Detection of IL-20R1 and IL-20R2 mRNA in C57BL/6 Mice Astroglial Cells and Brain Cortex Following LPS Stimulation

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ABSTRACT

Background: Astrocytes, which comprise ~90% of overall brain mass, are involved in brain immunity. These cells represent the non-professional class of CNS-resident APCs and may promote or inhibit CNS inflammation depending on the cytokines they secrete. IL-10 family of cytokines and their receptors, IL-20R1 and IL-20R2, may have a role in shifting astrocytes to a neuroprotective or neurodegenerative function. **Objective:** To address the expression of IL-20R1 and IL-20R2 cytokine receptors in astrocytes and brain cortex of C57BL/6 mice. **Methods:** We investigated the expression of IL-20R1 and IL-20R2 in C57BL/6 mice astroglial cells and brain cortex in response to lipopolysaccharide (LPS), using reverse-transcription polymerase chain reaction (RT-PCR) method. **Results:** Astrocytes were able to express IL-20R1 and IL-20R2 mRNA not only in response to LPS stimulation but also in the absence of LPS. Furthermore, we found the expression of IL-20R1 and IL-20R2 mRNA in the cortex of adult C57BL/6 mice. **Conclusions:** IL-20R1 and IL-20R2 are constitutively expressed in the brain. Since most neuropathological processes involve astrocytes and inflammatory cytokines, these findings have important implications for future therapeutic strategies.

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INTRODUCTION

Glial cells play an important role in modulating central nervous system (CNS) inflammation. Glial cell activation, as occurs in neurodegenerative diseases or after trauma, ischemia and infection is a major hallmark of the intracerebral inflammatory response. Inflammation in the brain leads to the activation of astrocytes, the most abundant glial cell type, which are crucial for the process of reactive gliosis and the formation of a glial scar (1).

Astrocytes produce neurotrophic and neuroprotective factors, guide neurons, regulate the extracellular pH and potassium levels, and participate in repair processes within the CNS (2). Indeed, astrocytes are implicated in immune reactions within CNS (3). Activated astrocytes express adhesion molecules and release chemokines and several inflammatory mediators including IL-1β, IL-6, and TNF-α in response to a variety of stimuli (3,4). Astrocytes could be involved in the down modulation of T cell auto-reactivity in the CNS (5). The contact with astrocytes has been shown to trigger the apoptotic death of T cells in vitro and in vivo. Moreover, Astrocytes participate in antigen presentation in the CNS and represent the non-professional class of CNS-resident APCs (6).

The IL-10 family of cytokine consists of several members: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29 (7,8). IL-10, IL-19, IL-20, and IL-24 are primarily secreted by activated macrophages, whereas T-cells are the main source for IL-22 and IL-26 (9,10). In contrast to the immunosuppressive functions of IL-10, other members of the IL-10 family possess different biological functions; for example IL-19 has pro-apoptotic functions in monocytes. IL-19 is capable of promoting TH2 immune deviation through a positive feedback loop (11). The IL-10 family of cytokines binds to heterodimer transmembrane receptor complexes that are composed of a long α-chain (R1-type; with a long cytoplasmic domain) and a shorter β-chain (R2-type; with a short cytoplasmic domain) (12-14). IL-20R1 and IL-20R2 genes are highly expressed in normal skin, testis, and lung (15). Immuno-histochemical staining has shown that IL-20R1 and IL-20R2 are expressed on basal and suprabasal keratinocytes in healthy skin and are up-regulated in psoriatic skin. These receptors induce the activation of signal transducers and activators of transcription 3 (STAT3) (16,17). Both IL-20 and IL-24 form complexes with the receptor pair IL-22R1/IL-20R2. In addition, IL-19, IL-20, and IL-24 also signal through formation of the complexes with IL20R1/IL-20R2 (18).

The primary hypothesis was that the IL-10 family of cytokine and their receptors could have a potential role in shifting the role of astrocytes to a neuroprotective or neurodegenerative property. Accordingly, we have recently found evidence concerning IL-19 mRNA expression in mice astroglial cells and brain cortex (data not shown).

