Interactions between Chemokines

REGULATION OF FRACTALKINE/CX3CL1 HOMEOSTASIS BY SDF/CXCL12 IN CORTICAL NEURONS

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The soluble form of the chemokine fractalkine/CX3CL1 regulates microglia activation in the central nervous system (CNS), ultimately affecting neuronal survival. This study aims to determine whether CXCL12, another chemokine constitutively expressed in the CNS (known as stromal cell-derived factor 1; SDF-1), regulates cleavage of fractalkine from neurons. To this end, ELISA was used to measure protein levels of soluble fractalkine in the medium of rat neuronal cultures exposed to SDF-1. Gene arrays, quantitative RT-PCR, and Western blot were used to measure overall fractalkine expression in neurons. The data show that the rate of fractalkine shedding in healthy cultures positively correlates with in vitro differentiation and survival. In analogy to non-neuronal cells, metalloproteinases (ADAM10/17) are involved in cleavage of neuronal fractalkine as indicated by studies with pharmacologic inhibitors. Moreover, treatment of the neuronal cultures with SDF-1 stimulates expression of the inducible metalloproteinase ADAM17 and increases soluble fractalkine content in culture medium. The effect of SDF-1 is blocked by an inhibitor of both ADAM10 and -17, but only partially affected by a more specific inhibitor of ADAM10. In addition, SDF-1 also up-regulates expression of the fractalkine gene. Conversely, exposure of neurons to an excitotoxic stimulus (i.e. NMDA) inhibits secretase activity and markedly diminishes soluble fractalkine levels, leading to cell death. These results, along with previous findings on the neuroprotective role of both SDF-1 and fractalkine, suggest that this novel interaction between the two chemokines may contribute to in vivo regulation of neuronal survival by modulating microglial neurotoxic properties.

Metabolically and electrically active neurons contribute to the maintenance of a healthy microenvironment through the release of factors that control immune cells, including microglia. Fractalkine, recently renamed CX3CL1, is a transmembrane chemokine abundantly expressed on neurons, which can also function as a soluble factor after cleavage of its extracellular portion containing the chemokine domain (1, 2). In the central nervous system (CNS), soluble fractalkine is known to interact with glial cells at distant sites that express its specific and only receptor, CX3CR1 (3, 4). Recent studies in transgenic mice lacking this receptor have demonstrated that fractalkine plays a crucial role in preventing microglia-induced neurotoxicity in different disease models (5). This is in line with previous in vitro studies indicating that fractalkine reduces neuronal damage caused by toxic insults and impairs microglial activation (6–10). Thus, regulation of fractalkine cleavage from neurons has a direct impact on neuronal survival both in vitro and in vivo. Reports in non-neuronal cells have shown that shedding of fractalkine from the plasma membrane is mediated by metalloproteinases of the ADAM (a disintegrin and metalloproteinase) family (11–14), namely ADAM10 and ADAM17, which are also found in the nervous system (15, 16). However, less is known about fractalkine homeostasis in the brain.

Another prominent chemokine in the CNS is SDF-1 (stromal cell-derived factor 1 or CXCL12, according to the new nomenclature), which regulates fundamental neuronal/glial functions, such as neurogenesis, neuronal migration, and synaptic transmission (17–19). SDF-1 is also involved in glia proliferation and growth of primary brain tumors (20). Unlike most chemokines, both SDF-1 and fractalkine are constitutively expressed in the developing and adult brain (21). In line with this, alterations of their function have been associated with many neuropathologies, including neuroAIDS (9, 22). The relative expression of these chemokines and their receptors by neuronal and non-neuronal cells suggests a role for both SDF-1 and fractalkine in neuronal-glial communication, as supported by in vivo and in vitro evidence (3, 23). Furthermore, data from different groups have shown that SDF-1 and fractalkine are both involved in neuroprotection and regulate excitatory neurotransmission (6, 24, 25). However, while the neuroprotective effect of fractalkine seems to be related to its ability to counteract microglia-induced neurotoxicity, SDF-1 can directly protect neurons via the activation of its specific receptor CXCR4, which is coupled to major survival pathways, such as the phosphoinositide 3-kinase (PI3K)-Akt pathway (26, 27). Interestingly, intracellular pathways stimulated by SDF-1 control transcription factors that up-express CX3CL1.

