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Effects of chemokine (C–C motif) ligand 1 on microglial function ☆

Nozomi Akimoto [a], Masataka Ifuku [b], Yuki Mori [a], Mami Noda [a,*]

[a] Laboratory of Pathophysiology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Laboratory of Integrative Physiology, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

Microglia, which constitute the resident macrophages of the central nervous system (CNS), are generally considered as the primary immune cells in the brain and spinal cord. Microglial cells respond to various factors which are produced following nerve injury of multiple aetiologies and contribute to the development of neuronal disease. Chemokine (C–C motif) ligand 1 (CCL-1), a well-characterized chemokine secreted by activated T cells, has been shown to play an important role in neuropathic pain induced by nerve injury and is also produced in various cell types in the CNS, especially in dorsal root ganglia (DRG). However, the role of CCL-1 in the CNS and the effects on microglia remains unclear. Here we showed the multiple effects of CCL-1 on microglia. We first showed that CCR-8, a specific receptor for CCL-1, was expressed on primary cultured microglia, as well as on astrocytes and neurons, and was upregulated in the presence of CCL-1. CCL-1 at concentration of 1 ng/ml induced chemotaxis, increased motility at a higher concentration (100 ng/ml), and increased proliferation and phagocytosis of cultured microglia. CCL-1 also activated microglia morphologically, promoted mRNA levels for brain-derived neurotrophic factor (BDNF) and IL-6, and increased the release of nitrite from microglia. These indicate that CCL-1 has a role as a mediator in neuron-glia interaction, which may contribute to the development of neurological diseases, especially in neuropathic pain.

1. Introduction

CCL-1, also known as thymus-derived chemotactic agent 3 (TCA-3), is secreted by activated macrophages, T-cell, mast cells and endothelial cells, and has important role as the chemoattractant for neutrophils and monocytes [1]. Although effects of CCL-1 on the peripheral immune cell are well characterized, the role of CCL-1 in the CNS remains unclear.

We have previously discovered that CCL-1 was produced mainly in the DRG and spinal cord after peripheral nerve injury and contributed to the development of neuropathic pain [2]. We also showed that the expression of the specific CCL-1 receptor CCR-8 was up-regulated in the ipsilateral superficial dorsal horn after spinal nerve ligation, not only in neurons but also in microglia and astrocytes. Microglial cells are generally considered as the primary immune cells in the CNS [3,4], and play important roles in the development of neuropathic pain [5]. One of the main functions of microglia in those pathological conditions is that they act as a source and a target of chemokine actions, also in an auto/paracrine fashion. Chemokines including CCL-2 (monocyte chemoattractant protein 1), CCL-21, or CX3CL1 (fractalkine) have been shown to serve as signals from endangered neurons to microglia [6]. Though microglial expression at transcriptional and/or protein level has been documented for many chemokine receptors [7], the functional role of CCL-1/CCR-8 has not been reported yet.

In the present study, we demonstrated that CCL-1 stimulated microglial migration, proliferation and phagocytosis, and release of BDNF and IL-6 in vitro, which may contribute to the neuropathic pain due to nerve injury.

2. Materials and methods

The study was approved by the Animal Research Committee of Kyushu University.

Abbreviations: BDNF, brain-derived neurotrophic factor; bp, base pair; CCL-1, chemokine (C–C motif) ligand 1; CCL-2, chemokine (C–C motif) ligand 2; CCL-21, chemokine (C–C motif) ligand 21; CCR-8, chemokine (C–C motif) receptor 8; cDNA, complementary DNA; CNS, central nervous system; CX3CL-1, chemokine (CX3C motif) ligand 1; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; DMEM, dulbecco's modified eagle medium; DRG, dorsal root ganglia; FBS, fetal bovine serum; GABA, γ-amino butyric acid; GFAP, glial fibrillary acid protein; Iba1, ionized calcium-binding adapter molecule 1; IL-1β, interleukin 1β; IL-6, interleukin 6; MAP2, microtubule-associated protein 2; mRNA, messenger RNA; NO, nitric oxide; NR2B, NMDA receptor 2B; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TCA-3, thymus-derived chemotactic agent 3; TNF-α, tumour necrosis factor-α.

