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Astrocytes Produce and Release Interleukin-1, Interleukin-6, Tumor Necrosis Factor Alpha and Interferon-Gamma Following Traumatic and Metabolic Injury

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ABSTRACT

The brain is no longer considered immune-privileged due to its capability of producing cytokines in response to neurotrauma; however, the cellular sources of cytokines have not been defined. This study focused on the production of four inflammatory cytokines, interleukin-1 (IL-1α), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and interferon gamma (IFN-γ) in primary culture of astrocytes under two different injury models which simulated *in vivo* mechanical trauma (scratch injury) and ischemia. Results demonstrated that astrocytes after scratch injury were positively immunostained with IL-1α, IL-6, and TNFα. A slot-blot study of culture media showed that the release of IL-1α, IL-6, TNFα, and IFN-γ by astrocytes subsequent to scratch and ischemic injury reached approximately twice the control values. The temporal expression of these cytokines was different for the two models. All four cytokines began to increase 1 h postscratch and remained at high levels throughout the experiment. In the ischemic model, however, the increase of cytokine expression was delayed until 4–8 h of ischemia, when sharp increases were seen in all four cytokines. In this culture system, the exogenous influence of blood-borne factors and leukocytes, which occur with *in vivo* trauma and ischemia, was eliminated. Accordingly, the cytokines detected in the culture media were derived from astrocytes. This study provides the first evidence that astrocytes, without the influence from other cell types, can produce and release cytokines following mechanical and ischemic injury.

Key words: astrocytes; cytokine; IFN-γ; IL-1α; IL-6; injury; TNFα

INTRODUCTION

THE CONCEPT that the brain is an immune-privileged site is undergoing radical change, as it is now known that it is capable of mounting an immunological response, characteristic of peripheral immune functions. The brain produces cytokines, antigen presenting cells and phagocytic cells in response to a wide variety of stresses and injuries (Benveniste, 1993; Schmidt et al., 1990). The increased production of inflammatory mediators—interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and interferon-gamma (IFN-γ)—has been demonstrated in brains afflicted by bacterial (Waage et al., 1989) or viral infection (Frei et al., 1989), immunological disorders, including multiple sclerosis (Merrill, 1992), acquired immunodeficiency syndrome (Merrill, 1992), and experimental allergic encephalomyelitis (Chung et al., 1991).

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Cytokines detected in the brain were thought to be produced primarily by activated lymphocytes and monocytes that have infiltrated the nervous system (Hickey, 1991). Other evidence, however, has shown that some endogenous cells such as microglia and astrocytes also produce cytokines under various injury conditions (Benveniste, 1993; Schmidt et al., 1990). Increased production of cytokines has also been demonstrated in noninflammatory neurological disorders such as ischemic infarction (Minami et al., 1992; Szaflarski et al., 1995) where few circulating leukocytes and no lymphocytes enter the brain.

Increased production of IL-1, IL-6, and TNFα occurred in the brain hours after trauma (Taupin et al., 1993) prior to the appearance of inflammatory cells and macrophages. Under these conditions the cytokine production was attributed to astrocytes and neurons. In cultured astrocytes, increased production of IL-1α and TNFα was induced by lipopolysaccharide (Chung and Benveniste, 1990), and that of IL-6, by fluid percussion trauma (Hariri et al., 1994; Tchelingerian et al., 1993) as well as by hypoxia-reoxygenation (Maeda et al., 1994).

This study focused on the production and release of IL-1α, IL-6, TNFα, and IFN-γ in cultured astrocytes subsequent to two models of injury: the first was a scratch wound model that mimicked a pure mechanical trauma (Yu et al., 1993; Ghirnikar et al., 1994; Hou et al., 1995); the second was a combination of hypoxia and reduced culture medium that contained no glucose, a situation that simulated *in vivo* ischemic injury (Kaku et al., 1991; Yu and Lau, 2000). The primary culture used for the study was a highly enriched preparation of astrocyte. Therefore, the cellular responses observed under these experimental conditions were assumed to be astrocytic.