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3 The abbreviations used are: CNS, central nervous system; ADAM, a disintegrin and metalloproteinase; DIV, days in vitro; FKN, fractalkine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR; SDF-1, stromal cell-derived factor-1; TACE, TNFα-converting enzyme; TNFα, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; NMDA, N-methyl-D-aspartic acid.
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regulate expression of the fractalkine gene, such as NFκB and p53 (28).

The present study investigates the cellular and molecular mechanisms involved in the regulation of fractalkine in the CNS and the role of SDF-1 in fractalkine cleavage. The data show that SDF-1 increases shedding of neuronal fractalkine, likely via up-regulation of neuronal ADAM17 (also known as TACE, *i.e.*, TNF-α-converting enzyme), and suggest that this novel SDF-1/fractalkine interaction may facilitate neuronal recovery after excitotoxic insults.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal Cultures**

Neurons were harvested from brain cortices of E17/18 Holtzman rat embryos as previously described (27, 29, 30). Cells were plated as reported below.

*Neuron/Glia Co-culture—*Cortical neurons were cultured in a serum-free medium using the bilaminar cell culture system, originally described by Banker and Cowan (31) and modified as in (29), in which pure neuronal cultures are grown in the presence of a separate glial feeder layer supporting their growth and differentiation. This model reproduces, to a certain extent, the in vivo environment (*i.e.*, the presence of neuronal and non-neuronal cells) and offers the major advantage that neurons can be separated from the glia at any time to analyze neuron-specific biological responses. We have extensively used the bilaminar culture system to study expression and function of chemokine receptors in neurons, including CXCR4. Briefly, cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum (HS), plated on poly-L-lysine-coated (0.5 mg/ml) 60-mm culture dishes (1.0 × 10⁶/dish, in 3 ml of DMEM medium/10% HS culture medium), and placed in the incubator (5% CO₂, 36.5 °C). After 3–4 h, cells were washed once with warm serum-free DMEM medium; 4 ml of serum-free DMEM (supplemented with 1% N2.1, and 50 mg/100 ml ovalbumin) were then added to each dish. A custom-made plastic coverslip containing a feeder layer of secondary glia was then added to each dish to provide trophic support to the neurons. Within twenty-four hours from plating, cytokine arabinoside C (10 μM) was added to the co-culture to inhibit glial proliferation in the neuronal layer (32). As previously reported (and also shown in the supplemental materials), more than 95% of viable cells in the neuronal layer are neurons (29, 32, 33).

*Glia-free Neuronal Culture—*Cells were plated at a density of 1.2 × 10⁶ in poly-L-lysine-coated 60-mm cell culture dishes in Neurobasal medium (3 ml) containing 2% B27 supplement and 2% horse serum, as previously reported (34) and originally described by Brewer et al. (35). After 2 h, cells were washed with warm Neurobasal medium, and 4 ml of Neurobasal medium (supplemented with B27, gentamycin, L-glutamine, and L-glutamic acid) was then added to each dish. Cells were cultured in the presence of cytokine arabinoside C (1 μM). This method yields an almost pure neuronal culture (>95% neurons), as assessed by staining for neuronal/glial markers (34, 35) (see also supplemental Fig. S1).

**ELISA**

Rat Fractalkine ELISA DuoSet (R&D Systems) was used to measure soluble fractalkine in the medium of neuronal (and/or glial) culture, as indicated by the manufacturer. Right before processing the total cell lysates for Western blotting, the medium was collected from each culture dish (usually, three dishes per experimental group) and stored at −80 °C until the time of the ELISA. All samples were run in triplicate. A Perkin-Elmer Victor II microplate reader was used for measurement of absorbance (wavelength: 450 nm and 550 nm). The 550-nm reading was subtracted from the 450-nm absolute reading, after subtraction of the blank (*i.e.*, wells containing blocker solution). A new standard curve was generated for each experiment and plotted using MatLab software. A 4-parameter curve was generated for calculation of the actual protein concentration.

