Journal of Life Science 2006 Vol. 16. No. 5. 840~849 ©JLS

Differential Expression of Chemokine MCP-1, MIP-1α, MIP-2 in Lipopolysaccharide-stimulated Neonatal and Adult Rat Brain

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Received June 29, 2006 / Accepted August 9, 2006

Severe brain injuries induced by toxin pose one of the most important problems on our health care because of their high morbidity and mortality, are implicated to leucocyte infiltration more premature or immature brain than mature brain. Chemokines are the induction meditators for infiltration of inflammatory cells to the inflammation sites. In order to study the mechanism of leucocyte infiltration, the expression of several chemokines, MCP-1, MIP-1α and MIP-2 was studied in lipopolysaccharide(LPS)-stimulated neonatal and adult brain. One week old Sprague-Dawley rats or adult male rats weighing 300-350 g were used for the experiment. After anesthetization, 1 μl LPS (0.5 mg/ml) subsequently was injected in the right caudate nucleus of the brain with stereotaxic frame. Animals were sacrificed at 6 hours, 24 hours, and 72 hours after injection. The present study was carried out using RT-PCR for the mRNA and immunohistochemistry for the expression of the proteins. In the neonatal rat brain, prominent interstitial edema with significant accumulation of leukocytes was detected at 24 and 72 hours after LPS injection. A semiquantitative analysis of RT-PCR revealed that the MCP-1, MIP-1α, and MIP-2 mRNA expression peaked at 24 hours in neonatal and adult rat brain. Neonatal rats showed about 2.6, 1.4, and 1.2 times more expression of the MCP-1, MIP-1α, and MIP-2 than that of the adult rats in the brain tissue. Immunohistochemical analysis also showed that MCP-1 immunoreactivity was paralleled with the RT-PCR results. MCP-1 protein was significantly detected at 24 and 72 hours in the brain parenchyma. MIP-1α protein was highly expressed at 24 hours. The results of leukocyte infiltration in H&E stain was parallelled with that of the immunohistochemistry. Chemokine proteins were markedly detected at 24 hours after injection of LPS and neutrophil influx into intraparenchymal was prominent at 24 hours. These results suggest that the leukocyte infiltration in the intracranial infection may be controlled by mechanisms influenced by chemokine producing cells in the central nervous system such as microglia, astrocyte and endothelial cell.

Key words – Brain inflammation, MCP-1, MIP-1α, MIP-2, immnune cell infiltration, edema

Introduction

Severe brain injuries pose one of the most important problems on our health care because of their high morbidity and mortality. Premature and immature brains are subject to the intracranial infection by several pathogens. Bacteria-toxin lipopolysaccharide (LPS) injected in the neonatal rat brain results in neuronal death more prominently than in adult rat brain. By figuring out this mechanism, we can understand the disastrous diseases caused by intracranial infection of pathogens in children. Diminished intelligence in children suffered from the infection of the ventriculoperitoneal shunt is a typical example of the complication of the intracranial infection.

It is well characterized that the brain inflammation or central nervous system (CNS) injury is different from that of the peripheral tissues[1]. Intraparenchymal injection of low-dosage LPS injected intraparenchymally causes little infilteration of leukocytes in inflammation site of the brain, however in the immature brain LPS leades significantly higher recuritment of leukocytes to the inflammation loci. Therefore, our hypothesis indicates that such effect of inflammation is age-dependent.

Chemokines are structurally similar proteins composed of around 60-140 amino acids. Some chemokines attract leukocytes into sites of tissue damage or infection. Others direct the traffic of leukocytes during their development or their recirculation through the tissues of the body. Chemokines are produced upon activation of the wide spectrum of cell types with immune pathogens, including T-cells, macrophage, fibroblasts, monocytes, endothelial cells, microglia and astrocytes. Four major subfamilies (CCC, CXC, C, and CX3C) are defined on the basis of pair

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of cysteine residues in a highly conserved motif [3,18]. The chemokine monocyte chemoattractant protein (MCP)-1/CCL2, RANTES/CCL5, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, and interferon γ inducible protein (IP)-10/CCL10 are produced by glia and infiltrating leukocytes during multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)[2]. MCP-1/CCL2 and its receptor, CCR2, have been implicated as pivotal mediators of leukocyte infiltration from blood to CNS tissue, whereas RANTES/CCL5-CCR5 interactions do not appear to be necessary for this response.

The present study was conducted to observe the expression level in mRNA and protein of MCP-1, MIP-1α (subfamilies of CC chemokine), and MIP-2 (CXC chemokine) chemokines in LPS-induced cerebral inflammation in neonatal immature brain to elucidate the mechanism of leukocyte accumulation which is a hallmark pathology in the inflammatory reaction.

Materials and Methods

Animal models

One week old Sprague-Dawley rats or adult male rats weighing 300-350 g were used for this experiment. LPS derived from Escherichia coli 026:B6 was purchased from Sigma (Saint Louis, MO, USA). More than five animals per group were used for the genetic variation.

