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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

I. 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.
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-diphenyl-tetrazolium 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-cm² 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 immuno-fluorescence 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 × 10⁵/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. 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'-ATGAAGTTTC-CTCTCTGCAAGAGACT, IL-6 antisense, 5'-CACTAG-GTTTGCCGAGTAGATCTC, IL-1β sense, 5'-ATGG-GCAACTGTTCCTGAACTCAACT, IL-1β antisense, 5'-CAGGCAGGTGATAGATTCTTTCCCTT, TNFα sense, 5'-ATGAGCAGAAAGCAGATGCCGC, TNFα antisense, 5'-CCAAAGTAGACCTGGCCCGACT [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...
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 1:105, and the antibodies completely abolished the effect of GM-CSF (100 U/ml) at a dilution of 1:103. 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. 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, and its receptors are expressed on these cells. Although astrocytes also express GM-CSF receptor mRNA [35], GM-CSF did not induce 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-glial 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.

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