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# Microglial Response Factor (MRF) -1: Constitutive Expression in Ramified Microglia and Upregulation upon Neuronal Death Induced by Ischemia or Glutamate Exposure

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**ABSTRACT**—We have isolated a new microglial gene, *mrf-1*, which is upregulated on microglia in response to apoptosis of granule neurons in cerebellar cell cultures. We examined whether or not upregulation of MRF-1 is observed in response to necrotic neuronal death both *in vivo* and *in vitro*. Though MRF-1 was detected on ramified/resting microglia in the brain of normal adult rats, activated microglia in the region of the brain where neuronal damage was induced by ischemia were strongly immunostained with anti-MRF-1 antibody. In the *in vitro* system, we confirmed, with immunocytochemistry or RT-PCR, that MRF-1 or *mrf-1* mRNA were constitutively expressed in ramified microglia at significant but lower levels than in amoeboid one. Moreover, by Northern blot, it was ascertained that expression level of *mrf-1* mRNA on microglia was markedly upregulated in response to glutamate-induced death of granule cells in a cerebellar cell culture. These results indicate the following: 1) expression of *mrf-1* in microglia may be markedly enhanced upon not only apoptotic but also necrotic neuronal death, and 2) MRF-1 is, thus, a useful marker for identifying all types of microglia *in vivo* and *in vitro*.

## INTRODUCTION

The *mrf-1* gene encodes 17 kDa protein consisting of 147 amino acids with a single calcium binding (EF-hand) motif (Tanaka *et al.*, 1998). The gene was isolated from activated microglia in response to apoptosis of cerebellar granule neurons in culture. Microglia/brain macrophages are involved in brain function under both normal and pathological conditions (Dickson *et al.*, 1993; McGeer *et al.*, 1993). In the adult brain, resident ramified/resting microglia become activated by altering their morphology to rod-shaped or amoeboid types in response to injury, infection, or inflammation of the nervous system (Gehrmann *et al.*, 1995). Activated microglia proliferate, engulf degenerating elements (Giulian *et al.*, 1989; Stoll *et al.*, 1989), and release either cytotoxic agents or cytokines (Thery *et al.*, 1991; Giulian *et al.*, 1994). We have shown previously that *mrf-1* is upregulated in response to apoptotic neuronal death and degeneration both *in vivo* and *in vitro* (Tanaka *et al.*, 1998). It has been thought that MRF-1 may play a role both in developmental programmed cell death and in recovery from brain injuries. Some homologues such as AIF-1 (Utans *et al.*, 1995) and Iba1 (Imai *et al.*, 1996) have been isolated. AIF-1 isolated from macrophage has the same amino

acid sequence as MRF-1, although the 5' noncoding region of AIF-1 mRNA is shorter than that of *mrf-1*. It is likely that the difference between *mrf-1* and AIF-1 mRNAs may reflect an alternative start site derived from a single gene (Utans *et al.*, 1995). MRF-1 is different from the Iba1 protein, which is also synthesized in microglia, in the upstream region containing the 5' noncoding region and some amino-acid sequence in the N-terminal region (Imai *et al.*, 1996). It is thought that these genes constitute a sub-family coding for one or two EF-hand-like motif(s), although their cellular functions are unknown so far.

Upregulated expression of these proteins was found in rat microglia on regions with induced neuronal damage *in vivo* (Ito *et al.*, 1998; Schluesener *et al.*, 1998; Tanaka *et al.*, 1998). Activated microglia, in response to neuronal damage of the axotomized hypoglossal nerve, show an upregulated expression of *mrf-1* mRNA and MRF-1 (Tanaka *et al.*, 1998). In another paradigm of axotomy, Ito *et al.* (1998) examined Iba1 expression of the activated microglia detected in the axotomized facial nucleus. Also, Schluesener *et al.* (1998) demonstrated that AIF-1 is heavily present in experimental autoimmune encephalomyelitis, neuritis, and uveitis. In this paper, we show that MRF-1 is constitutively expressed in ramified microglia at a low level, and its level is upregulated in response to necrotic neuronal death both *in vivo* and *in vitro*.

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## MATERIALS AND METHODS

### Induction of focal cerebral ischemia

Male adult rats (Wistar, 250–300 g; Funabashi Farm, Shizuoka, Japan) were used. The rats were anesthetized with 2% halothane in a mixture of 70% nitric oxide and 30% oxygen. Focal cerebral ischemia was induced by transient occlusion of the right middle cerebral artery as described previously (Nagasawa and Kogure, 1989; Kato *et al.*, 1996). Briefly, an 18-mm long, silicone-coated 4–0 nylon suture was inserted via the right internal carotid artery so that the origin of the middle cerebral artery was occluded with the suture. After 1 hr of occlusion, the suture was removed. The rats were sacrificed after 7 days of survival.