MATERIALS AND METHODS

Astrocytic cultures were prepared from the cerebral cortex of newborn mice as previously described (Yu and Lau, 2000). Dissociated brain cells were seeded in 35-mm Falcon culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) and maintained in minimum essential medium (MEM) with 10% fetal calf serum (Hyclone, Logan, UT) at 37°C in 5% CO_2. Confluent cultures were subjected to the scratch wound as previously described (Yu et al., 1993; Ghirnikar et al., 1994; Hou et al., 1995). In brief, the cultures were washed twice with MEM without serum and scratched with a sterile plastic pipette tip in 1 mL of serum-free medium. The scratch was performed by following a grid that resulted in an injury of 40% of the cells (Yu et al., 1993). The cultures were then incubated in a CO_2 incubator for 1, 2, 4, and 8 h before harvesting. Control medium for the scratch experiment was taken from the culture immediately after scratch, equivalent to 0 h. Control incubation was performed with cultures that had gone through all the preparation procedures except the scratching.

The ischemic model was established using an anaerobic chamber (Yu and Lau, 2000; Forma Scientific, Marietta, OH). The chamber was filled with a mixture of $H_2/CO_2/N_2$ in a proportion of 10/5/85. The oxygen level in the chamber was monitored throughout the experiment using an OM-1 Oxygen Meter (Microelectrodes Inc, Bedford, NH). Cultures were placed in the anaerobic chamber and washed twice with glucose-free MEM that had been degassed for 30 min and saturated with nitrogen. Eight hundred microliters of glucose-free medium was added to cover the cells in the culture dish. This amount of incubation medium was less than the amount used in other hypoxia/hypoglycemia culture models, and thus

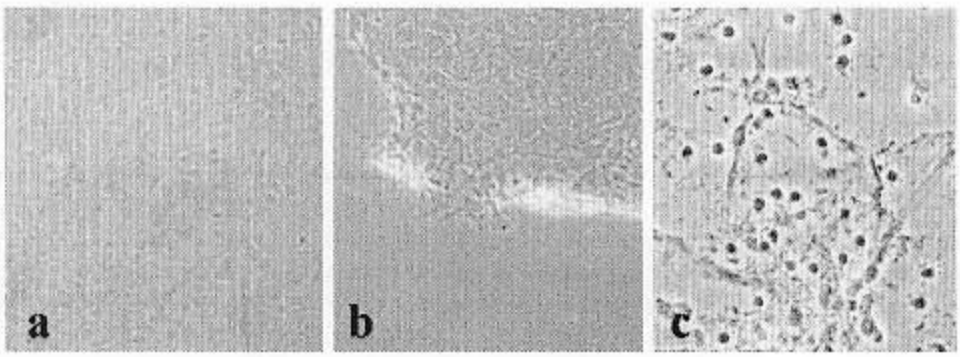

FIG. 1. Phase contrast micrographs showed the control (**a**), scratch (**b**), and ischemic (**c**) cultures of astrocytes. These cultures were at least 4 weeks old. Scratch cultures were taken 6 h after scratch. Some cells along the scratch began to send processes into the denuded area of the scratch. Ischemic culture showed huge morphological changes, indicating the nature of injury was different from the scratch injury. The cell death was quite even on the plate. The picture was taken 6 h after ischemic incubation.

