HIV-1 gp120 Proteins and gp160 Peptides Are Toxic to Brain Endothelial Cells and Neurons: Possible Pathway for HIV Entry into the Brain and HIV-Associated Dementia

Georgette D. Kanmogne, Ph.D., R. C. Kennedy, Ph.D., and Paula Grammas, Ph.D.

Abstract. Breakdown of the blood-brain barrier is commonly seen in patients with human immunodeficiency virus (HIV)-associated dementia, despite the lack of productive HIV-infection of the brain endothelium. Through this damaged blood-brain barrier, HIV and HIV-infected monocytes/macrophages infiltrate the brain and further infect microglia and brain macrophages. Neuronal cell death and dysfunction are the underlying cause of HIV-associated dementia, but no productive HIV-infection of neurons has been documented. It is likely that secreted viral products play a major role in blood-brain barrier damage and neuronal cell death. The aim of the present study was to examine the effect of HIV-1 gp160 peptides and gp120 proteins on brain microvascular endothelial cells and neurons from both human and rats. Four of the 7 gp160 peptides tested evoked significant neurotoxicity. Two different full-length recombinant HIV gp120 proteins (HIV-1 CM235 gp120 and HIV-1 MN gp120) also induced neuronal and brain endothelial cell death, and concentrations as little as 1 ng/ml evoked pronounced morphological changes in these cells and marked cytotoxicity. This study suggests that HIV proteins and peptides that are shed in vivo may be directly involved in blood-brain barrier damage and neuronal cell death in HIV-associated dementia.

Key Words: Brain endothelial cells; gp120 proteins; gp160 peptides; HIV-1; HIV-associated dementia; Neurons.

INTRODUCTION

The human immunodeficiency virus (HIV) invades the brain in the early stages of infection. This viral infection of the central nervous system (CNS) results in various clinical and pathological abnormalities, ranging from sub-clinical and mild cognitive motor deficits to dementia. HIV-associated dementia (HAD) usually occurs late in the course of HIV infection and is characterized by severe cognitive, motor, and behavioral deficits (1, 2). At the end-stage of HAD, patients are nearly vegetative, often mute, and unable to walk without support (1, 3). The pathogenesis of HAD is not well understood. Analysis of the cerebrospinal fluid (CSF) and autopsy studies of brains from AIDS patients reveals brain atrophy, white matter gliosis, neuronal and dendritic cell loss (2, 4–8) and breakdown of the blood-brain barrier (BBB) (9–11). It has been shown that through this damaged BBB, HIV and HIV-infected monocytes/macrophages from the bloodstream infiltrate the brain and further infect microglia and brain macrophages (10, 11).

Brain microvascular endothelial cells (BMECs) regulate immune and inflammatory reactions and play an important role in the pathogenesis of infectious diseases. Viral or bacterial infection often lead to increased expression of inflammatory proteins, chemokines, and pro-inflammatory cytokines by infected cells (11, 12). These inflammatory products upregulate the expression of adhesion molecules on BMECs (12–14) and mediate leukocyte interactions with BMECs (15) and cell transmigration across the brain endothelium (14, 15). Furthermore, some of these inflammatory products are neurotoxic and a damaged BBB may also provide access into the CNS.

It is believed that neuronal cell death and dysfunction are the underlying causes of HAD (4–8). However, whether HIV can infect neurons is controversial. A recent study shows the presence of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains (16). Some investigators report human neuronal cell lines and primary human neuroblasts are susceptible to HIV infection (17, 18); however, others find no evidence of productive HIV infection of neurons (2, 3, 19–22). Most investigators agree that HIV infection of microglia and brain macrophages directly or indirectly causes neuronal cell death (2–8, 22–24). HIV-1 infection of astrocytes has also been documented (25–28) and HIV gene products, as well as cellular metabolites, impair normal astrocyte function (29–31). Infection and/or dysfunction of astrocytes might cause neuronal dysfunction because astrocytes are important sources of growth factors and are crucial to the proper functioning of neurons (26).

HIV-infected microglia and monocytes/macrophages secrete high amounts of tumor necrosis factor alpha (TNF-α), a neurotrophic proinflammatory cytokine, as well as interleukin-1β, interleukin-6, platelet activating factor, and nitric oxide, all of which have been shown to cause neuronal injury (8, 23, 24). Other neurotoxic factors...
include excitatory amino acids, quinolinic acid, and glutamate, arachidonic acid metabolites, and elevated intracellular calcium induced by N-methyl-D-aspartate (NMDA) (8, 14, 16). Viral proteins, especially the HIV proteins gp120, Tat, Nef, and Rev have been shown to be neurotoxic (24, 32–43), and cytotoxic to endothelial cells (44, 45).

