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Research report

Selective induction of interleukin-6 in mouse microglia by granulocyte-macrophage colony-stimulating factor

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Abstract

Astrocytes produce granulocyte/macrophage colony-stimulating factor (GM-CSF) and support the survival and proliferation of microglia. To study the functions of GM-CSF in the central nervous system (CNS), we examined the effects of GM-CSF on cytokine production by glial cells. GM-CSF induced interleukin-6 (IL-6) production by microglia, but not by astrocytes, in a dose-dependent manner as assessed by bioassay and the detection of IL-6 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. GM-CSF did not induce tumor necrosis factor (TNF)α or IL-1 in microglia and astrocytes, whereas lipopolysaccharide induced all these cytokines. The induction of IL-6 by GM-CSF in microglia was completely inhibited by antibodies to GM-CSF. Neither IL-3 nor macrophage-CSF (M-CSF) induced IL-6 production in microglia. Given that IL-1 and TNFα, monokines derived from microglia, induce IL-6 production in astrocytes, but not in microglia, results indicate that astrocytes and microglia may mutually regulate IL-6 production by different cytokines.

Keywords: Microglia; Interleukin-6; Granulocyte/macrophage colony-stimulating factor; Cytokine; Astrocyte

1. Introduction

Microglia, or brain macrophages, produce a variety of immunoregulatory cytokines, including interleukin-1α (IL-1α), IL-1β, IL-5, IL-6, tumor necrosis factor-α (TNFα), and transforming growth factor-β (TGFβ) [5,11,31,32,34,36]. Astrocytes, another type of glial cell, also produce various cytokines including IL-1α, IL-1β, IL-5, IL-6, IL-8, TNFα, TGFβ, granulocyte- (G-CSF), macrophage- (M-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) [1,7,21,22,29,32,34,36,45,46]. Some populations of astrocytes and microglia may also produce IL-3 [6,9], although we could not confirm in our mouse system [29]. Recently, we also showed that microglia and astrocytes produce IL-10 [25]. These various cytokines affect the functions of glial or neuronal cells and form a unique cytokine network in the central nervous system (CNS). Astrocytes regulate the functions of microglia via cytokines, among which, colony-stimulating factors (CSFs) such as IL-3, GM-CSF and M-CSF are potent regulators of microglial function. Microglia express receptors for these CSFs [35]. M-CSF induces microglial proliferation and also activates lysosomal enzymes in these cells, effects that are inhibited by antibodies to M-CSF receptors [33]. GM-CSF also induces proliferation of but does not activate lysosomal enzymes in microglia [42,43]. IL-3 induces morphological changes in microglia and supports the survival of these cells in vitro [42,43]. We recently showed that IL-3 induces class II major histocompatibility complex (MHC) antigen expression on microglia, whereas GM-CSF inhibits in a dose-dependent manner the expression of class II MHC antigens on microglia induced by interferon-γ (IFNγ) [18].

Recent evidence suggests that the regulation of cytokine production differs among different types of cells. Mitogenic and antigenic stimuli induce IL-6 production in T cells [17]. Lipopolysaccharide (LPS), viral infection, and various cytokines also induce IL-6 production in a variety of cells, including monocyte-macrophages [13,26]. M-CSF and GM-CSF induce IL-6 production in human monocytes [27]. However, the effects of these CSFs on cytokine production in the CNS are as yet unknown. We have now investigated the effects of astrocyte-derived CSFs on cytokine production by microglia.

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2. Materials and methods

2.1. Reagents

Recombinant murine GM-CSF, IL-3, IL-4, IL-10, and TGFβ1, as well as human M-CSF and IL-6 were obtained from Genzyme (Boston, MA). Monoclonal antibodies to mouse GM-CSF were provided by Dr. J. Schreurs (DNAX Research Institute of Molecular and Cellular Biology, Inc. Polo Alto, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO), and *Escherichia coli* LPS was from Difco (Detroit, MI).

