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

Ning Tong,[‡] Seth W. Perry,[†‡¶] Qing Zhang,[‡] Harold J. James,[‡] Huang Guo,[‡] Andrew Brooks,[§] Harshawardhan Bal,[§] Sandra A. Kinnear,[‡] Steven Fine,[§] Leon G. Epstein,[**] Daniel Dairaghi,[††] Thomas J. Schall,[††] Howard E. Gendelman,[#] Stephen Dewhurst,[§] Leroy R. Sharer,[‡‡] and Harris A. Gelbard[2*†‡¶‖]

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.**

Human immunodeficiency virus-1 encephalitis (HIVE)[3] is a well-characterized neuropathologic entity associated with a progressive dementia in adults and encephalopathy in children. HIVE is characterized by productive infection of mononuclear phagocytes (MPs) with no evidence of cytolytic infection of neurons. However, vulnerable neurons will undergo apoptosis in brain regions where HIVE is present (1–3). Neurologic disease associated with HIVE is thought to occur because HIV-1-infected MPs produce soluble neurotoxic factors that contribute to neuronal dysfunction and death (4–7). However, the role of viral burden in the pathogenesis of neurologic disease is less clear. There is a greater correlation between neurologic disease and numbers of macrophages and microglia in inflammatory infiltrates than viral burden in patients with HIV-1 infection of the CNS (8).

Several classes of chemokine receptors (CCR5, CXCR4) may function as coreceptors for HIV-1 in the CNS and have helped to identify populations of CNS glia that are at risk for infection (9, 10). Macrophages in areas with the histopathologic correlates of HIVE express β chemokines, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, and RANTES, suggesting a role for chemokines in formation of inflammatory infiltrates and microglial nodules (9). Deletion of the CXCR4 gene in transgenic mice results in abnormalities of cerebellar neuronal migration (11). Thus, chemokine receptor activation may modulate development of the CNS and mediate immunologic communication between glia and neurons.

Fractalkine (FKN) is a novel chemokine derived from non-hemopoietic cells. It is a large (373 residues) protein that contains the chemokine domain attached to a mucin-like stalk (12). FKN exists in a soluble form with potent chemoattractant activity for T cells and monocytes, and in a membrane-bound form that is induced on activated endothelial cells, and promotes leukocyte adhesion (12). FKN is predominantly expressed in neurons in the CNS. Its receptor, CX_3CR1, is expressed in microglia (13). Administration of FKN substantially ameliorates gp120-induced neuronal apoptosis in cultured rat hippocampal neurons (14), which suggests that FKN may play an important role in modulating the neuropathogenesis of HIV-1. We demonstrate for the first time that FKN expression is

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[3] Abbreviations used in this paper: HIVE, HIV-1 encephalitis; ABC, avidin-biotin complex; PAF, platelet-activating factor; cPAF, carbamyl PAF; FKN, fractalkine; GFAP, glial fibrillary acidic protein; HIS, histidine; MP, mononuclear phagocytes; PE, progressive encephalopathy; PHFA, purified human fetal astrocytes; TRITC, tetramethylrhodamine isothiocyanate; ICC, immunocytochemistry; DAB, diaminobenzidine; ROD, relative OD.

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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 HIVE 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 symptoms of neurologic disease) with HIVE and one adult case (male, 41, 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 processed as previously 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 (Chemocentryx, 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 alkaline phosphatase or diaminobenzidine (DAB) chromagen. For FKN receptor ICC, tissue sections were incubated with either a human FKN variant that contained a small portion of the mucin stalk and a poly histidine (HIS) tag (Chemocentryx, 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 (Chemocentryx, 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 glutamatergic neurons. Glial 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; Dako) was used to identify MPs, and HIV-1 antiserum to p24 (1:500 in normal horse serum; American Bio-Technologies, Cambridge, 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). Ag retrieval was performed to obtain optimal signals from the Abs tested 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-immunopositive neurons were identified for the HIV^- group, 426 FKN immunopositive neurons were identified for the HIV^+ group, and 522 FKN immunopositive neurons were identified for the HIVE 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 in 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 (60 × 1.4 oil-immersion PlanApo objective). Confocal sections were scanned in the center of the tissue section and are represented as a stacked image of all sections (Olympus Fluoview Software). Wavelengths of 488 nm and 568 nm were used to excite fluorescein- and rhodamine-conjugated secondary Abs, respectively. Emission spectra were collected with 510-nm and 560-nm bandpass filters to achieve minimal overlap of fluorophore detection for accurate double labeling. Digitized images from representative fields were prepared from TIFF files (Adobe Photoshop 4.0, Seattle, WA).

