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Neuronal Fractalkine Expression in HIV-1 Encephalitis: Roles for Macrophage Recruitment and Neuroprotection in the Central Nervous System

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HIV-1 infection of the brain results in chronic inflammation, contributing to the neuropathogenesis of HIV-1-associated neurologic disease. HIV-1-infected mononuclear phagocytes (MP) present in inflammatory infiltrates produce neurotoxins that mediate inflammation, dysfunction, and neuronal apoptosis. Neurologic disease is correlated with the relative number of MP in and around inflammatory infiltrates and not viral burden. It is unclear whether these cells also play a neuroprotective role. We show that the chemokine, fractalkine (FKN), is markedly up-regulated in neurons and neuropil in brain tissue from pediatric patients with HIV-1 encephalitis (HIVE) compared with those without HIVE, or that were HIV-1 seronegative. FKN receptors are expressed on both neurons and microglia in patients with HIVE. These receptors are localized to cytoplasmic structures which are characterized by a vesicular appearance in neurons which may be in cell-to-cell contact with MPs. FKN colocalizes with glutamate in these neurons. Similar findings are observed in brain tissue from an adult patient with HIVE. FKN is able to potently induce the migration of primary human monocytes across an endothelial cell/primary human fetal astrocyte trans-well bilayer, and is neuroprotective to cultured neurons when coadministered with either the HIV-1 neurotoxin platelet activating factor (PAF) or the regulatory HIV-1 gene product Tat. Thus focal inflammation in brain tissue with HIVE may up-regulate neuronal FKN levels, which in turn may be a neuroimmune modulator recruiting peripheral macrophages into the brain, and in a paracrine fashion protecting glutamatergic neurons. The Journal of Immunology, 2000, 164: 1333–1339.
localized to cytoplasmic structures, that these structures have a vesicular appearance in glutamatergic neurons, and that FKN is up-regulated in neurons adjacent to macrophages and microglia in brain tissue from pediatric patients with HIV and progressive encephalopathy (PE), compared with pediatric patients with HIV-1 but no HIVE or PE, or to pediatric patients that were HIV-1 seronegative. Furthermore, FKN can function both as a potent mediator of monocyte trafficking across an endothelial cell/astrocyte bilayer, and as a neuroprotective agent when administered with either the HIV-1 neurotoxin platelet activating factor (PAF) or the regulatory protein Tat.

Materials and Methods

Postmortem tissue collection and immunocytochemistry (ICC)

Clinical and neuropathologic data on seven HIV-1-infected children and nine HIV-1-seronegative pediatric controls have been described (2, 15). HIV-1 infection was diagnosed by Centers for Disease Control (CDC) criteria (positive culture <15 mo; presence of HIV-1 Abs >15 mo) (16). One adult case (male, 42, i.v. drug abuser, with no symptoms of neurologic disease) with HIV-1 infection, but without HIVE, were also studied. Formalin-fixed paraffin-embedded tissue blocks were prepared as described from brain regions used in this study (15). Tissue sections from various brain regions of HIV-1-infected and uninfected patients were incubated with rabbit anti-FKN peptide (Chemo- centryx, San Carlos, CA) at 2.5 μg/ml overnight at 4°C. Various concentrations of rabbit anti-FKN peptide were tested to obtain optimal signal to background. Control slides from the same brain regions were incubated with a 22-residue blocking peptide containing the C-terminal sequence of FKN (20 μg/ml) (12), and coincubated with 2.5 μg/ml rabbit anti-FKN peptide, using the Vector ABC avidin-biotin complex with either an alka- line phosphatase or diaminobenzidine (DAB) chromagen. For FKN recep- tor ICC, tissue sections were incubated with either a human FKN variant that contained a small portion of the muncin stalk and a poly histidine (HIS) tag (Chemo centryx, 10 μg/ml, 1 h, at 22°C) or a chemokine portion of FKN lacking the polyHIS tag to serve as a control condition, followed by incubation with an anti-poly(HIS) mAb (Chemo centryx, 10 μg/ml, 1 h, at 22°C), using the Vector ABC system. Parallel tissue sections were stained with antisera to glutamate (1:500; Research Biochemicals, Natick, MA) to identify glutamatnergic neurons. Giall fibrillary acidic protein (GFAP) (1: 500 in normal goat serum; Dako, Carpinteria, CA.) was used to identify astrocytes while a mAb (EBM11) to the cell surface Ag CD68 (1:200 in normal horse serum) was used to identify macrophages, and HIV-1 antisera to p24 (1:500 in normal horse serum; American Bio-Technologies, Cam- bridge, MA.) was used to identify infected cells, using the Vector ABC system. In some experiments, double immunolabeling with antisera to FKN and antisera to CD68 or glutamate were performed as previously described (15). Neurons were collected, washed twice in serum-free medium, then resus- pared at 2 × 10^5 cells/12 mm, on glass coverslips precoated with poly( L-lysine) (Sigma, St. Louis, MO) in 24-well culture dishes. After 1.5 days in formalin-fixed brain tissues (15).