* Corresponding author. Fax: +81 92 642 6574.
E-mail address: noda@phar.kyushu-u.ac.jp (M. Noda).

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2.1. Cell culture

Mouse microglial cells were isolated from the mixed cultures of cerebrocortical and spinal cord from postnatal days 1–3 ddY mice (Kyudo, Tosu, Japan), as described previously [8,9]. In brief, tissue was trypsinized for 3 min and dissociated with a fire-polished pipette. Mixed glial cells were cultured for 9–12 days in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% Hyclone fetal bovine serum (FBS; Hyclone Labratories, UT, USA), 2 mM L-glutamine, 0.2% D-glucose, 5 μg/ml insulin, 0.37% $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a 10% CO_2, with medium changes every 3 days. Microglial cells were then separated from the underlying astrocytic layer by gently shaking the flask for 2 h at 37 °C in a shaker-incubator (120 rpm). After unattached cells were removed, microglial cells were isolated as strongly adhering cells. The purity of microglia was >98%, which was evaluated by staining with Iba1 (ionized calcium-binding adapter molecule-1), a marker for microglia/macrophage. Primary cultured neurons from the cerebral cortex were obtained from embryonic day 14–16 (E14–16) ddY mice as described previously [10]. Briefly, neurons were cultured at 37 °C in a 10% CO_2 incubator for 5–7 days with neurobasal medium (GIBCO, NY, USA) containing 2% B27 supplement (GIBCO) and 0.5 mM L-glutamine (GIBCO).

2.2. Immunocytochemical analysis

Primary cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 136.9 mM NaCl, pH 7.4) for 5 min, followed by treating with blocking solution (Block Ace; Dainippon Pharmaceutical, Japan) for 30 min at room temperature. Cells were incubated with primary antibodies: Iba1 (1:2000, Wako, Japan), CD11b (1:500, Serotec, UK), CCR-8 (1:500, Enzo, PA, USA), Ki67 (1:1000, Abcam, UK), NeuN (1:200, Millipore, CA, USA), MAP2 (1:1000, SIGMA, MO, USA), GFAP (glial fibrillary acid protein, 1:800, Millipore), phalloidin (mouse anti-Texas Red-conjugated phalloidin, 2 U/ml, Invitrogen, CA, USA) overnight at 4 °C. The cells were washed with PBS, and then incubated for 3 h at room temperature with secondary antibody (IgG-conjugated Alexa Fluor™ 488 or 568 or 593, 1:1000, Molecular Probes, OR, USA). The cells were washed with PBS and treated with DAPI (300 nM, Molecular Probes) for 10 min, then washed with PBS. Slides were coverslipped with permafluor aqueous mounting medium. The sections were analyzed using a confocal laser scanning microscope (LSM510META, Carl Zeiss, Germany).

