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ORIGINAL ARTICLE

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The expression of tumor necrosis factor-α in the rat brain after fluid percussive injury

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Abstract To investigate the role of tumor necrosis factor-α (TNFα) after traumatic head injury in rats, moderate brain injury of 1000 mmHg was generated by an original fluid percussion injury device. TNFα levels in cerebrospinal fluid (CSF) gradually increased during the first 1 h, rose to a maximal elevation at 3 h and 6 h and returned to basal values by 24 h. Horseradish peroxidase tracer experiments revealed that primary microvascular damage appeared as early as 15 min after impact, but rapidly recovered and 1 h after impact secondary microvascular damage occurred in the hippocampus and parasagittal cortex. By immunoelectron microscopy, TNFα reactions were detected in the lysosomes of microglia accumulated at the impact site of the cortex 30 min after impact, and 1 h after impact these reactions were mainly detected at the glial cells (such as microglia and astrocytes) in the hippocampus and parasagittal cortex. Therefore the delayed microvascular damage observed in sites remote from the impact may be induced by TNFα which is synthesized mainly by glial cells. The present study suggests that TNFα conveyed from the microglial cells is one cofactor contributing to the fluid percussive brain edema formation after moderate brain injury.

Key words Traumatic head injury · Tumor necrosis factor-α · Blood-brain barrier · Fluid percussion device · Microglial cell

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Introduction

Karger (1995a, b) reviewed the mechanism of gunshot ballistic injuries and suggested that direct hits to the central nervous system (CNS) induce an increase in secondary intracranial pressure or delayed brain edema. So we approached the problem of clarifying the mechanism of ballistic head injury with fluid percussion models which are well-recognized experimental models for traumatic head injury (Dixon et al. 1987; Sunami et al. 1989; Yamaki et al. 1994). Traumatic head injury is accompanied by secondary or delayed brain edema and these edema processes are also believed to participate in the pathogenesis of traumatic head injury (Ellis et al. 1989; Tanno et al. 1992a, b; Soares et al. 1992; Fukuda et al. 1993; Dietrich et al. 1994). In this respect, various experimental studies have been performed and recent evidence suggested that tumor necrosis factor-α (TNFα) is an important mediator of the metabolic sequelae and organ demise of traumatic head injury (Taupin et al.1993; Feuerstein et al.1994; Ross et al. 1994; Ott et al. 1994). Fan et al. (1996) reported that the expression of TNFα mRNA is acutely increased in the hippocampus and cortex following fluid percussive brain injury and suggested that the brain is able to regionally alter its gene expression for TNFα production. However, immunocytochemical studies on the synthesis and release of TNFα and how this mediator induces cerebral edemas have been limited (Tchelingerian et al. 1993; Liu et al. 1996). We know little about how the expression of TNFα in cerebral tissues results in cerebral edema and whether TNFα affects the deterioration of the blood-brain barrier (BBB) following fluid percussive brain injury. The aim of the present experiment was to show the immunolocalization of TNFα after traumatic brain injury by fluid percussion and the effects of TNFα on the deterioration of the BBB using horseradish peroxidase as a tracer.

Materials and methods

Male Wistar rats (Seiwa Experimental Animal Co., Ooita, Japan: n = 64, weighing 300–350 g were used. All animal experiments were performed in accordance with the Committee for experiments with animals at the School of Medicine, University of Occupational and Environmental Health, and performed along the guidelines of the Committee.

Surgical procedure

Animals were surgically prepared under sodium pentobarbital anesthesia (60 mg/kg intraperitoneally). After incision of the temporal muscle, a circular craniotomy, 5 mm in diameter, was drilled in the lower parietal region. A polyethylene tube filled with isotonic saline was placed against the intact dura, fixed securely to the skull with dental acrylic cement and connected to a fluid percussive device. The device used to produce experimental brain injury was a modified fluid percussion device produced by the department of neurological surgery of Chiba University (Sunami et al. 1989; Ozawa et al. 1991). The animals were then subjected to an impact pressure of 1000 mmHg for 20 ms.The device was removed and the skull hole was closed with dental acrylic cement. Normal body temperature was maintained with a hot plate. In sham-operated controls, all surgical steps were conducted, but rats were not traumatized.