Quantitative morphometry to measure the relative OD (ROD) of FKN-immunopositive neurons using computerized densitometry (Image Pro Plus for Macintosh, Vs. 3.1) was performed in the following fashion: ROD was corrected for incident light levels in each field. Maximum black levels (shutter closed condition) were the same for each field. FKN-immunopositive neurons in insular cortex were identified by observers blinded to the patient group using standard cytologic criteria. A total of 375 FKN-immuno- positive neurons were identified for the HIV+ group; 426 FKN immuno- positive neurons were identified for the HIV+ group, and 522 FKN immunopositive neurons were identified for the HIV- group. The ROD was integrated from the total surface area of each neuronal soma, and data were expressed as the mean ROD ± SEM. Significance was determined by paired t tests between groups.

Confocal microscopy

ICC for FKN and CD68 or glutamate was performed as described above using secondary Abs conjugated with FITC for either CD68 or glutamate and tetramethylrhodamine isothiocyanate (TRITC) for FKN. Images were recorded at intervals of 11.8 s increments of 0.2-μm slices for FKN and CD68 and intervals of 9–10.1 s in increments of 0.3-μm slices for FKN and glutamate on an Olympus (Spectra Services, Webster, NY) BX50WI microscope (5×-100× oil-immersion PlanApo objective). Confocal sec- tions were scanned in the center of the tissue section and are represented as a stacked image of all sections (Olympus Fluoview Software). Wave- lengths of 488 nm and 568 nm were used to excite fluorescein- and rho-
FKN expression was also markedly up-regulated in the neuropil of the HIVE brain (Fig. 1E). Brain tissues from patients with HIV-1 and HIV-1-seronegative patients were devoid of axonal staining for FKN (data not shown). Specificity of FKN immunostaining in neuronal cytoplasm and neuropil in HIVE tissue was shown by preincubation with a blocking peptide for FKN (Fig. 1F). With these conditions, FKN expression was reduced to near background levels.

Confocal microscopy of brain tissue sections stained for the macrophage and microglial marker CD68 and FKN revealed focal inflammatory infiltrates in cortex and basal ganglia where perivascular macrophages and activated microglia appeared to be in cell-to-cell contact with neurons expressing FKN in brains with HIV-1 (Fig. 2). Neuronal expression of FKN was localized to punctate structures with a vesicular appearance in the cytoplasm of the soma (Fig. 2) and in the neuropil, in agreement with the results obtained using conventional immunohistochemistry (Fig. 1) on HIVE brain tissue. Because Fig. 2 is a digital summation of stacked images obtained by successive scans in the z direction (i.e., through the entire thickness of the section), it does not depict that, in some sections, neuronal FKN immunostaining is discrete, but contiguous with CD68-positive microglia. Identical results were obtained in brain tissue from an adult patient with HIVE (data not shown). In contrast, CD68-positive macrophages and microglia were infrequently observed in proximity to FKN-expressing neurons in patients with either HIV-1, but not HIV-PE, or control patients without HIV infection (data not shown).