Little is known about the role of IL-10 family cytokines in the inflammatory process in the brain. The biological roles and regulation of the IL-10-related cytokines (IL-19, IL-20, IL-22, IL-24) and their receptors (IL-20R1, IL-20R2 and IL-22R) in the brain are not clear. IL-19 action is transduced by IL-20R1 and IL-20R2 which in turn affects immunological reactions. Whether astrocytes possess these receptors and hence respond to IL-19 is not known. As a first step to address the role of these cytokine and their receptors in the brain, we investigated the expression levels of IL-20R1 and IL-20R2 in astrocytes and cortex of adult C57BL/6 mice following LPS treatment using RT-PCR method.

MATERIALS AND METHODS

Primary Mixed Glial Cell Culture. Primary C57BL/6 mice astroglial cells were isolated as described by McCarthy and de Vellis (19). Briefly, cerebral cortices were excised from 1 to 5 day-old C57BL/6 pups and the meninges were carefully removed under a stereo microscope. Cortices were cut into small pieces and transferred to a tube containing serum-free complete Dulbecco's modified Eagle's medium (DMEM), HBSS-HEPES, trypsin and DNAase (Invitrogen, USA) and incubated for 5 min at 37°C.

Small particles of cortical tissue mechanically dissociated into a single cell suspension and cellular debris was removed by filtration through a 70 μm cell strainer. The primary glial cell cultures were resuspended and maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen, USA) and expanded in poly-L-ornithine-coated tissue culture dishes (Sigma-Aldrich, USA). The culture medium was changed next day and 6 days after and then twice a week with the DMEM supplemented with 10% FCS. Cells were allowed to grow to confluence (14-18 days).

The plates were shaken mechanically for 2 h at 150 rpm at 37°C in a temperature controlled orbital shaker to loosen microglia and oligodendrocytes growing on top of the astrocytic layer from the more adherent astrocytes. Then, microglia and oligodendrocytes were removed. The remaining adherent cells were detached with trypsin/EDTA, resuspended in fresh medium and reseeded in poly-L-lysin-coated tissue culture dishes (Sigma-Aldrich, USA). To remove any residual oligodendrocytes and microglial cells, the plates were shaken for 3 times as described above before harvesting.

Immunocytochemistry Assays for Glial Fibrillary Acidic Protein (GFAP). The purity of astrocyte cultures was determined by indirect immunocytochemical staining using rabbit anti-mouse GFAP, specific marker for astroglial cells, (Dakopatts antibody, Labome, UK). Astrocytes were grown on poly-L-lysin-coated cover slips. Cells were fixed in 4% Paraformaldehyde and blocked using goat serum, and incubated overnight at 4°C with rabbit anti-GFAP. The next day, cover slips were washed and incubated with green fluorescence Alexa Fluor 488 dye-labeled goat anti-rabbit IgG antibody (Invitrogen, USA) for 45 min at room temperature. Afterwards, astrocytes nuclei were stained with DAPI. The cover slips were mounted onto microscope slides using Mowiol mounting medium (Merck, USA). Observations were performed with fluorescence and Confocal microscopes.

Stimulation of C57BL/6 Mice Astroglial Cells with LPS. Astrocytes were treated with two doses of LPS from Escherichia coli serotype 0127:B8 (Sigma-Aldrich, USA): (a) 1 μg/ml for 8 h; (b) 10 μg/ml for 24 h. The control groups were not stimulated.

Intraperitoneal (i.p.) Administration of LPS. A single dose of 1.5 mg/kg of LPS in sterile, pyrogen-free 0.9% saline was randomly administered by i.p. injection to a combination of male and female mice for two time points (24 and 72 h). Four mice were used in each group. Control animals received volume-equivalent injections of sterile saline at the same times to rule out effects based upon the stress of the injection procedure itself.

Brain Tissue Preparation for RT-PCR. At the end of each time point, mice were euthanized with i.p. injection of chlorine (200 mg/kg) then they were transcardially perfused with cold sterile phosphate buffer saline (PBS). Brains were excised, dissected

and frozen on dry ice. The frontal cortex, containing the frontal association, dorso lateral orbital, ventral orbital and prelimbic cortices (first 1 mm of the frontal cortex), were collected and stored at -80°C until total RNA extraction.