**Western Blot**

Total cell lysates were obtained from neurons at indicated days *in vitro* (DIV), as previously reported (7, 27, 34). For equal protein loading, a bicinchoninic acid protein assay kit (Pierce) was used to calculate protein concentration in each sample. Proteins were resolved by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane for immunoblotting. The following primary antibodies were used: a polyclonal goat anti-fractalkine from R&D Systems (0.2 μg/ml) and a polyclonal rabbit anti-actin from Sigma (0.12 μg/ml). Anti-actin was used for loading controls. Protein G (50 ng/ml, Calbiochem), protein A (100 ng/ml, Invitrogen), or an anti-rabbit (1 ng/ml; Pierce) conjugated to horseradish peroxidase (HRP) along with Super Signal Reagents (Pierce) were used to detect specific bands. An anti-biotin HRP-conjugated antibody (6.6 ng/ml; Cell Signaling) was used for detection of the biotin-labeled molecular markers. Recombinant full-length fractalkine protein (R&D Systems) was used as a positive control (40 μl of a 0.08 μg/ml solution were loaded per lane). Images were acquired with the FluorChem 8900 system (Alpha-Innotech); the software U-SCAN-IT was used for densitometric analysis of the bands.

**RT-PCR, qPCR, and Microarrays**

Total RNA from neurons was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and quantitated by spectrophotometer. Reverse transcription of RNA to cDNA was performed using random hexamers (Roche Applied Science, Indianapolis, IN). Aldolase A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for RT-PCR, and GAPDH for quantitative PCR (qPCR). Primer sequences used for PCR are as follows: rat ADAM17 forward primer: 5’-CGT TGT TCT GCT TGT CCT GGT TTT CTC-3’, reverse primer: 5’-GGC CTT CTC ACT CCT G-3’; rat aldolase A forward primer: 5’-ACC AAT GGC GAG ACC ACT AC-3’, reverse primer: 5’-AAT TCG GTC CAC AAT GG-3’. Rat fractalkine and GAPDH primers were purchased from SABiosciences (Frederick, MD), and were used for both RT-PCR and qPCR. Amplification conditions are as follows: ADAM17/aldolase A: 4 min at 95 °C followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 1 min at 72 °C; Fractalkine/GAPDH: 15 min at 95 °C, followed by
40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7700 Sequence Detection System (Applied Biosystems). Samples were run in duplicate and normalized to GAPDH expression levels. For microarray analysis, RNA was extracted as described above and hybridized to rat p53 oligoarray (SABiosciences) as indicated by the manufacturer. Panels were normalized to housekeeping genes using the Interquartile method.

α-Secretase Activity Assay

The enzymatic activity of the α-secretase class of proteases was measured by the α-Secretase Activity kit from R&D Systems (Minneapolis, MN), which is based on the EDANS/DABCYL reporter system. Cell lysates (90 μg of protein) from treated and control neurons were loaded into a black 96-well plate containing the reaction buffer and the α-secretase specific substrate (i.e. the peptide YEVHHQKLV, corresponding to amino acids 681–689 of amyloid precursor protein) conjugated to the reporter molecules. Cleavage of the peptide by the α-secretase separates the EDANS and DABCYL reporter molecules generating a fluorescent signal, which was detected by a microplate reader (Victor II, Perkin-Elmer) following the protocol provided by the manufacturer. Recombinant TACE (R&D Systems) was used as positive control and to determine initial conditions for these experiments.

Cell Survival Assays

Neuronal survival was measured by different means including cleaved (i.e. active) caspase-3 immunostaining, fluorescein diacetate/propidium iodide/Hoechst 33342 staining, and lactate dehydrogenase (LDH) assay, as previously described (24, 27, 29, 30, 32). 5–10 fields were counted per coverslip, and three coverslips/treatment were used. LDH assays were performed in triplicate (40,000 cells/well).

Materials

Unless otherwise specified, reagents and media were purchased from Invitrogen, Gibco, or Sigma. GI254023X was a kind gift from Dr. D. J. Becherer of Glaxo Smith Kline, whereas the GM6001 was obtained from Chemicon. SDF-1 was purchased from Peprotech and recombinant fractalkine from R&D Systems.

