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Rapid microglial activation induced by traumatic brain injury is independent of blood brain barrier disruption

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Summary. Following CNS injury, microglia respond and transform into reactive species exhibiting characteristic morphological changes that have been termed "activated" or "ameboid" microglia. In an attempt to establish that microglial reactions induced immediately after injury are caused by intrinsic mechanisms rather than infiltration of blood and its constituents, oxygenized Ringer's solution was perfused into the cerebral circulation of rats so that the circulating blood could be eliminated prior to injury induction. Under artificial respiration, a catheter was inserted from the cardiac apex into the ascending aorta, and oxygenized Ringer's solution was immediately perfused with a pulsatile blood pump, resulting in wash out of the circulating blood from the brain within 1 min. Subsequently, a cortical contusion was induced in the unilateral parietal cortex using a controlled cortical impact (CCI) device. At 5 min following the injury, the brain was fixed by perfusion of fixative through the catheter and removed. Coronal vibratome sections were then processed for CR3 immunohistochemistry to examine the microglial activation. It appeared that microglial activation with both morphological transformation and an increase in CR3 immunoreactivity was induced throughout the hemisphere ipsilateral to the injury side exclusively, even in rats with elimination of circulating blood. The microglial reactions did not differ substantially from those observed in the control rats with extensive BBB disruption. The present results thus provide direct evidence that the microglial activation induced immediately after injury is independent of infiltration of circulating blood induced by concurrent BBB disruption.

Key words: Microglia, Blood brain barrier, Brain injury, CR3

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Introduction

Following injury, microglia are activated, can become ameboid and move into the damaged area, and are indistinguishable from blood-borne monocytes that invade the damaged site from the peripheral vasculature (Del Rio-Hortega, 1965; Giulian et al., 1989; Morioka et al., 1991). While microglial activation and proliferation are common events following traumatic brain injury (TBI), the precise mechanisms inducing the microglial activation are still a matter of debate. Recent studies have indicated that several molecules including cytokine(s), cations, and ATP display variable degrees of potential for inducing activation/proliferation of microglia (Walz et al., 1993; Giulian et al., 1994; Inoue, 2002; Davalos et al., 2005). Some studies have suggested that factors which may be released from the damaged brain tissues or extravasated plasma constituents resulting from blood brain barrier (BBB) disruption, can activate microglia following TBI (Jensen et al., 1997; Lu et al., 2001). However, we have observed previously that microglial activation could be induced even at 1 hour after TBI in rats, and such a glial reaction could be attenuated by the administration of kynurenic acid (KYN), a broad-spectrum EAA antagonist, at doses which inhibit the occurrence of neuronal depolarization (Katayama et al., 1995). Furthermore, microglial activation could be induced in a model of cortical spreading depression which consisted of a propagating wave front in the absence of neuronal injury (Gehrmann et al., 1993; Caggiano and Kraig, 1996). Moreover, previous studies have demonstrated the induction of rapid and widespread microglial activation in a brain slice TBI model which served to eliminate variables arising from the circulating blood (Koshinaga et al., 2000; Bellander et al., 2004). These findings suggest that the glial activation induced immediately after injury may be caused largely by intrinsic mechanisms within the brain tissue. The present study was undertaken in an attempt to establish that the rapid microglial activation seen immediately after injury

in vivo is independent of infiltration of circulating blood resulting from BBB disruption. For this purpose, oxygenized Ringer's solution was perfused into the cerebral circulation of rats so as to eliminate the influence arising from the circulating blood as well as the occurrence of anoxia-induced depolarization.

Materials and methods

Wistar rats were purchased from Charles River Laboratories (Saitama, Japan). Normal horse serum, normal mouse IgG and appropriate Vectastain kits and a biotinylated anti-rat IgG were obtained from Vector Laboratories (Burlingame, CA). OX42 (C3b complement receptor; CR3, a marker for microglia and monocytes) antibody was obtained from Serotech (Oxford, England). All salts and other reagents of cell culture grade were purchased from Sigma Chemicals (St. Louis, MO). The animal care and all experimental procedures were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, National Academy Press, Washington, DC, 2003) and the guidelines of the Nihon University Laboratory Animal Research Committee.