2.2. Cell culture

Primary mixed glial cell cultures were prepared as described previously [39], from newborn C3H/HeN mice (Charles River Japan, Sizuoka, Japan). In brief, after the meninges were removed carefully, the brain was dissociated by passing it through a 320-μm-pore nylon mesh. The cell suspension was washed with Hanks' balanced salt solution, triturated, and plated in 75-cm^2 culture flasks (Falcon 3024, Beckton-Dickinson, Lincoln Park, NJ) at a density equivalent to two brains per flask in 10 ml Eagle's minimum essential medium supplemented with 10% fetal calf serum, 5 μg/ml bovine insulin, and 0.2% glucose. Microglia were isolated on the 14th day by the 'shaking off' method previously described [40]; the purity of the cultures was 97 to 100% as determined by immunostaining with Mac 1 antibodies (Hybriteck, San Diego, CA, used in 1:50 dilution). Astrocyte-enriched cultures were prepared from the primary mixed glial cell cultures by repetitive exposure to trypsin and replating [41]; the purity of the cultures exceeded 95% as determined by indirect immunofluorescence staining with antibodies to glial fibrillary acidic protein [41].

2.3. Induction of cytokines in glial cells by CSFs

Microglia and astrocyte-enriched cultures were plated in 2.5 cm diameter culture dishes (Falcon 3001, Beckton Dickinson, Lincoln Park, NJ) at a density of 5×10^5/ml and incubated for 24 h in the absence or presence of graded concentrations of GM-CSF (0.1 to 100 U/ml), IL-3 (1 to 100 U/ml), M-CSF (1 to 100 U/ml), LPS (1 μg/ml) or IFNγ (100 U/ml). In some experiments, various concentrations of antibodies to GM-CSF were added to microglia together with GM-CSF. The cell supernatant was then collected and stored at −70°C until monokine activities were assayed. The time course of cytokine induction by GM-CSF was investigated by collecting cell supernatants at 2, 4, 8, 12, and 24 h after stimulation with GM-CSF. After the remaining cells were washed three times with phosphate-buffered saline, total RNA was isolated from the cells by the method of Chomczynski and Sacchi [4] with some modifications. RNA(1 μg) was subjected to first-strand cDNA synthesis at 37°C for 90 min with 50 U of recombinant moloney murine leukemia virus reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany) and 0.2 μg of DNA random hexamers. The reaction was terminated by boiling for 5 min, and 2 μl of the mixture were amplified by 30 of the polymerase chain reaction (PCR) cycles with 0.5 units of *Taq* polymerase (Promega, Madison, WI) and 0.5 μg each of the sense and antisense primers. The thermal cycle profile was 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. A portion (5 μl) of the PCR mixture was subjected to electrophoresis on a 2% agarose gel, which was then stained with ethidium bromide and photographed. The

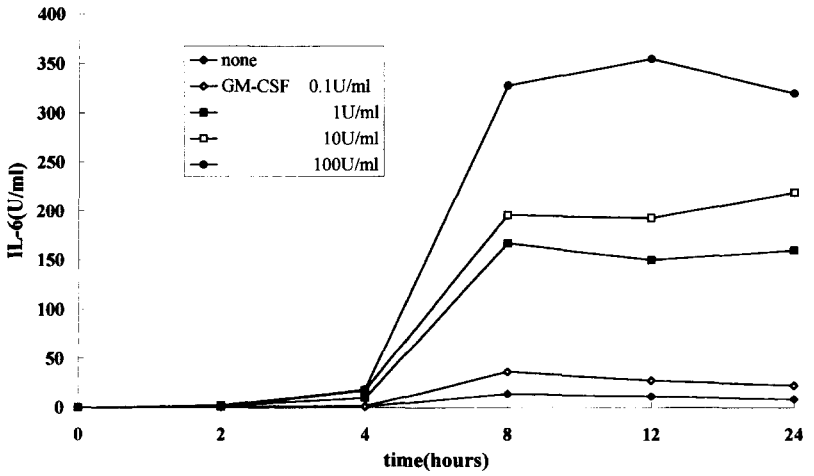

Fig. 1. Time course of GM-CSF-induced IL-6 production by microglia. Supernatants of microglia stimulated with indicated doses of GM-CSF were collected at indicated time (hours after stimulation), and were assayed for IL-6 activity as described in the text. Each point represents mean value of typical six samples from two different experiments. S.D. values were less than 10% of the means.

