Amelioration of Neurotoxic Effects of HIV Envelope Protein gp120 by Fibroblast Growth Factor: A Strategy for Neuroprotection

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Abstract. Approximately two thirds of patients with human immunodeficiency virus encephalitis (HIVE) show cognitive impairment and neurodegeneration, while one third are cognitively unimpaired and their neuronal populations are preserved. Thus, it is possible that these individuals might have the capacity to produce neurotrophic factors capable of protecting neurons against the deleterious effects of HIV. In this context, the main objective of this study was to determine whether fibroblast growth factor 1 (FGF1) is protective against HIV. For this purpose levels of FGF1 immunoreactivity were determined in the frontal cortex of 35 AIDS cases subdivided into 4 groups according to the presence or absence of HIVE and neurodegeneration. In cases without both HIVE and neurodegeneration, mild to moderate levels of FGF1 immunoreactivity were observed in pyramidal neurons, while in cases with HIVE but without neurodegeneration, levels were significantly elevated. In contrast, individuals with both HIVE and neurodegeneration showed low levels of neuronal FGF1 immunoreactivity.

Key Words: FGF1; gp120; HIV encephalitis; Neurodegeneration; Neuroprotection; Primary human neuronal cultures.

INTRODUCTION

Neurocognitive alterations in patients with AIDS continue to be a significant problem (1–4), yet to date there are no targeted therapeutic strategies to protect the central nervous system (CNS) and prevent neuronal damage and death due to the human immunodeficiency virus (HIV) infection. Pathologically, the brain is affected by spectrum of inflammatory changes, dendritic and synaptic damage, and neuronal loss (5). Increasing viral load is associated with worsening neuronal damage, which correlates with onset of early cognitive impairment (6, 7). However, the relationship between cognitive impairment, HIV encephalitis (HIVE), and neurodegeneration is complex since not all patients with HIVE show cognitive impairment and degeneration (8). This might indicate that this latter group of individuals has the capacity to produce neurotrophic factors able to protect neurons against the deleterious effects of HIV. Among them, recent studies suggest that fibroblast growth factor (FGF) might play a central role as neuroprotective factor (9–11) in a variety of disorders, including AIDS. For example, in patients with Kaposi’s sarcoma (KS), high expression of FGF (12) by the tumor is associated with a decreased risk for neuronal degeneration and neurological impairment (13).

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Other studies have shown that FGF modulates the HIV-related chemokine receptor CXCR4 (14, 15), and FGF2 is widely expressed by astrocytes in patients with HIVE (16).

There are 9 known (17) and up to 20 possible (18) members of the FGF family. They are 150–250 amino acids long and bind to 4 FGF receptors and heparin. In addition to playing a crucial role during normal development (19), FGFs maintain adult tissues and are pivotal in wound healing and repair (20, 21). High levels of FGF1 and FGF2 are present in the CNS (17, 22), and while FGF1 is produced by neurons, FGF2 is produced by astroglial cells. FGF1 and FGF2 are 55% homologous and do not have the N-terminal hydrophobic moiety secretory signal sequence, suggesting that these proteins are not secreted by the conventional endoplasmic reticulum-Golgi pathway (23, 24). In the CNS, the reported actions of FGFs include mitogenesis in astrocytes (25), oligodendrocytes (26), nerve fiber outgrowth (27), and promotion of survival of cerebral cortical and hippocampal neurons (28–30). The range of neuronal populations supported includes glutamatergic neurons and cortical calcium binding protein immunopositive neurons (31, 32); this latter population is susceptible to HIV-mediated damage (33).