Statistical Analysis

Each graph reports the mean ± S.E. of 3–4 independent experiments, unless otherwise specified. A one-way ANOVA followed by Newman-Keuls or Tukey comparative test was performed to test significance, as indicated. A p < 0.05 or less was considered statistically significant.

RESULTS

Accumulation of Soluble Fractalkine in Neuronal Cultures in the Presence and Absence of Glia—As reported in Fig. 1, fractalkine protein is expressed in both immature and differentiated cortical neurons, and its soluble form accumulates in the culture medium in a time-dependent fashion. In neuronal/glial cultures, soluble fractalkine levels steadily increased within the first 2 weeks (Fig. 1A) reaching a plateau at about 12 days in vitro (DIV); Western blot analysis using neuronal cell extracts from the same cultures showed that cell-associated fractalkine was expressed throughout the culture period (Fig. 1B). Furthermore, under these experimental conditions fractalkine was undetectable in the medium of the glial culture during the first few days as determined by ELISA, and it only reached ~0.7 ng/ml after the first week in culture (supplemental Fig. S2). This value corresponds to about 20% of soluble fractalkine in co-cultures of the corresponding age (Fig. 1 and supplemental Fig. S2). These findings suggest that fractalkine is continuously shed from the neuro-
nence of metalloproteinases as assessed by ELISA in both mixed (black bars) and pure (gray bars) neuronal cultures. The bar graph represents the mean ± S.E. of soluble fractalkine in the medium over a course of 24 h (*, p < 0.05 versus control). Data are derived from three independent experiments (each including 2–3 dishes per experimental group).

Role of ADAMs in Constitutive Cleavage of Fractalkine from Neuronal Membrane—To determine whether the increase in soluble fractalkine depends on activity of metalloproteinases normally involved in fractalkine cleavage in other cells, neurons were treated with inhibitors of ADAM10 (the enzyme implicated in constitutive shedding of fractalkine) and ADAM17 (an inducible enzyme also known as TACE) (11–13). Two different inhibitors were used: GI254023X, which preferentially blocks ADAM10, and GM6001, a general metalloproteinase inhibitor that also blocks ADAM17 (36). These experiments were performed in mixed as well as in pure neuronal preparations. The inhibitors were used at concentrations we previously tested (14), and which only minimally affected neuronal survival (supplemental Fig. S3). Inhibitors were added directly to the culture medium after approximately the first week in culture, and culture medium was collected 24 h later (Fig. 2). The results show that both inhibitors reduce accumulation of fractalkine in the culture medium to a similar extent, suggesting that ADAM10 is primarily involved in fractalkine shedding in resting conditions (Fig. 2), which is in agreement with other reports in non-neuronal cells (12). In the present studies, the reduction in soluble fractalkine caused by the metalloproteinase inhibitors reflects their effect over the last day in culture only. Thus, the quote of soluble fractalkine previously accumulated is not affected. Nevertheless, metalloproteinase inhibition caused a large decrease in soluble fractalkine levels after 24 h, suggesting a rapid turnover of soluble fractalkine. Also, additional studies show that increases in fractalkine concentrations were detected after only a few hours following a complete change of culture medium (supplemental Fig. S4). Such rapid fractalkine turnover further supports ADAM regulation as an efficient mechanism to control levels of the soluble chemokine.