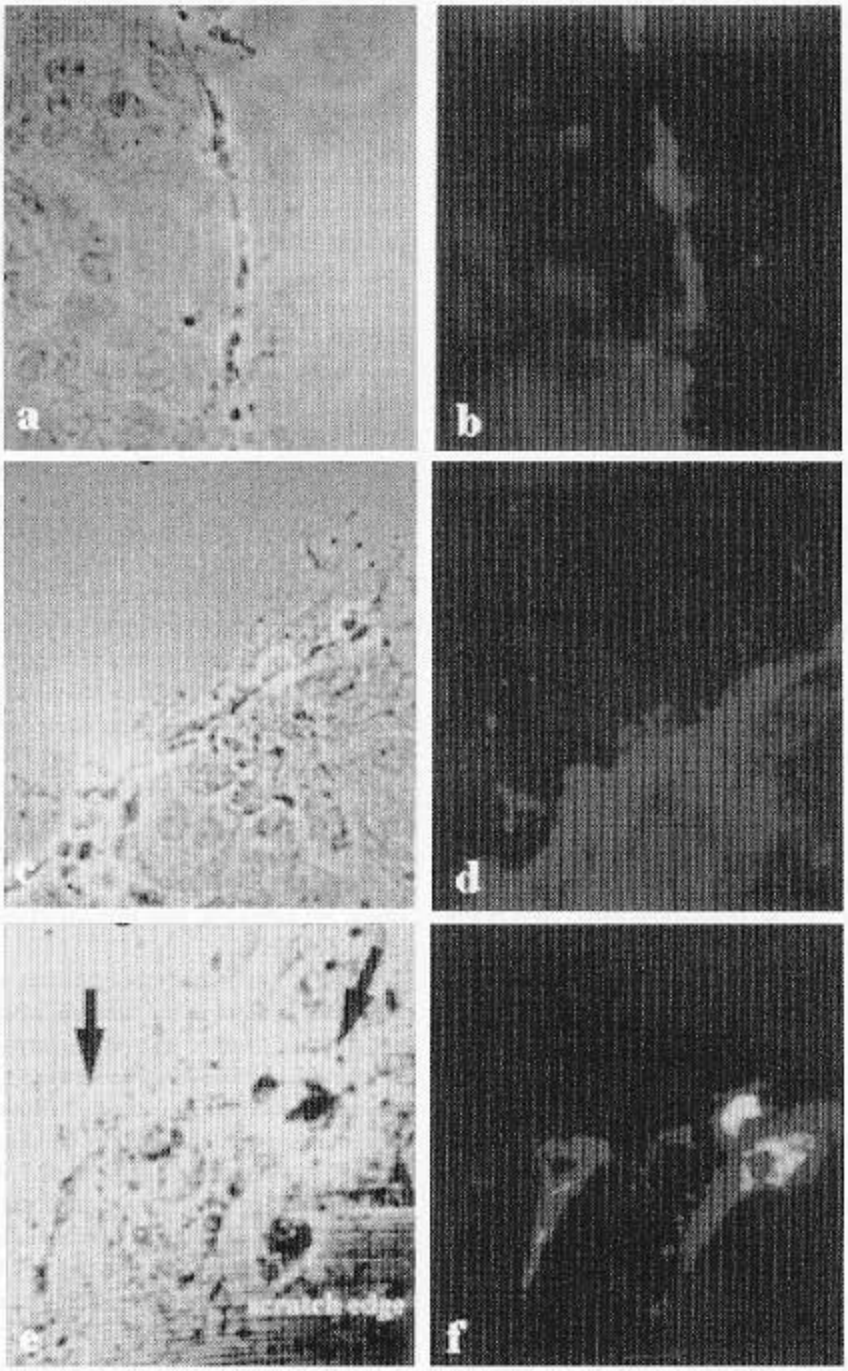

FIG. 2. Scratch astrocyte cultures stained for cytokines: IL-1α (**a,b**), TNFα (**c,d**), and IL-6 (**e,f**). a, c, and e were phase contrast micrographs; b, d, and f were fluorescent micrographs of the same field, respectively. b and d were taken from fluorescent microscope, and f was taken from confocal microscope. In f, the arrows indicated cells that were positively stained for IL-6. Similar cells were positively stained for GFAP in another experiment.

acquired a lower extracellular and intracellular volume ratio; a condition more comparable to the *in situ* ischemia (Ames and Nesbett, 1983; Yu and Lau, 2000). The culture dish wrapped with paraffin to prevent evaporation was placed inside an incubator located inside the anaerobic chamber at 37°C. Control cultures were incubated in parallel with the ischemic cultures but in 800 μL of normal MEM inside a normal CO_2 incubator.

Scratched cultures for immunostaining were washed twice with phosphate buffered saline (PBS), then fixed

with 4% paraformaldehyde (Sigma, St. Louis, MO) at room temperature. They were then washed with 0.1 M glycine in PBS for 5 min and permeabilized with 0.1% saponin (Sigma) in PBS for 10 min. The cultures were stained with cytokine antibodies. The cytokine antibodies were polyclonal anti-IL-1α, polyclonal anti-TNFα, monoclonal anti-IL-6 (Genzyme Corp., Cambridge, MA), and monoclonal anti-IFN-γ (BD PharMingen Inc., San Diego, CA). The antibodies were diluted 100 times in PBS/0.1% saponin (1% bovine serum albumin was included for monoclonal antibodies). After the addition of cytokine antibodies, the cultures were incubated at room temperature for 1 h (overnight for monoclonal antibody). All the primary antibodies were then visualized by 1 h incubation with rhodamine conjugated secondary antibody (Sigma) in 1:100 dilution. All stained cultures were mounted in Mowiol (Calbiochem, San Diego, CA) and observed under Confocal Laser Scanning Microscope (Bio-Rad Laboratories, Hercules, CA) or fluorescent microscope (Zeiss, Germany).