### Cell cultures

Amoeboid microglia were isolated and purified from a culture of rat cerebral cells according to the method of Suzumura *et al.* (1984) with some modification as described previously (Tanaka *et al.*, 1998). The cerebral cortices were dissected from neonatal rat pups (Sprague Dawley, SD). All meninges and blood vessels were removed and dissociated with Dispase (250 U/ml; Godo Shusei Co., Ltd.) in  $\text{Ca}^{2+}$ -free Krebs'–Ringer's bicarbonate buffer (KRBB) containing 0.1% BSA for 60 min at 37°C. They were then triturated. The dissociated cells were collected by centrifugation and resuspended in Dulbecco's modified Eagles' medium/Ham's nutrient mixture F-12 (DMEM/F12; Sigma Chemical Co.) containing 10% heat-inactivated fetal calf serum (hiFCS; J.R.H. Biosciences), 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma). The cells were plated on a poly-L-lysine (Sigma)-coated flask and cultured until confluency (~8–10 day) at 36°C in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air. Microglia were collected by shaking (60 rpm for 1.5 min) and centrifugation, and replated on 35-mm dishes or glass coverslips (diameter 12 mm; Matunami) ( $1 \times 10^4$  cell/cm<sup>2</sup>). The microglia constituted >95% of all cells on the culture plate after replating. For induction of ramification of microglia *in vitro*, purified microglia were cultured in serum free DMEM/F12 medium supplemented with N2 supplements (Bottenstein and Sato, 1979).

Ramified microglia were isolated from adult rat (Wistar, 5–6 weeks old) brains using isotonic Percoll (Merz *et al.*, 1987; Slepko and Levi, 1996) with some modification. Briefly, perfused brains were transferred to ice-cold KRBB, freed of meninges, minced, and treated with Dispase/DMEM/F12 (1 brain/6 ml) at 37°C for 60 min. After three washings with KRBB and trituration, the dissociated cells were filtered through lens paper. After the volume of the cell suspension was fitted to 21.4 ml with 10%-hiFCS DMEM/F-12 medium, the suspension was mixed with 8.6 ml of cold isotonic Percoll in KRBB and centrifuged at  $500 \times g$  for 10 min. The pellet was resuspended in 21.4 ml 10%-hiFCS DMEM/F-12 medium, mixed with 8.6 ml of isotonic Percoll, and recentrifuged. The pellet was then resuspended in a 10%-hiFCS DMEM/F-12 medium (1.5 ml/brain) and plated on 35-mm dishes (1 ml).

A cerebellar cell culture was prepared from the cerebella of P7 rats (SD) as described previously (Suzuki and Koike, 1997; Tanaka *et al.*, 1998). In brief, dissected cerebella were minced, treated with Dispase/MEM at 37°C for 30 min, and then triturated in KRBB. The dissociated cells were collected by centrifugation and resuspended in Eagle's MEM (Sigma) containing 10% hiFCS and penicillin/streptomycin. The cells were plated on poly-L-lysine-coated 60-mm dishes ( $1.0 \times 10^7$  cells/dish). For immunocytochemical analysis, polyethyleneimine-coated glass coverslips were used. Since a neuron-glia mix culture was used for experiments, 20 µM fluorodeoxyuridin (FudR) was added to the culture medium from 2 DIV to reduce proliferation of non-neuronal cells. A high concentration of  $\text{K}^+$  (at final 30 mM) was added to the culture medium from 2 DIV. The contamination of Vimentin-, GFAP-, or OX42-positive cells in the 7 DIV culture maintained with a high potassium medium containing FudR for 5 days was about 9.0, 2.0, or 4.5%, respectively.

### Histochemical detection of isolectin-B4 binding

Under deep pentobarbital anesthesia, rats were perfused transcardially with 4% paraformaldehyde (PFA)/0.1 M  $\text{Na}^+$ -phosphate buffer, pH 7.2. Fixed brains were embedded in paraffin wax to prepare paraffin sections (5 µm). Microglial cells were histochemically stained with peroxidase-labeled isolectin-B<sub>4</sub> from *Griffonia simplicifolia* (Sigma) as described previously (Streit, 1990; Kato *et al.*, 1995). Briefly, the sections were incubated with the isolectin (20 µg/ml) in PBS with cations overnight at 4°C. Then these sections were mixed with 3,3'-diaminobenzidine and  $\text{H}_2\text{O}_2$  for color development. Finally, the sections were exposed to counterstaining with Mayer's hematoxylin and eosin.

### Immunohistochemical analysis

The paraffin sections were immunohistochemically stained with antibodies raised against MRF-1 (0.1 µg/ml; Tanaka *et al.*, 1998) or a pan-macrophage/monocyte marker ED1 (1:500 dilution; Serotec). After being blocked with 10% normal serum, the sections were incubated with one of the antibodies overnight at 4°C. The primary antibodies were used in the presence of 0.3% triton X-100. For immunoperoxidase, sections were processed by the ABC method, using a Histofine kit (Nichirei) or Vectastain elite ABC kit (Vector). Finally, the sections were exposed to counterstaining with Mayer's hematoxylin and eosin.

### Immunocytochemical analysis

Cultured cells on glass coverslips were fixed with 4% PFA/ $\text{Na}^+$ -phosphate buffer. After washout of the fixative, the cells were preincubated with 10% horse serum/PBS for 1 hr. For detection of MRF-1, if necessary, the cells were previously treated with 0.2% triton X-100 for 5 min. The cells were then incubated with anti-MRF-1 antibody (rabbit IgG, 0.1 µg/ml) or OX42 (mouse IgG, 1:300 dilution; BMA Biomedicals Ltd.) for 1 hr at room temperature. The primary antibodies were detected using either biotinylated or fluorescence-conjugated IgG (FITC-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG, 1:20 dilution; ICN Pharmaceuticals, Inc.). The biotinylated antibodies were made visible by horseradish peroxidase-conjugated streptavidin (Nichirei) and aminoethercarbazol reaction product (Zymed). Finally, the cells were exposed to counterstaining with Mayer's hematoxylin solution. The fluorescences were analyzed with a laser scanning microscope (Fluoview; Olympus).

