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Short Communication

Macrophage Colony-Stimulating Factor Mediates Astrocyte-Induced Microglial Ramification in Human Fetal Central Nervous System Culture

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Colony-stimulating factors are important mediators of microglial growth and function. Microglia, derived from ameboid precursor cells, undergo a series of morphological changes in the central nervous system to become ramified, a morphological hallmark of differentiated microglia. The cellular mechanisms and environmental factors involved in microglial ramification in the developing brain are not known. In the present study, we examined the role of macrophage colony-stimulating factor (M-CSF) in the induction of ramification of microglia in human fetal glial cultures. Human fetal microglia underwent spontaneous conversion from ameboid to ramified shape when co-cultured with human fetal astrocytes. Concurrently, high levels of M-CSF also accumulated in cultures of unstimulated human fetal astrocytes. That the M-CSF was essential in inducing microglial ramification was demonstrated by almost complete inhibition of ramification by an antibody to human M-CSF receptor, c-fms. Furthermore, profound inhibition of microglial ramification was exerted by herbimycin A, a specific inhibitor of tyrosine kinase (a component of the signal cascade of c-fms), supporting the role of M-CSF/c-fms in the induction of microglial ramification. Our results suggest a pivotal role played by astrocytes and astrocyte-produced M-CSF in the induction of microglial maturation and differentiation in the developing human brain. (Am J Pathol 1994, 145:48–53)

Microglia, endogeneous brain mononuclear phagocytes, are an integral component of a central nervous system (CNS) neuroglial cellular network. They play a central role in certain viral infections and degenerative diseases of the CNS, in regulation of immune and inflammatory reactions, and in phagocytosis and host defense.[1] Most current evidence favors the idea that microglia are bone marrow-derived cells.[1–4] They express a phenotypic profile that closely resembles that of peripheral macrophages.[1–5] In the developing brain, microglia are concentrated in so-called "glial fountains" in the periventricular regions and show ameboid morphology; ramified microglia typically present throughout the mature adult brain are found in the cortical plate and underlying white matter in the developing brain.[1–7] It is thought that ramification, a morphological hallmark of differentiation in microglia, is induced by signals endogenous to the CNS after the precursor cells enter the brain. The identity of these signals and the mechanisms of induction of ramification are unknown.

To study conditions that promote microglial ramification, we used dissociated CNS cell cultures from second-trimester human fetal CNS.[8] With highly enriched microglia and mixed astrocyte-microglia cultures, we defined several conditions in which microglial ramification was observed.[9] As previously shown,[8] microglia from human fetal brain undergo progressive morphological changes from ameboid to ramified type principally under two conditions: coculturing with astrocytes and LPS stimulation of enriched microglial cultures. As alluded to in previous

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studies,[8, 10] the overlapped profile of these two culture conditions suggested to us that macrophage colony-stimulating factor (M-CSF) might be a mediator promoting microglial maturation in human fetal CNS cultures. In the present report, we show evidence that astrocyte-produced M-CSF is indeed essential for induction of microglial ramification.

Materials and Methods

Tissue

The research protocol used in this study was approved by the Albert Einstein College of Medicine Committee on Clinical Investigations and the Health and Hospital Corporation of the City of New York. Informed consent was obtained from the participants. Fetal brains were obtained at the time of elective terminations of pregnancy from normal women with no risk factors for HIV infection. The abortuses ranged in gestational age from 16 to 24 weeks.

Generation of Enriched Microglial, Enriched Astroglial, or Mixed Microglia-Astrocyte Cultures

