Asymmetrical release of interleukin-6 by cultured cerebral cortical astrocytes treated with lipopolysaccharide

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Background & objectives: The distribution of brain interleukin-6 (IL-6) may be asymmetrical both in cortex and hippocampus. While the brain asymmetry has been extensively investigated, the cellular origin of asymmetrical cytokine induction in the cortex has not been addressed. It was hypothesized that the immune function of glia cell to the inflammatory insults is asymmetrically distributed in the two brain hemispheres. To test this hypothesis, we examined the IL-6 secreting ability of the astrocytes in both the left and right neocortex treated with lipopolysaccharide (LPS) cultured in vitro.

Methods: Two groups of astrocytes cultured in vitro from the two cerebral cortices of the neonatal BALB/c mice were selected and experimental group was treated with LPS (10 µg/ml) for 24 h. IL-6 levels were measured in both LPS-treated and untreated astrocytes. To confirm the gene array data on the secretion of IL-6 by cortical astrocytes in the left and right hemispheres, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was conducted.

Results: A statistically significant difference between the levels of IL-6 in cortical astrocytes in the left and right hemispheres of culture supernatants was observed (P<0.05). Cortical astrocytes in the left hemisphere had significantly increased IL-6 mRNA levels compared with cortical astrocytes in right hemisphere (P<0.05).

Interpretation & conclusions: The results showed asymmetrical release of brain IL-6 by cerebral cortical astrocytes to the inflammatory insults both in protein and mRNA levels.

Key words: Astrocytes - asymmetry - cytokines - glial cells - interleukin-6 - lipopolysaccharide

In our previous studies1-3, evidences showed that IL-6 levels induced by lipopolysaccharide (LPS)-injection were asymmetrically distributed in the two brain hemispheres. Data indicated that IL-6 levels in the right cortices were higher for ambidextrous mice than for right/left-pawed mice. On the other hand, IL-6 levels in the left cortices for left-pawed mice were higher than that for both ambidextrous and right-pawed mice1-3. While brain asymmetry has been extensively investigated1, the cellular origin of asymmetrical cytokine induction in the cortex has not been addressed so far. Astrocytes are the major source...
 IL-6 is a likely candidate mediator of the effects of LPS on neuroendocrine secretion. It was hypothesized that the immune function of glial cells to the inflammatory insults are asymmetrical distributed in the two brain hemispheres. To address the cellular origin of asymmetrical cytokine induction in the cortex in response to LPS, we examined the IL-6 secretion abilities of the astrocytes between the left and right cortex treated with LPS cultured in vitro, exploring their roles in the relationship between the immune system and the brain asymmetry.

**Material & Methods**

The study was conducted in the Microbiol & Immunol Lab of Shantou University and the study protocol was approved by the Shantou University Medical College Sciences Ethics Committee. 

**Astrocyte isolation.** Glial cells were prepared from neonatal (<24 h old) BALB/c mouse (4 mice/group, purchased from Sun Yat-sen University, China) brains, as described earlier, for both control and experimental groups. The left and right cortices of neonatal BALB/c mouse were dissected free of meninges, minced with sterile surgical scissors, and separated into single cell suspensions by passage through stainless steel screens. The adherent fibroblast was removed after 30 min. The primary glial cell cultures were maintained in Dulbecco modified Eagle medium (DMEM-F12; Gibco, USA) supplemented with 10 per cent foetal calf serum (Gibco, USA) and adjusted to a final concentration of 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, Germany) in a humidified atmosphere of 5 per cent CO2/95 per cent air (CO2 incubator) at 37°C. After 10-14 days, the flasks were agitated on an orbital shaker (Forma Orbital Shaker, Thermo electron corporation, USA) for 2 h at 200 rpm at 37°C, and the nonadherent oligodendrocyte and microglial cells were removed. The astrocytes were trypsinized and expanded in complete medium. Astrocytes used in the current experiments were cultured for a total of 20-26 days. To remove any residual oligodendrocytes and microglial cells, the flasks were agitated for at least 3 times as described above before harvest. The purity of astrocytes was determined by immunohistochemistry in Fig. 1c, d with anti-glial fibrillary acidic protein (GFAP) antibodies (Wuhan Boster Biological Ltd., China) to identify astrocytes.