In this context, the main objective of this study was to determine if FGF1 is protective against the neurotoxic effects of HIV. For this purpose, levels of FGF1 immunoreactivity were determined in the frontal cortex of AIDS cases and correlated with levels of neurodegeneration. Furthermore, primary human neuronal cultures were treated with the neurotoxic HIV envelope protein-gp120 in the presence or absence of FGF1.
MATERIALS AND METHODS

Samples and Neuropathological Subgroups

A total of 35 individuals (28–54-years-old) enrolled in a longitudinal study at the UCSD/San Diego HIV Neurobehavioral Research Center (HNRC) were selected for the present study (7) (Table). In most cases, patients died as a result of acute bronchopneumonia and/or septicemia, with the autopsy performed within 24 h of death (Table). All cases had detailed antemortem, neuromedical, and neuropsychological examinations. Information obtained from the detailed neuropathological data available was used to exclude cases with opportunistic infections and malignant neoplasms in the CNS and anoxic brain injury. After a macroscopic examination, brain tissue was removed from the right dorsolateral mid-frontal cortex (Brodmann’s area 45 and 46), fixed overnight in 4% paraformaldehyde, and sectioned at 40 μm with a Leica VibeRatome 2000 for subsequent immunocytochemical analysis with antibodies against FGF1 (Sigma-Aldrich, St. Louis, MO) and microtubule-associated protein 2 (MAP2, dendritic marker) (Roche Biochemicals, Indianapolis, IN).

The AIDS cases were subdivided into 4 neuropathological groups depending on the presence/absence of HIVE and/or neuronal damage, and levels of FGF1 expression were compared among the groups. Briefly, as previously described (6), the diagnosis of HIVE was based on the presence of multinucleated giant cells (MNGCs), gp41-immunoreactive microglial cells, and determination of viral load; cases with a score of 3 or more were diagnosed as having HIVE (8). Neuronal damage was assessed, as previously described (6), by estimating the percent area of the mid-frontal cortex neuropil covered by MAP2-immunoreactive dendrites (6). Cases with a score of less than 19% were considered to have neuronal damage (ND). Previous studies have shown that while in control individuals with neurologic characterization the area of the neuropil covered by MAP2-immunoreactive dendrites is usually above 19.0%, individuals with HIVE displaying cognitive impairment usually had MAP2 values below 19.0% (6). Based on the criteria outlined above the following groups were obtained: 1) without both HIVE and neuronal damage (Group I, HIVE-/ND-) (n = 10); 2) without HIVE, with neuronal damage (Group II, HIVE-/ND+) (n = 6); 3) with HIVE, without neuronal damage (Group III, HIVE+/ND-) (n = 8); and 4) with both HIVE and neuronal damage (Group IV, HIVE+/ND+) (n = 11). The Table summarizes the clinicopathological characteristics of the cases.

### Analysis of FGF1 Immunoreactivity in the Frontal Cortex of AIDS Patients

Briefly, as previously described (15, 34), vibratome sections were incubated with the mouse monoclonal antibody against FGF1 (1:250, Sigma), followed by the FITC-tagged anti-mouse secondary antibody (1:75, Vector Laboratories, Burlingame, CA). Sections were mounted onto SuperFrost slides (Fisher Scientific, Tustin, CA) with VectaShield mounting medium (Vector) and imaged with the laser scanning confocal microscope (LSCM, BioRad 1024). For each case, 3 sections (4 fields each) were imaged. Essentially, FGF1-immunoreactive neurons were sampled within a volume by optically sectioning at 10 μm (7). Digitized images of the individual FGF1-immunolabeled neurons were traced and pixel intensity was calculated with the Image 1.43 program running on a Macintosh personal computer (15, 34). For each case, approximately 100 neurons per section were analyzed and the individual values were averaged. The 3-dimensional numerical densities of FGF1-immunostained neurons were estimated, as previously described (7), by the stereological “disector,” which consists of 2 parallel grids at a known distance apart. All objects falling within the space encompassed by the grids are counted. On the LSCM an image of neurons was obtained and then a second twin image was captured at the same x-, and y-coordinates, but at a greater depth of 2 μm. The number of objects present was then counted by superimposing the 2 images. The procedure was repeated 12 times per case, and the mean used in statistical analyses. For each case, approximately 100 neurons per section were analyzed and the individual values were averaged.