SDF-1 Up-regulates Fractalkine and ADAM17—In the last several years we have well characterized the effect of the chemokine SDF-1 on neuronal signaling (24, 27, 29, 34, 37). In agreement with data from other groups, our studies indicate that SDF-1 regulates a number of intracellular pathways involved in neuronal survival via activation of its specific G protein-coupled receptor, CXCR4. Under normal conditions, SDF-1 promotes neuronal survival and differentiation and reduces NMDA-mediated excitotoxicity (22, 24, 26, 27, 29, 30, 34). Moreover, microarray studies using a p53-specific gene array that included the fractalkine gene showed that SDF-1 significantly increases expression of fractalkine (Table 1, 18 h SDF-1). Thus, we hypothesized that SDF-1 positively modulates levels of neuronal fractalkine, further contributing to neuronal survival. PCR analysis (including quantitative real time PCR) was used to validate the initial microarray data and demonstrated that SDF-1 stimulates fractalkine expression in neurons (Fig. 3, A and B). However, cellular levels of fractalkine did not significantly increase in SDF-treated neurons, unless GM was also present (Fig. 3C), suggesting that SDF-1 may also stimulate fractalkine shedding from the neuronal membrane. In line with this prediction, content of soluble fractalkine in the medium of SDF-treated neuronal cultures (20 nM, 18–24 h) was found to be significantly higher than basal level, both in the presence and absence of glia (Fig. 3D). Interestingly, SDF-1 treatment did not increase soluble fractalkine levels in the medium of pure glia cultures (supplemental Fig. S2). To further study the effect of SDF-1 on neuronal fractalkine cleavage, we asked whether SDF-1 modulates ADAMs. Because the changes in soluble fractalkine in response to SDF-1 were usually
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Regulation of Fractalkine Cleavage by SDF-1—Recent studies suggest that cleavage of fractalkine might be an important mechanism of neuroprotection, as the chemokine inhibits microglia-mediated toxicity in vitro and in vivo (5, 10). Based on this evidence, we would expect fractalkine cleavage to be reduced in injured neurons. This led us to investigate the effect of neurotoxic insults, namely NMDA treatment, on content of soluble fractalkine in neuronal culture medium. Neurons were transiently exposed to NMDA (20 min, 100 μM) and then returned to their culture medium. This transient exposure to NMDA is known to lead to excitotoxicity and delayed neuronal death, which becomes apparent after several hours (i.e. 18–24 h) as reported by many groups, including ours (27, 30). Levels of fractalkine released in the culture medium were measured within 3–6 h post NMDA (Fig. 5A). These studies showed that NMDA treatment markedly reduced fractalkine content in the neuronal culture medium (Fig. 5A). The rapid effect of NMDA was not caused by a reduction in neuronal viability, as confirmed by cell survival assays (supplemental Fig. S5). Indeed, the decrease in soluble fractalkine precedes neuronal loss by at least 6 h. Additional controls were included to rule out that changes in soluble fractalkine were due to experimental artifacts, such as change of medium (supplemental Fig. S6A). Importantly, NMDA did not further decrease fractalkine levels in GM-treated cultures (Fig. 5A), suggesting that NMDA and the metalloproteinase inhibitor share similar mechanisms of action. To test this hypothesis we measured protease (i.e. α-secretase) activity in neurons exposed to NMDA, as previously reported (38). Both ADAM10 and 17 have α-secretase activity. In line with previous reports (38), NMDA significantly diminished neuronal α-secretase activity (Fig. 5B); this effect was sustained for at least 6 h after exposure to the excitatory amino acid and comparable to that of GM6001 (Fig. 5B). Finally, to determine whether SDF-1 is able observed after long-term treatment (i.e. more than 6 h), we postulated that the chemokine might regulate expression of ADAM17 rather than directly/rapidly stimulate its enzymatic activity. Therefore, we measured protein and RNA levels of ADAM17 in extracts from pure neuronal cultures treated with SDF-1 (20 nm; 3–6-24 h). The results of these experiments show that SDF-1 increases neuronal ADAM17 protein and mRNA in a time-dependent manner (Fig. 4). Interestingly, ADAM10 levels did not change (not shown). Both the mature form of ADAM17 and the precursor protein were up-regulated in SDF-treated neurons (Fig. 4); the effect on mature ADAM17 appears more robust and levels of the precursor protein return back to basal after 24 h. Levels of the mature protein are shown to plateau roughly 3 h before any observable changes in soluble fractalkine concentration, which may reflect time needed for the cleavage process and integration of other regulatory mechanisms, as well as the accumulation of noticeable increases in fractalkine levels. These results suggest that SDF-1 stimulates fractalkine cleavage by enhancing ADAM17 expression. Therefore, we tested the effect of SDF-1 in neurons treated with the general metalloproteinase inhibitor, GM6001. As shown in Fig. 4B, GM6001 prevented the increase in soluble fractalkine induced by SDF-1 whereas the chemokine was still able to stimulate fractalkine release in cells treated with GI250423X (which preferentially blocks ADAM10), albeit partially. SDF-1 increased soluble fractalkine content by almost 70% in GI-treated cultures (mean ± S.E. from 3 dishes: 69 ± 6). Overall, these findings show that SDF-1 stimulates both expression and cleavage of neuronal fractalkine and suggest that ADAM17 is primarily implicated in SDF-1-induced shedding.