Methods for production and characterization of human fetal CNS dissociated cultures have been described in detail elsewhere.[8] Briefly, cerebral tissues were freed from meninges, minced, and digested in Hanks' balanced salt solution containing 0.05% trypsin and DNAse for 45 minutes at 37 C by gentle shaking. After adding 10% fetal calf serum (FCS) (Whittaker, Walkersville, MD), the digest was passed through 230- and 130-μ nylon meshes (Tetko, Elmsford, NY) respectively, washed twice and resuspended in DMEM (with 4.5 g/L-glucose, L-glutamine and 25 mmol/L HEPES buffer) containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from GIBCO BRL, Grand Island, NY). Cells were seeded at 4×10^7/T 75 cm^2 tissue culture flask, and kept at 37 C in a 5% CO_2 humidified incubator. The cells were washed and fed with fresh medium on day 7. On day 14, flasks were gently tapped, and floating microglia were harvested, counted, and reseeded. Microglial cultures were washed twice with fresh media after 1 hour. Highly purified astrocyte cultures were prepared by repeated trypsinization of mixed cultures. Purity of the cultures was assessed by immunocytochemistry using antibodies to glial fibrillary acidic protein (GFAP, Boehringer-Mannheim, Indianapolis, IN) and CD68 (EBM-11, DAKO, Santa Barbara, CA) as markers for astrocytes and microglia, respectively. For mixed astrocyte-microglia cultures, pure microglia and astrocytes isolated as described above were mixed at a 1:4 ratio in suspension and seeded at 50,000 cells per well in 96 well plates and allowed to settle for 1 to 2 days. All experiments were performed in triplicate wells in 96-well microtiter plates (Nunc, Naperville, IL).

Induction of Ramification in Microglia in Enriched or Mixed Cultures

Enriched microglial cultures seeded at 20,000 cells/well in 96-well plates were stimulated with lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO) alone at 1, 10, 100, or 1000 ng/ml or in combination with a neutralizing antibody to human M-CSF receptor, c-*fms* (rat immunoglobulin [IgG]-1, 100 μg/ml in original concentration, Oncogene Science, Manhasset, NY[11]) at 1–4 μg/ml, or with an irrelevant rat IgG-1 at the same concentrations. Mixed microglia-astrocyte cultures were also grown in medium alone (control); with 1 μg/ml of anti-c-*fms*; with irrelevant rat IgG-1 (isotype control); or with tyrosine kinase inhibitor, herbimycin A (GIBCO BRL) at 250, 500, or 1000 ng/ml. Dimethylsulfoxide (vehicle for herbimycin A) at 0.2%, the highest concentration in herbimycin A wells, was also tested in mixed cultures. Additional controls consisted of highly enriched astrocyte cultures treated in parallel with anti-c-*fms* or herbimycin A (see below). Reagents were added twice a week with half-change of medium at 1 week. Experiments with co-cultures were ended 7 days, 10 days, or 14 days after the initial antibody/herbimycin A addition and examined with immunocytochemistry for CD68, a constitutive marker of human macrophage/microglia,[12] singly or with anti-GFAP antibody in double-staining protocols as previously described.[8, 10] Experiments were terminated when the majority of microglia in control cultures achieved ramification as observed in daily examination of cultures. Experiments with LPS-stimulated enriched microglial cultures were ended on day 7 by fixing the cells in ice-cold methanol; they were then evaluated by Hoffmann modulation optics microscopy.

Results

Induction of Ramification of Microglia with LPS

In more than five separate experiments it was demonstrated that addition of LPS to highly enriched mi-

croglia cultures induced rounded cells to become elongated and bipolar initially[8, 10] but ramified at 4 days or later (Figure 1, a and b). Multiple fine processes arose from small, granular cell bodies. The minimal effective dose of LPS was 1 ng/ml, and there was a dose-dependent increase in the number of ramified microglia between 1 and 100 ng/ml (data not shown). Anti-c-*fms* antibody, used at 1, 2, or 4 μg/ml in a single set of experiments in combination of 100 ng/ml of LPS, did not block the effect of LPS on ramification of microglia (see Discussion). Subsequent experiments were performed using 1 μg/ml of anti-c-*fms* and 100 ng/ml of LPS.