TNF assay

For the TNF assay 25 rats were utilized. Cerebrospinal fluid (CSF) was obtained from the cisterna magna at 0 h (pre-impact), 1 h, 3 h, 6 h and 24 h after impact. TNF-α levels of the CSF were determined by a use of the mouse TNF-α ELISA kit (no. 80–2903–00, Genzyme Corporation, Cambridge, Mass.) because previous investigators reported that this kit is a useful tool for the quantitation of rat TNF-α (Pizarro et al. 1993). The detection limit of the assay was 15 pg/ml. Data were expressed as mean ± SE for five rats. A non-paired *t*-test was used to determine significance by the Welch *t*-test and significance was accepted if $p < 0.05$.

Magnetic resonance analysis

For magnetic resonance(MR) analyses three rats were intraperitoneally anesthetised with sodium pentobarbital (60 mg/kg body weight) and analysed with T2-weighted MR imaging which was performed using a 4.7 Tesla MR system with an 400 nm horizontal bore and proton frequency of 200.0 MHz (SIS 200/400; Spectroscopy Imaging System Corp., Fremont, Calif.) before and 24 h after impact. The head of the animal was placed prone in a slotted tube resonator. Spin-echo MR images were obtained in orthogonal planes to confirm the desired positioning (acquisition parameters for T2-weighted images: TR = 2500ms; TE = 80ms; 256 × 192 matrix; field of view = 40 × 40 mm; 1excitation; and eleven 2-mm coronal slices with 0 mm center-to center spacing).

Tracer experiment

Tracer experiments were assessed before and 15 min, 30 min, 1 h, 3 h, 6 h and 24 h after impact. The rats (n = 21) were pretreated with diphenhydramine hydrochloride (0.5 mg/100 g; intraperitoneal injection) to prevent histamine release and received intravenous injections of 50 mg/kg horseradish peroxidase (HRP; type VI peroxidase; Sigma Chemical Co., St. Louis) via the tail vein according to the method of Karnovsky (1967). At 5 min after the HRP injection the animals were perfused with a solution of sterile saline, followed by a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PBS, infused from the left ventricle. The brain was removed and dissected for the light and electron microscopic visualization of HRP. The specimens for light microscopy were embedded in paraffin and stained with DAB substrate kit (Histofine SAB-PO Kit, Code 425011; Nichirei, Tokyo, Japan). The strategy used to select specimens for electron microscopy was to rely on the edema as a visual guide of the MR imaging. Specimens of parasagittal cortex, hippocampus and the impact site including the cerebral cortex were cut into approximately 30–40 μm sections on a microslicer (Dosaka EM, Osaka, Japan) and inserted into a sample mesh pack (Shiraimatsu, Osaka, Japan). Sections were treated with a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris buffer containing 0.05%H_2O_2 for 10 min at room temperature, thoroughly washed in 0.05 M Tris buffer, postfixed in 1% osmium tetroxide in 0.1 M PBS for 1 h at 4° C, dehydrated in an ascending ethyl alcohol series, embedded in Quetol 812, and examined under an electron microscope.

Immunocytochemistry

Antibodies

Polyclonal rabbit anti-mouse TNF-α antibody (Genzyme Corporation, Cambridge, Mass.) diluted to 1:100 in 0.1 M PBS was used. This antibody has demonstrated cross-reactivity with rat TNF-α (Merrick et al. 1992).