Cell culture and preparation of endothelial cell/astrocyte bilayers

Purified human fetal astrocytes (PHFAs) were isolated from second-trimester human fetal brain tissue obtained from elective abortions (performed in full compliance with both National Institutes of Health (NIH) and University of Rochester guidelines), using methods previously described (17). The purity of the PHFA cultures was determined by ICC for GFAP. Cultures were used for experiments only if >97% of cells were GFAP immunopositive. HUVECs (>99% factor VIII-immunoreactive) were obtained frozen from Cascade Biologics (Portland, OR). Twenty-four-well transwell inserts were first coated on the upper chamber side with rat-tail collagen (50 μg/ml; Collaborative Biomedical Products, Bedford, MA), then coated on both sides with human fibronectin (500 ng/ml; Life Technologies, Rockville, MD) (18). PHFAs were plated on the lower chamber side in an inverted position at 1×10^5 cells/100 μl for 2 h for cell adherence. The transwell was placed upright, and HUVECs were then plated at a density of 0.5×10^5 cells/200 μl as previously described (19, 20). Transwell cultures were maintained in Medium-200 (Cascade Biologics) at 37°C, in an atmosphere containing 5% CO_2 for ≥5 days before use, with daily changes of medium. For monocyte migration experiments, human monocytes were recovered from PBMCs of HIV- and hepatitis B-seronegative donors after leukapheresis and purified (>98%) by countercurrent centrifugal elutriation as previously described (4). Monocytes were adjusted to 1.5×10^5 cells/200 μl, and 200 μl was placed in the upper transwell. Doses of soluble human FKN (no mucin stalk; Chemocentryx), neutralizing mAbs (IgG1 and IgG2b, clones 21, 28, 29, 36, 37, and 43; Chemocentryx) to FKN, MCP-1 (R&D Systems, Minneapolis, MN), or vehicle were placed in the lower chamber of the transwell, and the chambers were returned to a 37°C, 5% CO_2 incubator for 4 h. Then 400-μl aliquots from the bottom transwell chamber were combined with FITC CD14 (PharMingen, San Diego, CA). The total number of monocytes migrating from upper to lower wells through the bilayer was counted for 60 s on a FACStar^{Plus} (flow cytometry) gating for monocytes (21). Ten replicates were performed for each concentration of FKN, and experiments were repeated three times.

Production of Tat

Recombinant HIV-1 Tat_{1-86} was expressed and purified as a thrombin cleaved GST fusion protein (22) and stored at −70°C. Tat_{1-86} was characterized and quantified using the Lowry method, SDS-PAGE, and by immunoblot analysis, using a polyclonal Ab (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (NIAID), NIH) (22). To control for contaminating bacterial proteins that might elicit neurotoxicity, an *Escherichia coli* strain containing a GST expression plasmid without the Tat_{1-86} sequence was used to prepare extracts, which were purified in the same manner as the Tat fusion protein and used as vehicle control.

Neuronal cell culture and quantitative analyses of cell death

Cerebellar brain tissue from 7 day Sprague Dawley rats was harvested according to the guidelines of the Animal Welfare Act (1987) and NIH policies. Briefly, cerebellum was trypsinized in 0.25 mg/ml and 0.1% DNase (∼10 ml volume/cerebellum), minced into 2 mm^3 pieces, triturated, and incubated for 20 min at 37°C (23). The tissue was filtered through nylon mesh, and the cell suspension was loaded over a two-step percoll gradient and centrifuged at 1000 × *g* for 15 min at 4°C to remove glia. Neurons were collected, washed twice in serum-free medium, then resuspended in fresh DMEM:F12 + 10% horse serum. Cells were triturated and plated 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 culture, fUDR (5-fluoro-deoxyuridine) was added to the cultures at 20 mg/ml and uridine at 50 mg/ml, to eliminate astrocytes. The purity of the neuronal population was verified by ICC for neuronal markers. Under these conditions, neuronal cultures have <3–5% glial contamination. Neurons were cultured for ≤7 days at 37°C in 5% CO_2. Serum-free DMEM:F12 was replaced every 3 days.

