Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury

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Abstract

Astrocytes form an integral part of the blood brain barrier and are the first cell type in the central nervous system to encounter insult if there is an ischemic attack. The immunologic reaction of astrocytes to an ischemic insult would be affective to the subsequent responses of other nerve cells. We previously showed that ischemia caused an increase in the levels of interleukin 1α (IL-1α), tumor necrosis factor α (TNFα), and interleukin 6 (IL-6) in the culture medium of mouse cerebral cortical astrocyte. We did not have evidence on the source of these cytokines. This study aimed to investigate the expressions of these cytokine mRNAs in the astrocytes under ischemia. Results demonstrated that ischemia could induce necrosis and apoptosis in astrocytes. By using the RT-PCR method, we demonstrated for the first time that the mRNA levels of IL-1α, TNFα and IL-6 in normal astrocyte was very low, but their expressions could be induced quickly under ischemia. These cytokines might be interactive as indicated by the difference in time course of their expressions, with IL-1α being the earliest and IL-6 being the latest. The result provided some understanding of the induction and progression of these immunologic responses in astrocytes under ischemia. It also supported our previous findings that astrocytes contributed to the cytokines released under ischemia. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Cerebral ischemia increases the expression of several groups of genes in the central nervous system (CNS) (Yu et al., 1995). The cellular sources of these gene expressions in the ischemic brain have not been defined. We have previously shown that astrocytes in primary culture under ischemia altered their gene expression in c–fos, hsp-70, GFAP, vimentin, and β-actin (Yu et al., 1995). This suggested that ischemia triggers dynamic changes in expression of genes in astrocytes which plays an important and determinant role in the damaging and recovering process of nerve cells after ischemia.

Astrocytes form an integral part, both structurally and functionally, of the blood brain barrier (BBB) and would be the first cell type in CNS to encounter an insult if there is an ischemic attack. Its response to the ischemic insult would be affective to the subsequent response of other nerve cells such as neurons, oligodendrocyte and microglia (Weiss and Berman, 1998). Although astrocytes have been viewed traditionally as supportive cells for the neurons in the CNS, mounting evidence indicates that astrocytes have a wide range of functions other than maintaining a balanced homeostatic environment in the CNS (Eng et al., 1995; Hertz, 1990). It has even been suggested that neuronal–glial interactions are necessary during the establishment of memory (Ng et al., 1997). Astrocytes have been reported to protect neurons against anoxic damage by accumulating glutamate and thus keeping the extracellular glutamate concentration lower than in isolated neuronal cultures (Huang et al., 1997). The production of neurotropic mediators and other immunologic agents from injured astrocytes reflected their critical role in the CNS response to ischemia. There-

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fore, it is important to study the induction and progression of immunologic response in astrocytes under injurious conditions.

We have previously shown that there was an increase in interleukin 1α (IL-1α), tumor necrosis factor α (TNFα), and interleukin 6 (IL-6) content in the culture medium of astrocytes after a scratch or an ischemic injury (Lau et al., 1996; Lau and Yu, 2000). Although some injured astrocytes were positively stained with antibodies for IL-1α, TNFα, and IL-6, there was no evidence to support the notion that these proinflammatory cytokines were synthesized by the astrocytes and were secreted to the culture medium under ischemia. This study aimed to investigate the expression of these cytokine mRNAs in normal and ischemic astrocytes using a reverse transcription — polymerase chain reaction (RT-PCR). The result might play an important role in redirecting immunological events observed under ischemia. It would also elucidate the role of astrocyte in the CNS response to ischemia.

2. Experimental procedures

2.1. Materials

LIVE/DEAD® Eukolight™ Viability/Cytotoxicity Kit was purchased from Molecular Probes Inc. OR, USA. In situ Cell Death Detection Kit, fluorescein was purchased from Boehringer Mannheim, Germany. LigA-Tor cloning kit was from R&D Systems, MN, USA. Wizard® *Plus* minipreps DNA purification system and AMV reverse transcriptase were purchased from Promega Corporation, WI, USA. Superscript preamplification system, Taq polymerase and TRIZOL reagent were purchased from Life Technologies, Inc, MD, USA. AutoRead DNA sequencing kit was purchased from Pharmacia LKB Biotechnology, Sweden. Diethyl pyrocarbonate was purchased from Sigma, MO, USA.