Immunostaining procedures

Immunoelectron microscopical experiments were assessed before and 30 min, 1 h, 3 h and 24 h after impact using the streptavidin-biotin technique. A total of 15 animals was perfused with 0.1 M PBS from the left ventricle, and then with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M PBS. The brain was removed and dissected into three parts (e.g. parasagittal cortex, hippocampus and impact site including the cerebral cortex). The specimens were cut into approximately 2 mm thick slices and postfixed in the same fixative for 1 h. Sections approximately 30–40 μm in thickness were made on the microslicer and inserted into the sample mesh pack. The endogenous peroxidase activities were blocked by incubation in a periodic acid solution (Histofine, Code29271; Nichirei, Tokyo, Japan) for 45 s. After treatment with normal goat serum at room temperature, sections were incubated overnight with the primary antibody at 4° C. Sections were incubated with a biotinylated secondary antibody (goat anti-mouse IgG F(ab′)2; EY Laboratories) diluted to 1:200 with 0.05 M Tris buffer for 1 h at 37° C, and then with a streptavidin-peroxidase complex (Histofine SAB-PO kits; Nichirei) diluted to 1:600 with 0.05 M Tris buffer for 1 h at 37° C. After incubation, sections were treated with DAB in 0.05 M Tris buffer containing 0.05%H_2O_2 at pH 7.6 for 10 min at room temperature. They were thoroughly washed in 0.05 M Tris buffer, postfixed in 1% osmium tetroxide for 1 h at 4° C, dehydrated in an ascending ethyl alcohol series, embedded in Quetol 812, and examined under the electron microscope.

For controls of each immunostaining, Tris buffer or normal serum was substituted for the primary antibodies.

Results

Figure 1 shows the time course of mean TNFα concentrations in CSF before and after impact. The TNF concentration gradually increased during the first 1 h, rose enormously to the maximal level (80.3 ± 40.4 pg/ml) at 3 h and (105.6 ± 30.6 pg/ml) at 6 h and then decreased to 14.6 ± 8.3 pg/ml at 24 h.

No visible signal intensities were detected by MRI in the brain pre-impact, but the localized area in the impact

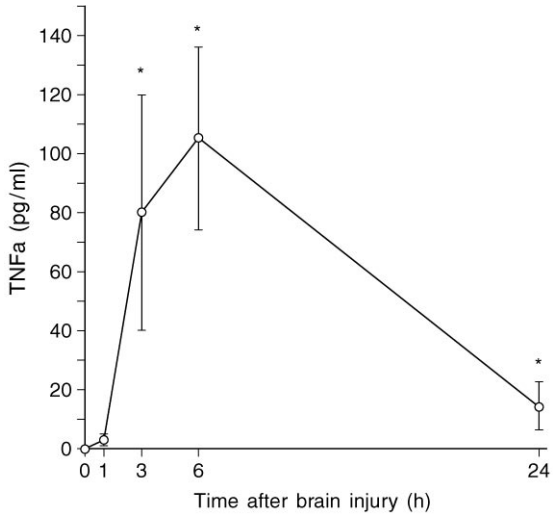


Fig. 1 Mean cerebrospinal fluid TNFα concentrations after treatment with impact measured by a use of the TNFα ELISA kit. *$p <$ 0.01 (versus 0 h: pre-impact). Data are expressed as the mean ± SE for 5 rats

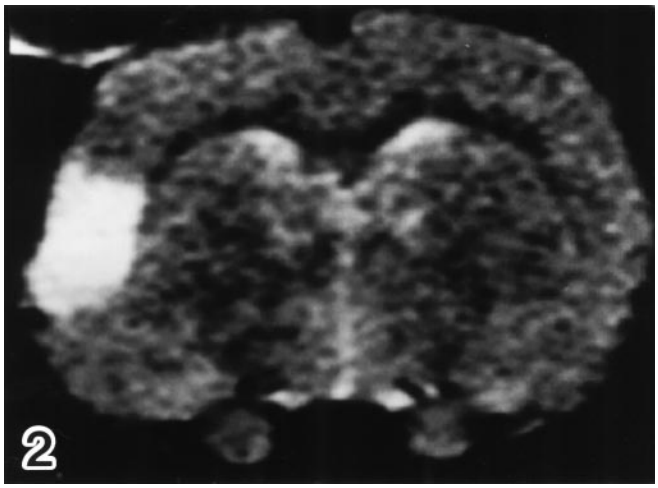


Fig. 2 Coronal T2-weighted image at the level of the anterior hypothalamus at 24 h after impact. The localized area in the impact site, parasagittal cortex and hippocampus increased signal intensities

site, superficial parasagittal cortex and hippocampus increased signal intensities at 24 h after impact (Fig. 2).