### Extraction and purification of RNA

Cells were washed with PBS and solubilized with 4 M guanidinium thiocyanate, pH 7.0, containing 25 mM sodium citrate, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol. The total RNA fraction was extracted from the microglia culture ( $1 \times 10^5$  cells) or cerebellar cell culture ( $1 \times 10^7$  cells) and precipitated by isopropanol. After centrifugation for 15 min, the pellet was washed with 75% ethanol and dried with flowing air.

### RT-PCR method

First-strand cDNA was synthesized from an equal amount of total RNA (0.5–1.5 µg) in a 20-µl reaction mixture using random hexamer as a primer with a superscript preamplification system (superscript II, 200 units; Gibco BRL) at 43°C for 50 min. After being treated with RNase H, 2 µl of the mixture was amplified by PCR with Taq DNA polymerase (Gene Taq NT, 1 unit; Wako Chemical Ltd.) in a 20-µl reaction mixture. The construction of a reaction mixture was followed: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% triton X-100, 1.5 mM  $\text{Mg}^{2+}$ , 25 µM dNTP mix, and 0.2 µM primers. The used primers were a 5' primer, 5' TCTGAGGAGCTATGAGCCAG 3' and 3' primer, 5' TCCACCTCCAATTAGGGCAAC 3' for *mrf-1*, or 5' primer, 5' AAGGTCGGTGTCAACGGATTGG 3' and 3' primer, 5' TGTAGGCCATGAGGTCCACCAC 3' for *G3PDH*. These upstream and downstream primers correspond to the sequences of a gene for rat *mrf-1* (Tanaka *et al.*, 1998) or *G3PDH*, respectively. Thus, the amplification was performed for 15–35 cycles with denaturation at 94°C, anneal-

ing at 60°C, and extension at 72°C for 1 min each (except for 5 min each at the first cycle). Finally, for complete elongation of synthesized strands, each sample was treated at 72°C for 15 min. The products were separated on a 7% acrylamide gel and stained with SYBR Green I (FMC BioProducts).

#### Northern blot

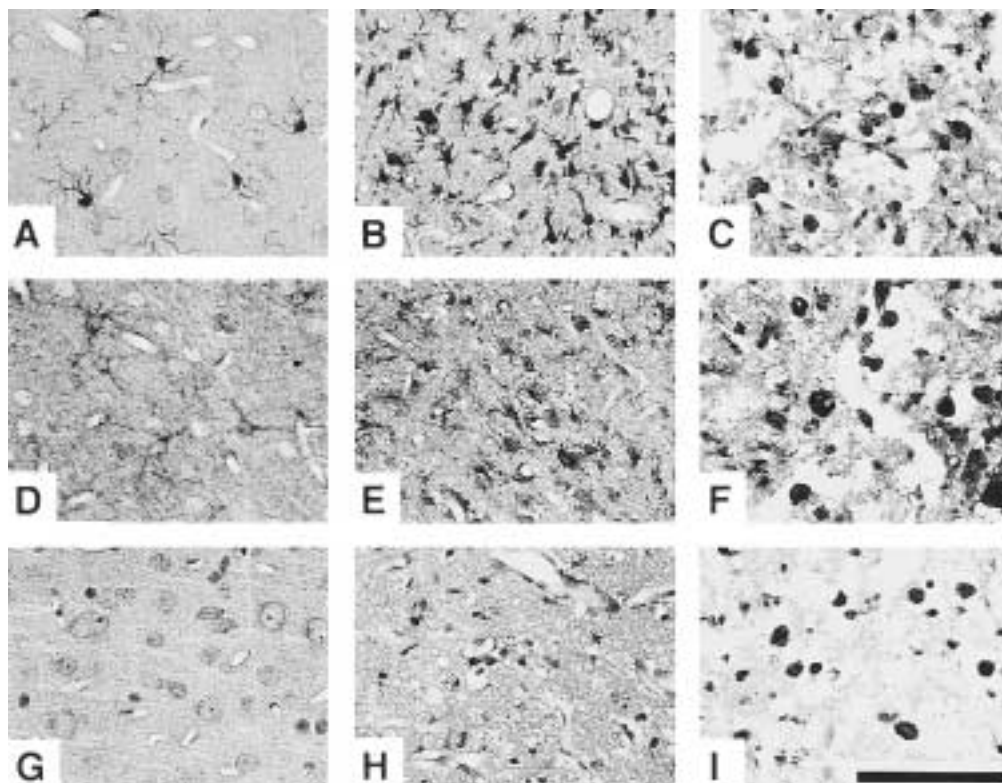
Total RNAs were loaded per lane onto a formaldehyde denaturing gel (1% agarose) and separated by electrophoresis. The RNAs were transferred to a nylon membrane (Hybond-N+; Amersham). Hybridization was performed in 50% formamide containing buffer at 42°C for 12–18 hr. The cDNA probes were labeled with  $^{32}\text{P}$ -dCTP to a specific activity of  $1\text{--}1.5 \times 10^9$  dpm/ng by the random priming method using the Ready-To-Go DNA Labeling Kit (Pharmacia Biotech). The *mrf-1* or *G3PDH* probe was prepared by PCR amplification using each pUC18 subclone as cDNA (421 bp or 983 bp, respectively, see the RT-PCR method section). The washed membranes were visualized, and the amount of radioactivity of specific transcripts was measured using a Bio-imaging Analyzer (BAS2000; Fuji Photo Film Corp.).