Slot-blot analysis was performed using a Bio-Dot apparatus (Bio-Rad Laboratories). Two hundred μl of samples was loaded onto a nitrocellulose membrane (Micron Separations Inc., Westboro, MA). After fixation, the blot was blocked with 5% nonfat dry milk in Tris-buffered saline Tween 20 (2% normal goat serum was included for polyclonal antibodies). Then the blot was incubated in the following primary antibodies: polyclonal anti-IL-1α (Genzyme Corp.) 1:250, polyclonal anti-TNFα (Genzyme Corp.) 1:250, monoclonal anti-IL-6 (Genzyme Corp.) 1:1,000 and, monoclonal anti-IFN-γ (BD PharMingen) 1:1,000. The blot was stained with HRP-conjugated secondary antibody (Amersham, Pharmacia Biotech, Buck, UK) 1:1,000 in Tris-buffered saline containing 2.5% milk. The signal was detected by ECL detection reagents and ECL Hyperfilm (Amersham). The film was scanned, and the signal intensities were measured by a Personal Densitometer (Molecular Dynamic Inc., Sunnyvale, CA). The change in signal intensity was estimated as ratios of the values in media of the injured and control cultures to the values in the media collected at 0 h treatment.

Each experiment was performed three times with duplicate cultures. All data were expressed as mean $\pm$ SEM. Statistical analysis was performed by ANOVA using SPSSWIN program. A confidence interval of 95%, that is, $p < 0.05$, was considered statistically significant.

RESULTS

Figure 1 showed some representative phase contrast micrographs of a normal astrocyte culture (Fig. 1a) and culture under the two injury models scratch and ischemic, respectively (Fig. 1b,c). As seen in Fig. 1a, the primary culture consisted of a confluent layer of astrocytes. All cells in the control preparation appeared intact and importantly the culture appeared to consist of a homogenous population of cells. With scratch injury, some astrocytes along the scratch wound started to extend processes into the denuded areas. In this model, the degree of injury was geographically distributed with the most severe injury associated with those cells along the scratch. Cells beyond the scratch appeared normal. In cultures under ischemia, no cell death was detected prior to 2 h of ischemia. The magnitude of cell death increased from 3 h upward and by 6 h, the integrity of many cells was altered (Fig. 1c). The morphology of the ischemic astrocytes was different from those seen in the scratch model. Many nuclei were condensed and the cytoplasm was disintegrated. Only 10% of the cells were considered viable at 8 h of ischemia.

The production of cytokines by astrocytes was demonstrated in the scratch model via immunostaining. The astrocytes along the scratch were positively stained for IL-1α (Fig. 2a,b), TNFα (Fig. 2c,d), and IL-6 (Fig. 2e,f). The immunostaining of IL-1α and TNFα was located primarily in cells paralleling the scratch. Some cells beyond the scratch edge also revealed strong TNFα staining. IL-6 immunostaining was also seen in some cells with typical astrocytic morphology close to the scratch. In this study, no astrocyte was immunostained with antibodies targeting IFN-γ.

Using slot-blot analysis cytokines were detected in the cell lysate but not in the medium of control culture (data not shown). Similar tests were performed on the media of control cultures that underwent the full preparative procedure including washing, without the induction of any injury procedure. Within the control incubation period, all cytokine levels in the scratch injury controls were low and did not shown any significant change. In the cultures prepared as controls for ischemia model, all the cytokine levels remained low, except with prolonged incubation when the levels of TNFα and IFN-γ were increased (data not shown).

