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The Chemokine Fractalkine Inhibits Fas-Mediated Cell Death of Brain Microglia

Stefen A. Boehme, Francisco M. Lio, Dominique Maciejewski-Lenoir, Kevin B. Bacon, and Paul J. Conlon

Fractalkine is a CX3C-family chemokine, highly and constitutively expressed on the neuronal cell surface, for which a clear CNS physiological function has yet to be determined. Its cognate receptor, CX3CR-1, is constitutively expressed on microglia, the brain-resident macrophages; however, these cells do not express fractalkine. We now show that treatment of microglia with fractalkine maintains cell survival and inhibits Fas ligand-induced cell death in vitro. Biochemical characterization indicates that this occurs via mechanisms that may include 1) activation of the phosphatidylinositol-3 kinase/protein kinase B pathway, resulting in phosphorylation and blockade of the proapoptotic functions of BAD; 2) up-regulation of the antiapoptotic protein Bcl-xL; and 3) inhibition of the cleavage of BH3-interacting domain death agonist (BID). The observation that fractalkine serves as a survival factor for primary microglia in part by modulating the protein levels and the phosphorylation status of Bcl-2 family proteins reveals a novel physiological role for chemokines. These results, therefore, suggest that the interaction between fractalkine and CX3CR-1 may play an important role in promoting and preserving microglial cell survival in the CNS. *The Journal of Immunology*, 2000, 165: 397–403.

Chemokines compose a superfamily of chemoattractant proteins that generally range from 7 to 14 kDa (1, 2). They have been shown to play an important role in governing the trafficking of leukocytes, as well as in modulating cell adhesion, T lymphocyte activation, phagocytosis, cytokine secretion, angiogenesis, viral pathogenesis, and proliferation. At present there are four subfamilies of chemokines, distinguished on the relative position of highly conserved cysteine residues in the amino terminus of the peptide. Fractalkine is the only chemokine identified to date that possesses a CX3C motif. Fractalkine is also unique in that the chemokine domain can be tethered to the cell surface via a mucin stalk attached to a transmembrane and intracellular domain (3, 4). Thus, fractalkine is biologically active as either a 95-kDa membrane-anchored protein, or a secreted chemokine upon protease cleavage from the mucin stalk. The tissue expression pattern of fractalkine is also unique, as it is also expressed in tissues of nonhemopoietic origin such as kidney, lung, and heart. Furthermore, it is highly and constitutively expressed in the CNS by neurons, and is up-regulated by injurious stimuli in neurons, or by TNF-α or IL-1β in astrocytes (5, 6). The fractalkine receptor, CX3CR-1, is highly expressed by microglia (5, 7–9). It has been shown to induce cell migration in primary microglial cells, as well as activate the p42/p44 mitogen-activated protein kinase and phosphatidylinositol-3 (PI-3) kinase/protein kinase B (PKB) signal transduction pathways (6).

Microglia have been shown to express both Fas (CD95) and Fas ligand (CD95L), and Fas-mediated apoptosis has been implicated in the pathogenesis of various CNS diseases, such as ischemia-reperfusion injury (10, 11), multiple sclerosis (12–14), and its rodent counterpart, experimental autoimmune encephalomyelitis (15–17). However, the exact role of Fas/Fas ligand in the pathology of multiple sclerosis/experimental autoimmune encephalomyelitis is unclear. Thus, as the Fas/Fas ligand interaction plays a role in maintaining hemopoietic homeostasis, it may also contribute to the pathogenesis of various disease processes (18). The binding of Fas ligand to the cell surface receptor Fas initiates a cascade of events leading to activation of various caspases in conjunction with modulation of Bcl-2 family proteins (19). The latter results in compromised mitochondrial function and integrity, which may contribute directly to the apoptotic process or be a result of caspase 8 and caspase 3 activation (19, 20). Specifically, the proapoptotic Bcl-2 family member BAD has the ability to complex with either Bcl-2 or Bcl-xL and antagonize their antiapoptotic function (21). This process is regulated in part by PKB/Akt, which can phosphorylate BAD at serine 136, rendering this proapoptotic protein inactive (22–25). Additionally, PKB/Akt can act via NF-κB to block apoptosis (26, 27). The proapoptotic protein BID is cleaved upon Fas ligand binding, allowing heterodimerization with Bcl-2 or Bcl-xL, and provides another pathway resulting in the abrogation of Bcl-2 or Bcl-xL antiapoptotic function (28).