The HIV envelope glycoprotein is synthesized as a polypeptide precursor of 160 kDa (gp160) and is subsequently cleaved into a surface exposed amino terminus subunit, gp120, and a carboxyl transmembrane subunit, gp41. During the infection of susceptible cells and the replication of HIV, direct cytopathic events can result in the release of HIV protein and peptide products into the surrounding environment. The objective of the present study is to determine if gp160 and fragments thereof could invoke toxicity against neurons and brain endothelial cells. In this report we demonstrate that culture supernatant from HIV-infected monocytes/macrophages, as well as selected HIV-1 gp160 peptides and intact gp120 proteins, induce the death of brain endothelial cells and neurons.

**MATERIALS AND METHODS**

**HIV-1 gp120 Proteins and gp160 Peptides**

HIV-1<sub>GAG</sub> gp120 and HIV-1<sub>MAC25</sub> gp120 proteins were obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH. Synthetic peptides homologous to HIV-1 envelope glycoprotein sequences were selected based on computer prediction of antigenic determinants as previously described (46–48). The Table depicts the amino acid sequences of the gp160 peptides used in this study. They were derived from the sequences of the HIV-IIIB (clone BH10) isolate (49); their selection was based on their conserved sequence homology between isolates, their hydrophilicity, their predicted beta-turn secondary structure, and the potential to be processed and presented to the immune system during HIV-1 infection or HIV-1 gp160 immunization (46, 48). A detailed method of gp160 peptides synthesis, purification, and quality control analysis has been described elsewhere (47, 48). Control non HIV-1 gp160 peptides corresponding to amino acid sequences derived from the envelope gene products of hepatitis C virus were also included.

**Neuronal Cell Cultures**

Cerebral cortical cells were prepared as previously described (50). Briefly, cerebral cortices were isolated from 17- to 18-day-old fetal rats, washed in Hank’s balanced salt solution and dissociated by trituration in 10 ml Brooks-Logan solution containing 0.025% trypsin. After 10-min incubation at room temperature, Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated horse serum (HIHS), 2 mM L-glutamine and 1 × antibiotic/antimycotic solution (Gibco BRL, Rockville, MD) was added to cells, to a total volume of 40 ml. The mixture was again incubated for 10 min at room temperature and centrifuged for 10 min at 800 g. The pellet was resuspended in fresh medium and centrifuged as above. The final pellet was triturated 10 times, filtered through 210 μm nylon mesh, and plated on 96- or 48-well poly-L-lysine-coated plates at a density of 3–5 × 10<sup>5</sup> cells/ml. After 3 days in culture, the medium was replaced with DMEM containing 5% HIHS and 10 μg/ml deoxyuridine. Neuronal cells were used for toxicity assays after 7 to 10 days in culture. The neuronal identity was confirmed by positive staining with neuronal specific enolase, as previously described (50).

Human cortical neurons (HCN-1A) were purchased from American Type Culture Collection (Manassas, VA) and seeded on 100 mm or 48-well culture plates. Neuronal cells were cultured in DMEM containing 4.5-g/l glucose, 2.2-g/l sodium bicarbonate, 10% fetal bovine serum (FBS) (HyClone, Logan, UT), and 4 mM L-glutamine (Gibco). Culture medium was changed twice per week and confluent cells subcultured at a split ratio of 1:2, using a solution of trypsin-EDTA (0.025% trypsin, 0.03% EDTA). All cells were maintained in an incubator at 37°C, 5% CO<sub>2</sub>. HCN-1A cells at passages 4 to 6 were used for toxicity assay.

**Brain Endothelial Cell Culture**

Rat brain endothelial cell cultures were obtained from enzymatic dissociation of isolated resistance vessels with collagenase. The endothelial cells were cloned from mixed cultures of endothelial and smooth muscle cells, using pencylinders as previously described (51). Cells were maintained in DMEM

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**Table Amino Acid Sequences of HIV-1 gp 160 Synthetic Peptides Used in this Study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid residues</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>112 (gp 120)</td>
<td>112–130</td>
<td>H-E-D-I-I-S-L-W-D-Q-S-L-K-P-C-V-K-L-T</td>
</tr>
<tr>
<td>304 (gp 120, V3)</td>
<td>304–321</td>
<td>(C-G-Y)-T-R-P-N-N-T-R-S-T-G-I-R-Q-R-G-P-G</td>
</tr>
<tr>
<td>735 (gp 41)</td>
<td>735–752</td>
<td>(Y)-D-R-P-E-G-I-E-E-E-G-E-R-D-R-D-R-S-(G-C)</td>
</tr>
</tbody>
</table>

<sup>a</sup> In parentheses are amino acids added for the purpose of conjugation or labeling (47). All peptide sequences match the corresponding sequences of the HIV-1 isolate IIIB.
supplemented with 10% FBS, 2 mM L-glutamine, and sub-cultured using phosphate buffered saline (PBS) containing 0.025% trypsin. Culture medium was changed twice a week, and endothelial cells at passages 12–15 were used in this study.