Cerebral perfusion with oxygenized Ringer's solution

Male Wistar rats (n=10) weighing 250-275 g were anesthetized with 1% halothane in a mixture of 66% nitrous oxide and 33% oxygen. The animals were placed in a stereotaxic frame, and a 6mm diameter craniectomy centered 4 mm posterior to the bregma and 3.5 mm from the midline was performed over the left parietal cortex. Subsequently, the rats were released from the stereotaxic frame and underwent a tracheostomy to maintain respiration with a ventilator. After making a sternotomy incision, catheterization was rapidly carried out into the cardiac apex and the tip of the catheter was positioned within the ascending aorta. Perfusion of oxygenized Ringer's solution was then initiated employing a pulsatile perfusion pump (Fig. 1). The Ringer's solution was maintained with 95% O_2 and 5% CO_2 at 37°C. The rate of perfusion was adjusted to approximately 2 ml/min. Next, the descending aorta was clamped with a surgical clip and the jugular veins were cut bilaterally in order to wash out the blood from the cerebral circulation. An electroencephalogram (EEG) was continuously monitored during the performance of these procedures, and we confirmed that no depolarizing event occurred for at least 15 min during the continuous perfusion of oxygenized Ringer's solution. In addition, the occurrence of cortical depolarization could be monitored from the cortical steady potentials (DC potentials). It has been widely recognized that steady potentials cause an initial slow positive shift followed by a sudden negative deviation after 3 min even in complete ischemia in the cat brain (Hossman and Kleihues, 1973). We confirmed that the oxygenized Ringer's perfusion could delay the occurrence of negative deviation for at least 15 min.

Cortical contusion

Subsequently, the rats were again placed in the stereotaxic frame, and cortical contusion was induced. The injury was inflicted using a controlled cortical impact (CCI) device, as described in detail elsewhere (Lighthall, 1988; Dixon et al., 1991). In the present experiments, a 5mm diameter injury tip with a round shape, 5m/sec impact velocity, 2mm penetration depth and 70degree impact angle for the injury tip were employed to induce the cortical contusion. Previous histological analysis had demonstrated that under such conditions of injury, the contusion does not obviously spread to the dorsal hippocampus underlying the lesion (Maeda et al., 1998).

The time between the initiation of perfusion and the induction of injury was set at 5 min in each rat. The perfusion of oxygenized Ringer's solution was maintained for at least 2 min, since it is sufficient to wash out blood from the cerebral circulation for 1 min. The cortical contusion was then induced. The control rats (n=8) were subjected to the same surgical procedures without perfusion of oxygenized Ringer's solution. At 5 min after the injury, sodium phosphate buffer containing 4% paraformaldehyde was perfused and fixed through the catheter inserted into the ascending aorta. The brains were removed and 50 mm-thick coronal sections were cut on a vibratome. The brain sections were processed immunohistochemically as described below. In addition, in order to compare the extent of the lesions macroscopically between non-perfused and perfused rats, brains of the respective groups were cut into a series of 2 mm-thick coronal

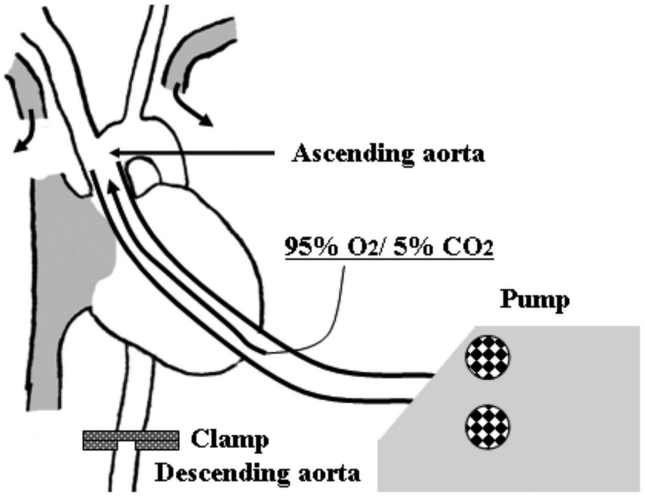

Fig. 1. Schema illustrating the system employed in the present experiments. This system enabled us to perfuse oxygenized Ringer's solution selectively into the cerebral circulation.

sections.