## RESULTS AND DISCUSSION

### Upregulation of *mrf-1* expression in response to ischemic neuronal death *in vivo*

In the brain of normal adult rats, MRF-1 positive cells were distributed homogeneously, having scant cytoplasm and numerous thin, branched processes, typical of ramified

morphology of microglia. This data indicates that ramified microglia constitutively express the MRF-1 protein and, thus, MRF-1 is an useful marker for them *in vivo*. Then, we have examined whether or not MRF-1 is upregulated in microglia/macrophages following focal cerebral ischemia in rats. Focal cerebral ischemia was induced as described previously (Kato *et al.*, 1996). In this experimental model, cerebral infarction developed in the areas supplied by the right middle cerebral artery, i.e., the lateral portion of the striatum and a part of the overlying neocortex. Paraffin sections were used for the visualization of microglia/macrophages using histochemistry with a common microglial marker, peroxidase-labeled isolectin-B<sub>4</sub> from *Griffonia simplicifolia* (Streit, 1990) and immunohistochemistry with anti-MRF-1 antibody (Tanaka *et al.*, 1998) or ED1 (Milligan *et al.*, 1991). In the sham-operated rat brains, resting microglia bearing ramified, thin processes were visualized with anti-MRF-1 antibody (Fig. 1A). Isolectin stained faintly a small number of them (Fig. 1D), and no microglial cells were stained with ED1 (Fig. 1G). After 7 days, a number of microglial cells that displayed a strongly activated morphology had accumulated in the marginal area surrounding the infarction. The cells exhibited enlarged cell bodies and contracted, stouter processes, and were strongly positive for anti-MRF-1 antibody (Fig. 1B) and isolectin (Fig. 1E). They were



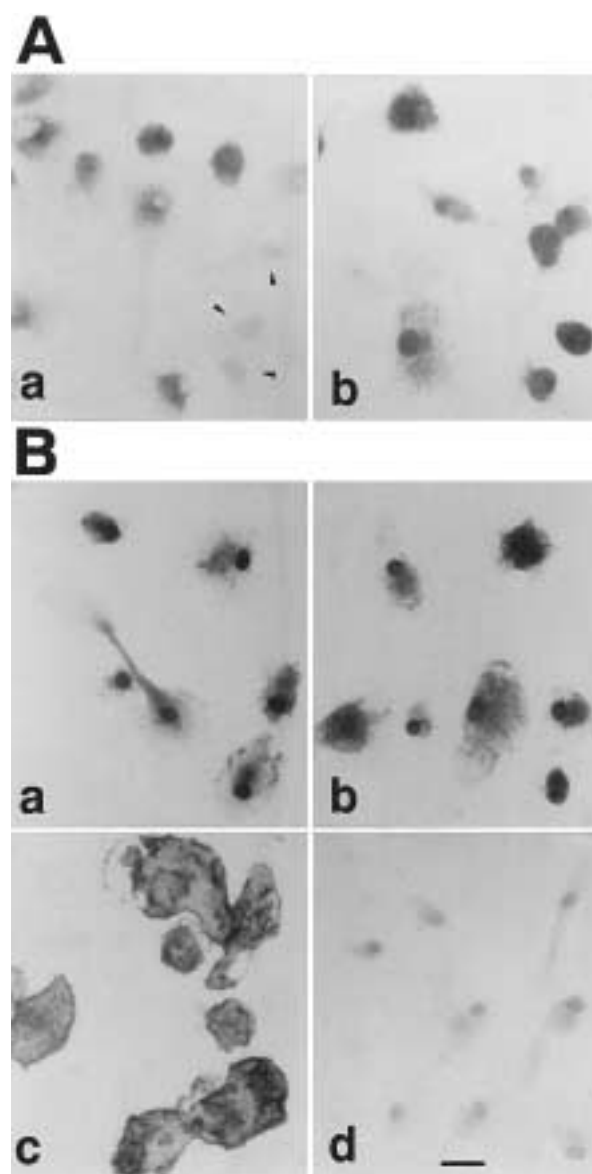
**Fig. 1.** Microglia/macrophages visualized with anti-MRF-1 antibody (A–C), isolectin-B<sub>4</sub> (D–F), and ED1 (G–I) in the rat brain. Counterstained with hematoxylin and eosin. A, D, G: sham-operation. B, E, H: seven days after 1 hr of right-middle cerebral artery occlusion, neocortex, peri-infarct area. C, F, I: seven days after 1 hr of middle-cerebral artery occlusion, neocortex, infarction. Resting microglia are visualized with anti-MRF-1 antibody (A), but only a small number of them are faintly stained with isolectin (D), and none are stained with ED1 (G). Activated microglia/microglia-derived macrophages in the peri-infarct area were strongly positive for MRF-1 (B), isolectin (E), and ED1 (H). Round, monocyte-derived macrophages covered the infarcted area. These cells were also positive for each marker (C, F, I). Bar = 50  $\mu\text{m}$ .