2.2. Primary culture of mouse cerebral cortical astrocyte

The primary cultures of astrocytes were prepared from cerebral cortices of new born ICR mice (Animal Care Centre, HKUST, Hong Kong) as described in Yu et al. (1986, 1993) with minor modification. In brief, cerebral cortices freed of meninges were cut into small cubes (<1 mm^3) in modified eagle's medium (MEM) (Life Technologies, Inc., MD, USA) with 7.5 mM glucose. The cubes were dissociated by vortexing for 1.5 min. The cell suspension was sieved through 70 μm and a 10 μm sterile Mesh® nylon filters (Spectrum Medical Industries, Inc., TX, USA). The suspension was diluted with MEM containing 10% fetal calf serum (Globepharm, UK) to 3×10^5 cells per

ml and 2 ml of which was seeded per 35 mm Falcon® culture dish (Becton Dickinson Labware, CA, USA). All cultures were incubated in a 37°C CO$_2$ incubator (Napco, Precision Scientific Inc., USA) with 5% CO$_2$ and 95% mixture of atmospheric air (vol/vol). The culture medium was changed after 1 day of seeding and subsequently 2 times per week. Cultures of at least 4 weeks old were used for the experiment.

2.3. Ischemic model

The model was created by incubating the cultures in an anaerobic chamber (Forma Scientific, Inc., OH, USA) as previously described by Kaku et al. (1991) and Juurlink and Hertz (1993) with modification. The chamber was made anaerobic by filling it with a mixture of H$_2$, CO$_2$ and N$_2$ (15:5:85). The oxygen level inside the chamber was monitored throughout the experiment with an OM-1 oxygen meter (Microelectrodes, Inc., NH, USA). In the anaerobic chamber, cultures were washed twice with glucose-free MEM that was degassed and saturated with N$_2$. After washing, 800 μl of the glucose-free MEM was added and the culture was wrapped with parafilm to prevent evaporation. This reduced amount of MEM was enough to cover the culture. The culture was incubated at 37°C in an incubator inside the chamber. Control cultures in reduced amount of normal culture medium were incubated in parallel but in a normal CO$_2$ incubator.

2.4. Cell viability assay

The cell viability assay was preformed with a LIVE/DEAD® Eukolight™ Viability/Cytotoxicity Kit (Molecular probes Inc., OR, USA). The TdT-mediated dUTP nick end-labeling (TUNEL) technique was performed with an In Situ Cell Death Detection Kit, fluorescein (Boehringer Mannheim, Germany). The procedure for the staining was provided with the kits from the manufacturer.

2.5. Extraction of RNA

The culture medium was drained and 1 ml of TRIZOL reagent (Life Technologies, Inc., MD, USA) was added to the culture. The cells were lysed by multiple pipetting and the lysate was transferred to a microcentrifuge tube. It was incubated at room temperature for 5 min before 0.2 ml of chloroform (Sigma, MO, USA) was added. The lysate was shaken vigorously for 15 s, then left under room temperature for 10 min. After centrifugation at 12,000 *g* at 4°C for 15 min, the top aqueous layer was transferred to a microcentrifuge tube and 0.5 ml of isopropanol was added. The samples were incubated at room temperature for

30 min before being centrifuged at 12,000 g at room temperature for 10 min. The supernatant was discarded and the pellet was washed once with 75% ethanol. The pellet was dried briefly under air and resuspended in 20 μl diethyl pyrocarbonate (Sigma, MO, USA) treated water.

2.6. Reverse transcription — polymerase chain reaction

Reverse transcription (RT) can be done either with AMV Reverse Transcriptase (Promega, WI, USA) or Superscript Preamplification System (Life Technologies, Inc., MD, USA). One tenth of the RT product was added to a reaction mixture containing $1 \times$ PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 ρmole target specific primers and 2 units of Taq polymerase (Life Technologies, Inc., MD, USA). The sequences of the primers used were: IL-1α forward: 5′CTC TAG AGC ACC ATG CTA CAG AC3′, IL-1α reverse: 5′TGG AAT CCA GGG GAA ACA CTG 3′, TNFα forward: 5′GCG ACG TGG AAC TGG CAG AAG3′; TNFα reverse: 5′GGT ACA ACC CAT CGG CTG GCA3′; IL-6 forward: 5′GTG ACA ACC ACG GCC TTC CCT ACT3′; and IL-6 reverse: 5′GGT AGC TAT GGT ACR CCA3′. The reaction was done by a thermal cycler (MJ Research, Inc., MA, USA). The amplification condition was the same for IL-1α, TNFα and IL-6, i.e., denature for 60 s at 94°C, anneal at 55°C for 120 s, then extend at 72°C for 120 s, except for TNFα the annealing was performed at 58°C for 90 s and extension was at 72°C for 140 s.