Visualization of the microglial reaction and extent of IgG extravasation

The sections were rinsed overnight in 50 mM Tris-HCl-buffered saline, pH 7.4 (TBS). They were then incubated in OX42 antibody (CR3; 1:3000) in TBS containing 0.25% Triton X-100 (TBS/TX), and 3% normal horse serum for 24 hours on a rotary shaker at 4°C. After rinsing three times for 15 min in TBS/TX, immunolabeled structures were visualized using the appropriate Vectastain kit according to the manufacturer's recommendations. The reaction product was visualized employing 3,3-diaminobenzidine (DAB) with 0.4% $NiCl_2$. In order to evaluate the relationship between the microglial reaction and BBB disruption, double staining for CR3 and rat IgG was performed. Following visualization of microglia, the sections were incubated in a biotinylated anti-rat IgG (1:200) in TBS/TX, and permeability of rat IgG was visualized as BBB disruption using only DAB as a chromagen. This double staining enabled us to observe final reaction products for both the CR3 and extravasated IgG in the same sections. The sections were then rinsed in TBS, mounted on gelatin-coated slides, dried overnight, dehydrated through a graded alcohol series, cleared in xylene, and coverslipped. The microglial reactions on the ipsilateral and contralateral sides of the cerebral hemispheres were evaluated microscopically and compared together with the extent of IgG permeability simultaneously.

Results

The macroscopical findings for a series of brain slices demonstrated that lesions with blood could be seen extensively in the cortex and underlying dorsal hippocampus in the control, non-perfused rats (Fig. 2, left line). In contrast, in the perfused rats, blood-containing damaged areas could not be identified macroscopically, indicating that the circulating blood had been successfully washed out prior to the injury (Fig. 2, right line), although an identical impact injury was applied to each group of rats.

In the present study, we chose to examine the upregulated expression of the complement CR3 receptor as a marker of the initial microglial activation. Further activation was also evaluated from the morphologic changes, since the microglia undergo an evolution to a globose form with short, round pseudopodia, termed ameboid microglia (Del Rio-Hortega, 1965; Graeber et al., 1988). The rationale for choosing the CR3 receptor expression was as follows: CR3 is expressed even in the normal condition, such as with the classic morphology of ramified microglia, while during the activation process, the CR3 expression increases rapidly (Akiyama et al., 1988; Koshinaga et al., 2000). Immediately after the injury in the control rats, widespread extravasation of IgG was evident in the cortex underlying the impact sites (Fig. 3A,B). In addition, large numbers of OX42-positive cells could be detected throughout both the gray and white matter in the whole sections (Fig. 3A). In the hemisphere contralateral to the injury side, CR3 immunoreactivity was present in the cells with classic ramified processes and the cell soma as seen in the normal brain (Fig. 3C,E). However, striking changes occurred exclusively ipsilateral to the injury side even at