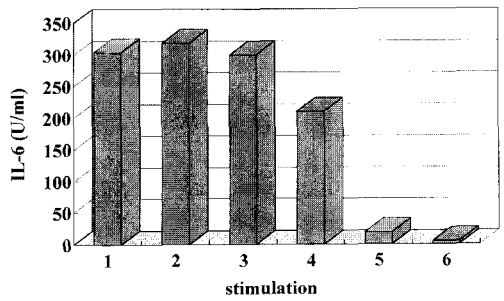

Fig. 2. Suppression of GM-CSF-induced IL-6 production by anti-GM-CSF antibody. Microglia were incubated for 24 h with 100 U/ml GM-CSF in the absence (1) or presence of $1:10^7$ (2), $1:10^6$ (3), $1:10^5$ (4), $1:10^4$ (5), $1:10^3$ dilution (6) of anti-GM-CSF antibodies. Data are means of quadruplicate samples; S.D. values were less than 10% of the means.

primers used were; IL-6 sense, 5′-ATGAAGTTCCTCTCTGCAAGAGACT, IL-6 antisense, 5′-CACTAGGTTTGCCGAGTAGATCTC, IL-1β sense, 5′-ATGGCAACTGTTCCTGAACTCAACT, IL-1β antisense, 5′-CAGGACAGGTATAGATTCTTTCCTTT, TNFα sense, 5′-ATGAGCACAGAAAGCATGATCCGC, TNFα antisense, 5′-CCAAAGTAGACCTGCCCGGACTC [24,34].

2.4. Assay for cytokine activities

IL-1 and IL-6 activities were determined by bioassay with the cytokine-dependent cell lines, D10N3 (provided

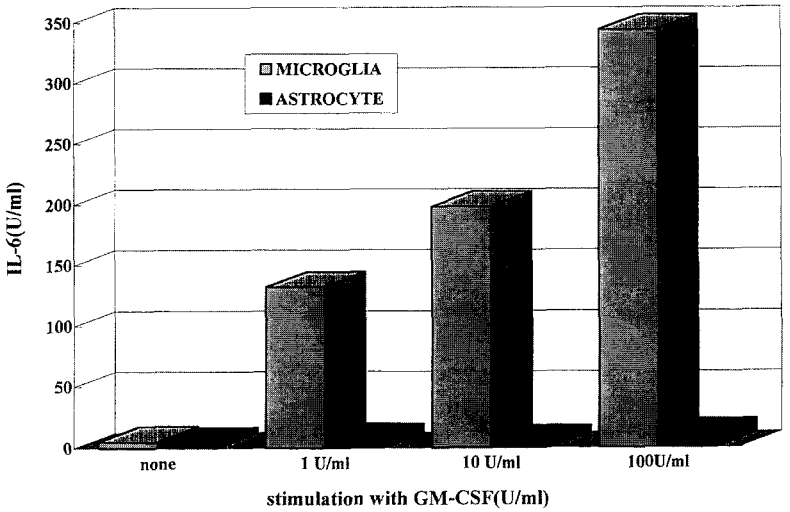

Fig. 3. Effect of GM-CSF on IL-6 production by microglia and astrocytes. Microglia and astrocytes were incubated for 24 h in the absence or presence of various doses of GM-CSF. Each column indicates mean value of six samples in a typical experiment; S.D. values were less than 10% of the means.