### Western Blot Analysis

Briefly, as previously described (34) snap-frozen samples were homogenized and separated into cytosolic and particulate fractions. From each group, 4–5 cases were selected based on the availability of frozen tissue. Twenty-five μg of each fraction was loaded onto 10% SDS/PAGE gels. Gels were blotted onto nitrocellulose paper, and incubated overnight with the monoclonal antibody against FGF1 (1:2000, Sigma), followed by rabbit anti-mouse IgG and 1251-protein A. Levels of FGF1 immunoreactivity were determined with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The experiment was repeated to assess reproducibility. To correct for variations in loading across samples, blots were also incubated with a mouse monoclonal antibody against actin (Chemicon, Temecula, CA).
and final values were expressed as ratios of (specific signal-background)/(actin signal-background).

Preparation and Maintenance of Primary Human Neuronal Cultures

Since previous studies have shown that primary fetal neuronal cultures are sensitive to the toxic effects of gp120, cultures were established from fetal tissue obtained from pregnancy terminations at King’s Healthcare NHS Trust (UK) with the approval of the ethical committees of the Bethlem and Maudsley NHS Trust and King’s Healthcare NHS Trust. Brain tissue was placed in holding medium (HM) and prepared for culture within 2–3 hours of clinical collection. The HM contains modified Hank’s Balanced Salt Solution (HBSS) without calcium chloride and magnesium sulphate, 2 mM glutamine, 10 mM HEPES buffer, and 20 μg/ml Gentamycin sulphate (35). Since fetal neurons are particularly sensitive to standard digestive enzymes, they were not used in preparation of single-cell suspensions from tissue (35). Tissue clumps were mechanically disaggregated with a scalpel and passed through a Pasteur pipette 10–15 times. Once the large tissue clumps settled to the bottom of the universal, the single-cell supernatant was passed through a sterile 200 μm nylon mesh (Stanier Co Ltd, Manchester, UK), ensuring that any remaining clumps were removed. The suspension was then diluted to 1x10^5–2x10^6 cells/ml in Neurobasal medium with 2 mM Glutamine, B27 supplement, and 10 μg/ml Gentamycin sulphate. This medium is specifically designed as a chemically defined medium for neuronal cultures which minimizes the growth of glial cells (36, 37). Cells were seeded as a chemically defined medium for neuronal cultures which contains 5% CO₂. For immunocytochemical analysis, cells were fixed for 25 min at room temperature with 4% paraformaldehyde. Cells were then incubated with the monoclonal antibodies against FGF1 (1:200, Sigma), MAP2 (1:50, dendritic marker, Roche), glial fibrillary acidic protein (GFAP, 1:500, astroglial marker, Chemicon) or SMI32 (1:1000, marker of unphosphorylated neurofilaments, Sternberger Monoclonals, Baltimore, MD). Cells were also immunolabeled with the rabbit polyclonal antibody against FGFRI (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Following an overnight incubation at 4°C in primary antibodies, coverslips were incubated with the FITC-conjugated secondary antibodies, air-dried, placed cell-side-up on slides and imaged with the LSCM as described previously (15, 34).

Immunocytochemical Characterization of Primary Human Neuronal Cultures

After 30 days in culture, 3x10⁵ cells were seeded onto 18 mm poly-L-lysine (70–150K, Sigma)-coated coverslips and grown for up to 7 days at 37°C in a humidified atmosphere containing 5% CO₂. For immunocytochemical analysis, cells were fixed for 25 min at room temperature with 4% paraformaldehyde. Cells were then incubated with the monoclonal antibodies against FGF1 (1:200, Sigma), MAP2 (1:50, dendritic marker, Roche), glial fibrillary acidic protein (GFAP, 1:500, astroglial marker, Chemicon) or SMI32 (1:1000, marker of unphosphorylated neurofilaments, Sternberger Monoclonals, Baltimore, MD). Cells were also immunolabeled with the rabbit polyclonal antibody against FGFRI (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Following an overnight incubation at 4°C in primary antibodies, coverslips were incubated with the FITC-conjugated secondary antibodies, air-dried, placed cell-side-up on slides and imaged with the LSCM as described previously (15, 34).