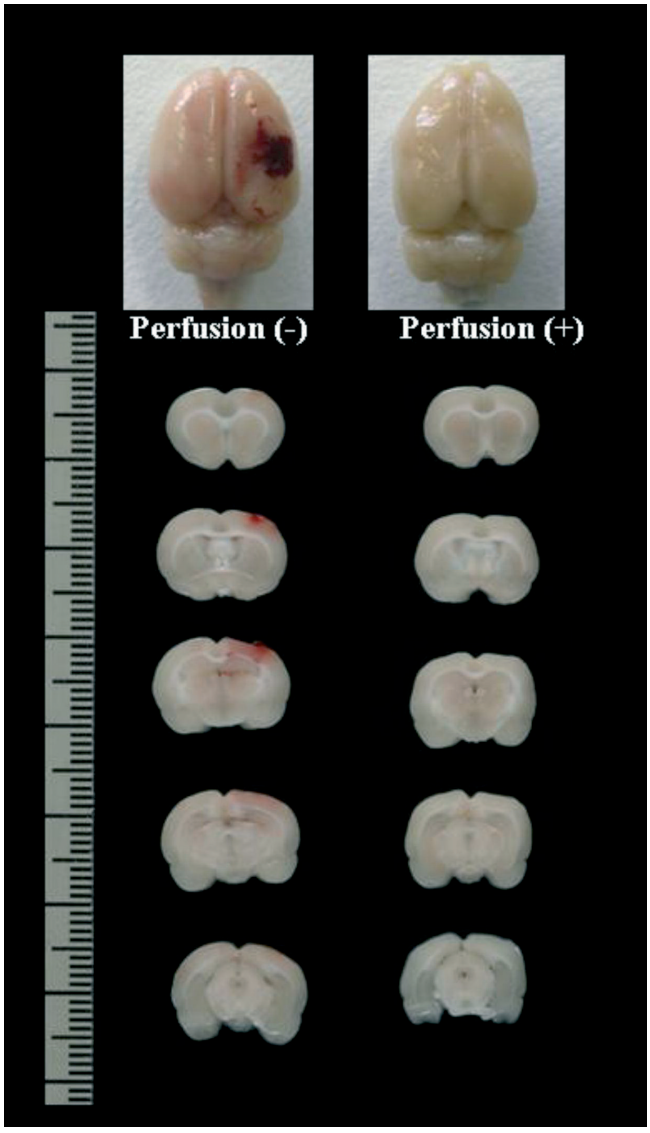

Fig. 2. Macroscopical findings for consecutive 2 mm-thick brain slices in a control rat (non-perfused) (left) and perfused rat (right) at 5 min after injury induction. Note that a lesion containing blood is seen extensively in the cortex and underlying dorsal hippocampus in the non-perfused rat (left line). In contrast, little intraparenchymal blood can be detected in the perfused rat, although an identical impact injury was applied to each group of rats.

5 min following the injury. Large numbers of cells with intense CR3 immunoreactivity appeared throughout the cortex. These cells were round and had short thickened processes, characteristic of activated microglia (Fig. 3D,F).

In the perfused rats, extravasation of IgG remained minimal and little immunoreactivity could be detected exclusively around the impact sites due to elimination of the circulating blood before the injury (Fig. 4A,B). In the hemisphere contralateral to the injury, microglia retained