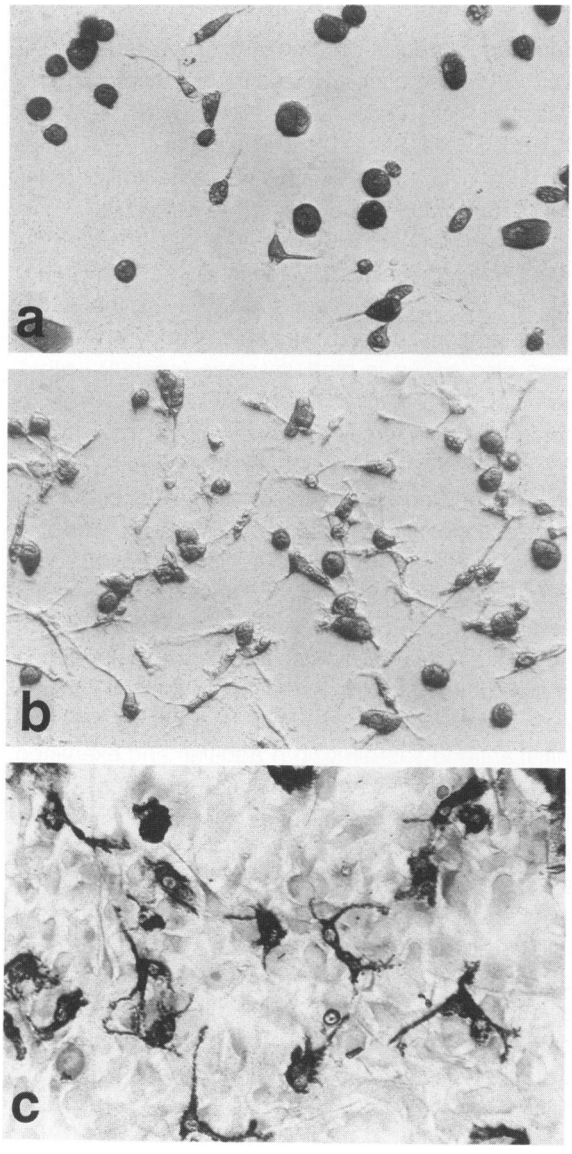

Figure 1. *Ramification of microglia in enriched human fetal microglial culture treated with LPS* (b) *or occurring spontaneously in co-cultures with astrocytes* (c). (a) *is a control enriched microglial culture without LPS showing conservation of ameboid shape* (a) *and* (b) *are photographs of cultures after fixation with methanol showing granular cell bodies of microglia;* (c) *is a double immunolabeling for microglia (CD68/DAB/brown in the original) and astrocytes (GFAP/X-gal/light blue in the original), and shows CD68$^+$-ramified microglia growing over a monolayer of GFAP$^+$ astrocytes. (For detailed methods, see Materials and Methods and refs. 8 and 13.)*

Induction of Ramification of Microglia by Co-Culture With Astrocytes

In mixed astrocyte-microglia cultures, microglia gradually became ramified over time (a 1–2-week period) (Figures 1c, 2a). Addition of anti-c-*fms* almost completely inhibited the ramification in microglia (Figures 2b, 3) leaving small, dark, granular cell bodies that over time became detached from the astrocyte monolayer (n = 5). To demonstrate the specificity of anti-c-*fms*, control cultures were treated with an irrelevant rat IgG-1 raised against neurofilament epitope (TA 26, a gift of V. Lee) and showed no difference from control cultures (Figure 3). In addition, mixed cultures treated with a monoclonal antibody to human granulocyte macrophage colony-stimulating factor (GM-CSF) (mouse IgG-1,Genzyme, Cambridge, MA) at 1 μg/ml or 10 μg/ml showed no blocking effect (data not shown; see Discussion). Addition of herbimycin A also inhibited the ramification of microglia at 250 and 500 ng/ml with more inhibition at the higher of the two doses (Figures 2c, 3; n = 3). Herbimycin A at 1000 ng/ml was shown to kill microglia either in mixed or enriched microglial cultures (see Discussion). The effect of anti-c-*fms* or herbimycin A was tested in highly enriched astrocyte cultures to see whether the effect on microglia by these reagents was indirect through stimulation of astrocytes. Neither of the reagents at the doses used in microglia experiments affected the astrocyte shape, GFAP immunoreactivity, or cell number in cultures (data not shown; see Discussion). This was in contrast to inhibitors of protein kinase C (H-7, Seikagaku America, Rockville, MD) or cyclic AMP-dependent protein kinases (H-8, Seikagaku America), both of which exerted a profound effect on astrocyte cell shape, changing from a flat to process-bearing type at a concentration of 20 μmol/L (lowest dose examined). This cell shape change prevented investigating further the (direct) role of protein kinases in the induction of microglial ramification in co-cultures. In addition, dimethylsulfoxide alone at 0.2% (control for herbimycin A) had no effect on the morphology of microglia in cocultures.