RNA Isolation from Astroglial Cells and Brain Samples. Total RNA was extracted from treated and control groups of astrocytes and brain samples using TRIzol (Invitrogen, USA) reagent according to manufacturer's instruction. RNA samples were treated with DNAase to degrade any traces of contaminating genomic DNA during extraction. Thereafter, RNA was quantified using a Nanodrop ND-1000 spectrophotometer and stored at -80°C until cDNA synthesis.

cDNA Synthesis. RNA was reverse transcribed using a RNT cDNA synthesis Kit (Applied Biosystems, USA) to prepare cDNA in accord with supplier's instructions. PCR amplification, using random primer sets, was conducted at a 25°C, 37°C and 85°C for 10 min, 2 h and 5 min, respectively. The PCR was performed by using a Gradient Thermal Cycler (Eppendorf, Germany).

RT-PCR Reactions for IL-20R1, IL-20R2, IL-1β, TNF-α, GFAP and β-actin. Amplifications of the cDNA templates were performed in 25 µl PCR mix containing 2 µl of cDNA, 5 µl PCR buffer, 50 mM dNTPs, 0.25 µl Taq DNA polymerase and 5 µl of Reverse and Forward primers (Table 1). The astrocytes cDNA were examined for expression of IL-20R1, IL-20R2, IL-1β and TNF-α mRNA. GFAP and β-actin housekeeping gene primers were also used as specific marker of astroglial cells and internal control of RT-PCR reactions; respectively. The PCR was carried out using a Gradient Thermal Cycler.

Electrophoresis. PCR products were loaded onto 1.5% agarose gels containing ethidium bromide, bands were separated by application of 90 V for 30 min.

Table 1. Nucleotide sequences of primer sets used for RT-PCR reactions.

Gene	Forward	Reverse
mIL-20R1	GGC ACA AGA GTC TTT GAA CCT ACT G	GAC AGT ATG CTC CTG ACC CAG G
mIL-20R2	GTC TGG ACA AGT CCG TTC ATG	CTA GGT GCA CCG GAA TGT C
mIL-1β	CTC CAT GAG CTT TGT ACA AGG	TGC TGA TGT ACC AGT TGG GG
mTNF-α	ATG AGC ACA GAA ACA TGA TCC GC	CCA AAG TAG ACC TGC CCG GAC TC
mGFAP	GGC GCT CAA TGC TGG CTT CA	TCT GCC TCC AGC CTC AGG TT
mβ-actin	GGG AAT GGG TCA GAA GGA CT	TTT GAT GTC ACG CAC GAT TT

Sequences are written from 5´nucleotide position (left) to 3´ nucleotide position (right) of the source sequence. m: mouse.

RESULTS

Purity of Enriched Astroglial Cell Cultures. The purity of astrocyte cultures was determined using indirect immunocytochemical staining with anti-GFAP antibody. Stained cells with specific marker for astrocytes were counted under fluorescent microscope. Purity of astroglial cells estimated approximately 95% as shown in Figure 1. Cells stained with rabbit anti-GFAP antibody (green) and Nuclei stained with DAPI (blue).

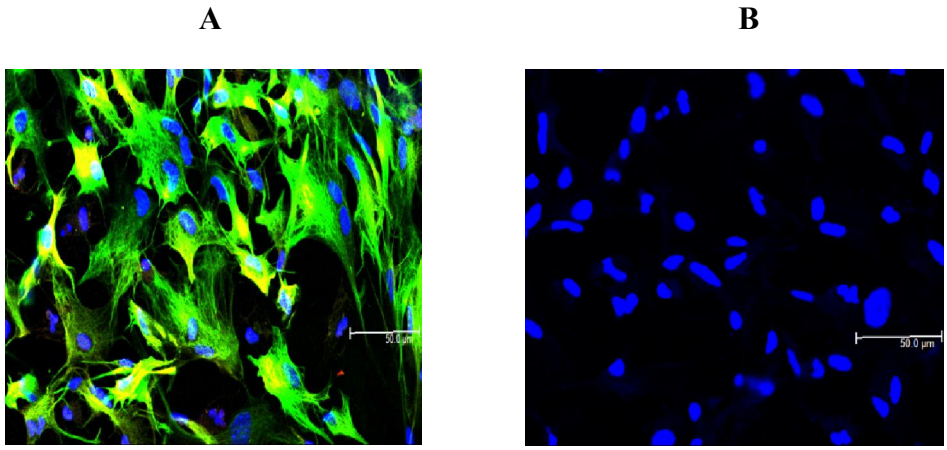

Figure 1. (A) Confocal Microscopy analysis of primary astroglial cells stained with rabbit anti-GFAP antibody (green). The cell nuclei stained with DAPI (blue). (B) Negative control stained with secondary antibody and DAPI.