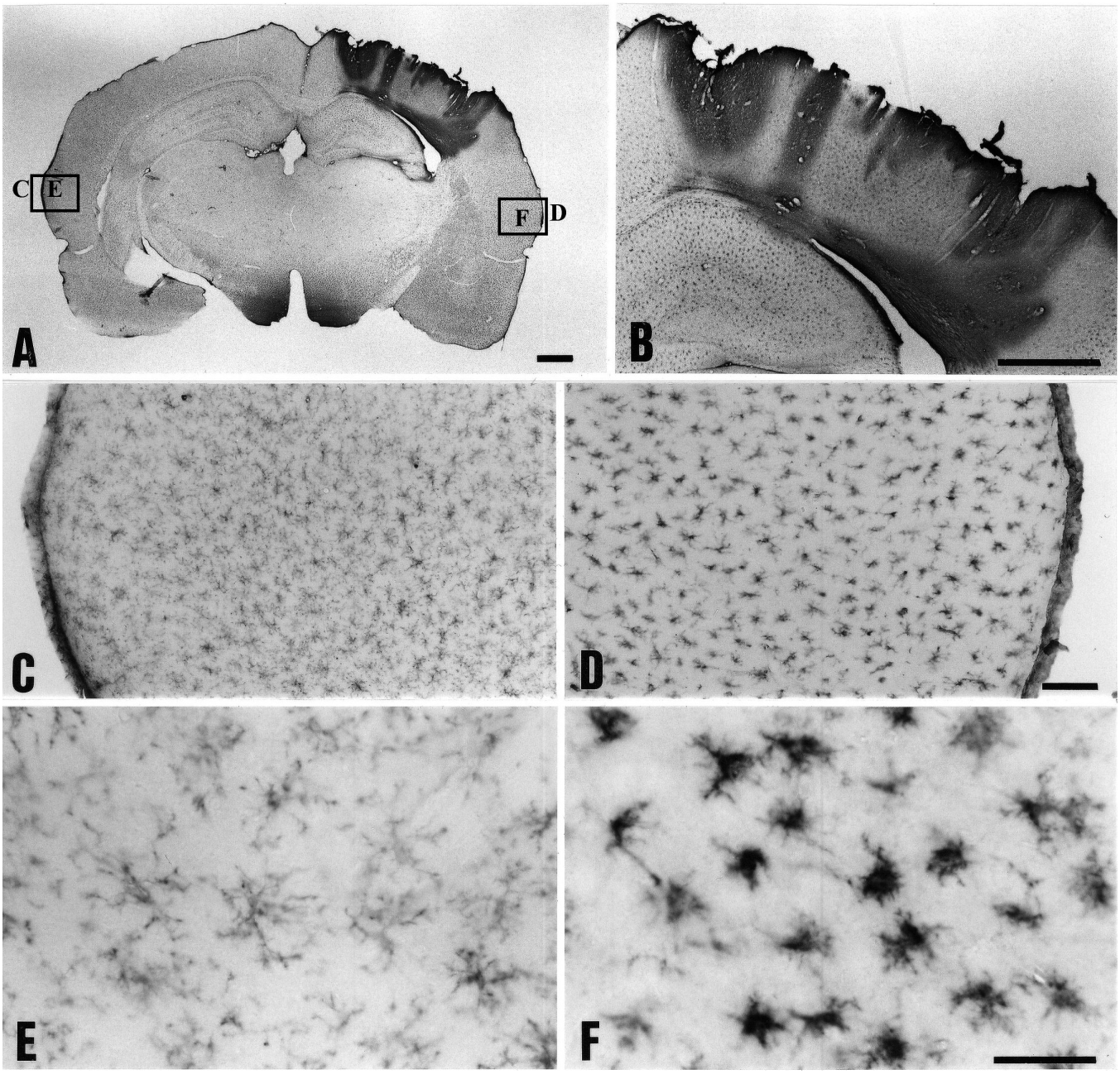

Fig. 3. Extravasation of IgG and CR3 immunoreactivity at 5 min after cortical contusion in control rats. **A.** Whole section. The boxes indicate the areas of higher magnification shown in **C-F**. **B.** Extent of IgG immunoreactivity which represents BBB disruption as visualized by the DAB reaction. Note that widespread IgG extravasation occurred in both the cortex and subcortex overlying the dorsal hippocampus. **C-F.** Microglial reactions in the cortex. Intense CR3 immunoreactivity is evident throughout the hemisphere ipsilateral to the injury side. The cells show a round cytoplasm with thickened processes, indicating transformation to activated or ameboid microglia (**D, F**). However, on the contralateral side, the microglia retain a ramified morphology and are indistinguishable from the resident ramified microglia seen in the normal brain **(C, E)**. Scale bars: A, B, 1.0 mm; C-F, 100 μm

a ramified morphology indicating that the process of undergoing perfusion with oxygenized Ringer's solution did not induce obvious changes in their morphology (Fig. 4C,E). Interestingly, the tissue damage appeared to be much smaller than in the control rats if the circulating blood was successfully eliminated, even though the