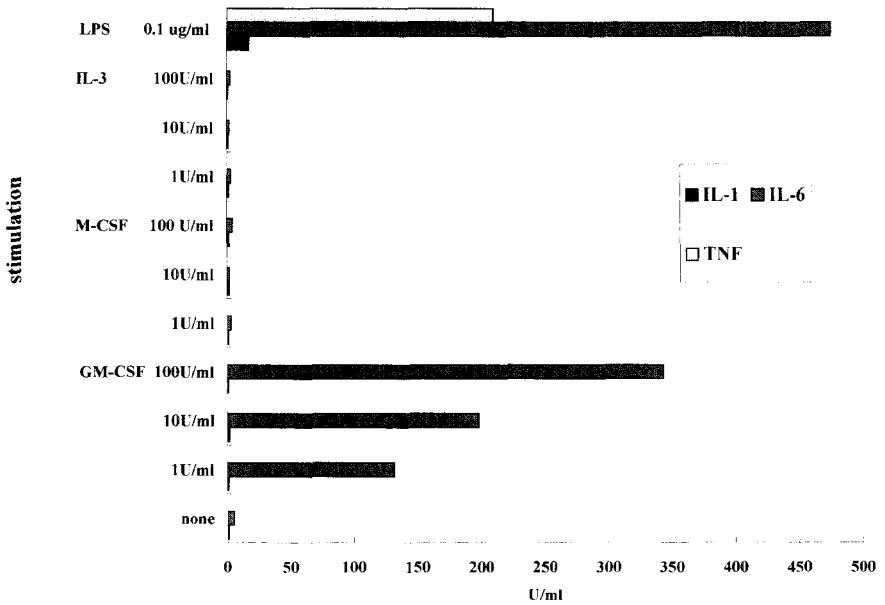

Fig. 4. Induction of monokines (TNFα, IL-6, and IL-1) by CSFs and LPS in microglia. Microglia were stimulated with the indicated doses of IL-3, GM-CSF, or M-CSF using LPS as a positive control. Supernatants were collected at 24 h after stimulation, then assessed for their TNF, IL-1 and IL-6 activity as described. Each column indicates mean value of six samples in a typical experiment (S.D. values were less than 10% of the mean values).

by Dr. K. Onozaki, Nagoya City University) [16] and MH-60 (from Dr. T. Hirano, Osaka University) [23], respectively, and MTT colorimetric assay [34,44]. TNF activity was determined by cytotoxicity to the L929 cell line as described [32].

2.5. Statistical analysis

All experiments were performed at least in triplicate. Data are presented as means ± S.D. and were analyzed by Student's *t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

GM-CSF, at doses of 0.1 to 100 U/ml, induced IL-6 production in microglia in a time- and dose-dependent manner, as assessed by bioassay with the IL-6 dependent cell line MH60 and MTT colorimetric assay (Fig. 1). At doses of 1 to 100 U/ml and time of > 8 h, the effect of GM-CSF on IL-6 production was statistically significant ($P < 0.001$) relative to unstimulated cultures at the corresponding times. The maximal effect of GM-CSF on IL-6 production was apparent after 12 h or 24 h of stimulation with each dose examined. The induction of IL-6 by GM-CSF was inhibited by antibodies to GM-CSF in a dose-dependent manner (Fig. 2); the inhibitory effects of the anti-GM-CSF antibodies was statistically significant ($P < 0.001$) at a dilution of $1{:}10^5$, and the antibodies completely abolished the effect of GM-CSF (100 U/ml) at a dilution of $1{:}10^3$. In contrast to its effect on microglia, GM-CSF did not induce IL-6 production in astrocytes. One to 100 U/ml GM-CSF did not induce IL-6 production by astrocytes (Fig. 3). Among the CSFs, only GM-CSF, but not IL-3 or M-CSF, induced the IL-6 production in microglia