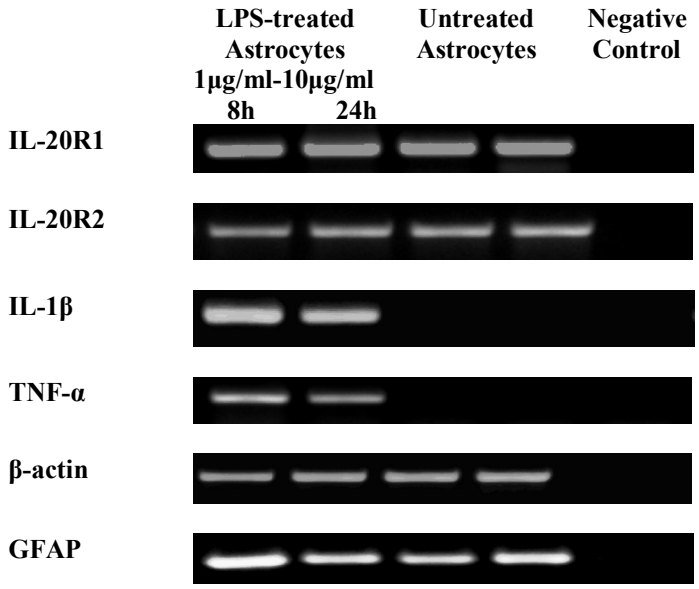

Figure 2. The expression of IL-20R1, IL-20R2, IL-1β, TNF-α, β-actin and GFAP mRNA were carried out using mRNA extracted from LPS-treated and untreated mouse astrocytes. β-actin and GFAP were used as internal control and specific marker of astrocytes, respectively.

Expression of IL-20R1, IL-20R2, IL-1β and TNF-α mRNA in LPS-treated Astroglial Cells. Mice enriched astroglial cells were cultured in the presence and absence of LPS for studying IL-20R1 and IL-20R2 mRNA. After treatment, IL-20R1 and IL-20R2 mRNA were constitutively expressed in LPS-treated and untreated astroglial cell cultures. Furthermore, IL-1β and TNF-α mRNA were expressed in LPS-treated astrocyte cultures but not in control cells. The results are shown in Figure 2.

Expression IL-20R1, IL-20R2, IL-1β and TNF-α mRNA in the Cortex of Adult C57BL/6 Mice. To assess the effects of LPS on IL-20R1 and IL-20R2 mRNA expression in the cortex, mice were injected i.p. with LPS. The findings of present study demonstrated that mice were able to produce IL-20R1 and IL-20R2 mRNA not only after administrating of LPS but also following normal saline administration; in fact these receptors are expressed constitutively in the cortex of adult mice. Moreover, IL-1β and TNF-α mRNA expression were detected in the cortex as compared with animals receiving only saline. Data are presented in Figure 3.

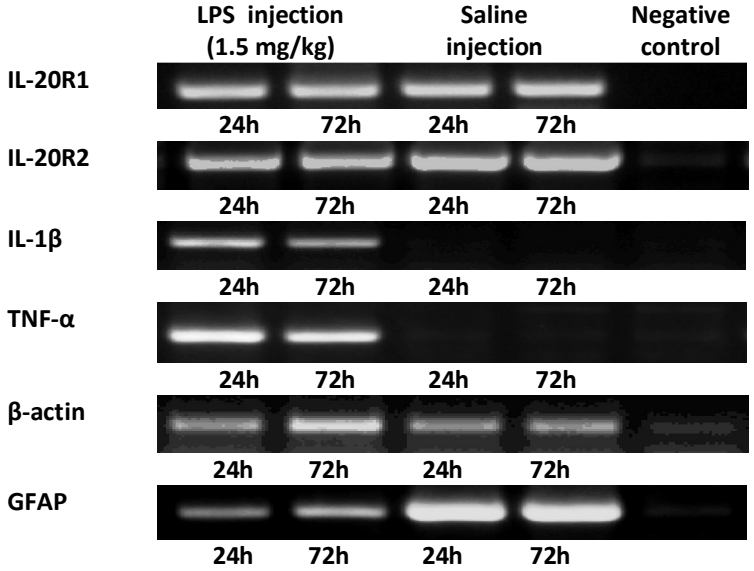

Figure 3. RT-PCR reactions for IL-20R1, IL-20R2, IL-1β, TNF-α, GFAP and β-actin were performed using mRNA isolated from cortex of adult C57BL/6 mice following intraperitoneal (i.p.) administration of LPS.