Analysis of Cell Viability in Primary Cultures Exposed to gp120 in the Presence/Absence of FGF1

In order to determine whether FGF1 is neuroprotective against HIV-gp120 neurotoxicity, cultures were first treated for 24 h with FGF1 (Sigma, 0.1 or 1.0 pg/ml). Then, gp120 (100 ng/ml, obtained from Dr. N. Haigwood through the NIBSC AIDS Reagents Project) was added to the media without washing out the FGF1. Cultures were maintained in FGF1/gp120 for 6 days and then analyzed for cell viability. Control groups consisted of treatment with FGF1 alone, gp120 alone, or no treatment. All experiments were done in triplicate. The doses of FGF1 and gp120 were based on preliminary studies where cultures were treated with increasing doses of FGF1 (0.1–100 pg/ml) or gp120 (1–1000 ng/ml) using the lactate dehydrogenase (LDH) release assay to determine cell viability. Furthermore, previous studies have shown that, at comparable levels, gp120 is toxic to neural and endothelial cell cultures and that these models are relevant for studies of neuroprotection and neurotoxicity associated with HIV (15, 40–44). The LDH release assay (CytoTox96, Promega, Madison, WI) is based on the fact that leakage of this enzyme into the extracellular fluid is detected when integrity of cellular membranes is compromised (45). Then, LDH release is inversely proportional to an intact mitochondrial membrane potential and proportional to total protein released from cells (46), providing an accurate index of membrane integrity and presence of cytotoxicity. Briefly, cells were grown in a 96-well plate and prior to each assay, 1 row of the plate was lysed with 20 μl of 9% Triton X-100 in 180 μl of culture media for 45 min at 37°C. The absorbance obtained from this selected row gives the maximal LDH release for the cells in each experiment (i.e. positive control with 100% toxicity). Once cells in this row were permeabilized, 50 μl of cellular supernatant from each well were placed in a fresh 96-well plate to estimate levels of LDH release. A row with 50 μl of pure culture medium without cells was used to determine background absorbance levels of the culture media. Fifty 50 μl of reconstituted substrate mix (i.e. diaphorase buffer) was added to each well and incubated for 30 min at room temperature in the dark. Subsequently, 50 μl of stop solution was added and the colorimetric change was measured as absorbance at 490 nm with a Dynatech MR700 plate reader. The viability data were transformed into % of the LDH released from control conditions for each experiment.

Statistical Analysis

All experiments were done blind-coded and the code was broken after results were obtained and data were analyzed using the StatView program (SAS Institute, Inc., Cary, North Carolina). For comparisons of levels of FGF1 immunoreactivity among the 4 neuropathological groups, one-way ANOVA with post-hoc Dunnett’s or Tukey-Kramer was performed. Similarly, for analysis of the in vitro results one-way ANOVA with post-hoc Dunnett’s was used. In addition, linear regression analysis was also performed. All results were expressed as mean ± SEM.

RESULTS

Patterns of FGF1 Immunoreactivity Are Altered in the Frontal Cortex of Patients with HIVE

In the 4 groups, the antibody against FGF1 immunolabeled the pyramidal neurons, but not the astroglial cells (Fig. 1). In order to assess differences among the 4
Fig. 1. Laser scanning confocal microscopic analysis of FGF1 immunoreactivity in the frontal cortex. A: In control Group I (HIVE-/ND-) and (B) Group II (HIVE-/ND+) pyramidal neurons showed mild FGF1-immunostaining. C: In cases from Group III (HIVE+/ND-) there was increased FGF1-immunoreactivity, in Group IV (HIVE+/ND+) there was mild neuronal immunolabeling (D). HIVE = HIV encephalitis, ND = neuronal damage. Magnification: ×930.