Human brain microvascular endothelial cells (HBMECs) were purchased from Cell Systems (Seattle, WA) and seeded on 65 mm or 48-well tissue culture plates coated with attachment factor (Cell Systems). Cells were cultured in CS-C complete media (Cell Systems) according to the manufacturer’s instructions, and confluent cells split at a ratio of 1:3 using the Passage Reagent Group solutions (Cell Systems). Culture medium was changed twice a week, and endothelial cells at passages 5–7 were used in this study. Cells were maintained at 37°C, 5% CO₂.

**RESULTS**

**Cytotoxicity of HIV-1 gp160 Peptides and gp120 Proteins on Neurons and Brain Endothelial Cells**

Confluent cells were washed 3 times with serum-free media and exposed to various doses of recombinant HIV-1 proteins or synthetic peptides (Table) for 24 h. Cytotoxic effects (i.e. changes in cell morphology and cellular disintegration) were assessed by phase-contrast microscopy. Cytotoxic effects are defined as changes in cell morphology and cellular disintegration.

**Cytotoxicity of HIV-1 gp160 Peptides and gp120 Proteins on Neurons and Brain Endothelial Cells**

Monocytes/macrophages were purified from fresh donor blood and infected with HIV-1 (BZ167, clade B and UG001, clade D), as previously described (22, 45). Confluent cultures of brain endothelial cells and neurons were exposed to cell-free culture supernatant of infected monocytes/macrophages collected between day 9 and day 20 post-infection. After 24 hours (h) exposure, cytotoxic effects were assessed by phase-contrast microscopy. Cytotoxic effects are defined as changes in cell morphology and cellular disintegration.

**Cytotoxic Effect Induced by Conditioned Media from HIV-Infected Monocytes on Neurons and Brain Endothelial Cells**

Exposure of human brain endothelial cells to culture supernatants from HIV-1 infected monocytes resulted in a marked cytopathic effect, as assessed by changes in cell morphology, cell detachment, and cellular disintegration. Figure 1 shows a phase-contrast micrograph of human brain endothelial cells after a 24-h exposure to culture supernatants of HIV-infected monocytes/macrophages collected at day 20 post-infection. Both culture supernatants from monocytes infected with clade B and clade D isolates induced high toxicity, as evidenced by cellular disintegration and morphological changes (Fig. 1A, B), with some cells detached from the plate. Culture supernatant from monocytes infected with clade B and clade D isolates also induced toxicity of human cortical neurons (not shown).

**Cytotoxicity of HIV-1 gp160 Peptides**

The results of experiments examining the effects of 7 different HIV-1 gp160 peptides (Table) on primary rat neuronal cultures demonstrate that 4 of the peptides evoked increased neurotoxicity (Fig. 2). Data are expressed as percent of total LDH (n = 7), and were analyzed by one-way ANOVA: f = 6.6, df = 9, p < 0.0001. Tukey’s test for multiple comparisons showed significant increase in neurotoxicity with peptides 639–652 and 846–860 (p < 0.05). At a concentration of 1 µg/ml, these peptides killed 30% to 42% of neuronal cells. Addition of peptides 616–632 and 735–752 at a concentration of 1 µg/ml also induced neuronal cell death ranging from 18% to 25%. Despite the high neurotoxicity evoked by peptide 846–860, an overlapping peptide 842–856 did not induce significant neurotoxicity. Also, exposure of neurons to HIV gp160 peptides 112–130 and 304–321 resulted in little neuronal cell death. Finally, control peptides, including 900 and 925 from the hepatitis C virus, did not cause any significant neurotoxicity (Fig. 2). The 3 HIV-1 gp160 peptides that demonstrated the highest level of neurotoxicity (616–632, 639–656, and 846–860) were further examined. At concentrations ranging from 0.1 to 10 µg/ml, these peptides induced neuronal cell death in a dose-dependent manner (Fig. 3).