In the scratch wound culture medium, a significant increase in the IL-1α level occurred beginning at 1 h postinjury, reaching 1.3 times of the control at 8 h (Fig. 3a, Table 1). In the ischemic culture, the IL-1α level remained the same at 1 h, dropped slightly from 2 to 4 h, and increased abruptly by 1.8 times at 8 h (Fig. 4a, Table 1). Compared with the changes in IL-1α in both models in their corresponding control incubations, the IL-1α levels in the media of injured cultures were increased significantly ($p < 0.05$).

One hour after scratch wound injury, the IL-6 level

rose to 1.2 times of the control and, at 2 h, reached its maximum level of 1.3 times of the control values. This was followed by a gradual decline to 1.2 times of the control at 8 h (Fig. 3b, Table 1). In the media of ischemic cultures, the IL-6 level remained close to normal until 2 h. The level significantly increased by 1.5 times at 4 h and abruptly to 2.6 times of the control value at 8 h (Fig. 4b, Table 1). Compared with changes in IL-6 in both models in their corresponding control incubations, the IL-6 levels in the media of injured cultures were increased significantly ($p < 0.05$).

In the media of scratch wound cultures, the TNFα level notably increased at 1 h, and at 4 h it reached a maximum level of 1.4 times of the control (Fig. 3c, Table 1). In the media of ischemic cultures, the level remained unchanged between 1 and 2 h. It then increased at 4 h and reached its maximum level of 2.2 times of the control value at 8 h (Fig. 4c, Table 1). Compared with the changes in TNFα in both models in their corresponding control incubations, the TNFα levels in the media of injured controls increased significantly ($p < 0.05$).

In the media of scratch-wound cultures, the IFN-γ level increased sharply by 1.6 times at 1 h and remained at a similar level up to 8 h (Fig. 3, Table 1). In the media of ischemic culture, the IFN-γ level showed little change for the first 2 h. The value increased over 1.6 times at 8 h (Fig. 4b, Table 1). Compared with the changes in IFN-γ in both models in their corresponding control incubations, the IFN-γ levels in the media from the injured cultures increased significantly ($p < 0.05$).

DISCUSSION

The results of this study demonstrate that astrocytes under mechanical and metabolic injury were capable of releasing IL-1α, IL-6, TNFα, and IFN-γ. The immunocytochemical studies clearly demonstrated that some astrocytes along the scratch edge were positively stained with antibodies to the cytokines. These astrocytes, when observed under time lapse video recording, were mobile (Yu et al., unpublished data). This indicates that some astrocytes were most likely activated by the scratch injury. Based on the staining result, we conclude that astrocytes could be induced to produce IL-1α, IL-6, and TNFα by mechanical injury. Although we could not identify any astrocytes positively stained for IFN-γ, others have demonstrated that astrocytes along lesions in multiple sclerosis showed IFN-γ immunoreactivity (Schmidt et al., 1990; Traugot and Lebon, 1988). The lack of IFN-γ positive staining in astrocytes in the scratch model may be related to the epitope specificity of the IFN-γ antibody used in this study.

These cytokines, including IFN-γ, were released into the culture medium by astrocytes subsequent to scratch wound and ischemic injury. In the astrocytic cultures, the exogenous influence of circulating factors and leukocytes, which occur in the *in vivo* models of trauma and ischemia, was eliminated. The cytokines released into the culture medium were therefore the sole product of injured astrocytes. This study thus provides the first evidence for the release of these cytokines from astrocytes under mechanical and metabolic injuries.

A difference in the timecourse of expression of the cytokines was observed between the two models. In the scratch wound model, all four cytokines began to increase 1 h postlesion and reached the peak levels at 4–8 h. In the ischemic model, however, the increase of cytokine expression was delayed until 4–8 h after ischemia when sharp increases were seen in all four cytokines. The distinct difference in the time of the onset of cytokine release could be related to the nature of the injury and the rate of cell death inherent in these two models. The scratch model (Ghirnikar et al., 1994; Hou et al., 1995; Yu et al., 1993) was designed to simulate traumatic in-