groups in levels of FGF1 immunoreactivity, analysis of pixel intensity was performed. This study showed that cases from Group III (HIVE+/ND-) displayed the highest levels of immunoreactivity compared to Groups I (HIVE-/ND-), II (HIVE-/ND+), and IV (HIVE+/ND+). Analysis of variance showed that, compared to Group I (control), levels of FGF1 immunoreactivity in Group III were significantly different (post-hoc Dunnett’s p < 0.05). No significant differences were observed among Groups I, II, and IV. In order to determine if alterations in the levels of FGF1 immunoreactivity were indeed associated with a neuropathological group not due to postmortem time (PMT), linear regression analysis was performed. This study showed no significant correlations between FGF1 immunoreactivity and PMT. To determine if the changes in the pixel intensity were accompanied by alterations in the numbers of FGF1-immunoreactive neurons, stereological assessment was performed. Comparisons among the 4 groups (by one-way ANOVA), showed no significant differences (Fig. 2B), indicating that differences in levels of FGF1 immunoreactivity were not related to neuronal density, but rather to FGF1 expression by individual neurons. To further validate this result, homogenates from the 4 groups were analyzed by Western blot. This study showed that FGF1 was identified as a double band at an estimated molecular weight of 18 to 22 kDa (Fig. 3A). This double band was more abundant in the cytosolic rather than particulate fraction (Fig. 3A). Semiquantitative analysis showed that, consistent with LSCM analysis, the levels of FGF1 immunoreactivity in Group III (ND-/HIVE+) (Fig. 3B), were significantly different when compared to control Group I (one-way ANOVA, post-hoc Dunnett’s p < 0.05), supporting the contention that FGF1 immunoreactivity correlates with neuronal preservation.

FGF1 Is Neuroprotective Against gp120 Toxicity in Human Primary Neuronal Cultures

To further evaluate the potential neuroprotective effects of FGF1, human primary neuronal cultures were used.
Immunocytochemical analysis showed that these cell preparations expressed FGF1 and FGF1R immunoreactivity (Fig. 4A, B) and neuronal markers such as neurofilament (SMI32) (Fig. 4C). Only occasional astroglial cells were encountered (Fig. 4D). While untreated neuronal cells (Fig. 5A) or cells treated with FGF1 alone (Fig. 5B) displayed normal structural features, cells treated with gp120 alone (100 ng/ml) showed extensive damage to their cell bodies and neuritic processes (Fig. 5C). Pretreatment with FGF1 prevented the neurotoxic damage induced by gp120 (Fig. 5D). Consistent with these findings, analysis of cell viability with the LDH assay showed that, compared to control Group I, gp120 alone resulted in significant LDH release—indicative of cell death (one-way ANOVA, post-hoc Dunnett’s p < 0.05). In contrast, cells pretreated with FGF1 (0.1 or 1 pg/ml) in the presence or absence of gp120 showed a significant reduction in LDH release (Fig. 5E) (one-way ANOVA, post-hoc Dunnett’s p < 0.05), supporting the notion that FGF1 is neuroprotective.

**DISCUSSION**

The present study showed that, compared to cases with both HIVE and neurodegeneration, cases with HIVE but no neurodegeneration displayed increased levels of FGF1 expression in pyramidal neurons of the frontal cortex. This might indicate that increased expression of FGF1 is neuroprotective against HIV-mediated neurotoxicity. Alternatively, it is possible that for a yet to be determined reason, FGF1-expressing neurons are selectively lost in HIVE cases. However, this is unlikely in our study since in the 4 groups evaluated the total numbers of FGF1-immunoreactive neurons were unchanged, while only the levels of FGF1-immunoreactivity were significantly different. Supporting the first possibility, previous studies have shown that FGF1 and FGF2 are neuroprotective in a variety of conditions, including excitotoxicity, ischemia, and denervation (9–11, 32, 34). Furthermore, AIDS patients with KS, a neoplasm known to produce high levels of FGF (12), have a lower risk of developing cognitive alterations (13). In addition, we showed that gp120 was toxic in primary human neuronal cultures and that FGF1 was protective. These findings are consistent with previous studies in primary neuronal cultures showing that gp120 is neurotoxic (42–44) and with studies where FGF1 was shown to be protective in neuronal cell lines exposed to gp120 (15). Taken together, this suggests that the neuroprotective effects exerted by FGF1 might translate into preserved or improved cognitive performance in AIDS patients. In contrast to this possibility, a recent study showed a significant increase in nerve growth factor (NGF) and FGF2 mRNA levels in the frontal cortex of cognitively impaired AIDS patients (16). This discrepancy might be explained by several factors: 1) we focused on FGF1 rather than FGF2; 2) levels of immunoreactivity versus mRNA levels were analyzed; and most importantly, 3) HIVE cases used in our study were further subdivided into those with/without neurodegeneration. We also showed that in cases with neurodegeneration (with or without HIVE), the levels of FGF1 immunoreactivity were low. The mechanisms for neurodegeneration in the group without HIVE (Group II) are under investigation. In this regard, we have recently shown that in CD4-positive individuals central microglial activation is triggered by systemic infection might promote neurodegeneration in the absence of HIV in the brain (47).
Fig. 3. Western blot analysis of FGF1 immunoreactivity in brain homogenates from the frontal cortex. A: In the cytosolic fraction, the FGF1 antibody recognized a double band at an approximate molecular weight of 18 to 22 kDa. B: PhosphorImager analysis of levels of FGF1 immunoreactivity showed a significant increase in cases from Group III, compared to the control group (Group I). * = p < 0.05 by one-factor ANOVA, post-hoc Dunnett’s.