DISCUSSION

Previous studies have shown that astrocyte and astrocyte-derived cytokines initiate and properly coordinate the immune responses of the CNS (20). Furthermore, these cytokines may be critical components in the mechanisms underlying neurodegeneration. Neuronal-glial interactions could be involved in determining the activation threshold of astrocytes to inflammatory cytokines (20).

Considering the potential role of astrocytes in neuroprotection and neuropathology we proposed that certain cytokines and their receptors may shift astrocyte fate to a desirable

or detrimental direction i.e., repair processes or neurodegeneration in inflammatory conditions. Formation of complexes with specific cell surface IL-10 family of cytokine receptor is the first step in the signaling pathways that involve these cytokines. To produce signaling complexes, two distinct receptor subunits (type I and type II) are needed. The type I receptors have longer intracellular domains that are able to recruit STATs. A receptor with a shorter intracellular domain that does not interact with STATs is named type II. The IL-20 subfamily (IL-19, IL-20 and IL-24) share receptor complexes; all of them are capable of signaling through IL20R1 and IL-20R2 and IL-20 and IL-24 can also use IL-22R/ IL-20R2 (21-23).

In the present study we identified that astroglial cells are able to produce IL-20R1 and IL-20R2 mRNA not only in response to LPS stimulation but also in unstimulated astroglial cells. Moreover, our results demonstrate that these receptors are expressed after i.p. administration of LPS and in healthy mice brain cortex. Therefore, it seems very likely that IL-20R1 and IL-20R2 genes are constitutively expressed in normal tissues such as skin, keratinocytes, testis, lung and brain (15-17). The presence of these receptors in astrocytes implies that cytokines of IL-20 subfamily can affect astrocyte function. Thus, the steady-state IL-20R1 and IL-20R2 expression in vivo and in vitro is also sufficient to initiate signaling of IL-19, IL-20 and IL-24.

A study by Lieberman et al. showed the expression of TNF-α, IL-1β and IL-6 proinflammatory cytokines genes in LPS-stimulated astrocytes (23). Astroglial cells are able to produce IL-1β in response to LPS (20). Furthermore, TNF-α is synthesized and secreted by astrocytes, microglia and neurons (24). Previous studies have also shown that LPS stimulates IL-1β synthesis in the brain (25). Excessive IL-1β expression is observed in neurodegenerative diseases, the main source being microglia and astrocytes (20). In the present investigation, to examine inflammation induction after LPS stimulation, we studied the expression of proinflammatory cytokines mRNA IL-1β and TNF-α in LPS-treated astroglial cells and after i.p. LPS administration in cerebral cortex. The results show that LPS-treated astrocytes can express inflammatory cytokines mRNA. Also, we have found IL-1β and TNF-α mRNA in cerebral cortex after i.p. injection of LPS.

The relevance of IL-20R1 and IL-20R2 in brain immunity remains unknown, especially with respect to the beneficial or detrimental influence on activity of astrocytes. The role of these receptors in neurodegenerative diseases has not yet been elucidated. These cytokines may induce different biological activities in the CNS, and modify the course of several neurodegenerative diseases depending on the stage of disease activity.

Further investigation should be performed to disclose the molecular basis of underlying IL-20R1 and IL-20R2 signaling pathway in astrocytes and the exact role of these receptors in regulation of astrocyte activity in brain immunity. Therefore, it is important to clarify the precise function of IL-20R1 and IL-20R2 and other members of IL-10 family receptors in the CNS in order to understand the mechanisms involved. This work has been carried out at the level of mRNA using RT-PCR reactions. More research on the level of IL-19 protein and mRNA in different inflammatory conditions using sensitive methods such as western blotting, ELISA or intracellular flowcytometry and Real Time PCR are also necessary.

Since most neuropathological processes include astrocytes and inflammatory cytokines, these findings have important implications for future research and therapeutic strategies.

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