To further characterize the identity of FKN-expressing neurons in brain tissue of children with HIVPE and adults with HIV-1 and HIVPE and dementia, confocal microscopy studies were performed with double immunostaining for FKN and glutamate (Fig. 3). A representative field is shown from the cerebral cortex of an adult patient with HIV-1. FKN expression was localized to punctate cytoplasmic structures in the neuronal soma. There was colocalization with cytoplasmic glutamate in FKN-expressing neurons. Superimposition of the summated confocal images revealed that FKN and glutamate may be localized to different cytoplasmic compartments in some of the neurons examined. Similar results were observed in pediatric patients with HIV-PE and PE (data not shown).

To investigate the distribution of receptors for FKN in brain tissue from pediatric patients with HIV-PE, HIV-1, or HIV-seronegative children, we employed a novel strategy because Abs to the human FKN receptor are unavailable. We used a poly(HIS)-tagged FKN variant containing a portion of the muin stalk to bind to FKN receptors in formalin-fixed brain tissue. A monoclonal anti-poly(HIS) Ab was incubated with the FKN receptor-FKN poly-(HIS)-tagged complexes. Representative results from these ICC studies are shown in Fig. 4. In pediatric brain tissue with the neuropathologic hallmarks of HIVPE, cytoplasmic staining for the FKN receptor was seen in neurons and microglia. A similar pattern of neuronal staining was observed in the HIV-1-infected pediatric brain tissue without HIV-PE, whereas limited neuronal staining of the FKN receptor was seen in pediatric brain tissue from HIV-seronegative children (data not shown). Brain tissue reacted with the chemokine-only portion of FKN lacking the poly(HIS) tag (control condition) was devoid of immunostaining when probed with the anti-poly(His) Ab (data not shown).

Because HIVPE is characterized by focal inflammatory infiltrates containing brain-resident macrophages, activated microglia, and microglial nodules, and because FKN receptor (CX3CR1) levels are up-regulated in activated microglia (13), we investigated whether FKN expression in the CNS plays a role in recruitment of monocytes from the periphery. We examined the ability of FKN to function as a chemoattractant in a human endothelial cell/astrocyte transwell bilayer. FKN induced a dose-dependent increase in
monocyte migration across this barrier, with maximal effect at 100 ng/ml (Fig. 5). FKN had a monocyte migration index of 2.85 (1–100 ng/ml). The interaction between FKN and monocytes was specific. Coincubation of FKN (10 ng/ml) with a neutralizing dose of a mixture of 5 mAbs to FKN (final concentration 1 μg/ml) completely abrogated FKN's ability to act as a chemoattractant (Fig. 5).

Neurologic disease in patients with CNS HIV-1 infection correlates with the relative numbers of brain-resident macrophages and microglia present in inflammatory infiltrates (8). Both in vitro and in vivo models of HIV-1 infection and antigenic stimulation of brain-resident macrophages demonstrate that the macrophage is a major source of neurotoxins. These include the HIV-1 gene products gp41, gp120, Tat, and cellular metabolites, including TNF-α, PAF, eicosanoids, free radical species derived from NO, and agonists for excitatory amino acid receptor subtypes (4–7, 27, 28). Since FKN served as a potent chemoattractant for monocytes

FIGURE 1. Cytoplasmic and neuropil FKN immunostaining. Panels in the upper row show FKN staining in (A) insular cortex of a pediatric patient that is seronegative for HIV; (B) insular cortex of a patient with HIV-1, but without HIVE; and (C) insular cortex of a patient with HIVE and PE, respectively. Quantitation of ROD in FKN-immunopositive neurons from each patient group is depicted in the graph below panels A–C. Lower panels show FKN expression in pencil fibers (axons) in white matter of putamen in D, a patient with HIV-1, but without HIVE; and E, a patient with HIVE and PE. Specificity of FKN immunostaining is demonstrated in F by preincubating parallel serial tissue sections from putamen in the same patient shown in E with a blocking peptide before application of FKN Ab. Note the marked reduction of cytoplasmic expression of FKN in neurons, and neuropil in the patient with HIVE. For all panels, the chromagen is Ni-enhanced DAB for FKN. Original magnification is ×160. Parallel tissue sections of perivascular inflammatory infiltrates have 10–20% p24-positive macrophages from the patient with HIVE (data not shown).