Intraparenchymal injection of LPS

LPS was intraparenchymally administrated. Briefly, neonatal rat was anesthetized with ether and 1 ㎕ LPS (0.5 mg/ml) subsequently injected in the right caudate nucleus at the point 2.5 mm right to bregma and 4 mm depth after fixation of the head with stereotaxic frame (David Kopf, Tujunga, CA, USA). Control animal were treated with saline.

Adult animals were anesthetized with intramuscular injection of Ketamine (1 g/kg). Animals thus treated were placed in the stereotaxic frame, an incision was made on the scalp, and a burr hole was made. Same amount and concentration of LPS as used in the neonatal brain was slowly injected intraparenchymally at the point 4.5 mm right to the bregma and 6 mm in depth. All injection was made slowly at least for 10 seconds. Animals were sacrificed at 6 hours, 24 hours,and 72 hours after injection.

The light microscopic examination of the brain

After anesthetizing the animals, the incision was made on the midline anterior chest. The exposure of the heart was followed by perfusion with 200 ml of 0.9% normal saline and 4% paraformaldehyde. The brain was embedded in paraffin, sectioned serially at the 10 ㎛ thickness, stained with hematoxylin and eosin.

RT-PCR for the measurement of chemokines (MCP-1, MIP-1α, and MIP-2) mRNA level

The expression of MCP-1, MIP-1α, and MIP-2 in the brain was assessed using RT-PCR. Brains were removed quickly and put into a Brain Matrix (Ted Pella, Redding, CA, USA). Serial coronal sections were prepared. The section that contained injected LPS was removed from Brain Matrix and dissected proximately 8 mm^3 cubic of injection site of caudate nucleus. Total RNA was isolated from the frozen specimens using TRIzol (Life Technologies, Rockville, MA, USA). Five microgram of total RNA isolated from tissues was transcribed into cDNA using Superscript (Life Technologies). The cDNA synthesis was carried out in a 20 μl reaction volume containing 5 μg total RNA, 500 mM dNTPs, 10 mM oligo (dT), 1X RT-PCR buffer, 0.8 unit of RNase inhibitor and 25 unit of Superscript. The reaction was performed in a GeneAmp PCR system (Perkin-Elmer, Wellesley, MA, USA) at 60℃ for 10 min and 42℃ for 50 min followed by termination at 70℃ for 5 min. Specific cDNA were amplified by PCR in the presence of AmpliTaq (Promega, Madison, WI, USA). The reaction was performed in a final volume of 50 μl, consisting of 5 μl of 10XPCR buffer, 3 μl of 25 mM $MgCl_2$, 2 μl mixture of 1 mM dNTP, 0.5 μl of AmpliTag Gold (5 U/μl), 2 μl of each primer (50 μM), 2 μl of cDNA, and ddH_2O added to achieve the final volume. The typical PCR reaction conditions were 5 min at 95℃, 35 cycles at 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min, and after the cycles at 72℃ for 10 min and then remaining at 4℃. PCR products were subjected to 2% agarose gel. To ensure that equal amounts of reverse-transcribed cDNA applied to the PCR reaction, the primer pairs for β-actin were also included in the PCR reaction as a technical control. Oligonucleotide primer sets were generated to amplify fragments of the rat cDNA sequences. The sequence is the following.

MCP-1: 5′-GTCTCTGTCACGCTTCTGGGCCTG-3′,
5′CTACAGAAGTGCTTGAGGTGGTTGTGG-3′
MIP1-α; 5′-GCTGTTCTTCTCTGCACCATG-3
5′-CAGTGATGTATTCTTGGACC-3′
MIP2; 5′-GCTCCTCAATGCTGTACTGGTC-3′
5′-GGACGATCCTCTGAACCAAG-3′
***β-Actin;* 5′-TTGTAACCACCTGGGACGATATGG-3′**
5′-GATCTTGATCTTCATGGTGCTAG-3′

β-Actin mRNA was assayed concurrently to evaluated the equivalence of RNA content among samples. DNA density was anaylzed with MacBAS V2.5, (FUGI, Japan). Graphs were made in terms of the ratios of the value of MCP-1, MIP-1, and MIP-2 when amount of β-actin put 100%.

The immunohistochemistry of MCP-1, MIP-1α, and MIP-2

The section of the brain used for the histological evaluation was treated with xylene and ethanol for the removal of paraffin and dehydration. The sections were washed with 0.1MTris-buffered saline (TBS with 1% Triton X-100, pH 7.4) and boiled with 10 mM citrate buffer (pH 6.0) solution for 10 minutes. The sections were washed with TBS and treated with normal goat serum for 40 minutes at room temperature to block the nonspecific reaction. The MCP-1, MIP-1α, and MIP-2 (1:150, rabbit anti-MCP-1, MIP-1α, and MIP-2 polyclonal antibody, SantaCruz, CA, USA) were diluted with TBS buffer containing 0.1% bovine serum albumin.

