS in microglia of the peri-ischemic area reached a peak level at 48 to 72 hours after reperfusion (Figure 4Ab, 4Ac). On the other hand, in FcγR−/− mice, iNOS was detected only in endothelial cells of the ischemic core area (Figure 4Ae to 4Ah). Immunoblots of iNOS were detected as a protein band at 130 kDa. In WT mice, the intensity of the iNOS band in the stroke side was stronger \((P < 0.005)\) than the corresponding site in FcγR−/− littermates (Figure 4B, 4C).

**Activation of Bone Marrow–Derived Macrophages in FcγR−/− Mice**

At 7 days after reperfusion, double immunostaining for EGFP and Iba-1 showed many morphologically phagocytic EGFP/Iba-1–positive cells in the ischemic core and many amoeboid-like EGFP/Iba-1–positive cells in the transition area of WT/EGFP chimera mice. These findings indicated activation and migration of EGFP-positive bone marrow–derived microglia/macrophages in WT/EGFP chimera mice (Figure 5b, 5c). In contrast, microglial staining was comparatively less in FcγR−/−/EGFP chimera mice (Figure 5e, 5f), and only a few ramified Iba-1/EGFP–positive cells with branching processes were detected in the transitional area. In WT/EGFP chimera mice, some EGFP/iNOS-positive cells were detected in the transition area, but many EGFP-positive intrinsic microglia did not exhibit iNOS and were observed in endothelial cells (Figure 5g, 5h). The activation and migration of iNOS in microglia of the transition area was detected (Figure 5i), and nitrotyrosine staining was observed in the luminal surface of vessels at ischemic lesions (Figure 5j), while there were few nitrotyrosine-positive cells in FcγR−/−/EGFP chimera mice (data not shown). These nitrotyrosine-positive microglia did not stain for EGFP.

**Discussion**

In the present study we analyzed the effects of brain ischemia on the functional contribution of FcγR by using FcγR−/− mice. The major finding of the present study was that FcγR−/− mice were protected from progression and expansion of infarct volume after focal cerebral ischemia followed by reperfusion.
To our knowledge, there are no studies that proposed the involvement of a FcγR-dependent pathway in the pathogenesis of cerebral ischemia/reperfusion injury. FcγR promotes phagocytosis, antibody-dependent cell-mediated cytotoxicity, activation of inflammatory cells, and antibody-dependent immunity.12 In the present study, using FcγR−/− mice, we demonstrated that FcγR contributed to the activation of microglia, induction of iNOS followed by generation of reactive oxygen species, and infiltration of bone marrow-derived macrophages during cerebral ischemia/reperfusion. To our knowledge, this is the first report on the functional role of FcγR on microglia/macrophages in cerebral ischemia/reperfusion injury.

Cerebral ischemia/reperfusion injury is associated with the development of inflammatory response, including pathological contributions from vascular leukocytes and endogenous microglia.5 In the ischemic brain, microglia/macrophages are the major source of inflammatory cytokines.1,13 Therefore, inhibition of microglial activation can protect against stroke-associated pathological changes.14 After ischemia, microglial activation results in a series of functional and morphological modifications that involve proliferation.15 The present results showed that microglial activation was markedly suppressed in ischemic lesions from the early stage of reperfusion in FcγR−/− mice compared with WT mice. Our results provide strong evidence that FcγR plays a crucial role in the initiation and progression of neuronal damage by activation and proliferation of microglia.

In WT mice, iNOS immunoreactivity was observed in activated microglia and reached a peak level at 48 to 72 hours after reperfusion. However, in FcγR−/− mice, iNOS immunoreactivity was not detected in microglia but only in endothelial cells. In addition, iNOS-positive microglia and nitrotyrosine-positive microglia were observed in WT/EGFP chimera mice but not in FcγR−/−/EGFP littermates. In our bone marrow transplantation model, induction of iNOS does not occur on the invading macrophages. Therefore, the possible mechanisms involved in the reduction of infarction volume during brain ischemia/reperfusion include the suppression of activation of microglia followed by induction of iNOS and peroxynitrite production through the FcγR-dependent pathway. In contrast to neuronal NOS, which generates NO early after onset of ischemia,16 iNOS appears somewhat later in inflammatory cells and contributes to the evolution of brain injury. Furthermore, suppression of iNOS expression has been demonstrated to play a major role as a protective agent in several experimental models, such as iNOS null mice,16,17 treatment with antisense oligodeoxynucleotide to iNOS,18 administration of iNOS inhibitors,19 and mild hypothermia.20 In the present study, therefore, it is possible that the potential neuroprotective role of the FcγR-dependent pathway is mediated in part by the suppression of
iNOS upregulation and peroxynitrite production in activated microglia.

In our bone marrow transplantation model, although FcγR was present in the donor EGFP-positive cells, activation and migration of EGFP-positive bone marrow–derived macrophages were markedly reduced in FcγR−/− mice compared with WT mice. Microglial activation has been observed as early as 6 hours after insult, followed by subsequent macrophage transmigration. A previous study reported that reactive microglia showed increased expression of FcRs and that engagement of FcγR triggered inflammatory, cytolytic, or phagocytic activities. The mechanism of migration and infiltration of bone marrow–derived cells into infarcted areas is a topic of debate and remains unclear but may involve the FcγR-dependent pathway for macrophages or some activating signals from activated microglia. Taken together, our results indicate that the signaling pathway through the FcγR on residual microglia may play an important role in the migration and activation of bone marrow–derived macrophages.

In this study we demonstrated that FcγR deficiency decreased the inflammatory responses through microglial activation, iNOS induction, and bone marrow–derived macrophage infiltration after transient focal cerebral ischemia/reperfusion. Therefore, the neuroprotective effect of FcγR deficiency may be primarily attributable to suppression of inflammatory cell activation and infiltration. Our data showed that anti-inflammatory therapy through the FcγR may be useful for neuroprotection after cerebral infarction. Suppression of the FcγR-dependent pathway may provide an approach to potentially reduce ongoing damage during reperfusion in stroke patients.

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