also ED1 positive (Fig. 1H); since its positive signals are limited on cell bodies, the stained regions are a little smaller than that of anti-MRF-1 antibody or isolectin. This result suggests that they had been transformed into activated microglia/macrophages. On the other hand, round, mononuclear macrophage-like cells covered the infarcted area. These cells were strongly positive for anti-MRF-1 antibody (Fig. 1C), isolectin (Fig. 1F), and ED1 (Fig. 1I). They were most likely monocyte-derived macrophages. Thus, MRF-1 was expressed in microglial cells at any stage of activation in response to cerebral ischemia as well as in blood-borne macrophages. Furthermore, MRF-1 was apparently upregulated when microglial cells were activated following ischemia.

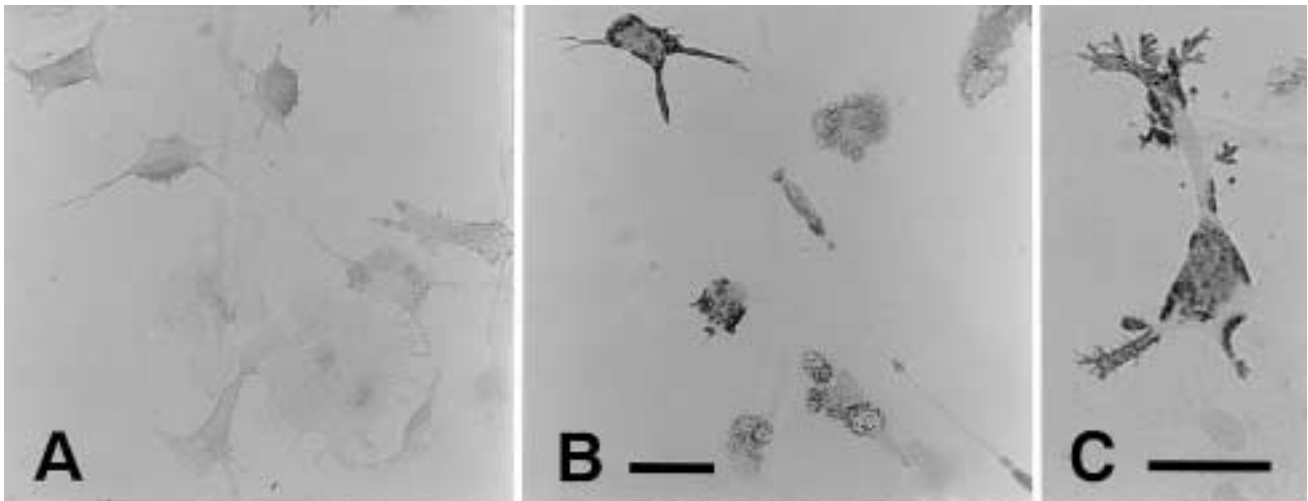
#### Detection of *mrf-1* RNA and MRF-1 on purified microglia *in vitro*

When microglia derived from the cortex of rats are cultured in the presence of serum, most of all cells show an amoeboid morphology. We performed immunostaining of the cultured microglia with anti-MRF-1 antibody or OX42. When fixed microglia were directly used for the immunostaining with anti-MRF-1 antibody, the positive signals of MRF-1 were detected on limited regions of cytoplasm of many, not all, of the microglial cells (Fig. 2A-a) (Tanaka *et al.*, 1998). When fixed microglia were, however, previously treated with triton X-100, all of them were immunostained with anti-MRF-1 antibody (Fig. 2A-b). Moreover, MRF-1 positive signals were detected on both the nucleus and the whole cytoplasm. The shape of the microglia stained with anti-MRF-1 antibody was slightly less clear than that of microglia stained with OX42 because the antibody binds to a cytoplasmic protein, while OX42 detects the microglial membrane protein (Robinson *et al.*, 1986) (Figs. 2B-a and -c). However, we found that immunostaining with anti-MRF-1 antibody have a benefit concerning to the stability on the detection of microglia (Fig. 2B). When microglia were immunostained with OX42 at 2 days after fixation, OX42 barely detected them (Fig. 2B-d). In contrast, immunoreactivity of MRF-1 was very stable after fixation (Fig. 2B-b). Giulian *et al.* (1995) reported that some of the microglia transforme from amoeboid- to ramified-type in a serum-free medium *in vitro*. We confirmed that ramified microglia express MRF-1 *in vitro*. Six days after purified microglia were exposed to a serum-free medium, about half of them developed some thin processes (Fig. 3A). When the microglia were treated with anti-MRF-1 antibody and MRF-1 was detected immunocytochemically, both the process-bearing and amoeboid microglia were apparently stained (Figs. 3B and C). The result shows that ramified microglia express MRF-1 not only *in vivo* but also *in vitro*. This immunocytochemical data was consistent with the data of mRNA analysis of cultured microglia by the RT-PCR method. Slepko and Levei (1996) showed that ramified microglia dissociated from the adult rat brains by using isotonic Percoll (Merz *et al.*, 1987) progressively transform to amoeboid/activated microglia over several days in the presence of serum *in vitro*. We analyzed the mRNA level of *mrf-1* on both the ramified microglia and