For the detection of MCP-1, the sections were incubated with rabbit anti-rat MCP-1 antibody for 12 hours at 4℃, and then washed with TBS buffer. To detect MCP-1, the sections were incubated with AlexFluor goat anti-rabbit (1:150, monoclonal, DAKO, USA) for 40 minutes at room temperature, and analyzed using a immunofluoresence microscopy. MIP 1-α and MIP-2 were performed with the same procedures as MCP-1.

Statistical analysis

All data was expressed as mean and ± SEM. Parametric data among the MCP-1, MIP-1α, MIP-2 and saline control groups were evaluated with ANOVA followed by Duncann's between-group comparison (SPSS 11.0). A value of $P<0.05$ was considered to be statistically significant.

Results

The light microscopic examination

The brain tissue of adults rats treated with LPS showed interstitial edema without accumulation of leukocytes (Fig. 1). In the neonatal rat brain, prominent interstitial edema with significant accumulation of leukocytes was detected at 24 and 72 hours after LPS injection (Fig. 1; A, and B).

LPS induces the expressions for MCP-1, MIP-1α, and MIP-2 mRNA

The mRNA expression of members of the CC chemokines, MCP-1 and MIP-1α, was significantly different in neonatal and adult brain during experimental time-course. A semiquantitative analysis of RT-PCR revealed that the MCP-1 mRNA expression arose at 6 hours, peaked 24 hours, and declined to 72 hours after injection in both groups (Fig. 2). The transient expression of the mRNA encoding MCP-1 following the LPS treatment was induced significantly at 24 hour. In other words, neonatal rats showed about 2.6 times more expression of the MCP-1 than that of the adult rats in the brain tissue. The expression level of MCP-1 returned to basal level at 72 hour. This result indicates that mRNA level of MCP-1 peak at 24 hours.

The expression of MIP-1α mRNA was peaked at the 24 hours after LPS injection in neonatal as well as adult brain. Fig. 3 illustrates that MIP-1α mRNA of neonatal and adult brain were expressed about 3 and 4 times higher compared with controls, respectively during 24 hours. The relative quantities of MIP-1α were 245.82 ng/μl and 170.74 ng/μl in neonatal and adult brain, respectively. The increase of MIP-1α mRNA in neonatal rat brain was about 1.4 times to adults. The expression level was decreased after 72 hours.

In order to compare CC chemokine expression, we tried to detect CXC chemokine expression. MIP-2 is one of CXC chemokine members. After treatment of LPS in neonatal and adult brain, the expression pattern of MIP-2 mRNA was similar to those of MCP-1 and MIP-1α mRNA. MIP-2 mRNA expression arose at 6 hours, peaked 24 hours, and declined to 72 hours after injection (Fig. 4). Relative quantity of MIP-2 in neonatal brain was 209.34 ng/μl and in adult 164.06 ng/μl. The increase of MIP-2 mRNA in neonatal rat brain was about 1.2 times to adults.

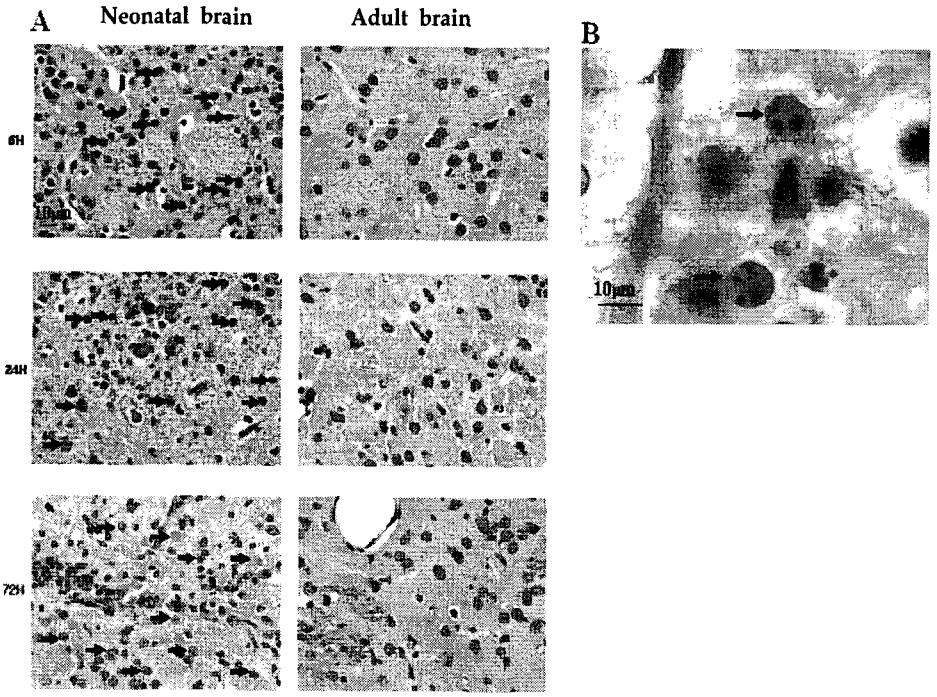

Fig. 1. Histological evaluation of the caudate nucleus of neonatal and adult rat brain after the stereotaxic injection of 0.5 μg LPS. A: SD-rats were sacrificed and the brains were removed at 6, 24, and 72 hours post-LPS injection. Inflammatory cells, predominately neutrophils, are located in the caudate nucleus. B: Neutrophil infiltraion in caudate nucleus at 24 hours after injection of LPS in neonatal brain. H&E stain.