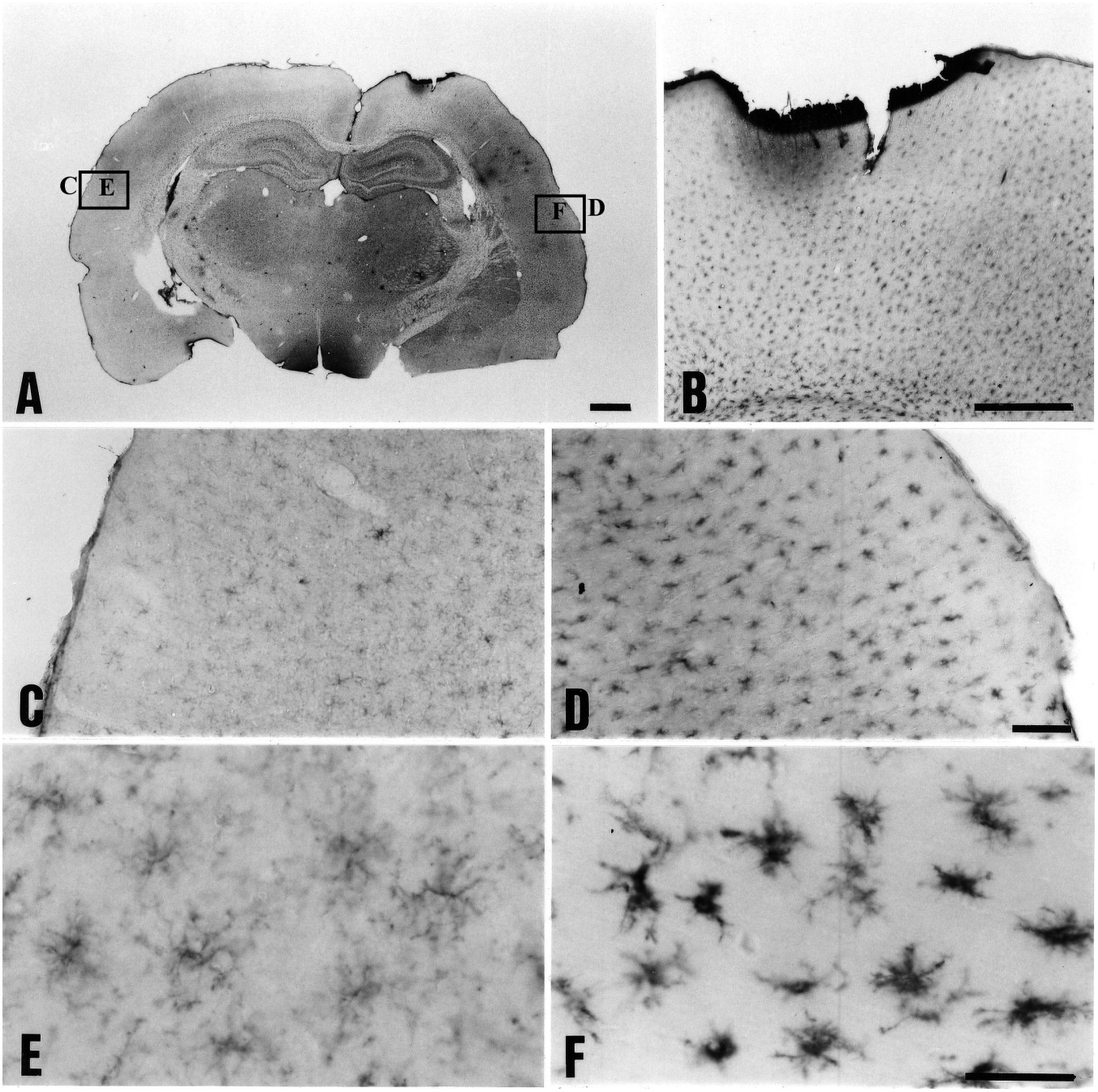

Fig. 4. Microglial reaction at 5 min after impact injury in rats which were perfused with oxygenized Ringer's solution prior to the injury. **A.** Whole coronal section showing the cortical contusion in the right cerebral cortex. **B.** Higher magnification of the impact site. Note that the extravasation of IgG is markedly suppressed and little immunoreactivity can be observed due to elimination of the circulating blood. The boxes indicate the areas of higher magnification shown in **C-F**. **C-F.** Microglial reactions on each side of the cortex. Note that intense CR3 expression appeared exclusively in the hemisphere ipsilateral to the injury side **(D, F)**. On the contralateral side, the microglia retained a ramified morphology **(C, E)**. These findings were reproducibly observed in all rats (n=8) which were perfused with oxygenized Ringer's solution prior to injury and processed immunohistochemically. Scale bars: A, 1.0 mm; B, 500 μm; C-F 100 μm

cortical impact injury was identical. However, activated microglia with an increased CR3 expression were evident in the hemisphere ipsilateral to the injury side (Fig. 4D,F). The morphology and staining pattern of the microglia corresponded with those seen in the control rats. The present results thus clearly indicated that the microglial activation induced immediately after injury is not subject to the involvement of variables arising from the circulating blood and its constituents.