FIGURE 2. FKN and CD68 expression in cerebral cortex of a pediatric patient with HIVE and PE. Confocal sections (0.2-μm slices using a ×60 objective) from the middle of the tissue section are represented as a stacked image (total section thickness = 5 μm). (Olympus Fluoview Software). Wavelengths of 488 nm (argon) and 568 nm (krypton) were used to excite FITC- (green = CD68) and TRITC- (red = FKN) conjugated secondary Abs, respectively. Note the juxtaposition of CD68-expressing microglial processes with FKN-expressing neurons in A. FKN expression is localized to punctate structures with a vesicular appearance in neuronal soma (B); single immunostaining for FKN.
across the endothelial cell/astrocyte bilayer, we investigated whether FKN could also induce production of HIV-1-induced neurotoxic substances from monocytes. Both soluble and immobilized FKN, at doses ranging from 1–1000 ng/ml, failed to induce production of TNF-α, NO, or superoxide in cultures of primary human monocytes or monocyte-derived macrophages (data not shown).

These data, and data from a recent report by Meucci et al. (14) demonstrating that coadministration of FKN ameliorated gp120-induced toxicity to hippocampal neuronal cultures, suggested that increased expression of FKN in neurons from patients with HIVE and PE may represent a compensatory neuroprotective mechanism against HIV-1-induced neurotoxins. We tested the ability of soluble FKN or a 22-residue peptide analogue to the C-terminal region of FKN to ameliorate the neurotoxicity of two HIV-1 neurotoxins, Tat and PAF, that play key roles in initiating neuronal apoptosis (23, 29). Coincubation of soluble FKN at 100 ng/ml (the dose that induced maximal numbers of monocytes to migrate across an in vitro endothelial cell/astrocyte bilayer) was able to significantly decrease the amount of neuronal apoptosis induced by carbamyl PAF (cPAF) (48% reduction) or Tat (35% reduction) in highly purified cultures of rat cerebellar granule neurons with less than 3–5% glial contamination (Fig. 6A). Coincubation of the 22-residue peptide (100 ng/ml) was able to significantly decrease the amount of neuronal apoptosis induced by cPAF (37% reduction) or Tat (50% reduction) (Fig. 6B). These findings suggest that the C terminus of FKN plays a significant role in neuroprotection. Here cPAF was used because it is resistant to inactivating acetylhydrolases present in brain (30). Administration of either soluble FKN or the 22-residue FKN peptide alone were not significantly neurotoxic relative to control conditions. Dose-response analyses confirm that soluble FKN and the C22 analogue of FKN (FKN22) have similar effective doses (K_i for soluble FKN = 14.45 ± 5.5 ng/ml vs 55.71 ± 16.7 for the C22 FKN peptide) for ameliorating cPAF-mediated neurotoxicity in rat cerebellar granule neurons (Fig. 6C). Transformation of the data to Hill plots reveals that the nH for both soluble FKN and FKN22 is 1.0, suggesting that positive or negative cooperativity is not involved in this biologic effect.

Discussion

Postmortem studies suggest that macrophage infiltration and microglial activation contribute to the pathogenesis of neurologic disease associated with HIV-1 infection of the CNS (8). The molecular mechanisms for macrophage recruitment and microglial activation that damage populations of vulnerable neurons in the brains of patients with HIV are unclear. Studies have shown that chemokine receptors such as CCR5, CCR3, and CXCR4 are upregulated in patients with HIV-1 infection of the CNS and may serve as coreceptors for HIV-1 infection (10). The role(s) of chemokine signaling in mediating inflammation in patients with HIV are largely unknown. MIP-1α (macrophage inflammatory protein 1α) and RANTES from macrophages are present in inflammatory infiltrates in brain tissue with the histopathologic correlates of HIVE (9). Recently, Conant et al. (31) have shown that the HIV-1 regulatory protein Tat can stimulate astrocytes to release MCP-1 in