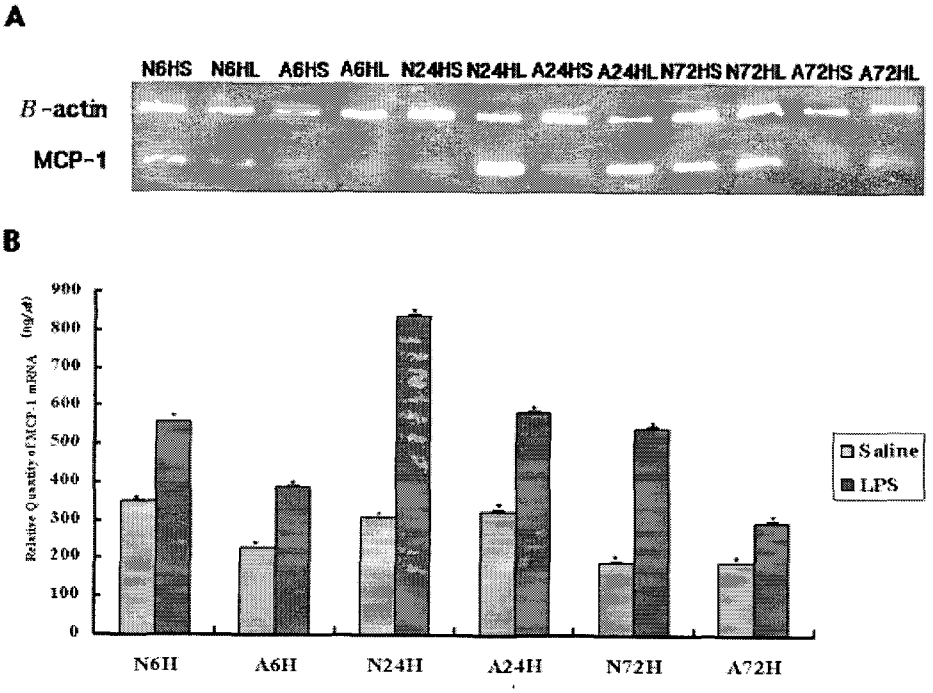

Fig. 2. RT-PCR revealed expression of MCP-1 mRNA in LPS injected neonatal and adult brains. A pannel illustrates a fluorometric scan of the PCR products, visualized in EtBr-stained agarose gels. B pannel is the corresponding graphic representation of MCP-1 band intensity in arbitrary optical density (OD) units, normalized, based on β-actin mRNA. MCP-1 mRNA expression is markedly up-regulated in the neonatal 24 hours group. (ANOVA, $p<0.05$) N; neonate, A; adult, L; LPS. and S; saline.