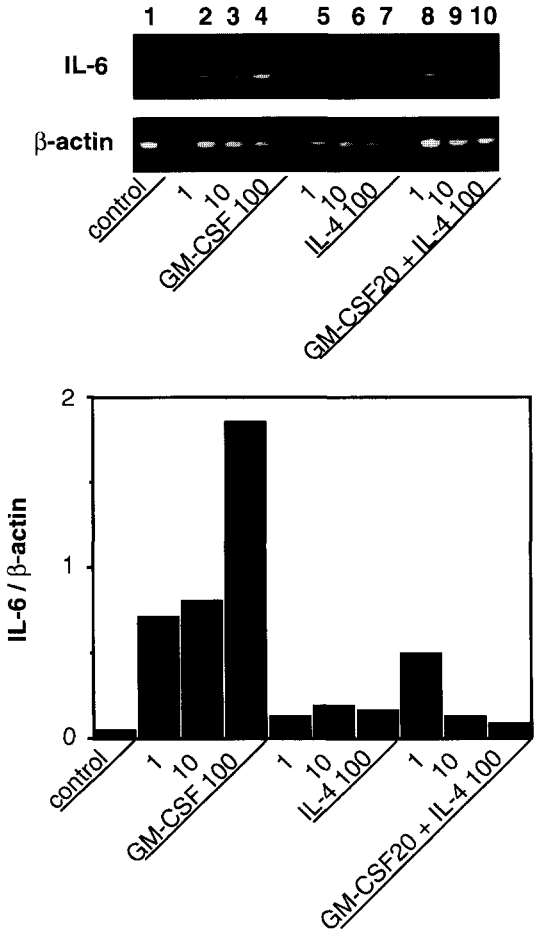

Fig. 6. Induction of IL-6 mRNA in microglia. Upper: RT-PCR analyses for expression of IL-6 mRNA and β-actin in microglia stimulated with 1–100 U/ml GM-CSF (lanes 2–4), 1–100 U/ml IL-4 (lanes 5–7) and 20 U/ml GM-CSF and 1–100 U/ml IL-4 (lanes 8–9). Lower: closed columns indicate mean values of the ratio of IL-6 mRNA/β-actin mRNA, calculated by each value obtained from the TIAS-200 image analyzer.

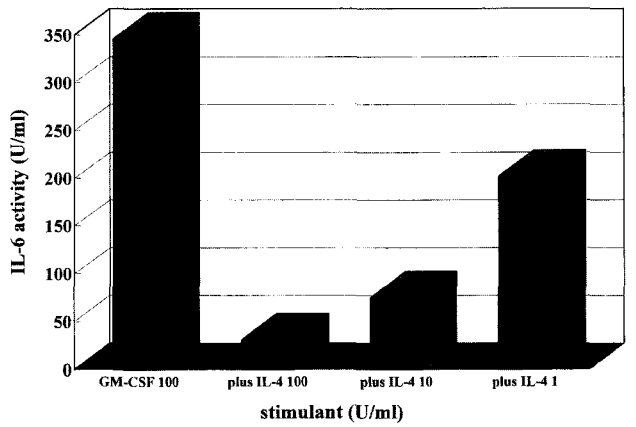

Fig. 5. Suppression of GM-CSF-induced IL-6 production by IL-4. Each column indicates mean value of six samples in a typical experiment. Bars indicate standard deviations. One to 100 U/ml IL-4 significantly ($P < 0.001$) suppressed IL-6 production by microglia stimulated with 100 U/ml GM-CSF for 24 h.

(Fig. 4). The induction of monokine production by GM-CSF in microglia was specific for IL-6; GM-CSF had no effect on the production of IL-1 or TNFα, whereas LPS, used as a positive control, induced all three monokines (Fig. 4). The induction of IL-6 production in microglia by GM-CSF was inhibited by IL-4 in a dose-dependent manner (Fig. 5). GM-CSF induced the expression of IL-6 mRNA in microglia in a dose-dependent manner (Fig. 6). The inhibition of GM-CSF-induced IL-6 production by IL-4 was also confirmed at the mRNA level (Fig. 6). IL-4 by itself did not induce IL-6 activity (Fig. 5) or IL-6 mRNA (Fig. 6).