Discussion

It has been demonstrated that microglia can respond rapidly to injurious stresses arising from various kinds of CNS injury, and transform into reactive species which have been termed activated or ameboid microglia (Kreutzberg, 1987). Following injury, microglia rapidly change their morphology from resident ramified to round activated or ameboid forms, and secrete certain kinds of molecules which could potentially exacerbate the secondary damage via inflammation processes (Suzumura et al., 1990; Giulian et al., 1994; Bruce-Keller, 1999; Block and Hong, 2005). However, recent studies have revealed that microglial activation can be observed even in the uninjured brain and may reflect environmental changes occurring around the cells (Gehrmann et al., 1993; Caggiano and Kraig, 1996). The earliest stimulant(s) which can induce microglial activation following TBI *in vivo* remain obscure. *In vitro* studies have shown that molecules such as IFN-γ, IL-3, IL-4, colony-stimulating factors (CSFs), and ATP exert effects on microglia for activation/proliferation (Giulian et al., 1989; Suzumura et al., 1990; Waltz et al., 1993; Inoue, 2002). Growing evidence has indicated that these factors may contribute to the process of microglial reactions after a variety of kinds of CNS injury (Block and Hong, 2005). Since the circulating blood potentially contains a large amount of cytokines or molecules which could activate microglia, it can be assumed that these factors might initiate microglial activation when vasculature damage occurs (Jensen et al., 1997; Lu et al., 2001). However, it remains uncertain whether or not these molecules arising from the circulating blood do contribute to the occurrence of microglial activation *in vivo*.

Some reports have indicated that microglial activation can be detected in areas that are more extensive than the extent of BBB disruption following ischemic injury (Morioka et al., 1991; Gehrmann et al., 1992). Furthermore, it has been demonstrated that microglia can transform into macrophage-like cells in the absence of monocytes and other blood constituents by employing a brain slice paradigm following ischemic insult (Shipley and Zimmer, 1994). Kettenmann et al. has reported that microglia have been found to possess a unique potassium channel pattern *in vitro* which renders them more sensitive to changes in extracellular potassium than are any other cell types in the brain (Kettenmann et al., 1990). On this basis, two studies have shown that microglia respond to neuronal depolarization and are associated with increased potassium fluxes across the membranes in the absence of neuronal damage as demonstrated in a model of cortical spreading depression (CSD) (Gehrmann et al., 1993; Caggiano and Kraig, 1996). In agreement with these findings, we have previously observed that the microglial activation with increased CR3 expression of microglia occurring at 1 hour following TBI can be inhibited when KYN, a broad-spectrum EAA antagonist, is perfused by microdialysis at doses which inhibit trauma-induced depolarization (Katayama et al., 1995). Furthermore, we have reported that microglial activation can be induced in an in vitro slice injury model which serves to eliminate influences arising from the circulating blood (Koshinaga et al., 2000). Thus, there is abundant indirect evidence to suggest that microglial activation is largely unrelated to infiltration of circulating blood and invasion of mononuclear phagocytes, although direct evidence has not hitherto been provided. In order to obtain such direct evidence, the present experiments employed perfusion of oxygenized Ringer's solution into the ascending aorta so that the circulating blood could be eliminated prior to injury induction. Rapid and widespread microglial activation was observed throughout the ipsilateral hemisphere induced by TBI even after the circulating blood had been replaced with oxygenized Ringer's solution. There was a possibility that BBB disruption might contribute in part to the induction of microglial activation, at least near the lesion sites where the vascular damage might be significant. We carefully assessed the degree of microglial activation from both the observed morphological changes and increased expression of CR3 in the perfused rats, but it appeared that neither of them exhibited a substantial difference between sites near to and distant from the lesions. Our findings thus suggest that the rapid and widespread microglial activation induced immediately after TBI is exclusively caused by intrinsic mechanisms rather than infiltration of circulating blood.

The results of the present study demonstrated that the size of lesions seen in the perfused rats was much smaller than that of the non-perfused rats following injury, indicating that the BBB disruption begins even at 5 min after the injury. Such an apparent difference can be explained by the fact that the extent of BBB disruption does not necessarily reflect the exact size of the tissue damage. The lesions induced by impact injury are thought to consist of mechanical injury, bleeding, and edema formation arising from vascular damage including BBB disruption. In the perfused rats, only mechanical injury was applied to the brains without infiltration of blood components, so that the size of the lesions might be much smaller than in non-perfused rats. The BBB disruption induced by the injury might thus play an important role in extending the lesion volume. However, the present results clearly demonstrated that the insult given by the cortical impact injury was

sufficient to induce a microglial reaction immediately following the injury, and this was independent of the BBB disruption.

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