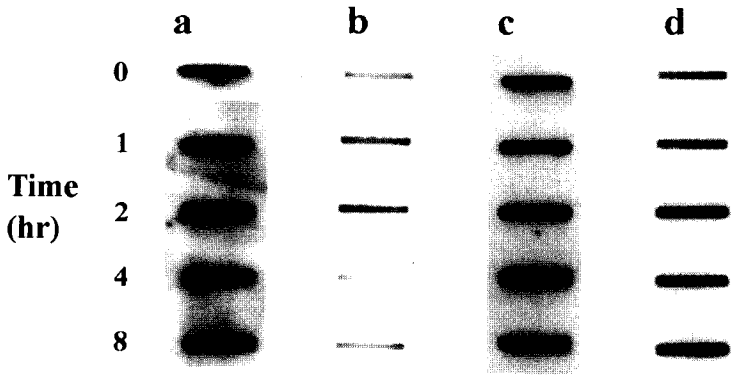

FIG. 3. Slot-blot analysis of changes of cytokine levels in astrocytic culture media after scratch injury. (**a**) IL-1α. (**b**) IL-6. (**c**) TNFα. (**d**) IFN-γ. The slot-blot shown was selected from six blots in three separated experiments.

Table 1. Changes of the Levels of Different Cytokines in Astrocyte Culture Media After Scratch and Ischemic Injury

	IL-1α		*IL-6*		*TNFα*		*IFN-γ*	
Time (h)	*Scratch*	*Ischemia*	*Scratch*	*Ischemia*	*Scratch*	*Ischemia*	*Scratch*	*Ischemia*
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
1	1.175 ± 0.006	1.021 ± 0.082	1.261 ± 0.145	1.010 ± 0.152	1.244 ± 0.159	1.003 ± 0.020	1.596 ± 0.284	1.036 ± 0.042
2	1.189 ± 0.055	0.940 ± 0.118	1.312 ± 0.126	1.209 ± 0.158	1.308 ± 0.141	1.035 ± 0.075	1.637 ± 0.079	1.124 ± 0.015
4	1.235 ± 0.072	0.927 ± 0.107	1.265 ± 0.085	1.490 ± 0.124	1.413 ± 0.160	1.689 ± 0.011	1.641 ± 0.095	1.229 ± 0.115
8	1.322 ± 0.064	1.865 ± 0.290	1.159 ± 0.019	2.513 ± 0.229	1.695 ± 0.030	2.289 ± 0.437	1.695 ± 0.030	1.687 ± 0.067

Densitometric analysis of cytokine slot blots from Figures 3 and 4. The measured intensities were expressed as ratio compared to 0 h. Data represented the mean ± SEM taken from three different experiments with duplicate cultures in each experiment.

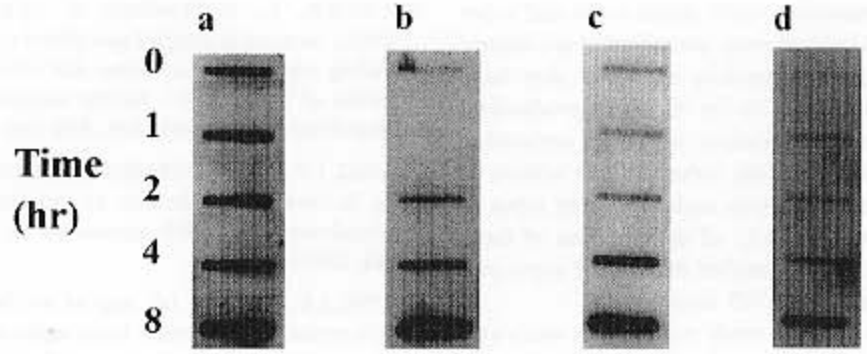

FIG. 4. Slot-blot analysis of changes of cytokine levels in astrocytic culture media under ischemia. (**a**) IL-1α. (**b**) IL-6. (**c**) TNFα. (**d**) IFN-γ. The slot-blot shown was selected from six blots in three separated experiments.

sult, and the injury and death imposed on many astrocytes were of rapid onset along the scratch. The scratching injury therefore rapidly release intracellular cytokines from these astrocytes into the medium. Cells distant from the scratch edge were not physically injured (Yu et al., 1993). This was supported by the location of immunopositive staining of cytokines only in astrocytes along the scratch. It is also possible that the cytokines leaked from these injured astrocytes could also prompt the neighboring cells to produce more cytokines, and such paracrine action possibly contributed to the consistent high levels of cytokines in the media of the scratch astrocytic culture throughout the experimental period.