Decrease in Soluble Fractalkine Precedes Neurotoxicity—Recent studies suggest that cleavage of fractalkine might be an important mechanism of neuroprotection, as the chemokine inhibits microglia-mediated toxicity in vitro and in vivo (5, 10). Based on this evidence, we would expect fractalkine cleavage to be reduced in injured neurons. This led us to investigate the effect of neurotoxic insults, namely NMDA treatment, on content of soluble fractalkine in neuronal culture medium. Neurons were transiently exposed to NMDA (20 min, 100 μM) and then returned to their culture medium. This transient exposure to NMDA is known to lead to excitotoxicity and delayed neuronal death, which becomes apparent after several hours (i.e. 18–24 h) as reported by many groups, including ours (27, 30). Levels of fractalkine released in the culture medium were measured within 3–6 h post NMDA (Fig. 5A). These studies showed that NMDA treatment markedly reduced fractalkine content in the neuronal culture medium (Fig. 5A). The rapid effect of NMDA was not caused by a reduction in neuronal viability, as confirmed by cell survival assays (supplemental Fig. S5). Indeed, the decrease in soluble fractalkine precedes neuronal loss by at least 6 h. Additional controls were included to rule out that changes in soluble fractalkine were due to experimental artifacts, such as change of medium (supplemental Fig. S6A). Importantly, NMDA did not further decrease fractalkine levels in GM-treated cultures (Fig. 5A), suggesting that NMDA and the metalloproteinase inhibitor share similar mechanisms of action. To test this hypothesis we measured protease (i.e. α-secretase) activity in neurons exposed to NMDA, as previously reported (38). Both ADAM10 and 17 have α-secretase activity. In line with previous reports (38), NMDA significantly diminished neuronal α-secretase activity (Fig. 5B); this effect was sustained for at least 6 h after exposure to the excitatory amino acid and comparable to that of GM6001 (Fig. 5B). Finally, to determine whether SDF-1 is able...
to counteract the effect of NMDA on soluble fractalkine levels, SDF-pretreated neurons were exposed to NMDA as described above and processed for ELISA. In these experiments SDF-1 only slightly counteracted NMDA-induced inhibition of soluble fractalkine (Fig. 5A). As also reported by others (38), NMDA did not affect ADAM17 protein levels (supplemental Fig. S6B). Thus, NMDA may act downstream of SDF-1 directly inhibiting metalloproteinase activity in line with the results of the enzymatic activity assays.

Fractalkine Prevents NMDA-induced Neuronal Cell Death—The demonstrated sensitivity of fractalkine cleavage to known neuroprotective and neurotoxic stimuli suggested an ability of the chemokine to acutely affect neuronal survival. Thus, to evaluate the physiological importance of regulated fractalkine cleavage we investigated the effects of soluble fractalkine on neuron loss induced by NMDA. The bilaminar co-cultures were used to provide the full complement of CX3CR1-containing cells. Cultures were briefly exposed to NMDA (20 min, 100 μM), with or without exogenous soluble fractalkine (100 nM, 30 min before, during 20 min NMDA treatment, and after for

FIGURE 4. SDF-1 increases expression of neuronal ADAM17. Increased expression of ADAM17 and its precursor protein in protein extracts from pure neuronal cultures treated with 20 nM SDF-1 for 24 h starting at 6 DIV (A, top) is shown. Expression of ADAM17 mRNA was also increased with similar treatment, shown here as a 415-bp fragment (A, lower). The bar graph (B) shows the effect of SDF-1 (20 nM, 24 h) on the content of soluble fractalkine in the medium of 7 DIV neurons in the presence and absence of 25 μM GM6001 (pure neuronal cultures; *, p < 0.05 versus untreated control or SDF alone; ∧, p < 0.05 versus untreated control; n = 3).