**Treatment procedures:** The cortical astrocytes were trypsinized for 5 min at 37°C and washed three times on 20-26th day for both controls and experimental groups. Astrocytes were re-seeded with complete medium at a concentration of 5 x 104 cells/ml in 6-well plates and the cells were allowed to recover from trypsinization by incubation in complete medium for 24 h at 37°C. The medium was removed and astrocytes were washed three times with DMEM without foetal calf serum. The astrocytes of experimental groups were treated with 10 µg/ml LPS (Escherichia coli serotype O55:B5; Sigma Chemical Co., USA) for 24 h and the control groups were not treated with LPS in DMEM-F12 in the absence of serum.

**Cytokine assays:** Culture supernatants after LPS activation (experimental groups) and no LPS activation (control groups) were collected and saved at -80°C until assayed. Commercial ELISA (R&D Systems, USA) kits were used to determine culture supernatant protein levels of the cytokines IL-6 according to the manufacturer’s protocol.

**Reverse transcription-polymerase chain reaction (RT-PCR):** Total cellular RNA was obtained from the experimental and control astrocytes using Trizol (Invitrogen, USA) reagent according to the Invitrogen’s protocol. First-strand cDNA was generated from 1 µg of total RNA from the astrocytes by using oligo(dT)15 primer and a protocol for RT for PCR Kit (Promega, USA). Each PCR(GeneAmp PCR System 9700, USA) was conducted in a 20-µl volume with 100 pmol of each 5’ and 3’ gene-specific primer and 1 µl of diluted cDNA. The primers were synthesized by Invitrogen...
PCR cycle conditions were 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Levels of β-actin were used to control for sample amount. The PCR products were resolved by staining with ethidium bromide and were run by electrophoresis in 1.5 per cent agarose gels. Results were visualized under UV illumination using a Gel Doc apparatus (Gene Genius Bioimaging system, British Syngene, British). Images of the PCR bands were analyzed semi-quantitatively using Quantity One 1-D analysis software (Bio-Rad, USA). The sequences of the cytokine primers and the expected product sizes were as follows: IL-6, 5′ primer (5′ CGG AGA GGA GAC TTC ACA GAG GA 3′) and 3′ primer (5′ GGA GAG CAT TGG AAA TTG GGG 3′); 426bp, GenBank accession no. NM-031168). β-actin, 5′ primer (5′ GGC ATA CAG GGA CAA CAC AG 3′) and 3′ primer (5′ ATT CCG ATA ACG AAC GAG AC 3′); 163bp, GenBank accession no. NM-031144).

Data analyses: Data were analyzed using one-way ANOVA. All experiments were repeated six times. For mouse measurements, astrocytes from the four neonatal BALB/c mouse left and right cortices for each preparation were harvested and cultured both in experimental groups and control groups.

Results

The primary left and right glial cell cultures were maintained after removing the adherent fibroblast. Glial cells were flat, thinly spread out, tripolar and polygonally shaped when plated either in plastic tissue culture flasks or on glass coverslips with a small amount on day 9. (Fig. 1a)

The purest astrocytes were determined after being agitated at for least 3 times on 20th day (Fig.1b). The expression of GFAP in cultured cells was detected by immunohistochemistry. Positive immunostaining was detected in the cytoplasm of more than 96 per cent cells (Fig.1c, 1d).

After astrocytes were treated with LPS for 24 h, a difference was observed in IL-6 levels between left and right cortices. IL-6 levels were significantly more secreted from the left cortical astrocytes culture supernatants compared with those from the right and the left control groups (P<0.05). Control groups had lower levels of IL-6 compared to experimental group (Fig. 2)

To confirm the gene array data on the secretion of IL-6 by left and right cortical astrocytes, semi-quantitative RT-PCR was conducted. Astrocytes stimulated for 24 h with LPS dramatically upregulated the levels of

Fig.1a. The morphology of cortical glial cells cultured in vitro on day 9, detected by inverted microscope (×50); b. The morphology of cortical astrocytes on day 20, detected by inverted microscope (×50); c. The expression of glial fibrillary acidic protein (GFAP) in cultured cells was detected by immunohistochemistry. Positive immunostaining was detected in the cytoplasm of more than 96 per cent cells (ICC, ×200). d. Negative control. No positive staining was observed in the cytoplasm of fibroblasts by immunohistochemistry (ICC, ×200).
IL-6 mRNA both in the left and right cortices. The left cortical astrocytes had significantly increased IL-6 mRNA levels when astrocytes were stimulated with LPS, compared to the cortical astrocytes in the right hemisphere and left control group; \( P<0.05, P<0.001 \) respectively). Right cortical astrocytes had higher IL-6 mRNA levels than right control \( (P<0.05) \). (Fig. 3a)