2.3. Real time reverse transcription (RT) polymerase chain reaction (PCR)

Cultured microglia cells were plated in 60 mm dishes (10^6 cells/dish) and incubated for 24 h and collected after treatment of CCL-1 (R&D systems, MN, USA). They were subjected to total RNA extraction according to the protocol of the manufacture and purified with QIAamp RNA Blood Mini (Qiagen, Valencia, CA, USA). The amount of total RNA concentration was measured using Smart Spec™ 3000 (Bio Rad, Tokyo, Japan). Total RNA (175 ng) was converted to cDNA by reverse transcription, using random 9 mer (Takara, Otsu, Japan) and RNA PCR kit (Takara). The forward and reverse primers and the estimated product size were as follows: CCL-1 mRNA (5′-TTCCCCTGAAGTTTATCCAGTGTT-3′; 5′-TGAACCCACGTTTTGTTAGTTGAG-3′), 124 bp; CCR-8 mRNA (5′-ACGTCACGATGACCGACTACTAC-3′; 5′-GAGACCACCTTACACATCGCAG-3′), 301 bp; β-actin mRNA (5′-TTGCTGACAGGATGCAGAAGGAG-3′; 5′-GTGGACAGTGAGGCCAGGAT-3′), 127 bp; IL-10 mRNA (5′-GTCATCGATTTCTCCCCTGTG-3′; 5′-CCTTGTAGACACCTTGGTCTTGG-3′), 93 bp; CCL-2 mRNA (5′-CGGAACCAAATGAGATCAGAA-3′; TGTGGAAAAGGTAGTGGATGC-3′), 26 bp; TNF-α mRNA (5′-CCACCACGCTCTTCTGTCTAC-3′, 5′-TGGGCTACAGGCTTGTCACT-3′) 848 bp; IL-1β mRNA (5′-CTCCATGAGCTTTGTACAAGG-3′; 5′-TGCTGATGTACCAGTTGGGG-3′) 245 bp; IL-6 mRNA (5′-ACACTCCTTAGTCCTCGGCCA-3′; 5′-CACGATTTCCCAGAGAACATGTG-3′) 129 bp; BDNF mRNA (5′-TGCAGGGGCATAGACAAAAGG-3′; 5′-CTTATGAATCGCCAGCCAATTCTC-3′), 110 bp. All primers were purchased from Sigma Aldrich Japan (Tokyo, Japan). PCR amplification was undertaken for Thunderbird Sybr qPCR Mix (Toyobo, Osaka, Japan) in Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Japan, Tokyo, Japan). Each reaction volume consisted of 12.5 μl Thunderbird Sybr qPCR Mix, 0.05 μl 50×ROX reference dye, 1 μl mix of forward and reverse primers (0.3 μM each), and 11.45 μl RNAase free water containing cDNA (17.5 ng). PCR was done by 15 s denaturation at 95 °C, and annealing/extending at 60 °C for 40 cycles. Each mRNA expression level was normalized by β-actin. The mRNA expression was calculated relative to β-actin using the $\Delta\Delta C_T$ algorithm.

2.4. Microchemotaxis assay

Microglial chemotactic migration was tested with 48-well microchemotaxis Boyden chamber (Neuroprobe, MD, USA) [11]. Upper and lower wells were separated by polycarbonate filter (8 μm pore size; Poretics, CA, USA). Primary cultured microglial cells (200×10^4 cells/ml) in 50 μl of serum-free DMEM were added to the upper wells, and the lower wells contained the same medium with or without (control) CCL-1 and other drugs if mentioned. The chamber was incubated at 37 °C and 10% CO_2 for 90 min. Cells remaining on the upper surface of the membrane were removed by wiping, and migrated cells were subjected to Diff-Quik stain kit (Sysmex Corp., Kobe, Japan). Rate of microglial migration was calculated by counting cells in four random fields of each well.

2.5. Motility experiment

Experiments were performed as described [9]. Cultured microglial cells were seeded on glass-bottom dishes (Matsunami, Osaka, Japan) at a density of 4×10^4 cells/dish in serum-free DMEM. Motility of microglia under controlled temperature (37 °C) and gas (10% CO_2/90% humidity) was monitored with a time-lapse videomicroscopy system (Nikon Instec, Fukuoka, Japan). The video camera (Nikon inverted microscope, TE-2000-E) was controlled by Luminavision software (MITANI, Osaka, Japan). Images were acquired at 1 min intervals for 1 h in the presence or absence (control) of drugs, and stored on a computer and analyzed by Dipp-Motion 2D (DITECT, Tokyo, Japan). Serum-free DMEM was used as control.

2.6. Phagocytosis assay

Microglial cells seeded on the slide glass (4×10^4 cells/dish) were pre-incubated with 4 μg/ml fluorescent zymosan beads (Zymosan A (*S. cerevisiae*) BioParticles, Alexa Fluor 488 conjugate, Invitrogen, UK) in serum-free DMEM for 1 h, then incubated with CCL-1 with or without other drugs for another 1 h. Beads-treated microglial cells were fixed with 4% paraformaldehyde, treated with anti-Iba1 (1:2000, overnight), then incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1000). Series of Z-stack images (every 0.6 μm) were examined with an All-in-One fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan) for 3D-analysis of each phagocytic cell.