The number of cycle performed for each of them was 30.

2.7. DNA sequencing

After PCR amplification, the products were cloned using LigATor cloning kit (R&D Systems, MN, USA). The detailed procedures were described in the instruction manuals. The successful clones were white in color and some of them were cultured. The plasmid DNA was extracted and purified by the Wizard ® *Plus* Minipreps DNA Purification System (Promega, WI, USA) as described in the instruction manual provided with the kit. The inserts were sequenced by an Auto-Read sequencing kit (Pharmacia LKB Biotechnology, Sweden) and an ALF automated sequencer (Pharmacia LKB Biotechnology, Sweden).

3. Results

3.1. Cell death under ischemia

Astrocytes under ischemia showed severe cell death at 6 h (Fig. 1). Cell death began at 3 h but only dramatically increased from 4 to 6 h. There was no obvious cell death in astrocytes under less than 2 h of ischemia as indicated by the cell viability test. In Fig. 1(A) and (B) were taken from the same field. Astrocytes with red fluorescent nuclei were dead and some of them still had the green fluorescent cytoplasmic attachment. The TUNEL staining (Fig. 1(D)) in astro-
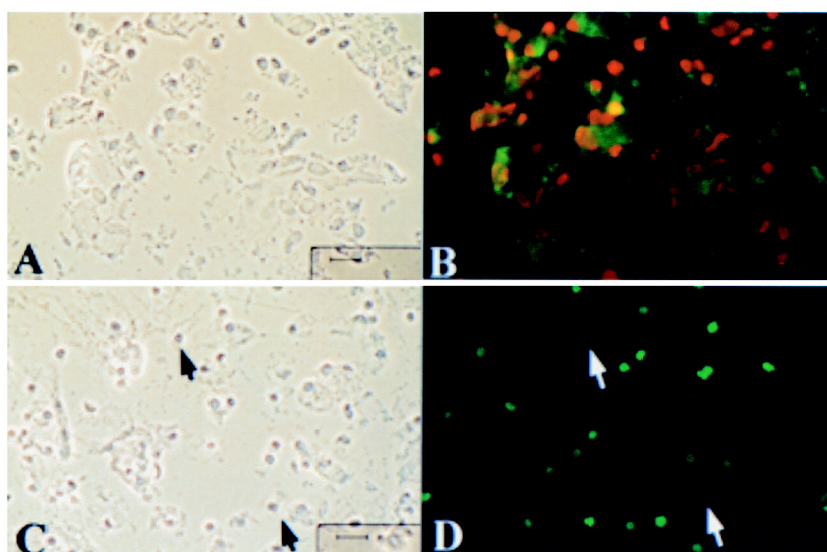

Fig. 1. Ischemia induced severe cell death in astrocytes. (A) and (B) were from the same field. (A) was phase contrast micrograph of astrocytes under 6 h of ischemia and (B) was a fluorescent micrograph showing cells treated with LIVE/DEAD ® Eukolight ® Viability/Cytotoxicity Kit, where dead cell nuclei were stained orange and cytoplasm stained fluorescent green. (C) and (D) were from the same field. (C) was phase contrast micrograph of astrocytes under 6 h of ischemia and (D) was a fluorescent micrograph showing cell stained with TUNEL. Arrows indicating cell nuclei did not stain with TUNEL. Scale bar = 20μm.
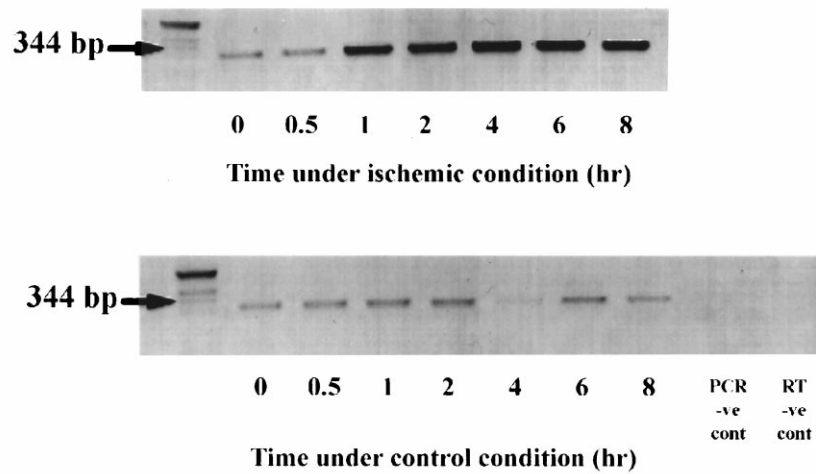
Fig. 2. Expression of IL-1α mRNA in astrocytes under ischemic (A) and control (B) conditions. The RT-PCR products were amplified by the IL-1α primers and analyzed by agarose gel electrophoresis. Lane 1 on (A) and (B) are the 1 kb DNA ladder. PCR −ve cont was PCR negative control and RT −ve cont was RT negative control. The experiments were repeated three times with a total of six cultures.