**Cytotoxic Effect and Cytotoxicity of gp120 Proteins**

Full-length recombinant HIV-1 gp120 proteins also induced neuronal and brain endothelial cell death (Figs. 4, 5). In contrast to the peptides where 0.1 µg/ml evoked only low levels neurotoxicity (Fig. 3), full-length HIV-1MN gp120 killed 60% of neurons at 0.1 µg/ml (Fig. 4). At 0.5 µg/ml to 1 µg/ml, HIV-1MN gp120 evoked maximal neurotoxicity (75%–83%) (Fig. 4). HIV-1MN gp120 is a full-length, glycosylated recombinant protein from the HIV-1 isolate strain MN. This gp120 protein was also very potent in causing human brain endothelial cell death. Phase-contrast microscopy of confluent human brain-derived endothelial cells treated with as little as 1 ng/ml protein showed pronounced morphologic changes indicative of cytopathic effect (Fig. 5).
When exposed to HIV-1MN gp120 protein concentrations of 10 ng/ml, most of the cells in the plate were rounded and refractive. At 1 µg/ml, essentially all endothelial cells had disintegrated and detached (Fig. 5). Similarly, a second HIV-1 gp120 protein from a different viral isolate (CM235) induced marked cytotoxicity in cultures of brain endothelial cells and cortical neurons from both human and rats, as assessed by LDH released (Fig. 6). Concentrations of 0.01, 0.1, 1, and 10 µg/ml HIV-1CM235 gp120 induced cytotoxicity in neurons ranging from 36% to 55%. These same concentrations induced cytotoxicity in brain endothelial cells that ranged from 28% to 80% (Fig. 6).

**DISCUSSION**

In this study, we initially observed that culture supernatant from HIV-infected monocytes/macrophages induced a marked cytopathic effect on human neurons and brain endothelial cells. During the course of HIV infection, viral proteins are constantly shed in the surrounding medium (52, 53) and could be responsible for the observed cytotoxicity. In addition, apoptotic endothelial cells have been found in postmortem brain tissues of AIDS patients (54) and in the brain of SIV-infected macaques (55). Endothelial cells and neurons generally have similar properties and characteristics in mammals (56, 57) and there is evidence that HIV proteins affect both...
Cytotoxicity of HIV-1 gp160 peptides on rat cortical neurons. Cells were exposed for 24 h to 1 μg/ml peptides and cytotoxicity assessed by measuring LDH released. Peptides 616–632, 639–656, 735–752, and 846–860 induced toxicity on neuronal cells. *p value <0.05 compared to the control. On the contrary, peptides 112–130, 304–321, and 842–856 did not induce any significant toxicity on neuronal cells. Control peptides 900 and 925 are from the hepatitis C virus.

Cytotoxicity of HIV-1 gp160 peptides 616–632, 639–656, and 846–860 on cerebral cortical neurons. Confluent cells were exposed for 24 h to culture medium containing 10, 1, and 0.1 μg/ml peptide respectively, and cytotoxicity assessed by measuring LDH released. All 3 peptides induced neuronal cell death in a dose-dependent manner.

Cytotoxicity of HIV-1 MN gp120 protein on cerebral cortical neurons. Confluent cells were exposed for 24 h to culture medium containing respectively 0.1, 0.5 or 1 μg/ml gp120 protein or gp160 peptide and cytotoxicity assessed by measuring LDH released. HIV-1_MN gp120 protein induced significant neuronal cell death.

A number of different hypotheses have been postulated to explain the mechanisms of HIV entry in the brain through the BBB. Some authors previously suggested that this could occur by HIV-1 infection of brain endothelial cells (59, 60). Results from our laboratory, as well as by other investigators showed that no productive HIV infection of human brain endothelial cells occurs either in vitro or in vivo (22, 61, 62). Therefore, mechanisms other than direct viral infection of the endothelium must play a role in HIV-1 entry in the brain. In the present study, we report that HIV-1 gp120 proteins and gp160 peptides are toxic to brain endothelial cells from both human and rat. The gp160 peptides used in this study were derived from the sequences of HIV-1IIIB (BH10) isolate (49); the HIV-1_CM235 gp120 and HIV-1_MN gp120 proteins were full-length recombinant proteins from the HIV-1 strains CM235 and MN, respectively. Both gp160 peptides and gp120 proteins were toxic to brain endothelial cells at low concentrations. HIV-1 gp120 proteins were previously shown to be cytotoxic to other human endothelium, including human lung microvascular endothelial cells (45) and human umbilical vein endothelial cells (44), with concentrations of 0.1 ng/ml causing a 7.8-fold increase in cell death. These previous studies found that gp120-induced endothelial cell death occurs by apoptosis and is mediated through the CCR5 or CXCR4 receptor (44, 45). The data described herein are the first to demonstrate HIV-1 gp120 cytotoxicity to brain endothelial cells.