In sham-operated control animals, there was no diffuse staining of HRP. At 15 min after impact, diffuse staining of HRP occurred at the impact site (Fig. 3a), in the deep cortical layer adjacent to the external capsule, hippocampus (Fig. 3b) and mid-brain. At 30 min after impact, diffuse staining of HRP occurred at the impact site only. At 1 h after impact, diffuse staining of HRP occurred at the impact site and superficial parasagittal cortex (Fig. 3c). Diffuse staining of HRP occurred at the impact site only, 3 h after impact and persisted up to the 24 h after impact.

The results of the tracer experiments with electron microscopy were almost identical to the tracer experiments with light microscopy. The leakage of tracer was observed

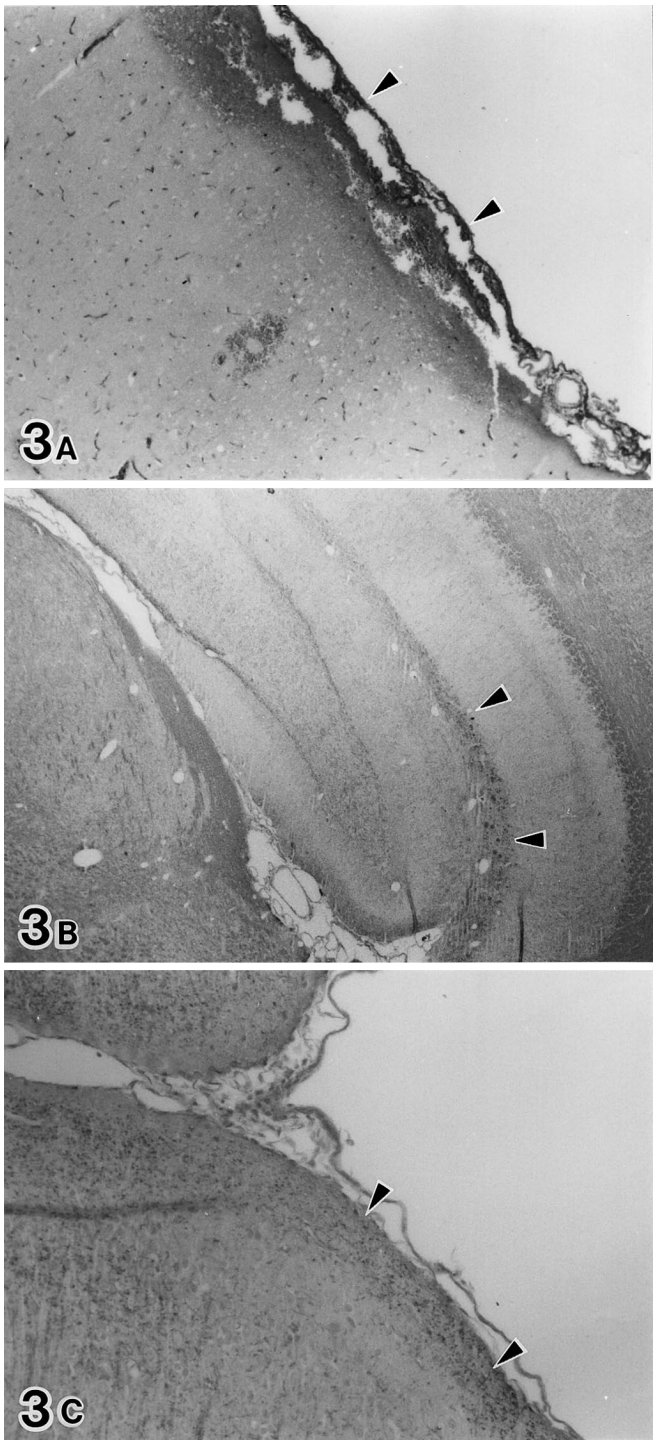
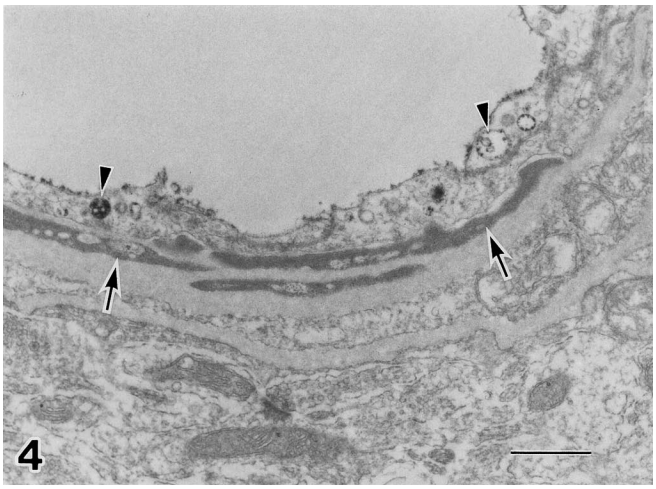
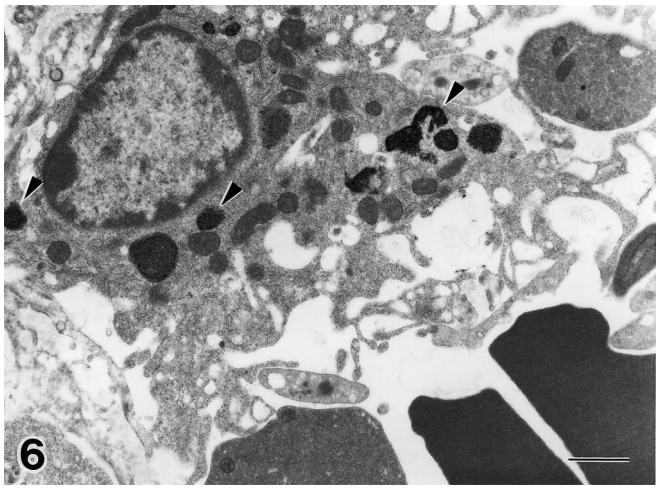