Discussion

This report is part of an ongoing investigational effort to understand and elucidate the mechanisms of microglial differentiation and maturation in the develop-

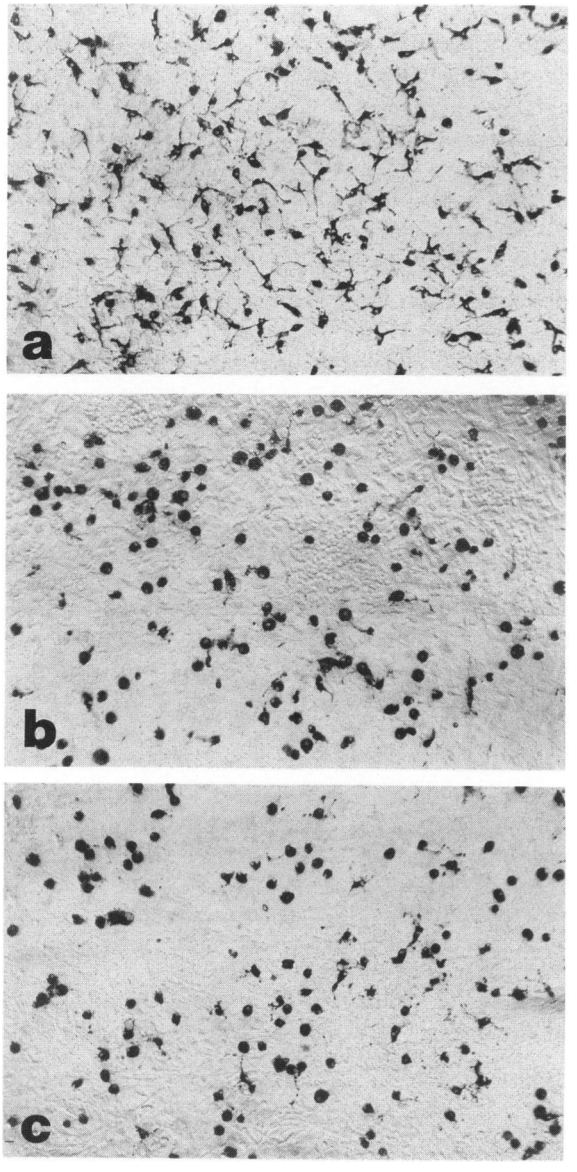

Figure 2. *Anti-c-fms or herbimycin A inhibits ramification of microglia in astrocyte-microglia co-culture. Astrocyte-microglia co-cultures showing numerous CD68⁺-ramified microglia in control culture* (a), *dramatic inhibition of ramification in cultures treated with 1 μg/ml of anti-c-fms* (b), *or 500 ng/ml of herbimycin A* (c). (*For detailed methods, see Materials and Methods.*) *Photographs were taken with Hoffmann modulation optics after immunostaining of co-cultures in 96-well plates for a microglial marker, CD68. Unstained monolayer of astrocytes is apparent in the background.*

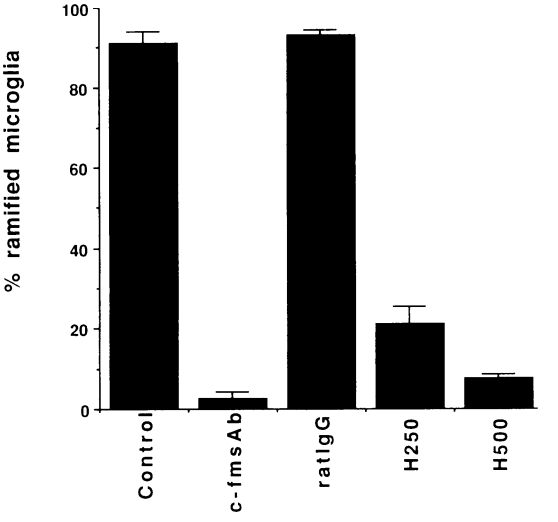

Figure 3. *Quantitative analysis of percent ramified microglia in co-cultures treated with various reagents (control: none added, anti-c-fms at 1 μg/ml, irrelevant isotype control [rat IgG-1], or herbimycin A at 250 ng/ml or 500 ng/ml). CD68 immunostained cultures were evaluated for relative numbers of ramified and ameboid microglia and percent ramified microglia was scored as number of ramified CD68⁺ cells/number of total CD68⁺ cells in each well. Data are mean ± standard deviation from triplicate values. Results are representative of at least three separate experiments.*

ing human brain. Previous studies from our laboratory have shown that M-CSF (also called CSF-1) is produced by human fetal astrocytes in the basal state, and the protein steadily accumulates in the culture.[10] We have also shown that M-CSF alone is a weak mitogen for fetal human microglia and that M-CSF promotes microglial survival and inhibits the expression of class II MHC antigen on microglia,[10] suggesting a role for M-CSF in the maintenance of the differentiated state of microglia and possibly contributing to the immunologically privileged state of the CNS.