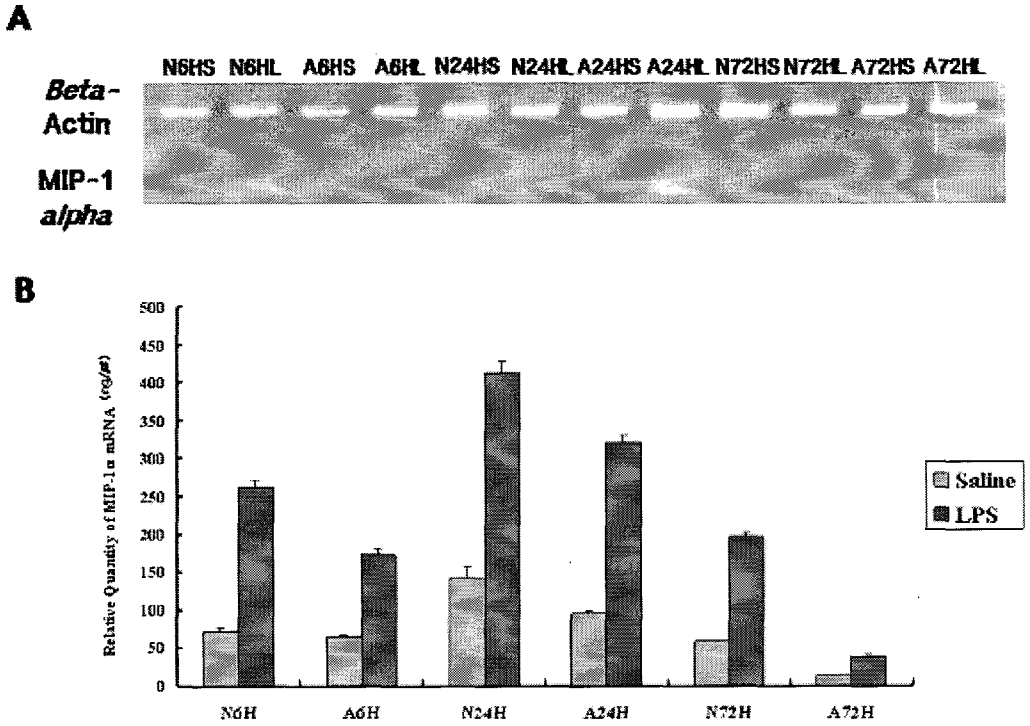

Fig. 3. RT-PCR revealed expression of MIP-1α mRNA in LPS injected neonatal and adult brains. A pannel illustrates a fluorometric scan of the PCR products, visualized in EtBr-stained agarose gels. B pannel is the corresponding graphic representation of MIP-1α band intensity in arbitrary optical density (OD) units, normalized, based on β-actin mRNA. MIP-1α mRNA expression arose at 6 hours, peaked at 24 hours, and declined to 72 hours after LPS injection in neonatal group, while little expression in control group (ANOVA, $p<0.05$).

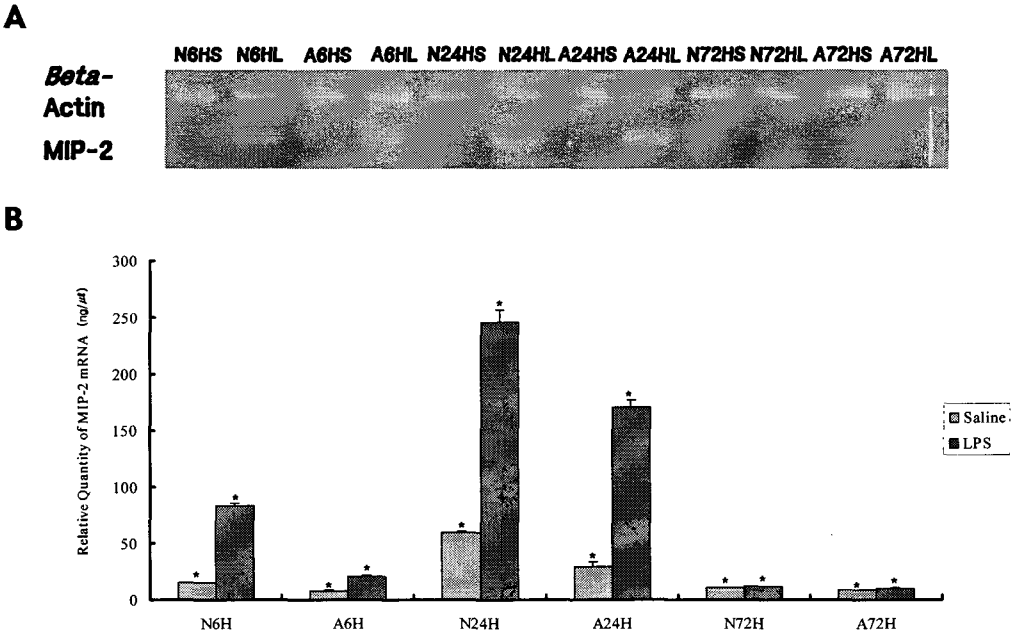

Fig. 4. RT-PCR revealed expression of MIP-2 mRNA in LPS injected neonatal and adult brains. A pannel illustrates a fluorometric scan of the PCR products, visualized in EtBr-stained agarose gels. B pannel is the corresponding graphic representation of MIP-2 band intensity in arbitrary optical density (OD) units, normalized, based on β-actin mRNA. MIP-2 mRNA expression is markedly up-regulated in the neonatal 24 hour group (ANOVA, $p<0.05$).

Immunohistochemistry

We tired to analyze the tissue using immunohistochemistry to view the MCP-1, MIP-1α, MIP-2 protein level compared to mRNA level in the neonatal rat and adult rat brain. Immunohistochemical analysis showed that MCP-1 immunoreaction was paralleled with the RT-PCR results. MCP-1 protein was significantly detected at 24 and 72 hours in the neonatal rat brain parenchyma (Fig. 5). MIP-1 α protein was highly expressed at 24 hours in the neonatal rat brain (Fig. 6). These result showed that neonatal rat brain is subject to invasion by pathogen compared to adult rat brain.

The results of leukocyte infiltration in H&E stain were parallelled with that of the immunohistochemistry. Chemokine proteins were markedly detected at 24 hours after injection of LPS and neutrophil influx into intraparenchymal was prominent at 24 hours.