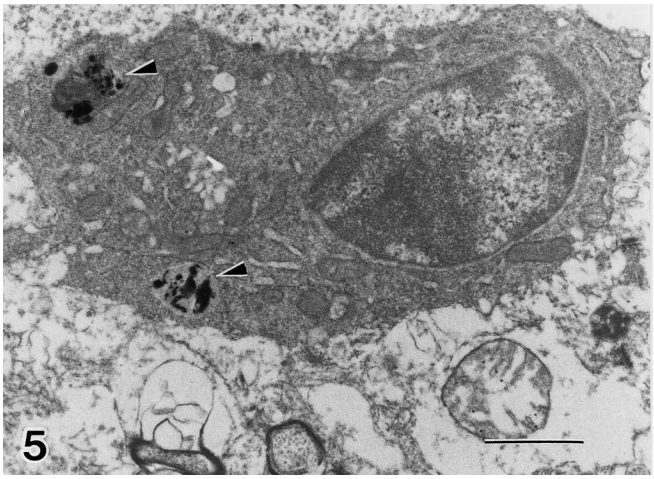
Fig. 3A–C **A** At 15 min after impact, diffuse staining of HRP (*arrowheads*) occurred in the impact site. **B** At 15 min after impact, diffuse staining of HRP (arrowheads) occurred in the hippocampus. **C** At 1 h after impact, diffuse staining of HRP (arrowheads) occurred in the superficial parasagittal cortex

in the hippocampus at 1 h after impact. The tracer was regularly found in the basal lamina and extracellular spaces (Fig. 4).