4. Discussion

IL-6 has important functions in a variety of biological processes [14]. In the CNS, IL-6 promotes the survival and

differentiation of cholinergic neurons [12] and modulates the production of neurotrophin [7]. Both IL-6 mRNA and IL-6 receptor mRNA are present in the striatum of rat brain during postnatal development [8], although we did not detect IL-6 mRNA in the developing mouse cerebral cortex [24]. High levels of IL-6 transcript are present in the adult rat hippocampus [8,37], suggesting that IL-6 may function as a neurotrophic factor in both the developing and adult brain in vivo. The concentration of IL-6 is increased in the cerebrospinal fluid of individuals infected with human immunodeficiency virus [20], of patients with HTLV-1 associated myelopathy [28] and of patients with systemic lupus erythematosus [15]. IL-6 was detected in association with amyloid plaques in the cortex and hippocampus of individuals with Alzheimer's disease, whereas it was undetectable in control brains [2,38]. IL-6 may thus be induced in certain pathological conditions and participate directly in the pathological process in either inflammatory or neurodegenerative diseases in the CNS.

IL-6 is produced by astrocytes and microglia in the CNS, and its receptors are expressed on these glial cells as well as neurons [1,3,34,35]. In addition, neurons themselves may produce IL-6 [37,47]. As in the immune system, IL-6 production is regulated differently in astrocytes and microglia. For example, IL-1 and TNFα induce the production of IL-6 in astrocytes but not in microglia [34]. Substance P also induces IL-6 production in astrocytes [10]. In human monocytes, IL-1, IFNγ, M-CSF, and GM-CSF all induce IL-6 production [27]. However, with the exception of GM-CSF, these cytokines failed to induce IL-6 in microglia, the counterpart of monocytes/macrophages in the CNS. We show now, for the first time, that GM-CSF, which is produced by astrocytes in the CNS in response to appropriate stimuli [29], induces IL-6 production in microglia. Because (i) microglia express GM-CSF receptor mRNA [35], (ii) the effect of GM-CSF was completely inhibited by anti-GM-CSF antibodies, and (iii) other CSFs did not induce IL-6 production in microglia, the induction of IL-6 by GM-CSF appears to be a specific function of GM-CSF mediated by GM-CSF receptors on these cells. Although astrocytes also express GM-CSF receptor mRNA [35], GM-CSF did not induce the production of IL-6 in astrocytes. The current data, together with our previous observation that IL-1 and TNFα specifically induce IL-6 production in astrocytes, but not in microglia [34], suggest that astrocytes and microglia may mutually regulate IL-6 production in the opposing cell type by different cytokines. The functions of the induced IL-6 in the CNS remain to be elucidated. However, it may exert neurotrophic effects, acting directly on neurons or indirectly via induction of neurotrophic factors as mentioned above. Alternatively, it may play a role on neuron-glia interaction by activating glial cells.

GM-CSF exerts a variety of functions in the CNS. It reportedly functions as a neurotrophic factor [19], and induces the proliferation and the morphological transformation to rod-shaped cells of microglia [42,43]. Recently, we also showed that GM-CSF inhibits the IFNγ-induced expression of class II MHC antigen on these cells [18]. We did not detect GM-CSF mRNA in the developing mouse cortex in vivo [24], and unstimulated glial cells did not produce detectable amounts of GM-CSF [29]. Therefore, GM-CSF may neither function as a neurotrophic factor nor induce IL-6 production in the normal developing brain. However, both GM-CSF mRNA and protein are inducible in astrocytes by stimulation with LPS [22,29,45]. Axotomy of facial nerves results in a rapid increase in GM-CSF receptors in the facial motor nucleus, which suggests that GM-CSF may play a role in the pathophysiology in this area after axotomy [30]. Because microglia have very similar characteristics to monocytes/macrophages and are thought to play important roles in the development of various neurological disorders, the effects of GM-CSF on microglia may be crucial in the pathophysiology of these cells. These observations suggest that GM-CSF, when produced in the CNS, may function either as a proinflammatory cytokine by inducing the proliferation and activation of microglia and by inducing another proinflammatory cytokine, IL-6, in microglia, or as an inhibitory cytokine by suppressing immunoregulatory functions of microglia.

Acknowledgements

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