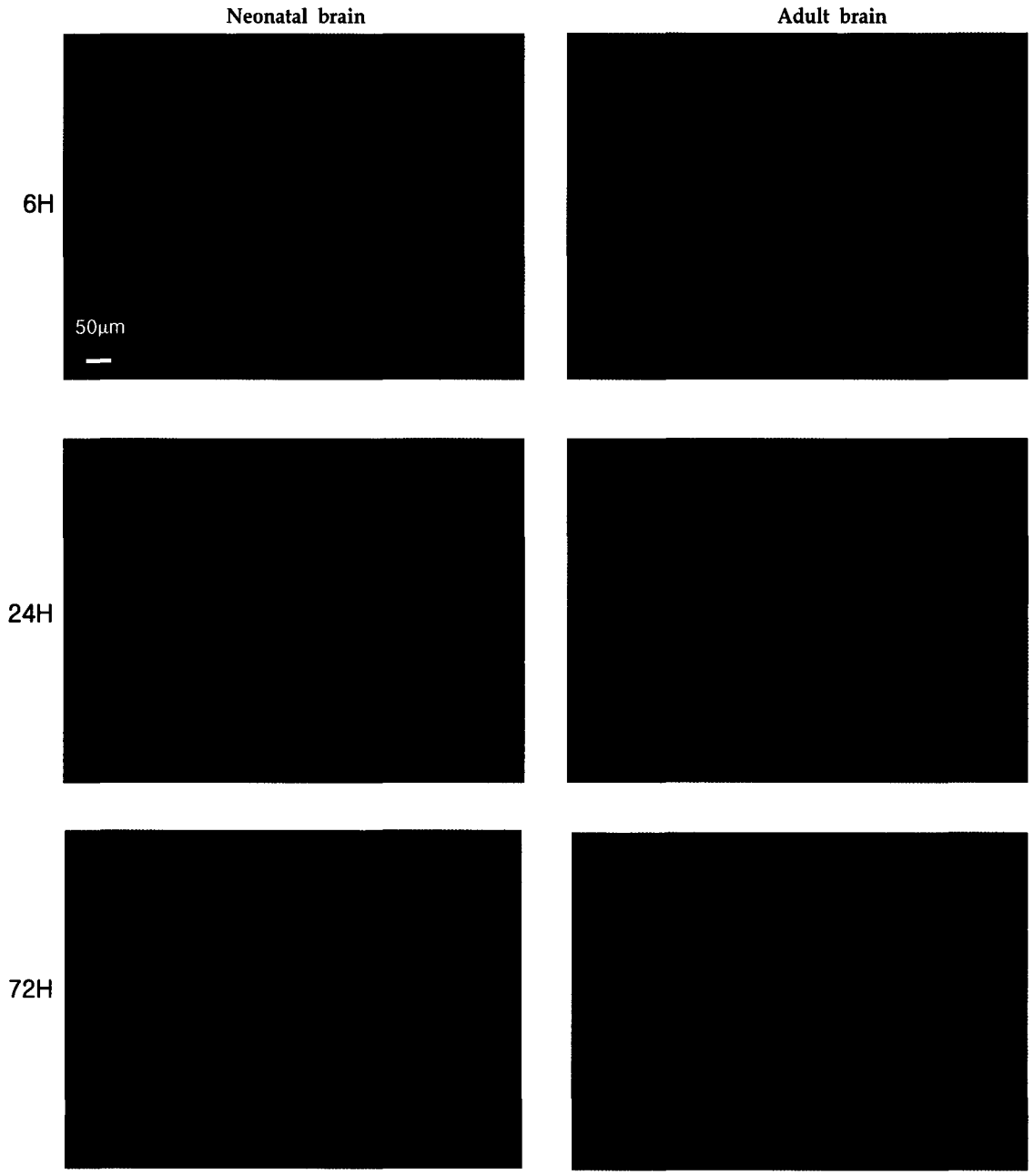

Fig. 5. Immunostaining for MCP-1 at 6, 24, and 72 hours after LPS injection revealed expression of MCP-1, most prominent in neonatal 24 hour group. Immunostaing was observed at the caudate nucleus area. Weak MCP-1 positive staining was detected at the same area in the LPS-injected adult rat brain (X400).

Discussion

Intracranial infection in children results in serious sequel such as diminished intelligence, epilepsy, and focal neurologic deficit. Patients with the history of shunt infection caused by ventriculoperitoneal shunt are known to have lower intelligence than patients without infection. According to McLone *et al*[14], in their myelomeningocele series, mean IQ of the hydrocephalus patients with infected ventriculoperitoneal shunt was 73, while patients without infection was 95. The low intelligence was more prominent in patients infected with gram negative bacilli. Elucidating the mechanism of neuronal damage in the intracranial infection of the immature brain is mandatory to save the children's brain from intelligence deficit in intracranial infection. The inflammatory reactions of the CNS are unique. In response to intraparenchymally injected LPS, which is a potent inflammation inducing agent derived from gram negative bacilli, the brain reacts differently from peripheral tissues[1]. Andersson *et al*[1] have reported that the injection of LPS into the brain did not elicit edema or neutrophil recruitment in rats. Monocyte was recruited several days later. LPS at high doses induced the infiltration of the inflammatory cells in the CNS. However, these mechanisms were known to be age-dependent. The neutrophil infiltration was more prominent in neonatal brain while it was sparse in adult brain in low dose LPS-induced inflammation. The infection in the brain induces the more prominent neuronal death in neonatal immature brain than that in adult. It is suggested that research on the differences of the mechanism of the inflammation in the neonatal brain from adult will contribute to protecting immature brain from neural damage in the intracranial infection.