The expression of fractalkine by neurons, and CX3CR-1 by microglial cells suggests that a paracrine interaction exists; however, no definitive CNS function has yet been elucidated. We have recently shown that fractalkine activates the PKB/Akt signaling pathway in primary microglia (6). As PKB/Akt has been shown to be a survival factor in a number of systems, we asked whether fractalkine stimulation could promote survival of primary brain microglia, and furthermore block Fas-mediated microglial cell death. We show in this work for the first time that the chemokine fractalkine can increase microglial cell survival and block Fas-induced programmed cell death of primary cells. This novel function provides crucial insight into the role fractalkine plays in...
regulating CNS homeostasis, as well as demonstrating evidence of neuron/microglia cross-talk.

Materials and Methods

Materials

All reagents were obtained from Sigma (St. Louis, MO), unless otherwise specified. Animal experimentation was approved by the Institutional Animal Care and Use Committee (IACUC) before implementation.

Primary microglial cell cultures

Microglial cell cultures were established as previously described (6). Briefly, cortices from newborn Sprague Dawley rats (Charles River, Boston, MA) were isolated, mechanically dissociated, and plated at a density of one brain/75 flask (Costar, Charlotte, NC) in DMEM (Mediatech, Tunis, CA) containing 10% FCS (HyClone, Logan, UT). Once confluent, the cells were left for 5–7 days without changing the media to favor microglia proliferation. The mixed glial cells were then shaken for 6–20 h at 225rpm. The supernatant, containing an enriched population of microglia, was pelleted and the cells were replated in DMEM +10% FCS. After 2 h, the cells were manually shaken and the medium was replaced with DMEM +10% FCS containing 200U/ml of both GM-CSF (R&D Systems, Minneapolis, MN) and M-CSF (R&D Systems). The adherent cells (>95% pure microglia) were grown for an additional 48 h before assaying. At this point, they were detached with Versene (Life Technologies, Gaithersburg, MD), pelleted and the cells were replated in DMEM +10% FCS, and cultured in experiments, as described. It should be noted that no exogenous growth factors were added during the experimental procedures, except where indicated (Fig. 1).

Cell death detection ELISA

A total of 5 × 10^4 microglia/well was seeded into a 96-well flat-bottom plate (Costar) following the described conditions, and each condition was done in triplicate. Before the addition of cells, tissue culture plate wells were coated by the aid of the indicated concentration of fractalkine (chemokine portion only; R&D Systems) diluted in PBS (Mediatech), and incubated at 4°C overnight. Control wells received PBS. Following incubation, the wells were washed once with PBS; the cells were then added and cultured for the indicated time periods. Soluble Fas ligand was obtained from Upstate Biotechnology (Lake Placid, NY). Eighteen hours postplating, the cells were harvested, and the oligosomal DNA was quantitated using the Cell Death Detection ELISA (Roche Diagnostics, Indianapolis, IN), according to the manufacturer’s protocol.

Photomicroscopy

Microglial cells were cultured in 24-well plates at 5 × 10^3 cells/well for 18 h under the various conditions. They were then isolated using Versene, pelleted onto slides, and fixed using 4% paraformaldehyde, followed by a −20°C methanol wash. The cells were then stained with DAPI (Calbiochem, La Jolla, CA) at a 1 μg/ml concentration for 15 min, and rinsed in PBS. Photomicrographs were taken using a Nikon Eclipse TE300 microscope.

Western blot analysis

Microglia were seeded into 24-well plates at a concentration of 5 × 10^5 well at the described conditions. Following an 18-h incubation, the microglia were harvested with Versene, pelleted onto wells coated with 100 nM fractalkine, or cultured in the presence of 200 U/ml M-CSF and GM-CSF (R&D Systems). Mitochondrial function was used as a measure of cell survival, and after 44 h of culture, an MTT assay was performed, according to the manufacturer’s instructions (Roche Diagnostics). After 24 h, parallel cultures were pulsed with 1 μCi of [%H]Thymidine (6.7 mCi/mmol; New England Nuclear, Boston, MA), and [%H]Thymidine incorporation was measured by betaplate scintigraphy at 48 h. Each experimental point was done in quadruplicate, and five independent experiments were performed. Dr. Lili Feng (Scripps Research Institute, La Jolla, CA) kindly provided the neutralizing anti-CX3CR1 Ab.