By immunoelectron microscopy, there were no immunoreactions of TNFα detected in sham-operated con-







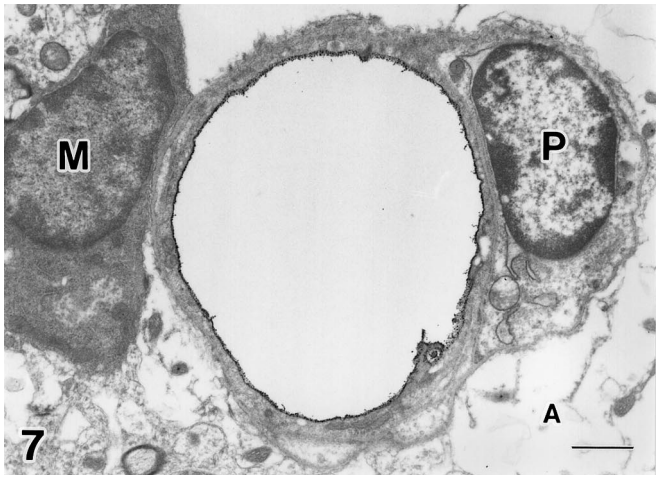


Fig. 4 Electron micrograph of tracer experiments showing capillary in the hippocampus at 1 h after impact. The endothelial cells appear to be edematous, and there is an increase in the number of vacuoles and pinocytotic vesicles (*arrowheads*) with the tracer in their interior compared with the control. Electron dense deposites of the tracer exist in the subendothelial layer including the basal lamina (*arrows*). Bar = 500 nm

Fig. 5 Electron micrograph showing immunoreactions of TNF in lysosomes (*arrowheads*) of a microglia at the impact site of cortex at 30 min after impact. This rounding of mature microglia reflect a metamorphosis to an activated state. Bar = 1 μm

Fig. 6 Electron micrograph showing immunoreactions of TNF in lysosomes (*arrowheads*) of a macrophage in the hemorrhagic contusion site of the cortex at 1 h after impact. Bar = 1 μm

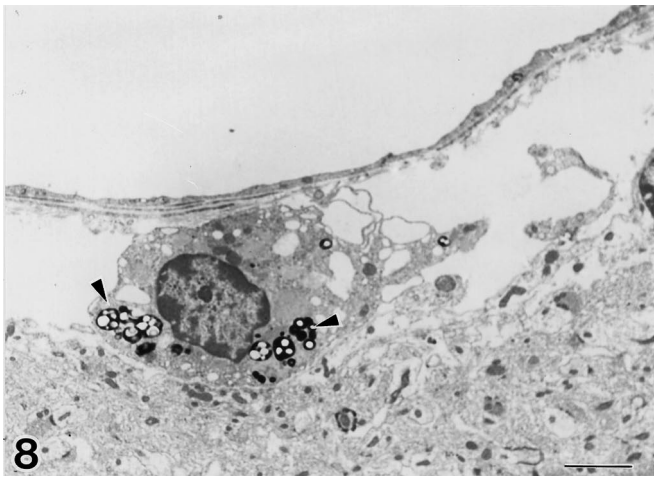
Fig. 7 Electron micrograph showing immunoreactions of TNF on the apical plasma membrane of the capillary endothelium in the hippocampus at 1 h after impact. The vascular feet of astrocytes (A) around the capillary show remarkable edematous changes. P: pericyte M: microglia Bar = 1 μm

trol brain tissue, but were occasionally detected in the lysosomes of microglia (Fig. 5) accumulated at the impact site of cortex at 30 min after impact. At 1 h after impact, microglias accumulated at the impact site of cortex and inflammatory cells such as macrophages (Fig. 6) were occasionally seen in the hemorrhagic contusion site of the cortex. Lysosomes of such cells were immunoreactive for TNFα. In addition immunoreactions of TNFα were detected at the luminal surface of the capillary endothelium (Fig. 7), the lysosomes of inflammatory cells such as intravascular leukocyte and perivascular monocyte-derived cells (Fig. 8) which were infrequently seen, and the lysosomes of accumulated microglias, perivascular astrocytes and neuronal cell in the hippocampus (Fig. 9) and parasagittal cortex. In these site, edematous changes occurred in the neuronal cell (Fig. 10) and the vascular feet of astrocytes. The appearance of inflammatory cells frequently increased with the increase in time after impact.

These immunoreactivities were more frequently observed at 3 and 6 h after impact and persisted up to the 24 h after impact.