In situ detection of apoptotic neurons by TUNEL stain

Neurons cultured on 12mm poly(L-lysine)-coated coverslips were treated with vehicle or reactions, and apoptotic cells were stained using an in situ

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TdT-mediated digoxigenin-TUNEL assay method (Oncor, Gaithersburg, MD) as previously described (22). Cell cultures were fixed in 4% paraformaldehyde, rinsed with PBS, postfixed with a 100% ethanol:acetic acid solution (2:1), and rinsed with PBS. Neurons were pretreated with 2% H_2O_2 to quench endogenous peroxidase, before the addition of TdT. Anti-digoxigenin-peroxidase was added and catalytically reacted with 0.05% DAB in PBS. TUNEL-stained neurons were counted from 15 randomly selected fields. Each field of at least 100 neurons was counted for immunoreactive cells. Data are expressed as the percentage of TUNEL-positive neurons per high power field. For dose-response studies, data were analyzed using Sigmaplot (Vs. 3.0) to obtain the resulting sigmoidal curves using the following equation: $Y = (V_{max} \times (1 - (x/(x + K_i))) + \text{os}$. Inhibitor values ($K_i$) for soluble FKN (sFKN) and the C22 analogue of FKN (FKN22), as well as Hill coefficients (nH) were then determined from the resulting dose-response curves.

Measurement of TNF-α, NO, and superoxide anion

TNF-α was measured in primary human monocyte or monocyte-derived macrophage cultures exposed to FKN species by ELISA (R&D Systems) as previously described (24). NO production was estimated by Griess reaction as previously described (25). Superoxide anion was measured in cultures of human monocytes or monocyte-derived macrophages (4, 15) using the Lumimax superoxide anion detection kit (Stratagene, La Jolla, CA). Briefly, cultures exposed to either soluble or immobilized FKN were then reacted with PMA, a bioactive peptide (fMLP), or opsonized zymosan capable of inducing superoxide anion. Luminol and the proprietary enhancer were added to a final concentration of 100 mM and 125 mM, respectively. Results (compared with a standard curve) were read on a luminometer.

Results

Immunocytochemical analysis of cerebral cortex and basal ganglia revealed the greatest levels in cellular expression of FKN in pediatric patients with HIVE (HIVE is defined by morphologic demonstration of multinucleated giant cells and expression of p24 in macrophages and microglia in focal inflammatory cell infiltrates; Ref. 26 and data not shown) and neurologic disease referred to as PE**,** compared with patients with HIV-1 alone and patients who were seronegative for HIV. Representative high power fields from a patient seronegative for HIV (Fig. 1*A*.), a patient with HIV-1 without either encephalitis or PE (Fig. 1*B*.), and a patient with HIVE (Fig. 1*C*.) are shown in Fig. 1. Little or no FKN was detected in astrocytes, macrophages, microglia, or oligodendrocytes. In all patient groups, neurons were the predominant cell type to express FKN, which was localized to the cytoplasm. In the patient with HIVE, immunopositive neurons were present in the insula of the temporal lobe (Fig. 1*C*), caudate, putamen, claustrum, and globus pallidus. In the caudate and putamen of the HIVE case, small neurons were immunopositive for FKN, but large neurons did not express FKN (Fig. 1, *E* and *F*). A similar pattern of FKN staining was seen in the insular cortex, globus pallidus, and claustrum of the patient with HIV-1, but not HIVE (Fig. 1*B*). To quantitate the relative difference in FKN immunostaining between patient groups, we performed computerized morphometry of the ROD of immunostaining in neurons expressing FKN. The mean ROD of FKN immunostaining in neurons from HIVE brain tissue was 0.709 ± 0.011 vs 0.590 ± 0.005 for neurons in HIV-1 brain tissue vs 0.347 ± 0.004 for neurons in HIV^- brain tissue (all comparisons between groups significant at $p < 0.001$). Thus, mean ROD of FKN immunostaining was 2.04 times greater than the mean ROD of FKN immunostaining in neurons from HIV^- brain tissue, but only 1.2 times greater than the mean ROD of FKN immunostaining in neurons from HIV-1 brain tissue (Fig. 1).