Statistical analysis

Two-tailed Student’s t test was used to determine whether differences observed were significant. A p value <0.05 was considered significant.

Results

Fractalkine acts as a survival factor for primary brain microglia

In analyses of fractalkine effects on primary rat microglial cells in vitro, we observed that cells grown in the presence of immobilized fractalkine (as described in Materials and Methods) showed increased viability over time when compared with unstimulated cells by MTT assay (Fig. 1). Both microglial cell survival and mitochondrial function were enhanced in the presence of fractalkine, and a specific blocking Ab to the receptor, CX3CR1, abrogated this effect. No comparable effects were seen with 20 other chemokines tested (data not shown). In contrast to microglia treated with both M-CSF/GM-CSF, fractalkine-treated cells proliferated minimally (Fig. 1; [%H]thymidine). These data suggest that fractalkine serves a unique function compared with other chemokines, that of a survival factor for microglial cells in vitro.

Fractalkine can block Fas-mediated apoptosis of microglia

To further analyze the survival-promoting effects of fractalkine, we asked whether fractalkine could antagonize Fas-induced programmed cell death. Microglia were stimulated with plate-bound fractalkine (immobilized chemokine domain alone) and various
FIGURE 1. Fractalkine promotes microglial survival. MTT assay (■) and [\( ^{3}H \)] thymidine incorporation (▲) measuring cell viability and proliferation, respectively. Microglia were either untreated (control) or treated for 48 h with 100 nM fractalkine (chemokine domain), or 200 U/ml M-CSF and GM-CSF in the presence or absence of neutralizing concentrations of CX3CR-1 Ab. MTT was added for the last 4 h of the experiment to measure mitochondrial metabolic function as an indicator of cell survival. Cells were pulsed after 24 h, and [\( ^{3}H \)] thymidine incorporation was measured at 48 h to assess proliferation. All results have been normalized to control levels (100%) ± SEM, and a representative of five separate experiments is shown. Statistical differences were significant between the various samples; *, denotes \( p < 0.001 \), and **, indicates \( p < 0.005 \).

Stimulation of microglia with fractalkine leads to PKB/Akt activation and phosphorylation of BAD

The significance of these effects on cell survival correlates with in vitro biochemical signaling. Previously, we demonstrated that fractalkine activates the PI-3 kinase/PKB pathway in primary microglia (6), and this might account for fractalkine acting as a survival factor. Therefore, we tested whether blockade of this pathway by the PI-3 kinase inhibitor LY294002 could reverse the protective effect of fractalkine (31). The addition of 1 μM LY294002 to microglial cell cultures effectively blocked the kinase activity of PKB (Fig. 3A); however, it only partially antagonized the protective effect of fractalkine (Fig. 3B). Similar effects were observed using another PI-3 kinase antagonist, wortmannin, at several concentrations (data not shown). This observation illustrates that activation of the PI-3 kinase/PKB pathway plays a role in fractalkine’s ability to block Fas-mediated apoptosis, although only partial protection was seen under various doses, suggesting multiple pathways mediate fractalkine’s protective effect.

To further biochemically characterize the role of the PI-3 kinase/PKB pathway, microglia were treated for 18 h with fractalkine and Fas ligand, separately or in combination, BAD protein was immunoprecipitated, and phosphoserine levels were examined by Western blot analysis (Fig. 4A). This revealed that microglial cells treated with fractalkine had substantially higher levels of phosphoserine-BAD protein, indicative of inhibition of its apoptotic function. When the immunoblot was reprobed, comparable levels of BAD protein were found under all conditions tested (Fig. 4B). Additionally, we tested whether BAD could heterodimerize with Bcl-2 under these conditions; Western blots of pulldown experiments using a Bcl-2-GST fusion protein were probed for BAD. The levels of BAD protein precipitated with the Bcl-2 fusion protein were reduced in untreated, fractalkine-treated, or fractalkine and Fas ligand-treated microglia, compared with Fas ligand-stimulated microglia, further suggesting that BAD had been phosphorylated and lost its ability to heterodimerize (Fig. 4C).