FIGURE 5. NMDA treatment reduces levels of soluble fractalkine. Soluble fractalkine levels (A) were measured in the medium of pure neuronal cultures (9–11 DIV) exposed to NMDA (100 μM, 20 min). Before NMDA treatment, neurons were treated with vehicle, the metalloproteinase inhibitor GM6001 (25 μM) or SDF (20 nM). Culture medium was changed in all neuronal cultures prior to the start of the experiment. Data are shown as percentage of their respective controls, i.e. basal or SDF-treated cells; *, p < 0.05 versus respective control. Neurons treated with NMDA also display decreased activity in the α-secretases as determined by an in vitro activity assays (B). The bar graph represents the mean fluorescence value for each sample (three independent experiments, 2 dishes per experimental group; *, p < 0.05; **, p < 0.01 versus control). As expected, a ~50% inhibition was also found in neurons treated with GM6001 (C; 2 or 3 dishes/group).
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The present study shows that healthy and active neurons in culture normally release fractalkine via the constitutive activation of metalloproteinases and suggest that homeostatic, glia-dependent mechanisms contribute to stimulation of fractalkine expression and cleavage. These results emphasize the role of fractalkine and microglia in neuroprotection (5, 39), as well as the notion that neurons are the primary source of this chemokine under physiological conditions (40). In addition, our studies identify a novel function of SDF-1 as regulator of fractalkine homeostasis and suggest that SDF-1 could control both expression and cleavage of fractalkine in vivo. Both constitutive and stimulated fractalkine cleavage would ultimately limit activation of microglia and support neuronal survival as previously reported (5–10). Evidence indicates that fractalkine provides a tonic inhibitory signal that controls neighboring microglia resulting in reduced release of pro-inflammatory cytokines and increased production of neurotrophins (41); it controls glutamatergic activity by inhibiting excitatory postsynaptic currents (6, 25, 42); it negatively regulates neuronal migration while promoting their adhesion to ECM (43). The extent to which each of these mechanisms is involved in the physiological role of fractalkine may vary at different stages of life and/or during disease states. The stimulation by SDF-1 and the subsequent increase in soluble fractalkine would serve similar neurotrophic purposes (with a major emphasis on inhibition of microglia/excitatory activity) under pathological conditions where microglia tend to be overactive and excitatory neurotransmission is potentiated. In addition, soluble fractalkine could directly stimulate pro-survival pathways in neurons that may express CX3CR1 under specific conditions (7), though in vivo CX3CR1 seems to be normally only expressed by microglial cells. The data here point to a major role of ADAM17 in this regulatory process; this is in line with a previous study reporting the stimulatory effect of SDF-1 on tumor necrosis factor-α (TNFα) release from astroglia (23), which is known to depend on TACE/ADAM17. However, the involvement of additional proteases cannot be excluded. The ability of SDF-1 to activate transcription factors involved in regulation of the fractalkine gene (like p53 and NFκB) would explain the results on fractalkine expression, though the relative contribution of these transcription factors will have to be determined.

Unlike other chemokines, both fractalkine and SDF-1 are constitutively expressed in the CNS. Thus, the interaction between the two chemokines adds further complexity to the mechanisms of neuronal-glial communication and their involvement in neuronal survival. A recent study reported the effect of SDF-1 on the adhesive properties of fractalkine in hippocampal and cerebellar neurons (43), which suggests that SDF-1 may regulate fractalkine function at different levels, though the final outcome in terms of neuronal function and survival may depend on the neuronal phenotype and/or cell types involved.