Fig. 4. Immunocytochemical characterization of primary human neuronal cultures. A: FGF1-immunoreactive neurons in primary cultures. B: Neuronal cells showing abundant FGFR1 immunostaining. C: Consistent with their neuronal origin cells displayed anti-SMI32 immunolabeling and (D) only occasional glial cells displaying GFAP immunoreactivity. Scale bar = 20 μm.
Fig. 5. Neuroprotective effects of FGF1 against gp120 in primary human neuronal cultures. Phase contrast microscopy of primary cultured neurons untreated (A), pretreated with FGF1 alone (0.1pg/ml) (B), treated with gp120 alone (100 ng/ml) (C), and pretreated with FGF1 (D), followed by gp120. E: Results from the LDH release assay to evaluate cell viability showed that addition of FGF1 24 h prior to gp120 exposure prevented toxicity. Scale bar: A–D = 20 μm.
While mechanisms by which FGF1 might be neuroprotective against HIV are not completely clear, at least 2 possibilities have been proposed: One, there may be antagonism of excitatory amino acid toxicity (48) by regulating expression of glutamate receptors since it is postulated that gp120 interacts with N-methyl-D-aspartate (NMDA) glutamate receptors (49). Two, it has recently been shown that FGF down-modulates cell surface expression of CXCR4 receptors, which are co-receptors for HIV cellular entry (15). Furthermore, CXCR4 expression by neurons appears to be an important mediator of gp120 neurotoxicity (44). Alternatively, neuroprotective effects of FGF1 may be mediated by the signalling pathway downstream from FGFR1. In this regard, FGF1 and FGF2 bind to FGFR1 leading to dimerization of the receptor with phosphorylation and activation of tyrosine kinase (50). There is a range of signal transduction molecules activated by the FGFR1 dimer (50). These include phospholipase C-γ, Src family kinase, Src homology 2 (SHP-2), focal adhesion kinase (FAK), phosphatidylinositol 3’ kinase (PI3-kinase), FGF receptor substrate 2 (FRS2), which is a recently characterized 90 kDa adaptor molecule, and Grb-2, which activates Ras (51). FGF induces sustained activation of MAP kinases such as extracellular signal-regulated kinase (erk) erk1 and erk2, which are positioned downstream of Ras in the pathway (52). MAP kinase activation may be important in mediating a number of neurotrophic effects, although independent pathways could also be activated (53). Furthermore, FGF binding activates p90 (rsk), which in turn stabilizes membrane-associated β-catenin (54). Degradation of β-catenin is promoted by glycogen synthase kinase-3 (GSK3) β and FGF inhibits endogenous GSK3β, possibly by p90 (rsk) (54). While activation of GSK3β might lead to cell death, inhibition of this enzyme is associated with cellular survival (55). Therefore, FGF1 might be neuroprotective via regulation of GSK3β pathway. Further supporting a role of this pathway in HIVE, a recent study showed that in HIV-infected cells, the trans-activator molecule Tat promotes cell death activation of GSK3β (56).

In summary, FGF1 might be neuroprotective against HIV via regulation of the intracellular signalling pathway important for cell survival.

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