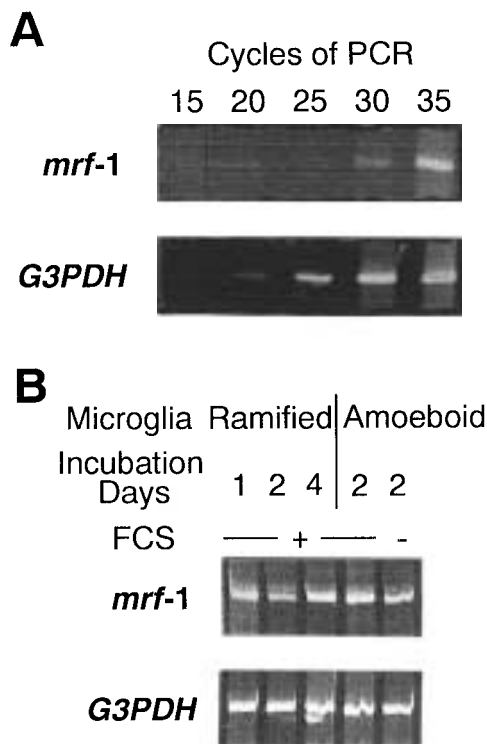


**Fig. 2.** *In vitro* immunostaining of amoeboid microglia. A: Immunostaining of cultured microglia with anti-MRF-1 antibody. Purified microglia were further cultured in normal medium for 1 day and fixed with PFA. Some of the fixed cells were treated with triton X-100 (b). Then the cells were immunostained with anti-MRF-1 antibody and counterstained with Mayer's hematoxylin solution (a, b). Arrowheads in a show undetected microglia with anti-MRF-1 antibody. B: Immunoreactive stability of MRF-1. The fixed cells were immunostained with anti-MRF-1 antibody (a, b) or OX42 (c, d), and counterstained with Mayer's hematoxylin solution 0 (a, c) or 2 (b, d) days after fixation, respectively. When the fixed cells were immunostained with anti-MRF-1 antibody, they had been previously treated with triton X-100. Bar=30  $\mu$ m.

amoeboid microglia which were incubated in a serum-free condition. *mrf-1* or *G3PDH*, as control, on cDNAs derived from cultured microglia were amplified dependent on the PCR cycles for 25–35 or 20–30, respectively (Fig. 4A). Then, we compared the amounts of *mrf-1* or *G3PDH* message in several culture conditions with 30 or 25 cycles of PCR, respectively. As shown in Fig. 4B, when adult ramified microglia were incu-



**Fig. 3.** Detection of MRF-1 in ramified/resting-like microglia *in vitro*. Purified microglia were cultured in a serum-free (N2) medium for 6 days and fixed. Some of microglia transformed from amoeboid to ramified form. The fixed microglia were immunostained with OX42 (A) or anti-MRF-1 antibody (B, C) and counterstained with Mayer's hematoxylin solution. No treatment with triton X-100. Bar=30  $\mu$ m.