Previous studies have demonstrated that SDF-1 promotes neuronal survival by stimulating the chemokine receptor CXCR4 on neurons (24, 27, 44). Activation of neuronal CXCR4 by its natural ligand leads to stimulation of pro-survival pathways and prevents neurotoxicity induced by different insults, including NMDA-mediated excitotoxicity (24, 30, 37, 45). Thus, regulation of fractalkine expression by SDF-1 could be involved in the beneficial action of the latter chemokine under specific circumstances. However, based on the present study, SDF-1 would not be able to prevent NMDA-induced inhibition of fractalkine cleavage but could rather slowly restore ADAM17 function by stimulating its protein synthesis de novo, thus helping neurons recover from the inhibition of metalloproteinases caused by a previous NMDA stimulation. Indeed, overstimulation of NMDA receptors reduces cleavage of fractalkine from neurons by causing a long-lasting inhibition of protease activity; this would compromise the ability of fractalkine to inhibit microglia-induced neurotoxicity (5). These findings relate to an...
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...important aspect of immune regulation in the CNS (i.e. neuronal suppression of immune function by viable neurons) that is supported by in vivo/in vitro studies and is also in line with the regulation of glutamatergic synapses by fractalkine (25, 38, 46). The relation between fractalkine and glutamatergic neurotransmission is further supported by our finding that soluble fractalkine prevents neurotoxicity induced by excessive NMDA, in line with previous reports (5, 7, 9, 25), suggesting a novel mechanism by which NMDA may contribute to neuron loss. On the other hand, the contribution of different types of glutamate receptors to the regulation of neuronal fractalkine cleavage may vary significantly.

Indeed, a previous report had shown that exposure of neuronal cultures to toxic concentrations of glutamate reduces levels of cell-associated fractalkine (47) leading the authors to conclude that glutamate-stimulated fractalkine shedding is involved in recruitment of toxic microglia. Though this report was very innovative at the time as it showed the potential role of fractalkine in neuronal survival, the overall conclusions are in disagreement with new, compelling in vivo studies (5) and other in vitro evidence (6–10), all supporting a neuroprotective role of fractalkine against microglia toxicity. Furthermore, as discussed by the same authors (47) inhibition of fractalkine cleavage does not confer neuroprotection and activation of metalloproteinase per se does not trigger neuronal death, as one would expect if soluble fractalkine was involved in neurotoxicity. In addition, glial contamination of the neuronal cultures may also account for some of the effects reported as cytotoxic arabinoside C was added to the culture only after 3–5 DIV (47). In fact, microglial cathepsin S can stimulate fractalkine cleavage from neurons (48). One intriguing hypothesis is that stimulation of metabotropic receptors is involved in a early (transient) increase in fractalkine cleavage caused by glutamate, an event that could possibly represent a neuroprotective mechanism, whereas a predominant NMDA receptor-mediated response would have opposite results (on both survival and cleavage) due to prolonged inhibition of metalloproteinases. This scenario would at least partially reconcile the contradictory results discussed above and would be supported by (i) the established neuroprotective effect of certain metabotropic glutamate receptors (49–51), (ii) additional reports showing inhibition of neuronal metalloproteinases by NMDA (38), (iii) the involvement of NMDA in ADAM10 trafficking (46), and (iv) the neurotoxicity induced by prolonged (>24 h) exposure to metalloproteinase inhibitors in mixed neuronal/glial cultures (supplemental Fig. S3).

In summary, our results suggest that ADAM10 is mainly implicated in constitutive cleavage of neuronal fractalkine whereas exposure of neurons to SDF-1 up-regulates the inducible metalloproteinase ADAM17 and increases levels of soluble fractalkine. The data also indicate that soluble fractalkine content in healthy neuronal cultures positively correlates with neuronal differentiation and survival. In contrast, neurotoxic insults that activate NMDA receptors result in an inhibition of protease activity and a reduction in soluble fractalkine, followed by cell death. The overall conclusion of the studies discussed here is that cleavage of fractalkine is a dynamic process that regulates neuronal survival through continuous neuron-glia and glia-glia interactions. SDF-1 modulation of fractalkine levels implements the multi-cellular nature of this process by introducing the astroglia component (a major source of SDF-1 in the CNS) and adding a further level of regulation through ADAM17 expression. The fine details and molecular mechanisms of such complex phenomenon remain to be elucidated, but are expected to provide important insights into the concerted role of these chemokines in pathological states such as neuroinflammation and cancer.

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