cytes under 6 h of ischemia demonstrated that many but not all of the dead cells were apoptotic. In cultures under 4–6 h of ischemia, many cells died of necrosis. These dead cells were not seen in Fig. 1 because they were removed from the cultures during the washing and staining procedure.

3.2. Cytokine gene expression

The expressions of IL-1α, TNFα and IL-6 mRNAs in astrocytes under ischemia and control incubation were compared by amplifying the mRNA signals using RT-PCR. Fig. 2 showed the expression of IL-1α mRNA in cultured astrocyte under ischemic (A) and control (B) incubations. From the controls, there was a basal level of IL-1α expression that remained unchanged throughout the 8 h of incubation. In cul-

tures under 0.5 h of ischemia, the mRNA level was the same as in the control. However, the signal began to increase dramatically at 1 h and reached a higher level at 6 h; it then decreased slightly at 8 h. The RT and PCR negative controls indicated no contamination during the RT-PCR processes.

Fig. 3 showed the expression of TNFα mRNA in cultured astrocytes under ischemic (A) and control (B) incubations. There were TNFα mRNA expressions being detected at 0 h in both ischemic and control cultures. These background signals were quite high. In the controls, TNFα mRNA signal increased at 0.5 h, then the level decreased at 1 h and remained at the same low level until 4 h. It disappeared in the controls after 6 h of incubation. Under ischemia, the level of TNFα gene expression did not change very much from 0 to 0.5 h. At 1 h of ischemia, the signal began to
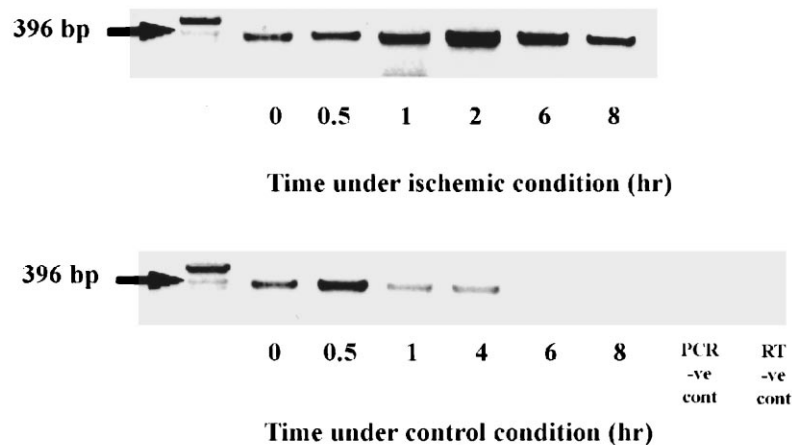