FKN expression was also markedly up-regulated in the neuropil of patients with HIVE, compared with controls. In support of this finding, white matter pencil fiber bundles (i.e., axons) in the putamen of the patient with HIVE were strongly immunopositive for FKN, which may account, in part, for the darker appearance of the neuropil in the HIVE brain (Fig. 1*E*). 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. 1*F*). 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 HIVE (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 HIVE and 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 HIVE and PE and adults with HIVE 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 HIVE. 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 HIVE and PE (data not shown).

To investigate the distribution of receptors for FKN in brain tissue from pediatric patients with HIVE, 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 mucin 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 HIVE, 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 HIVE, 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 HIVE is characterized by focal inflammatory infiltrates containing brain-resident macrophages, activated microglia, and microglial nodules, and because FKN receptor (CX_3CR1) 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

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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).

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

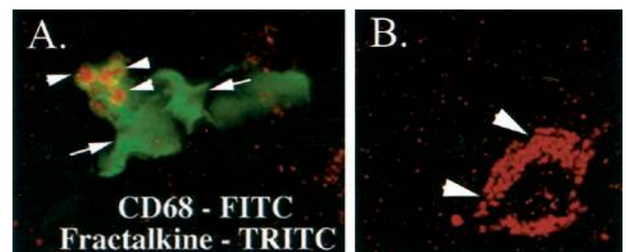

FIGURE 2. FKN and CD68 expression in cerebral cortex of a pediatric patient with HIVE and PE. Confocal sections (0.2-μm slices using a ×60 objective) from the middle of the tissue section are represented as a stacked image (total section thickness = 5 μm). (Olympus Fluoview Software). Wavelengths of 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).

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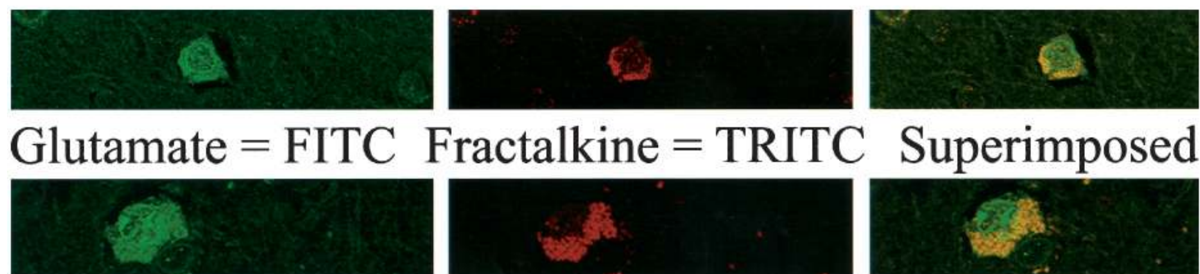

FIGURE 3. FKN and glutamate expression in cerebral cortex of an adult patient with HIVE and neurologic disease. Confocal images were recorded at intervals of 9–10.1 s in increments of 0.3-μm slices. Images are represented as a stacked image of all sections scanned (total section thickness = 10.2 μm) (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*).

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. 6*A*). 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. 6*B*). 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. 6*C*). 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.

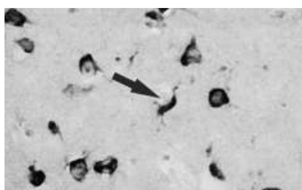

FIGURE 4. Example of cytoplasmic FKN receptor immunostaining. Here, FKN receptor staining is present in neurons and microglia (arrow) present in cerebral cortex of a pediatric patient with HIVE and PE. The chromagen is Ni-enhanced DAB for FKN receptor. Original magnification is ×160. Parallel tissue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

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 HIVE 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 HIVE 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