Fractalkine stimulation can modulate expression of Bcl-2 family proteins

As the regulatory pathway of BAD phosphorylation did not appear to be the sole regulator of apoptosis (Fig. 3B), the role of other Bcl-2 family proteins was explored by measuring levels of the
antiapoptotic protein Bcl-x<sub>L</sub> and the proapoptotic protein Bax. The intracellular ratio of these proteins is thought to dictate cell fate (32). Our results show that Bcl-x<sub>L</sub> is up-regulated in microglia treated with fractalkine for 18 h; conversely, Bax protein levels are reduced (Fig. 5, A and B). Additionally, levels of the proapoptotic protein BID were examined (28). BID has been shown to be a critical mediator in the mitochondrial amplification loop, resulting in Fas-induced apoptosis (33). We observed BID to be cleaved and

**FIGURE 2.** Fractalkine blocks Fas-induced programmed cell death. 

**a.** Photomicrographs (×1000) of DAPI-stained microglial nuclei that were untreated, treated with 100 ng/ml soluble Fas ligand, 10 nM immobilized fractalkine (chemokine domain), or both for 18 h. Arrows indicate cells with clear morphological nuclear changes associated with apoptosis, as the apoptotic nuclei have condensed chromatin and nuclear blebbing. 

**b.** Oligosomal DNA content was measured in microglia challenged with various doses of soluble Fas ligand receiving either no treatment (●), 10 nM plate-bound fractalkine (chemokine domain; ▲), or soluble 10 nM eotaxin (△) for 18 h. The results shown are the mean of four independent experiments ± SEM. 

**c.** Fractalkine protection of Fas ligand-treated microglia is dose dependent. Microglia were treated as above with various doses of plate-bound fractalkine, and oligosomal DNA content was determined after 18 h. Representative results from three independent experiments are shown ± SEM.
FIGURE 3. The PI-3 kinase/PKB pathway is activated in fractalkine-stimulated microglia and plays a role in fractalkine inhibition of Fas-mediated microglial cell death. A, PKB immunoprecipitates from microglia treated with either PBS or 10 nM plate-bound fractalkine with various concentrations of the PI-3 kinase inhibitor LY294002 were used in in vitro kinase reactions using histone 2B as an exogenous substrate for PKB. To control for equal amounts of Akt/PKB immunoprecipitated, the immunoblot was probed with anti-Akt/PKB polyclonal Abs (lower panel). B, Quantitation of oligosomal DNA detected from either untreated microglial cells or microglia stimulated in the presence or absence of 1 μM LY294002 and 10 nM immobilized fractalkine. All conditions received 50 ng/ml of soluble Fas ligand for 18 h. Results shown are the mean from three separate experiments ± SEM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Cells in:</th>
<th>G₁</th>
<th>S</th>
<th>G₁/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-treated</td>
<td>7.21 ± 0.15</td>
<td>61.14 ± 0.65</td>
<td>25.1 ± 6.39</td>
<td>8.64 ± 0.45</td>
</tr>
<tr>
<td>Fractalkine</td>
<td>1.93 ± 0.23</td>
<td>80.85 ± 1.68</td>
<td>11.69 ± 1.45</td>
<td>6.32 ± 2.46</td>
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<tr>
<td>Fas ligand</td>
<td>20.62 ± 0.23</td>
<td>58.66 ± 6.39</td>
<td>15.86 ± 0.45</td>
<td>6.01 ± 0.27</td>
</tr>
<tr>
<td>Fractalkine/Fas</td>
<td>4.24 ± 0.20</td>
<td>71.66 ± 5.35</td>
<td>19.27 ± 5.28</td>
<td>6.41 ± 0.18</td>
</tr>
</tbody>
</table>

*Microglial cells were either untreated, or stimulated with 10 nM fractalkine, 100 ng/ml soluble Fas ligand, or both for 18 h, and harvested. The DNA content was determined by staining ethanol-fixed cells with propidium iodide and flow cytometric analysis. The <G₁ cells contain hypodiploid DNA content indicative of apoptosis. The values obtained are from three experiments (±SEM). Differences between the <G₁ fraction of the various samples were significant (p < 0.005).