Fig. 3. Expression of TNFα mRNA in astrocytes under ischemic (A) and control (B) conditions. The RT-PCR products were amplified by the TNFα primers and analyzed by agarose gel electrophoresis. Lane 1 in both (A) and (B) was 1 kb DNA ladder. PCR −ve cont was PCR negative control and RT −ve cont was RT negative control. The experiments were repeated three times with a total of six cultures.
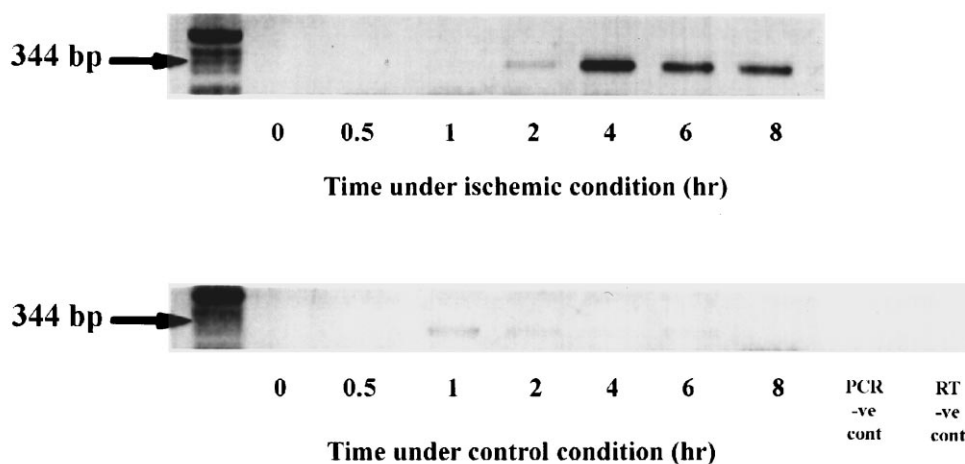
Fig. 4. Expression of IL-6 mRNA in the cultured astrocyte under ischemic (A) and ischemic (B) conditions. The RT-PCR products were amplified by the IL-6 primers and analyzed by agarose gel electrophoresis. Lane 1 in (A) and (B) was 1 kb DNA ladder. PCR −ve cont was PCR negative control and RT −ve cont was RT negative control. The experiments were repeated three times with a total of six cultures.

increase and reached the maximum at 2 h. It decreased between 6 and 8 h. The RT and PCR negative controls indicated no contamination during the RT-PCR processes.

Fig. 4 showed the changes of IL-6 mRNA level in cultured astrocytes under ischemic (A) and control (B) conditions. In the control experiment, no IL-6 mRNA could be detected except at 1 h of incubation. The level was very low as compared to IL-1α and TNFα. In the ischemia experiment, no IL-6 mRNA signal was detected at 0, 0.5 and 1 h. The signal for IL-6 mRNA expression was detected at 2 h of ischemia, but the level was very low. The signal dramatically increased between 2 to 4 h and gradually decreased between 6 and 8 h of ischemia. The RT and PCR negative controls indicated no contamination during the RT-PCR processes.

3.3. Confirmation of the PCR products

To confirm that the PCR products were amplified from the corresponding cytokines cDNA, they were cloned and sequenced. The DNA sequences were then aligned with the known cytokines cDNA sequences obtained from the Genebank. Fig. 5(A) showed the alignment between the cloned PCR product amplified by the IL-1α primers and the mouse IL-1α cDNA (Lomedico et al., 1984; Genebank accession number: X01450). The extremely high homology indicated that the fragment was amplified from IL-1α cDNA. Fig. 5(B) showed the alignment between the cloned PCR product by the IL-6 primers and the mouse IL-6 cDNA (Grenett et al., 1990; Genebank accession number: X54542). The extremely high homology indicated that the fragment was amplified from IL-6 cDNA.

4. Discussion

Ischemia is a pathophysiological condition accompanied by the lack of oxygen and nutrients, and the accumulation of toxins. Several in vitro models have been established (Pellegrini-Giampietro et al., 1990; Juurlink and Hertz, 1993; Ogata et al., 1995; Yu et al., 1992, 1995). In this study, ischemia was created in an anaerobic chamber and cultures were incubated in a reduced volume of glucose-free medium. The reduction of the volume of the medium can abide the model to have the accumulation of toxins as one of the causes of injury, a condition more competent to the physiologic ischemia (Lau and Yu, 2000; Yu et al., 1992, 1995). Most important of all, this experimental design allows us to investigate the response of an isolated population of astrocytes to ischemia insult without the influence of other cell types such as neurons, microglia, or endothelial cells.

We showed that ischemia induced necrosis and apoptosis in astrocytes (Fig. 1). In this model system, astrocytes experienced a terminal death process. Many astrocytes became necrotic during the ischemia incubation. Many of them were not shown on the phase contrast micrographs (Fig. 1(A) and (C)) as they were removed during the staining procedure. The TUNEL staining indicated that many cells underwent apoptosis (Fig. 1(D)). As shown in Fig. 1(C), these TUNEL positive cells seemed to die of necrosis — secondary necrosis. It is because the experiment was designed that the injury imposed on astrocytes was accumulative. Cells that had the apoptotic pathway initiated could not generate sufficient energy to complete the energy dependent apoptosis, and thus died of a secondary necrosis.