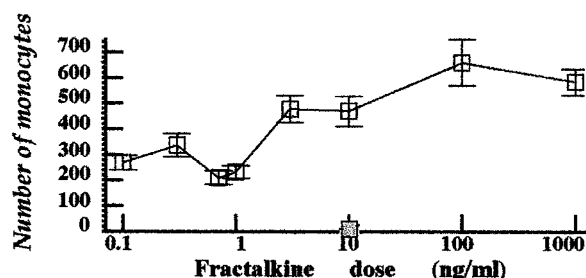

FIGURE 5. Monocyte chemotactic activities of soluble FKN and MCP-1 across an in vitro human endothelial cell/astrocyte bilayer. Transwells containing endothelial (HUVEC) cell monolayers in the upper chamber membrane and astrocyte (PHFA) monolayers in the lower chamber membrane were prepared as described 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.

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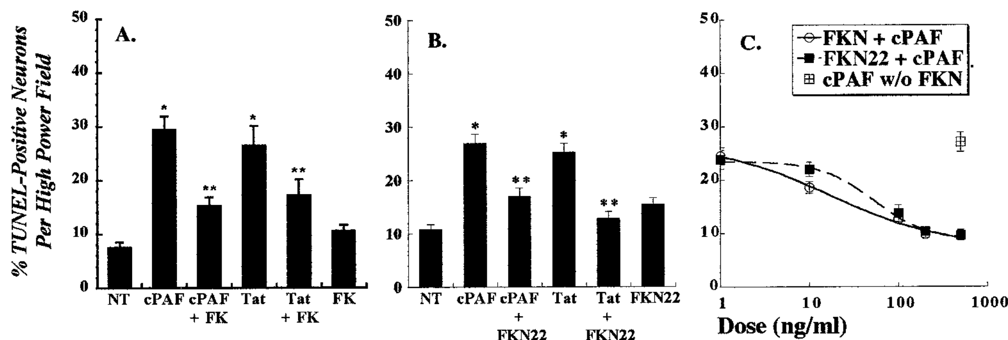

FIGURE 6. Soluble FKN (FK) and a 22-residue FKN peptide can ameliorate cPAF or HIV-1 Tat-induced neuronal apoptosis. Cultures of rat cerebellar granule neurons were grown for 7 days (*Materials and Methods*) before treatment with either vehicle, cPAF (125 ng/ml, Biomol, Plymouth Meeting, PA), or recombinant Tat_{1-86} in the presence and absence of either soluble FKN (*A*) or a 22-residue peptide containing the C terminus of FKN (FKN22; Ref. 12) (100 ng/ml; *B*) for 24 h. *C*, Dose-response curves for soluble FKN and FKN22 in ameliorating neurotoxicity from cPAF (250 ng/ml) in 7-day-old rodent cerebellar granule neuronal cultures. Cultures were fixed, TUNEL-stained, and analyzed for TUNEL-positive neuronal cells (see *Materials and Methods*). *, $p < 0.01$ from vehicle control; **, $p < 0.01$ from either cPAF- or Tat-treated cultures. Data shown are from a single, representative experiment repeated three times.

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, CX_3CR1 in activated microglia (13), we investigated whether FKN was up-regulated in brain tissue from patients with HIVE, 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 HIVE, compared with brain tissue from patients seronegative for HIV-1 or patients with HIV-1 but not HIVE. It is technically difficult to quantify FKN expression in HIVE 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 HIVE, 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 (CX_3CR1) are increased (13). In this model it is unclear whether the total protein changes, but lower m.w. species (presumably secreted FKN) increase when measured by Western blot. Our data suggest that levels of FKN expression in neurons correlate with inflammation in patients with HIVE. 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 HIVE (28).

Our confocal data demonstrate that increased expression of FKN in vesiculoid cytoplasmic structures in neurons in brain tissue with HIVE 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 CX_3CR1 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. Data from in vitro studies, and our data demonstrating colocalization of FKN and glutamate in neurons in brain tissue of patients with HIVE, suggest that FKN may function as a neuroprotective chemokine via a paracrine mechanism. FKN may activate CX_3CR1 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 HIVE, FKN may function as a neuroimmune modulator that is released from cytoplasmic neuronal structures with a vesicular appearance into the neuropil, and may

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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 HIVE 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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