FIGURE 3. FKN and glutamate expression in cerebral cortex of an adult patient with HIVE and neurologic disease. Confocal images were recorded at intervals of 9–10.1 s in increments of 0.3-μm slices. Images are represented as a stacked image of all sections scanned (total section thickness = 10.2 μm) (as described in Fig. 2). Here FITC identifies (green = glutamate) and TRITC identifies (red = FKN) conjugated 2” Abs, respectively. Note colocalization of FKN and glutamate in neurons in two different, representative fields (upper and lower panels).

FIGURE 4. Example of cytoplasmic FKN receptor immunostaining. Here, FKN receptor staining is present in neurons and microglia (arrow) present in cerebral cortex of a pediatric patient with HIVE and PE. The chromagen is Ni-enhanced DAB for FKN receptor. Original magnification is ×160. Parallel tissue sections of perivascular inflammatory infiltrates have ~10–20% p24-positive macrophages (data not shown).

FIGURE 5. Monocyte chemotactic activities of soluble FKN and MCP-1 across an in vitro human endothelial cell/astrocyte bilayer. Transwells containing endothelial (HUVEC) cell monolayers in the upper chamber membrane and astrocyte (PHFA) monolayers in the lower chamber membrane were prepared as described in Materials and Methods. Aliquots of human monocytes were added to the upper chamber of transwells, incubated with vehicle control or increasing doses of FKN, or FKN (10 ng/ml) in the presence of a mixture of neutralizing mAbs to FKN (1 μg/ml final concentration), subsequently followed by FACS analyses of monocyte migration to the lower chamber. Results are from a typical experiment with 10 replicates of each dose of FKN, with data expressed as number of monocytes ± SEM. Experiments were replicated at least three times from different monocyte donors. Note that FKN’s effect (10 ng/ml) on monocyte migration can be completely abolished by coincubation with neutralizing mAbs.
vitro. Levels of MCP-1 are elevated in brain and cerebrospinal fluid (CSF) of patients with HIV-1-associated dementia. These data lend credence to the hypothesis that HIV-1 gene products can initiate a cycle of inflammation through chemokine production and subsequent signaling.

Since a possible functional interaction exists between neuronally expressed FKN and its receptor, CX3CR1 in activated microglia (13), we investigated whether FKN was up-regulated in brain tissue from patients with HIV-1, and whether it functioned in monocyte recruitment into the CNS. Figs. 1 and 2 show that FKN expression is up-regulated in neurons and in the neuropil of brain tissue from patients with HIV-1, compared with brain tissue from patients seronegative for HIV-1 or patients with HIV-1 but not HIV-1. It is technically difficult to quantify FKN expression in HIV-1 tissue because it is unknown at the time of dissection whether focal inflammatory infiltrates are present in the brain regions of interest (blocks of fresh-frozen tissue corresponding to the formalin-fixed tissue blocks used in this study were not available for these studies), which precludes Western blot analyses. In all cases, FKN immunostaining was abolished upon addition of a blocking peptide (based on the C terminus of FKN) to the anti-FKN Ab used in these studies. Thus, the staining results were specific. The explanation for the presence of extracellular FKN is unknown. Since data from confocal studies show that FKN is present in punctate structures with a vesicular appearance in neuronal soma and the neuropil, FKN may be released into the neuropil as a neural-immune modulator to signal brain-resident macrophage and microglia. These data show that CD68-expressing perivascular macrophages and activated microglia are in close proximity to, and may actually be in cell-to-cell contact with, neurons expressing FKN (Fig. 2), in agreement with another model of neuronal FKN signaling to microglia after neuronal injury (13).