The destruction of blood-brain barrier with kainic acid did not always recruit leukocyte. It represents the sparse inflammatory cell infiltration is not wholly due to the intact blood brain barrier[1,19]. The salient feature of inflammation is the association of leukocyte infiltration. The maintenance of leukocyte recruitment during inflammation requires intermolecular communication between infiltrating leukocytes and the endothelium, resident stromal, and parenchymal cells. These events are mediated via the generation of early response cytokines, e.g. interleukin (IL)-1 and tumor necrosis factor (TNF), the expression of cell-surface adhesion molecules, and the production of chromaticity molecules, such as chemokines[4].

The chemokine family consists of more than 40 members and is subdivided into four groups: α(CXC), β(CC), δ (CX3C), and γ(C), according to the number of amino acids separating two cysteine residues within a highly conserved region of the chemokine. Chemokine receptors are classified similarly according to which group of chemokines they bind and are designated CXCR-1, CXCR6, CCR1, CCR11, CXCR1 and XCR1[3,18]. Although chemokines were first identified in relation to inflammation, they also have important roles in controlling cell migration within tissues during developemnt, angiogenesis, and tissue repair[12]. For example, chemokines such as IL-8 (CXCL8) and MCP-1 (CCL2), which are induced during inflammation, are also expressed transiently during CNS development where they are thought to control migration of microglial precursors[13].

The CXC or α-family, represented by IL-8 and MIP-2, attracts mainly polymorphonuclear granulocytes, and neutrophils that have a leading role in the early chemotaxic

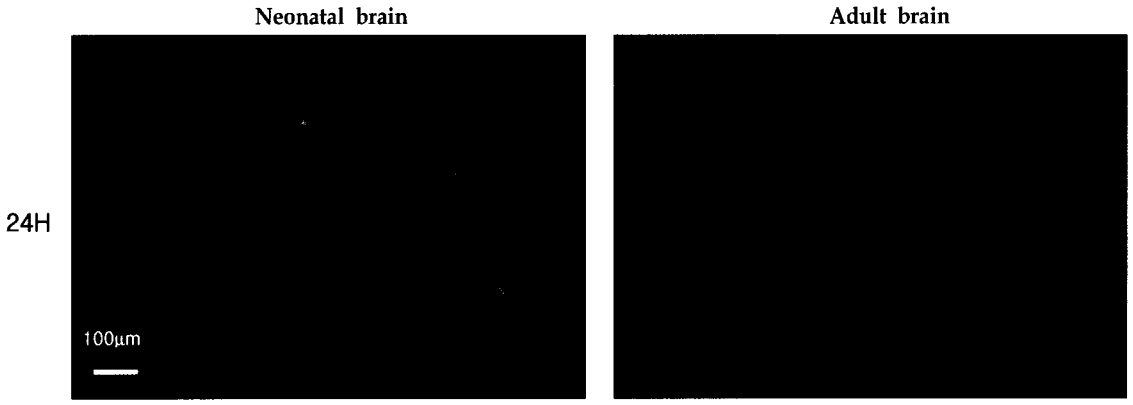

Fig. 6. Immunohistochemistry for MIP-1α at 24 hours after LPS injection (X400).

events during inflammation 7, 8]. MCP-1 is one of the principal CC chemokine and is rapidly synthesized by endothelial cells in response to leukocyte-derived IL-1β to allow the monocyte recruitment and rolling prior to transmigration and arrival at the site of inflammation[5,16]. MIP-1 is a member of the CC or β-family of chemokines and was originally identified as a LPS-inducible heparin-binding protein migrating as an 8000 mol. wt doublet, processing inflammatory and neutrophil chemokinetic properties[22]. MIP-1α and MIP-1β, the two components of the doublet, have been resolved, both peptides appear to be closely related with about 70% homology of their amino acid sequence and show mostly monocyte attractant activity *in vitro*. Like other chemokines, MIP-1 proteins act through activation of G-protein-coupled, seven transmembrane receptors. MIP-1α binds and signals through the CC-chemokine receptors, CCR1, 4, and 5, whereas MIP-1β seems to be a selective agonist for CCR5. Although these homologous peptides exert general overlapping properties in terms of their inflammatory activity and pyogeneicity, MIP-1α and MIP-1β exhibit differences in their specific biological activity[10]. Only MIP-1α stimulates the secretion of TNF-α, IL-1α and IL-6 from macrophages, whereas MIP-1β does not produce this effect. MIP-1α also plays a critical role in the pathogenesis of EAE, and animal model for multiple sclerosis[11].