(A)

		10	20	30	40
cloned PCR product	<u>CTCTAGAGCA</u>	<u>CCATGCTACA</u>	<u>GAC</u>TTTTACA	CTTTTTCCAC
mouse IL-1α cDNA	<u>CTCTAGAGCA</u>	<u>CCATGCTACA</u>	<u>GAC</u> TTTACA	CTTTTTCCAC

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                    50          60          70          80
          AGCCACGAAG CTCTCCGTAC ATTCCTGCAC TTGGGAGCCC
                           *          *
          AGCCACGAAG CTCTCTGTAC ATTCCTGTAC TTGGGAGCCC

                    90         100         110         120
          TTTCATCATG ATCTTAATCT GTGCTGTTTA CTTTGTGCAT
                                      *             *
          TTTCATCATG ATCTTAATCT GTACTGTTTA CTTTGTTCAT

                   130         140         150         160
          CTAAAATGAT AATTGAGTCA GTCTTTCTCC CTCCCGTCCT
                                         *          *
          CTAAAATGAT AATTGAGTCA GTCTTTTTCC CTCCCATCCT

                   170         180         190         200
          TAAAGCTGTC TGGGTATTCT TACATCATTC AGTCTCACCT

          TAAAGCTGTC TGGGTATTCT TACATCATTC AGTCTCACCT

                   210         220         230         240
          GTAACTAACA CCAACCATCT AAAGATGGAA AGAGCTTAAC

          GTAACTAACA CCAACCATCT AAAGATGGAA AGAGCTTAAC

                   250         260         270         280
          TGTGACAACA ACATCACTGA TACCTGAAGT TTCTTTTCTA
                 *               *
          TGTGACCACA ACATCACTGT TACCTGAAGT TTCTTTTCTA

                   290
          GAATGTAAGT GTTTCCCC

          GAATGTAAGT GTTTCCCC
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Fig. 5. Alignment between the cloned PCR products (upper sequence) and the mouse cDNA (lower sequence) obtained from Genebank. The underlined region was the primer sequence and * indicates mismatches. (A) was IL-1α (Genebank accession no.: X01459; and (B) was IL-6 cDNA (Genebank accession no.: X54542).

It is believed that the basal expression of cytokine is absent or is of very little amount in the normal brain. We measured the expression of IL-1α, TNFα and IL-6 mRNA in the control cultures. The high background signal for TNFα mRNA in the controls and cultures had 0–0.5 h ischemia, indicating that the expression of this gene is very sensitive. It could be induced by the minor disturbance created in the preparation of astrocytes for experiment. This background signal vanished later in the control incubation, indicating that the induction was transient. This high sensitivity in TNFα mRNA expression was not observed in IL-1α and IL-6. It might imply that the induction of TNFα expression could be independent from IL-1α. The return of TNFα to an undetectable level in the controls supports the observation of a lack of cytokine expression in the brain under normal condition.

There is no doubt that astrocytes are capable of exerting immunologic responses. In an injured brain, cytokines are usually detectable before the invasion of white blood cells, indicating that the brain cells must have synthesized them. Astrocytes form an integral part of BBB. They would be the first of all nerve cells

(B)

	10	20	30	40
cloned PCR product	<u>GTGACAACCA</u>	<u>CGGCCTTCCC</u>	<u>TACT</u>TCACAA	GTCCGGAGAG
mouse IL-6 cDNA	<u>GTGACAACCA</u>	<u>CGGCCTTCCC</u>	<u>TACT</u>TCACAA	GTCCGGAGAG