Discussion

Previous HRP tracer experiments using fluid percussion models have indicated that early microvascular damage is induced by direct head injury and delayed microvascular damage observed in sites remote from the impact sites such as the hippocampus and parasagittal cortex may be induced by endogenous neurotoxic mediators (Fukuda et al. 1993; Dietrich et al. 1994). The results of our present



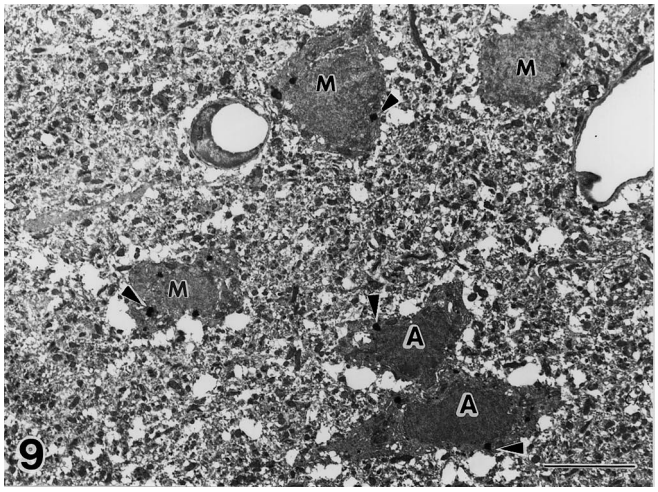


Fig. 8 Electron micrograph showing immunoreactions of TNF in lysosomes (arrowheads) of a perivascular monocyte-derived cell in the hippocampus at 1 h after impact. An inflammatory cell such as monocyte-derived cell has extravasated through the venular endothelium. Bar = 2 μm

Fig. 9 Electron micrograph showing immunoreactions of TNF in lysosomes (*arrowheads*) of glia cells such as microglia and astrocyte in the hippocampus at 1 h after impact. M: microglia A: astrocyte Bar = 5 μm

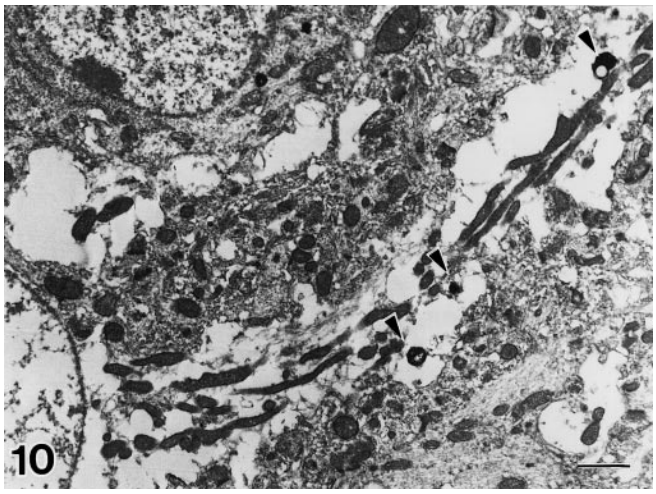


Fig. 10 Electron micrograph showing immunoreactions of TNF in lysosomes (*arrowheads*) of a neuronal cell in the hippocampus at 3 h after impact. The edematous changes occurred in the dendrite. Bar = 1 μm