In the ischemic model, astrocytes could withstand a relatively long period of ischemia exposure. With less than 2 h of ischemia, no detectable cell death occurred in the cultures. After 4–6 h of ischemia, the cells began to show severe depletion of energy metabolites (Fu and Yu, 1995); after 8 h, only 10% of cultured astrocytes were still viable (Yung et al., 1998). Because the ischemia model was designed to simulate terminal cell death under ischemia, the injury imposed on the astrocytes was progressive and cumulative with time. This difference in the nature of injury was ascertained by the release pattern of the cytokines. The first two cytokines released at 2 h of ischemia were IL-6 and IFN-γ, which are known to have neuroprotective effects (Balasingam et al., 1994; Geiger et al., 1997; Maeda et al., 1994; Toulmond et al., 1992). At 4 h of ischemia, TNFα was released into the medium, while IL-1α was not released until at 8 h of ischemia. This pattern suggests that the release mechanisms for IL-1α were different from the other cytokines under ischemia, suggesting that IL-1α was not released by intact astrocytes but leaked out from the damaged or dead astrocytes in 8 h ischemic culture. This is also supported by the observation in the scratch model that the early detection of IL-1α might be related to the membrane-oriented nature of the injury. The unfavorable environment created by the low medium volume in cultures of ischemia experiment could explain the high levels of TNFα and IFN-γ detected in the media in the later periods of the control incubation. Furthermore, these cultures were incubated in serum free medium, which also may cause injury and trigger the release of these cytokines under control incubation.

The production and release of inflammatory cytokines from injured astrocytes in culture indicates that astrocytes are capable of taking part in the immune response to injury. These cytokines have pleiotropic and overlapping functions and play fundamental roles in the induction and regulation of immune and inflammatory response (Arai et al., 1990; Benveniste, 1993; Rothwell and Hopkins, 1995). IL-1, IL-6, TNFα, and IFN-γ induced ICAM-1 expression and antigen presentation by astrocytes (Frohman et al., 1989) and the activation of T lymphocytes (Fierz et al., 1985). TNFα altered vascular permeability and enhanced adhesion of leukocytes to the endothelial surfaces (Pohlman et al., 1986). They were shown to exhibit cytotoxic activities and were held responsible for tissue damage and demyelination in EAE (Selmaj and Raine, 1988). IL-1–receptor antagonists IL-1ra inhibited neurodegeneration induced by focal brain ischemia (Bourdiol et al., 1991). IL-1, IL-6, and TNFα induce (Balasingam et al., 1994; Selmaj et al., 1990), but IFN-γ inhibits (Pawlinski and Janeczko, 1997) proliferation of astrocytes. On the other hand, IL-1 and TNFα have been demonstrated to induce or modulate genes that are known to have neuroprotective roles such as nerve growth factor (Lindholm et al., 1987), platelet-derived growth factor (Silberstein et al., 1966), heat shock protein (D'Souza et al., 1994) and nitric oxide synthase (Hewett et al., 1993). IL-6 is considered to promote neuronal survival and inhibit NMDA mediated neurotoxicity and ischemic neuronal damage (Bourdiol et al., 1991; Maeda et al., 1994; Toulmond et al., 1992). IFN-γ induces the expression of many genes (Kuchinke et al.,

1995) including immediate early genes c-fos and c-jun (Rubio, 1997). The contradictory pictures that are emerging suggest that the inflammatory cytokines may have opposing actions depending on the dosage of production, local circumstances and interaction with other molecules. The temporal difference in the induction and release of these cytokines from astrocytes under different types of injury revealed the complexity of the response of these cytokines to injury. It also ratified the role of astrocytes in the immunoresponse in CNS injury.

Findings presented in this study suggest that astrocytes participate in the response to pathophysiological condition by releasing cytokines. Astrocytes play a specific role in blood-brain barrier (BBB). This makes them the first neural cells facing any ischemic insult and other insults related to BBB. The cytokines released from injured or activated astrocytes may prime other astrocytes and neural cells for subsequent roles in diverse adaptive response including defense against infection, immunological response, wound repair, and nerve regeneration. Most important of all, we have shown that astrocytes are capable of responding directly to injury by the production and release of proinflammatory cytokines.

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