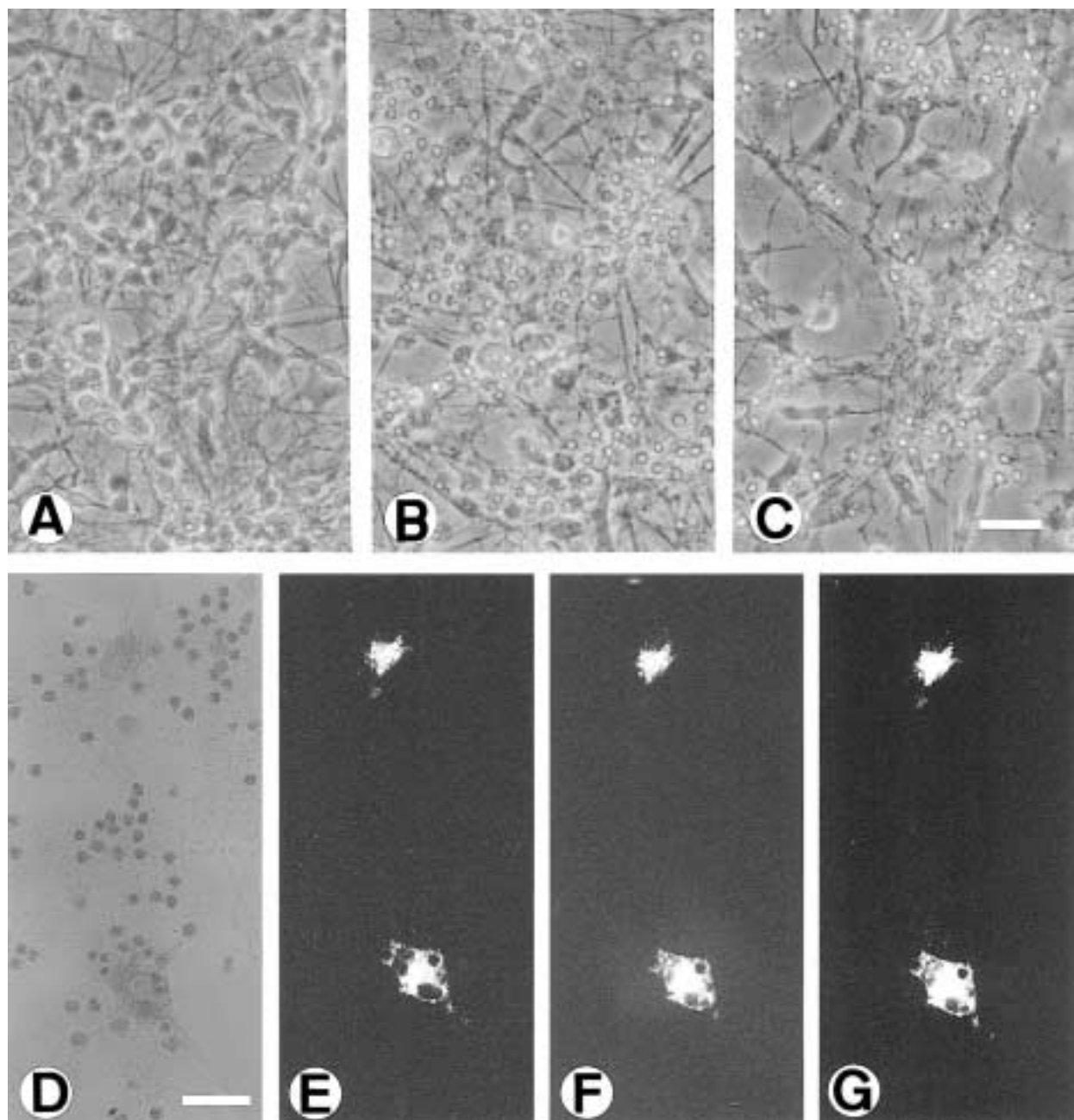


**Fig. 4.** Analysis of the amounts of *mrf-1* mRNA on cultured microglia by RT-PCR method. A: Standard amplification of *mrf-1* and *G3PDH* cDNAs. Both *mrf-1* and *G3PDH* in cDNAs derived from cultured microglia were amplified by PCR of 15–35 cycles with each specific probes (see the materials and methods section). B: Decreased expression levels of *mrf-1* mRNA on ramified microglia *in vitro*. Purified microglia from either adult rats (lanes 1–3) or mixed culture of glia (lanes 4 and 5) were incubated in the presence (lanes 1–4) or absence (lane 5) of serum for the indicated number of days. All RNA was extracted from each culture, and 0.5  $\mu$ g of it was used for a reaction with reversetranscriptase to produce cDNAs. An equal volume of the cDNA was analyzed for quantification of *mrf-1* or *G3PDH* mRNA by PCR of 30 or 25 cycles, respectively. The amplified DNA was separated on a 7% acrylamide gel and stained with SYBR Green I.

bated in the presence of serum for 4 days (lane 3), their *mrf-1* mRNA apparently increased more than that of the cultured microglia derived from the same population and cultured for 1 or 2 days (lane 1 or 2, respectively). In contrast, amoeboid microglia purified from a mixed culture of glia decreased in the expression level of *mrf-1* after they were shifted to a serum-free condition (Fig. 4B, lanes 4 and 5). For these cDNA preparations, no apparent difference was observed on *G3PDH* mRNA (Fig. 4B). These data suggest that *mrf-1* is expressed in ramified microglia and the expression level is less than that of amoeboid microglia.

#### Upregulation of *mrf-1* expression in response to necrotic neuronal death *in vitro*

Many researches suggest that ischemia induces neuronal necrosis rapidly (for review, Chalmers-Redman *et al.*, 1997). The necrosis may be mediated through an excitatory amino acid, such as glutamate, toxicity. It has been reported that the distribution of neuronal necrosis detected by cresyl violet staining on the cerebral cortex of a rat ischemic brain is mostly identical to the areas with increased OX42 immunoreactivities (Kato *et al.*, 1996). It is known that rapid, not delayed, neuronal death induced by glutamate *in vitro* is necrosis (Dessi *et al.*, 1993; Ankarcrona *et al.*, 1995; Armstrong *et al.*, 1997). The response of microglia to the necrotic neuronal death was examined in a cerebellar cell culture. When cerebellar cells dissociated from P7 rats were cultured in a high  $K^+$  condition for 5 days, granule cells in the culture matured and became high sensitive to glutamate (Fig. 5A–C) (D'Mello, 1993; Tanaka *et al.*, 1998). As shown in Fig. 5B, upon expose of glutamate (300  $\mu$ M), most of all the granule neurons showed shrunken cell bodies within several hours. We extracted RNAs from the cerebellar cell cultures 0–24 hr after glutamate treatment and analyzed them by Northern blot. As shown in Fig. 6, the levels of *G3PDH*, showing the amount of RNA analyzed, did not change apparently. In contrast, the level of *mrf-1* mRNA gradu-