In the present investigation, the role of M-CSF in the morphological differentiation of microglia was examined in two experimental paradigms in which ramification of human fetal microglia was induced. The culture conditions conducive to ramification of microglia were co-cultivation with astrocytes or stimulation of pure microglia with LPS, which suggested to us that M-CSF, present in both conditions, may be the key factor in promoting ramification. Since addition of recombinant M-CSF or astrocyte-conditioned medium alone did not induce ramification of microglia in enriched cultures,[8] the strategy was to block the process using a neutralizing antibody to the M-CSF receptor, c-*fms*. The result showed that induction of ramification of microglia by astrocytes but not by LPS was almost completely blocked by an antibody to human CSF-1 receptor. The specificity of the antibody was demonstrated by the failure of irrelevant isotype-matched antibody or anti-GM-CSF antibody to produce similar effects (GM-CSF is not detected in unstimulated human fetal astrocyte cultures using sensitive enzyme-linked immunoadsorbent assay, S. C. Lee, unpublished observation).

In rodents, microglia but not astrocytes have been shown to express c-*fms* and to respond to CSF-1 *in vitro*.[13, 14] The rat antibody to human c-*fms* used in this study has been shown to inhibit M-CSF binding

to receptor-bearing cells and to abrogate M-CSF-dependent colony formation by bone marrow macrophage precursors. This antibody does not show agonist activity.[11] Although we initially proposed that the LPS-induced ramification of human fetal microglia might also be mediated through M-CSF,[8, 10] anti-c-*fms* at doses that efficiently blocked the induction of ramified microglia by astrocytes failed to do so in pure microglial cultures. This may suggest that effective ligand-receptor interactions are maximized in cells that simultaneously express the growth factor and its receptor and that these cells may be partially refractory to such inhibition. In fact, it has been shown that the concentrations of antibodies necessary to inhibit the colony formation by cells co-transfected with human M-CSF and c-*fms* genes are much higher than those required to inhibit colony formation of c-*fms*-bearing cells in response to exogenously-added M-CSF.[11] Alternatively, the failure of anti-c-*fms* to inhibit LPS-induced microglial ramification may suggest the presence of multiple different mechanisms for the induction of ramified microglia. The LPS paradigm also suggests a potential for activated microglia *in situ* to become ramified resident microglia after injury subsides. Certainly the biphasic response to LPS, which consists of an early activation phase characterized by release of numerous inflammatory cytokines and growth factors by microglia,[10, 15] followed by a later ramification as demonstrated in this study, supports that hypothesis. Currently the molecular events underlying this biphasic response are unknown.

The M-CSF receptor is encoded by the c-*fms* proto-oncogene and is one of a family of growth-factor receptors that exhibits a ligand-activated tyrosine-specific protein kinase activity.[16, 17] The demonstration that herbimycin A, a tyrosine kinase inhibitor, inhibits the ramifying process of microglia co-cultured with astrocytes, provides additional support for the role of microglial c-*fms* in this process. Endogenous phosphotyrosine immunoreactivity has indeed been demonstrated in ramified microglia in mature rat brain and has been suggested as a selective marker for microglia.[18]

The involvement of astrocytes in the induction of spontaneous ramification of microglia growing over the astrocyte monolayer is highly intriguing. Since astrocyte-conditioned medium alone failed to induce microglial ramification, the result is best interpreted as microglia requiring additional signals from astrocytes as well as M-CSF. However, the situation is also reminiscent of a recent proposal that the initiation of cellular responses by marrow precursor cells may depend primarily on locally-produced M-CSF, and circulating M-CSF may be of no physiological importance.[19] It appears that M-CSF may act through a paracrine mechanism in which M-CSF is directly transferred from the cells to M-CSF-responsive cells in the vicinity.[19] Therefore, we can envisage a scenario in which, in normal developing and mature brains, locally-produced M-CSF may be an essential differentiation and survival factor for microglia by working through c-*fms*. In addition to previous studies demonstrating M-CSF-mediated downmodulation of class II MHC expression on microglia as well as enhanced microglial survival, the results shown here suggest that M-CSF is a pivotal agent in microglial maturation.

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