tracer experiments are similar to these reports and revealed that primary microvascular damage appears as early as 15 min after impact. This primary BBB damage acutely recovered and the diffuse staining of HRP disappeared except at the impact site at 30 min after impact. At 1 h after impact, secondary microvascular damage occurred at the hippocampus and parasagittal cortex. TNFα has been implicated as a major proinflammatory cytokine that is elevated after head injury (Goodman et al. 1990; Taupin et al. 1993; Ross et al. 1994; Feuerstein et al. 1994) and plays a role in the pathology of a brain injury. Our present immunocytochemical study showed that activated microglia release TNFα as early as 30 min after impact at the impact site. Microglia are the brain's own kind of macrophages and secrete a variety of biologically active factors, including TNFα (Sheng et al. 1995; Chao et al. 1995). Streit and Kincaid-Colton (1995) suggested that microglia are extremely mobile and are transported easily to injured areas within the brain, and our present study revealed that microglia are the first cell type to arrive at an injury site. Therefore, the fact that TNFα was released by microglia is very important. TNFα often helps to recruit other components of the immune system to a site of injury causing an increase in inflammation and glial cells activation (Kahn et al. 1995; Chao et al. 1995), and TNFα can also directly damage the BBB (Terada et al. 1992; Megyeri et al. 1992; Liu et al. 1996). The present study indicated that TNFα was detected 1 h after impact at the glial cells such as astrocytes, and inflammatory cells such as intravascular leukocyte and perivascular monocyte-derived cells in the hippocampus and parasagittal cortex. Fan et al. (1996) reported that the expression of TNFα mRNA is acutely increased in the hippocampus and parasagittal cortex 1 h after fluid percussive brain injury and suggested that TNFα mRNA is synthesized directly by brain tissue. Previous studies with this model have demonstrated that inflammatory cells were only occasionally seen adhering to the endothelium of venules and these cells appeared to be mononuclear 45 min after impact (Dietrich et al. 1994). Aihara et al. (1995) suggested that macrophages in the subarachnoid space may have migrated into the adjacent injured cortex. Soares et al. (1995) reported that intravascular leukocytes appeared at the impact site and hippocampus 2 h after impact. Our present experiments gave similar results and 1 h after impact the inflammatory cells were infrequently seen and mainly observed in the accumulated microglia in the remote impact sites. In addition immunoreactions for TNFα were detected at the apical plasma membrane of the capillary endothelium and lysosomes of the edematous changes occurred in the vascular feet of astrocytes 1 h after impact. Tsujimoto et al. (1985) suggested that these reactions on the surface of the endothelium are specific receptor sites

for TNF and these cytokines have chemotactic factors for adhesion in the process of contact between endothelial and inflammatory cells. So these reactions of the apical plasma membrane may induce the later appearance of the inflammatory cells which frequently increased with the increase in time after impact. Since Stolpen et al. (1986) suggested that TNF induces a deterioration in the molecular arrangement of the tight junctional proteins of the vasucular endothelium, the leakage of the HRP throughout the tight junctions of the blood-brain barrier (BBB) in our experiments may imply an increase of the permeability due to the deterioration of BBB. These facts indicate that the microvascular damage in the hippocampus and parasagittal cortex 1 h after impact may be induced by TNFα which is synthesized mainly by perivascular glial cells including microglia and astrocytes.According to the immunoelectron microscopic results, lysosomes of the inflammatory cells were intensely immunoreactive for TNF. Thus TNF, produced and stored in these cells, may also accelerate the cytotoxic changes of the remote impact site as previously described by others (Rothstein and Schreiber 1992; Nagano et al. 1992; Kita et al. 1993).

Our present TNF assay study demonstrates that TNFα is elevated in CSF following head injury, but the levels of TNFα gradually increased during the first 1 h and maximally increased at 3 h and 6 h after impact. The present immunoelectron microscopical study indicates that the immunoreactions of TNFα were detected early at the impact site in microglia 30 min after impact and at 1 h after impact were mainly detected at the glial cells. The appearance of these inflammatory cells was infrequently observed 1 h after impact and frequently increased in addition to the time after impact. So the present immunoelectron microscopical results indicate that the increase in TNFα levels of CSF may original from inflammatory cells. Previous studies demonstrated that the local tissue level of TNF is more important than circulating levels (Plata-Salaman et al. 1988; Tracey et al. 1990; Ross et al. 1994) and our study confirmed these findings.

The degeneration of neuron and neural pathways has been reported in previous studies with this model (Povlishock et al. 1992; Dietrich et al. 1994; Aihara et al. 1995; Soares et al. 1995). The results of the present study are similar and revealed that neuronal cell damage appears 1 h after impact and the immunoreactions of TNFα were also detected. The present study suggests that TNF contributes to the destruction of the neuron and neural pathways and this is supported by electron microscopic immunocytochemical evidence of TNF labeling.

In conclusion, we would like to mention that the present study suggests that TNFα conveyed from the microglia cell is one cofactor contributing to the fluid percussive brain edema formation after moderate brain injury.

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