Brain endothelial cells regulate immune and inflammatory reactions and constitute the BBB. In this study, we demonstrate that nanomolar concentrations of gp120 proteins and gp160 peptides induce marked cytotoxicity on BMECs in vitro. In the bloodstream of HIV-infected human (36–38, 40, 42, 58) and rodent-derived cells (32–35, 37, 41, 43). We therefore tested the effect of HIV-1 gp120 proteins and gp160 peptides on brain endothelial cells and neurons from human and rats in order to elucidate the pathway through which HIV invades the brain and results in HAD. Our assumption was that any adverse effect of these viral proteins and peptides on the brain endothelium, which results in the disruption of the BBB integrity, could create a pathway for HIV entry into the brain. As the result of HIV replication and subsequent cytopathic effects on susceptible cells, HIV protein products and processed or degraded peptide fragments could be released. If these HIV products were toxic to endothelial cells and could alter cell morphology, they could disrupt the BBB and possibly contribute to HAD.
patients there is constant circulation of both free virus and free viral proteins (52, 53) and the brain endothelium is constantly exposed to these viral products. Thus, it is likely that the resulting damage to the brain endothelium in vivo is more severe than the level of cytotoxicity we observed after 24 h cell exposure to HIV-1 gp120 proteins and gp160 peptides. This constant assault on the brain endothelium could result in the injury and/or death of brain endothelial cells and a damaged BBB, allowing other free virus and HIV-infected cells from the bloodstream to infiltrate and enter in the brain. In fact, it has been shown that HIV-1 invades the BBB via a paracellular route mediated by TNF-α (62). Similarly, analysis of cloned sequences of HIV-1 gp160 genes from post-mortem tissues of a patient with HAD showed evidence of monocyte trafficking into the brain (63). These data showing increased HIV transit across the BBB support the importance of our observations that HIV-1 proteins and peptides cause BBB damage.

Exposure of rat and human neuronal cells to HIV-1 gp120 proteins or gp160 peptides also resulted in a marked cytotoxicity. Two of the 7 gp160 peptides tested (peptides 639–656 and 846–860) induced statistically significant cytotoxicity in neurons when compared to control peptides (Fig. 2). Two other peptides (peptides 616–632 and 735–752) also induced cytotoxicity in neurons. Compared to control peptides, the neurotoxicity induced by the 2 latter peptides was not statistically significant (Fig. 2), but could be biologically significant. These peptides correspond to amino acid sequences of the HIV transmembrane glycoprotein gp41 (47, 48, 64) and are relatively conserved among HIV-1 isolates. Previous studies showed that peptides 616–632 and 735–752 induce neutralizing antibodies against HIV-1 in vitro (64, 65). Peptides 735–752 and 846–860 have been shown to induce an immune response directed against a native envelope glycoprotein epitope of HIV (47), and to suppress normal human lymphocyte proliferation and natural killer
HIV-1 gp120 proteins were also shown to be neurotoxic, including Tat (37, 40, 43), Nef and Rev (24), and gp41 (69). There are reports of direct association between gp41 levels in the brain and HIV dementia (69–71), as well as evidence of a strong correlation between AIDS dementia and the presence of macrophages in the brain parenchyma (72).

Studies on postmortem brain tissues showed that in the CNS, HIV-1-infected microglia and macrophages are concentrated around microvessels (73); these infected cells can produce HIV and shed viral products for an extended period, with continuous damage to the surrounding brain vessels. Furthermore, brain macrophages and microglia can be activated by secreted products released from infected cells, which results in upregulation of chemokines and cytokines such as IL-6 and TNF-α, neuroinflammation, and increased neuronal cell injury (2, 8, 11, 12, 23, 24). This is evidenced by studies where neuronal apoptosis was found to be more severe in atrophic brains of AIDS patients and the extent of cell death correlated with the profile of microglial activation (24). To be successful, therapeutic interventions against HAD should aim at preventing neuronal cell death by 1) protecting the BBB integrity to prevent the passage of HIV and HIV-infected cells into the brain, 2) preventing viral infection of macrophages and microglia and the release of neurotoxic viral products, and 3) preventing macrophage and microglial activation and subsequent release of neuroinflammatory products. This report provides direct evidence for the cytotoxic effects of HIV-1 gp120 proteins and HIV-1 gp160 peptides on human and rat brain endothelial cells, as well as their neurotoxic effects in vitro.

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