Unlike HIV-1, facial nerve axotomy is a model of microglial activation without a strong inflammatory response. Here FKN mRNA is decreased, and perineuronal microglia expressing the FKN receptor (CX3CR1) are increased (13). In this model it is unclear whether the total protein changes, but lower m.w. species (presumably secreting FKN) increase when measured by Western blot. Our data suggest that levels of FKN expression in neurons correlate with inflammation in patients with HIV-1. To further explore the relationship between FKN mRNA and protein levels in areas of focal brain inflammation, it will be necessary to use animal models for lentiviral infection such as simian immunodeficiency virus (SIV) or the SCID mouse model of HIV-1 (28).

Our confocal data demonstrate that increased expression of FKN in vesiculoid cytoplasmic structures in neurons in brain tissue with HIV-1 are consonant with the idea that FKN, in addition to other chemokines such as MCP-1 (31), may be another signal for monocyte recruitment into the CNS. This hypothesis is confirmed by the data in Fig. 5, which show that FKN is a potent chemoattractant in an in vitro model of an endothelial cell/astrocyte bilayer. FKN’s role as a chemoattractant is confirmed by coincubation of FKN with neutralizing mAbs to FKN with no subsequent monocyte migration across endothelial/astrocyte transwell membranes (Fig. 5). Further studies are necessary to resolve the relative contribution between soluble and membrane-bound FKN in initiating monocyte recruitment and microglial activation into the CNS.

The neuroprotective role of FKN is shown by the marked reduction in neuronal apoptosis after coincubation of FKN with cPAF or Tat (Fig. 6). Excessive production of PAF in the CNS is regulated by TNF-α and correlates with neurologic disease and immunosuppression (7, 29) and PAF may play a pivotal role in induction of neuronal apoptosis via glutamatergic mechanisms (7). The HIV-1 regulatory protein Tat also induces neuronal apoptosis, in part through a mechanism that involves production of TNF-α and activation of glutamatergic mechanisms (22, 24). These data, combined with the ability to ameliorate gp120-induced neuronal apoptosis in rat hippocampal cultures with and without glia (14), suggest that FKN-mediated activation of CX3CR1 receptors results in amelioration of neuronal apoptosis from structurally diverse HIV-1 neurotoxins that signal through different pathways. Reduction of neuronal apoptosis induced by either cPAF or Tat resulted from coincubation with either soluble FKN or the C-terminal containing peptide, suggesting that this region is important in neuroprotection. Our data demonstrate colocalization of FKN and glutamate in neurons in brain tissue of patients with HIV-1, suggest that FKN may function as a neuroprotective chemokine via a paracrine mechanism. FKN may activate CX3CR1 receptors located directly on neurons as opposed to microglia, since the rodent CGN cultures used in our experiments lack significant numbers of astrocytes or microglia. One intriguing area for further investigation is whether FKN regulates the release of presynaptic glutamate and thus controls the amount of glutamate available in the synapse to induce excitotoxic stimulation of vulnerable neurons.

These data suggest that, in HIV-1, FKN may function as a neuroimmune modulator that is released from cytoplasmic neuronal structures with a vesicular appearance into the neuropil, and may
participate in recruiting peripheral monocytes into the CNS, and possibly mediate cell-to-cell contact between FKN and neurons and microglia adjacent to focal inflammatory infiltrates. Despite the fact that macrophage recruitment into the CNS may increase the potential for productive HIV-1 infection, our data suggest that FKN may serve a neuroprotective role by interfering with HIV-1 neurotoxin induction of neuronal apoptosis. However, it is unlikely that FKN blocks neuronal apoptosis in HIV by direct antagonism of HIV-1-induced neurotoxins, since PAF, Tat, and gp120 (14) can initiate neuronal apoptosis by distinct signaling pathways that may involve both glia and neurons. A more likely possibility is that FKN blocks apoptosis at a level that is downstream from neuronal or glial signaling by these neurotoxins or related intermediaries, possibly by interfering with glutamate release. Equally intriguing, FKN may promote production of glial protective factors that influence neuronal survival in neurons exposed to HIV-1 neurotoxins. Thus strategies to modulate or stimulate FKN signaling may have important therapeutic implications in the treatment of HIV-1 associated neurologic disease.

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