FIGURE 4. BAD protein is serine phosphorylated upon fractalkine stimulation in microglia. Western blot analysis of BAD expression and posttranslational phosphorylation in microglia treated for 18 h with either 10 nM plate-bound fractalkine (chemokine domain), 100 ng/ml soluble Fas ligand, or in combination. A, BAD protein was immunoprecipitated from fresh lysates, and the immunoblot was probed with anti-phosphoserine Ab (n = 3; a representative Western blot is shown for A, B, and C). B, The same immunoblot was subsequently reprobed with anti-BAD Abs as a control to demonstrate similar levels of BAD protein as a control to demonstrate similar levels of BAD protein were immunoprecipitated in each sample (n = 3). This approach was taken because the cross-reactivity of the anti-phospho-BAD Abs tested was suboptimal against rat cell lysates. C, Western blot analysis of BAD protein levels using a Bcl-2-GST fusion protein in a pulldown experiment. Microglia were treated as above, and the fresh cell lysates were incubated with 1 μg of a Bcl-2-GST fusion protein linked to agarose beads and precipitated. Western blots were subsequently probed with anti-BAD Abs, indicating the relative amounts of BAD protein heterodimerized with Bcl-2 (n = 3). Histograms of the densitometry readings are shown for each blot, and are normalized to untreated samples.
activated in Fas ligand-treated cultures of microglia; however, the amount of activated protein was significantly reduced in cells stimulated with fractalkine (Fig. 5C). These results illustrate that fractalkine inhibition of Fas-induced microglial cell death is mediated at least in part through specific antiapoptotic signaling. Taken together with the survival effect measured by mitochondrial function (MTT assay, Fig. 1), these observations suggest that one mechanism by which fractalkine is acting to suppress Fas-mediated apoptosis of activated CD4+ T lymphocytes occurs predominantly via a Fas ligand/Fas interaction, and blocking the cells in the G1 phase of the cell cycle can abrogate this effect (35–37). This observation raises the question of whether fractalkine may also be acting via the cell cycle to inhibit Fas-mediated cell death, and this hypothesis is currently being tested.

The Fas ligand/Fas pathway has been shown to be involved in various CNS diseases (10–17). Normally, Fas is only weakly expressed in the brain, but is up-regulated in brain tissues from stroke, multiple sclerosis, and Alzheimer’s patients, suggesting it may play a role in various CNS pathological states (10, 38). In these diseases, there is a concomitant release of Fas ligand into the CNS environment by various cell types, including microglia (10, 14). We show in this work that fractalkine can inhibit Fas-mediated death of primary microglial cells in vitro. Although fractalkine is constitutively expressed in the brain, deleterious stimuli further induce fractalkine expression by neurons (5). Additionally, the proinflammatory cytokines TNF-α and IL-1β trigger fractalkine expression by astrocytes (6). Taken together, these observations suggest a critical role of fractalkine in various CNS disease states, and one function may be to promote microglial cell survival.

Microglia are brain-resident macrophage, and they perform a critical role as phagocytic cells. Furthermore, their anatomic location close to the blood brain barrier, and their ability to secrete inflammatory cytokines, reactive oxygen intermediates, NO, and inflammatory cytokines, reactive oxygen intermediates, NO, and the later phase of tissue repair. Microglia are brain-resident macrophage, and they perform a critical role as phagocytic cells. Furthermore, their anatomic location close to the blood brain barrier, and their ability to secrete inflammatory cytokines, reactive oxygen intermediates, NO, and proinflammatory cytokines, reactive oxygen intermediates, NO, and this hypothesis is currently being tested.

In light of microglial function in the CNS both in homeostasis and disease states, our results demonstrate a novel and potentially critical function of fractalkine. These results illustrate a physiologically relevant role for this constitutively expressed chemokine in the brain. In summary, the data presented demonstrate a novel biological precedent for a chemokine, and provide insight into basic cellular interactions between neurons and microglia in both normal and pathological conditions within the CNS. These observations may, in turn, open up new avenues for therapeutic gain in reducing CNS damage in various diseases by modulating the fractalkine/CX3CR-1 interaction.

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References