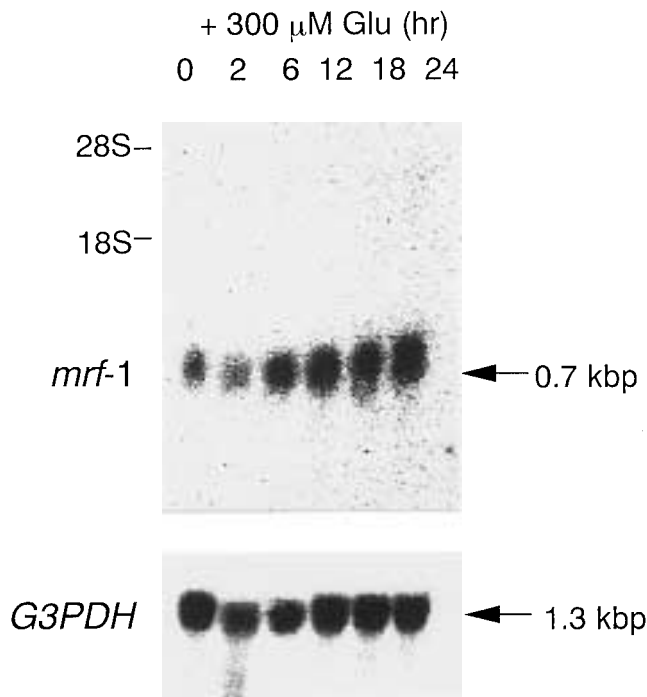


**Fig. 5.** Glutamate-induced death of matured granule neurons. After cerebellar cells were cultured in high  $K^+$  (30 mM) medium for 5 days (A), they were exposed to glutamate (300  $\mu$ M) for 1.5 (B) or 18 (C) hr, and photographed with a phase-contrast microscope. In C, some of dead neurons had detached from the surface of the dish. Also, after the cerebellar cells treated with glutamate (18 hr) were fixed with PFA and treated with triton X-100, they were immunostained with both anti-MRF-1 rabbit IgG and OX42 (mouse). The primary antibodies were detected with fluorescence-conjugated secondary antibodies (FITC-, anti-rabbit; TRITC-, anti-mouse). Finally, the cells were stained with hematoxylin and photographed with a bright light (D) or a laser (E-G) on the same field: D, hematoxylin stained nuclei; E, MRF-1 positive cells; F, OX42 positive microglia; G, double positive cells. Bars=30  $\mu$ m.

ally increased for at least 24 hr. When purified microglia were exposed to glutamate (30–300  $\mu$ M) in 10% FCS-MEM medium, the level of their *mrf-1* mRNA did not change for at least 24 hr (data not shown). On the culture of cerebellar cells treated with glutamate, MRF-1 positive signals were also detected in OX42 positive microglia only (Figs. 5D-G). In Fig. 6, the relative amount of *mrf-1* mRNA per total RNA increased to 530% of control at 24 hr. Since the amount of total RNA

isolated from a culture dish decreased to 54% at 24 hr because of neuronal cell death, the relative amount of *mrf-1* mRNA per culture dish becomes 280% at 24 hr. It is known that granule neurons dissociated from the P7 rat cerebella undergo apoptosis during 4–6 DIV in normal  $K^+$  medium (Gallo *et al.*, 1987; Tanaka *et al.*, 1998). The induction level of *mrf-1* mRNA in response to glutamate toxicity was similar to that of microglia in response to the apoptosis of immature granule





**Fig. 6.** Upregulated expression of *mrf-1* mRNA in cultured microglia response to glutamate-induced necrosis of matured granule neurons. Five days after sister cultures of cerebellar cells were incubated in the presence of high  $K^+$  (30 mM), glutamate (300  $\mu$ M) was added to the each culture medium directly. At indicated times, each culture was treated with GTC solution for RNA extraction. The total RNA (20  $\mu$ g) derived from each culture was separated on a 1% agarose gel and transferred to a nylon membrane. Either *mrf-1* or *G3PDH* mRNA was detected on the same membrane by  $^{32}$ P-labeled *mrf-1* or *G3PDH* probe, respectively. The positions of the rRNA are indicated on the left. The amount of separated rRNA in each lane was almost equal when the transferred membrane was stained with methylene blue (data not shown).

neurons in a normal  $K^+$  culture (Tanaka *et al.*, 1998). These data suggest that microglia may respond to necrotic neuronal death and upregulate MRF-1 both *in vivo* and *in vitro*.

It is thought that microglia play an important role both in the development of the brain and in recovery from brain injuries including ischemia. In these cases, both neurons and glia are known to undergo cell death prominently. In the region where neurons or the nervous systems are destroyed by stimulation, injury, or disease, it is observed that microglia become transformed from a resting to an activated state and the activated microglia are proliferated. We, along with others (Ito *et al.*, 1998; Schluesener *et al.*, 1998), confirmed that MRF-1 or its homologues are upregulated in the activated microglia on the damaged tissue after nerve injury. This phenomenon suggests that the physiological function of MRF-1 and its homologues is possibly important in the damaged region of the brain; however, its role has not been determined so far. Chen *et al.* (1997) searched for intestinal peptides with effects on insulin release and isolated a polypeptide daintain/AIF-1 from porcine intestines. They suggested that daintain/AIF-1 may be synthesized as a prohormone and release as a cleaved form. MRF-1 and its homologues may be involved in several func-

tions of microglia in brain.

In summary, *mrf-1* is constitutively expressed in ramified/resting microglia, and its expression level may be upregulated in activated microglia in response not only to apoptotic but also to necrotic neuronal death. These results, also, indicate that MRF-1 is an useful marker for identifying all types of microglia *in vivo* and *in vitro*.

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