**Discussion**

Brain asymmetry is known to exist in numerous animal species, such as mammals and reptiles\(^1\). Lateralization of the brain is known to be responsible for the differences in cognitive and motor tasks. Interestingly, lateralization is also related to the function of neuroendocrine and immune systems. In fact, both the functional asymmetries and biochemical differences are different between the two sides of hypothalamus. The right half of the hypothalamus may play a dominant role in the control of reproductive functions\(^15\). In our study the opposing effects of right and left cortical lesions were observed when measured 10 wk after surgery. Proliferation of T-cells induced by mitogens was decreased after ablation of the left fronto-parieto-occipital cortex. However, mitogenesis was enhanced after asymmetrical right ablation in female C3H mice\(^16\).

IL-6 is one of the most important cytokines present in the brain of normal animals\(^17\). IL-6 exhibits a beneficial effect on the CNS because of its neurotrophic properties; however, overexpression is generally detrimental since it adds to the pathophysiology associated with CNS disorders\(^18\).

Evidence accumulated from clinical research and *in vitro* experiments suggest that elevated IL-6 levels potentiate neural injury in Alzheimer’s and Parkinson’s disease\(^19,20\) and also in other conditions where CNS inflammation is observed\(^21\). IL-6 is not only produced by monocytes, fibroblasts, endothelial cells, and T lymphocytes but also by microglia and astrocytes\(^22-24\). Righi *et al*\(^25\) generated clones of microglial cells which produced IL-6 as assessed by biological assays and by Northern blot analysis. Moreover, the addition of TNF-\(\alpha\) or IL-1\(\beta\) plus IL-6/solubility IL-6 receptor led to synergistic increases in IL-6 expression by astrocytes\(^7\).

IL-6 has been shown to be asymmetrically distributed in cortex after LPS injection\(^1-3\). Astrocytes, the major glial cell type of the CNS, and the major source of IL-6 in CNS injury are known to play a major role in maintaining the blood brain barrier (BBB)\(^9,26\). They act in providing nutrition and encapsulate inflammatory lesions, and are likely to influence immune responses in the CNS. Indeed, IL-6 and astrocytes are both closely associated with CNS inflammation, and as such, are important in the exploration of the cellular origin of the asymmetrical levels of IL-6 in the cerebral cortices. IL-6 mRNA is found in normal brain tissue and that IL-6 protein is present in several brain structures, including the cortex, hypothalamus and hippocampus\(^27,28\) in adult mice. These reports have shown that IL-6 was present under physiological conditions in the normal brains of adult mice. In our study, astrocytes were isolated from neonatal mice cortices, IL-6 levels of control groups remained lower both in terms of protein and mRNA levels in the left and right cortices. Adult mice are
exposed to more inflammatory factors than neonatal mice in the process of growth. Therefore, using neonatal mice to study may be more representative of the raw state of immune system.

There was a significant difference in the IL-6 secretion ability of the astrocytes between the left and right cortex cultured in vitro. Under physiological conditions, IL-6 levels of the control groups remain low both in left and right cortices. However, after 24 h treatment with LPS, IL-6 levels became elevated, which agrees well with previous reports. In addition, our results also showed asymmetrical distribution of release of IL-6 by cerebral cortical astrocytes with LPS treatment both in terms of protein and mRNA levels. Our results also indicated that RT-PCR was more sensitive than ELISA for the cytokine assay. We have to continue to increase the number of cases to prove whether there are difference existed between right and left control. However, for each preparation of astrocytes that responded, cytokine levels were elevated, suggesting that IL-6 secretion from the left cortical astrocytes was significantly higher than that from the right.

In conclusion, our results showed a significant difference in the secretion ability of astrocytes isolated from left and right cortices. Whether it is caused by the difference in genetic make up of the astrocytes or the culture environment is still a question. Since the difference in genetic make up of the astrocytes or the difference in the secretion ability of astrocytes isolated from left and right cortices cannot be clarified, we are only beginning to comprehend the asymmetrical secretion of IL-6 induced by LPS in the cortical astrocytes. Further experiments are needed to explain these observations.

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