	50	60	70	80
	GAGACTTCAC	AGAGGATACC	ACTCCCAACA	GACCTGTCTA
	GAGACTTCAC	AGAGGATACC	ACTCCCAACA	GACCTGTCTA

	90	100	110	120
	TACCACTTCA	CAAGTCGGAG	GCTTAATTAC	ACATGTTCTC
	TACCACTTCA	CAAGTCGGAG	GCTTAATTAC	ACATGTTCTC

	130	140	150	160
	TGGGAAATCG	TGGAAATGAG	AAAAGAGTTG	TGCAATGGCA
	TGGGAAATCG	TGGAAATGAG	AAAAGAGTTG	TGCAATGGCA

	170	180	190	200
	ATTCTGATTG	TATGAACAAC	GATGATGCAC	TTGCAGAAAA
	ATTCTGATTG	TATGAACAAC	GATGATGCAC	TTGCAGAAAA

	210	220	230	240
	CAATCTGAAA	CTTCCAGAGA	TACAAAGAAA	TGATGGATGC
	CAATCTGAAA	CTTCCAGAGA	TACAAAGAAA	TGATGGATGC

	250	260	270	280
	TACCAAACTG	GATATAATCA	GGAAATTTGC	TATTTGAAAT
	TACCAAACTG	GATATAATCA	GGAAATTTGC	TAT**TGAAAAT

	290	300	310
	TTCCTCTGGT	CTTCTGGAGT	ACCATGCTAC C
	TTCCTCTGGT	CTTCTGGAGT	ACCATGCTAC C

Fig. 5 (*continued*)

in the CNS to encounter the insult during an ischemic attack; thus astrocytes could be the cells to initiate the immunologic cascade in the brain under an ischemic attack. This is supported by the evidence that ischemia expressed immunologic mediators earlier than macrophages and reactive microglia in the ischemic brain tissue (Gourmala et al., 1997; Weiss and Berman, 1998). In this study, all three cytokines were found to be expressed in a very short time after the imposition of the ischemic condition. This agreed with the in vivo studies on rat cerebral cortex that the expressions of these cytokines mRNA took place at 3–4 h under

ischemia (Szaflarski et al., 1995; Wang et al., 1995). The PCR products were amplified from the corresponding cytokines cDNA because only one band with the expected size could be identified after the gel electrophoresis analysis for each PCR product. Nevertheless, a sequencing test was performed on two selected PCR products. The results clearly showed their correspondence to cytokine cDNAs. The time course for the expressions of these cytokine mRNAs was different. The expression of IL-1α was induced at 1 h and the level was maintained high through out the ischemic incubation. The peak level of TNFα expression was

not reached until 2 h after ischemia. Whether the expression of TNFα is induced by IL-1α or ischemia still needs to be clarified in the future. The IL-6 expression did not elevate until 4 h of ischemia. The late expression of IL-6 supports that IL-6 is involved in the later stage of the immune response triggered by IL-1α and TNFα.

Some previous studies indicated that similar astrocytes under ischemia could secrete these cytokines into the culture medium (Lau et al., 1996; Lau and Yu, 2000). Others have shown that astrocyte release IL-1α, TNFα and IL-6 after being stimulated by lipopolysaccharide (Chung and Benveniste, 1990; Fontana et al., 1982). IL-1α has been shown to have diverse functions in the CNS. They can induce the proliferation of astrocytes (Giulian and Lachman, 1985; Selmaj et al., 1990). Relton and Rothwell (1992) have shown that the IL-1 receptor antagonist can reduce neuronal death induced by ischemia or excitotoxin. IL-1α is believed to be one of the key factors in triggering brain immune responses by inducing the expressions of IL-6 (Benveniste et al., 1990, Frei et al., 1989) and TNFα (Bethea et al., 1992; Chung and Benveniste, 1990). In neuron, TNFα can modulate calcium channels (Soliven and Albert, 1992), decrease catecholamine secretion (Soliven and Albert, 1992), alter synaptic transmission (Tancredi et al., 1992) and protect CNS neurons against metabolic-excitotoxic insults and promote calcium homeostasis (Cheng et al., 1994). IL-6 can induce astrocyte to secrete nerve growth factor (Frei et al., 1989). The findings from this study as well as the many functions of IL-1, TNFα and IL-6 sustain the essential role of astrocytes in the immunologic response in brain under ischemia.

The expressions of proinflammatory cytokine mRNAs in ischemic astrocytes supported our previous findings that the cytokines released under ischemia were synthesized by the astrocytes. This also affirmed astrocytes' ability to initiate immunologic cascade under ischemic attack. The expression of these cytokines might be interactive as indicated by the difference in time course of their expressions. Using RT-PCR, we showed for the first time that mRNA level of cytokines in normal astrocytes was very low, but it could be induced to rise very rapidly under ischemia. The result provides some understanding of the induction and progression of such immunologic responses in astrocytes.

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