Babcock et al[2] reported glial cells directed leukocytes to sites of axonal injury in CNS. Axonal injury provoked the expression of CC and CXC chemokines, including RANTES/CCL5 and MCP-1/CCL2. Kinetic studies and lesions in CCL5-deficient mice suggest that leukocyte recruitment did not depend on RANTES/CCL5-CCR5 interaction. Early expression of MCP-1/CCL2 and abrogation of lesion-induced leukocyte infiltration in mice deficient in its receptor, CCR2, support a critical role for this chemokine. Microglia and astrocytes were prominent sources of MCP-1/CCL2. Innate glial responses direct leukocyte entry to the injured CNS by means of chemokine production[2]. Glia produces chemokines in various models of CNS injury. RANTES/CCL5, MCP-1/CCL2, IP-10/CXCL10, MIP-1α/CCL3, and MIP-1β/CCL4 were elevated after cuprizone-induced demyelination[15]. Aspiration lesions induced MCP-1/CCL2 expression in the visual cortex and in the thalamic nuclei before retrograde degeneration[17]. Acute excitotoxic injury elicited by local injection of NMDA or kainic acid induced MCP-1/CCL2 expression by macrophage/microglia and astrocytes[6,21]. Other studies of CNS inflammation have documented endothelial or neuronal expression of MCP-1/CCL2[9,21]. According to Bobcock[2], the MCP-1/CCL2 is produced by astrocytes and microglia. The different patterns of these chemokines reflect tissue injury patterns and neurochemical milieu.

In our experiment the expression of mRNA of chemokine was age-dependent. Low dose LPS injection into the adult cerebral parenchyma provoked little expression of the chemokines in adult brain, while in immature brain the expression was significantly higher. These results suggest that the leukocyte infiltration in the intracranial infection was controlled by mechanisms influenced by chemokine producing cells in the central nervous system such as microglia, astrocyte, and endothelial cell. Clarifying the role of these chemokine-producing milieu in immature brain and modifying chemokine activity in the disease processes will provide a promising perspectives in protecting neuronal damage in various disease processes including intracranial infection.

The data revealed that the expression of mRNA chemokines was age-dependent after LPS injection into the cerebral parenchyma. The chemokine expression of immature brain was significantly higher than mature brain. Clarifying the role of these chemokine-producing milieu mechanisms in the immature brain and modifying chemokine activity during the disease process may provide a tool for preventing neuronal damage in various disease processes including intracranial infection.

Acknowledgment

This study was financially supported by the Dong-eui University in 2006 (2006AA180)

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초록 : LPS 유도에 의한 신생쥐에서 chemokine의 단계별 발현

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외부 병원체의 침입에 의한 병원독소로 발병된 뇌염증에서 미성숙뇌는 성숙뇌에서 보다 많은 백혈구의 침윤을 일으킨다. 케모카인은 뇌염증부위로 염증세포의 침윤을 매개하는 물질이다. 본 연구는 미성숙뇌의 뇌염증에서 백혈구침윤의 기전을 연구하기 위하여 내독소로 유발된 뇌염증에서 케모카인의 일종인 MCP-1, MIP-1α, MIP-2의 발현을 연구하였다. 신생쥐와 성숙쥐의 미상핵 국소에 LPS (0.5 μg/μl)를 정위주사 한 후 시간대별로 RT-PCR과 면역조직화학검사을 통하여 각각 mRNA상과 단백질상에서의 발현을 비교분석하였다. 광학현미경상 LPS 주입 후 6 시간 후부터 주사부위의 염증세포의 침윤이 시작되었으며 24 시간 후에 가장 뚜렷한 현상을 보였다. RT-PCR 결과, MCP-1, MIP-1α, MIP-2 mRNA 발현은 24 시간 때에 최고치를 나타냈었다. 미성숙뇌의 각 케모카인의 mRNA발현은 성숙뇌에 비해 MCP-1은 약 2.6배, MIP-1α는 약 1.4배, MIP-2는 약 1.2배 발현양이 더 많다. 면역조직화학검사는 광학현미경결과와 RT-PCR결과와 상응하여 각 케모카인들이 24 시간 때에 가장 양성반응이 뚜렷이 나타났다. 이러한 현상은 뇌염증에서 백혈구의 침윤은 케모카인을 생성하는 소교세포, 성상세포, 내피세포 등의 영향을 받는 기전에 의하여 조절되었다는 것을 나타내며 이들 케모카인 형성환경의 역할을 명확히 밝히고 질병의 진행에서 케모카인의 활성을 조정하는 방법을 연구하면 향후 뇌염증을 포함한 여러 가지질환에서 신경세포의 손상을 막는 치료법의 개발이 가능하게 될 것이다.