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Platelet-derived growth factor protects neurons against gp120-mediated toxicity

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The human immunodeficiency virus (HIV)-1 envelope glycoprotein gp120 has been implicated in mediating neuronal apoptosis, a hallmark feature of HIV-associated dementia (HAD). Mitigation of the toxic effects of gp120 could thus be a potential mechanism for reducing HIV toxicity in the brain. In this study the authors hypothesized that neurotrophic factor, such as platelet-derived growth factor (PDGF), could protect the neurons against gp120-mediated apoptosis. SH-SY5Y cells treated with gp120 exhibited increased cell death when measured by lactate dehydrogenase (LDH) and deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay, with concomitant loss of neurites and increased cell rounding. Pretreatment with PDGF-BB, however, reduced gp120-associated neurotoxicity and rescued the neurite outgrowth. Additionally, gp120-mediated activation of caspase-3 was also significantly reduced in cells pretreated with PDGF-BB. Antiapoptotic effects of PDGF-BB were also confirmed by monitoring levels of anti- and proapoptotic genes, Bcl-xL and Bax, respectively. Furthermore, PDGF-mediated protection against gp120 involved the phosphoinositide (PI) 3-kinase/Akt pathway. Taken together these findings lead us to suggest that PDGF-BB could be considered as a therapeutic agent that can mitigate gp120-mediated neurotoxicity in HAD.
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Keywords: Gp120; HIV dementia; neurons; PDGF

Introduction

It is estimated that almost 25% of untreated human immunodeficiency virus (HIV)–infected individuals and ~7% of HIV-infected patients treated with highly active antiretroviral therapy develop HIV-associated dementia (HAD) (Budka, 1991; McArthur *et al*, 1993; Sacktor *et al*, 2001; Spencer and Price, 1992), a neurodegenerative syndrome that is clinically characterized by progressive cognitive, motor, and behavioral abnormalities (Lipton and Gendelman, 1995; Price *et al*, 1991). Pathological manifestation of the syndrome, HIV encephalitis (HIVE), is accompanied by prominent microglial activation, formation of microglial nodules, perivascular accumulations of mononuclear cells, presence of virus-infected multinucleated giant cells, and neuronal damage and loss (Bell, 1998; Gendelman *et al*, 1994; Nath, 1999). The primary cell types infected by HIV-1 in the brain are macrophages/microglia and, to a lesser extent, astrocytes, but not neurons (Kaul *et al*, 2001). One broad explanation frequently advocated explaining the loss of neurons in this disease is that cellular and/or viral proteins released from the infected cells have a direct toxic effect on the neurons (Adamson *et al*, 1996; Brenneman *et al*, 1988; Chen *et al*, 2000; Dreyer *et al*, 1990; Hof *et al*, 1998; Kruman *et al*, 1998; New *et al*, 1997; Patel *et al*, 2000).

Among the HIV proteins that are shed in the brain from virus-infected cells, HIV envelope glycoprotein gp120 is shown to have neurotoxic effects

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both *in vitro* and *in vivo* (Bansal *et al*, 2000; Lipton *et al*, 1991; Meucci and Miller, 1996). In cell culture gp120 is able to induce apoptosis of neurons both in the presence and in the absence of microglia. Transgenic mice expressing gp120 in the brain also manifest neuropathological features similar to those observed in acquired immunodeficiency syndrome (AIDS) patients (Mucke *et al*, 1995). Several independent findings have shown that gp120-mediated neurotoxicity occurs via apoptosis through the seven-transmembrane domain chemokine receptor CXCR4 (Davis *et al*, 1997; Herbein *et al*, 1998; Hesselgesser *et al*, 1998a, 1998b; Klein *et al*, 1999; Kozak *et al*, 1999), involving the activation of proapoptotic caspase-3. Gp120-mediated caspase-3 activation has been specifically demonstrated in rat cerebellar granule cells (Campus *et al*, 2000), human embryonic kidney (Biard-Piechaczyk *et al*, 2000), and endothelial cells (Ullrich *et al*, 2000). Reciprocally, inhibition of caspase-3 has been shown to protect neurons from apoptosis. Hence, factors that inhibit caspase-3 activation could be valid candidates for rescuing neurons from gp120 toxicity.

Belonging in this category of caspase inhibitors are various growth factors in the central nervous system (CNS) that are neurotrophic and have been shown to block apoptosis, thereby serving as survival factors during normal nervous system development (Bachis and Mocchetti, 2005; Cameron *et al*, 1991; Cameron and Rakic, 1991; Sanders *et al*, 2000; Vogelbaum *et al*, 1998a, 1998b). In particular, brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), and insulin-like growth factor have been shown to protect neurons from gp120-mediated apoptosis (Bachis and Mocchetti, 2005; Kulik *et al*, 1997; Sanders *et al*, 2000). In the present study, we explored the role of yet another neurotrophic factor, platelet-derived growth factor (PDGF), that has been documented to be critical for the development of brains of postnatal rats (Smits *et al*, 1991). PDGF is a family of five dimeric ligands (PDGF-AA, -AB, -BB, - CC, and -DD) assembled from four gene products (PDGF-A–D) that act via two classical receptor tyrosine kinases, PDGF-αR and PDGF-βR (Bergsten *et al*, 2001; Heldin *et al*, 2002; Li *et al*, 2000). Members of the PDGF family have multiple roles during embryogenesis and in a variety of pathological situations in the adult. The focus of this study was to examine whether PDGF could protect the neurons against gp120-mediated toxicity.

Results

PDGF-B chain is down-regulated in neurons in the brains of macaques with SHIV encephalitis

Before attempting to explore the role of PDGF-B chain in gp120-mediated toxicity, it was critical to first assess the expression of this factor in the brains of simian human immunodeficiency virus (SHIV)-infected macaques with and without encephalitis. We immunostained sections of basal ganglia region of brains of rhesus macaques with and without the CNS disease with the PDGF-B chain antibody. The basal ganglia was chosen for immunostaining due to the predilection of the virus for this region (Trotot and Gray, 1997). These studies presented in this report were focused on PDGF-B chain and not PDGF-A chain expression, because the former has been implicated as major player in neuronal fitness (Giacobini *et al*, 1993; Nikkhah *et al*, 1993). As shown in Figure 1A, neurons in the brain sections from uninfected macaques stained strongly for the PDGF-B chain and maintained their architecture. In contrast, the corresponding encephalitic brain section demonstrated very weak staining for PDGF-B chain protein and this correlated with degeneration of neuronal processes (circled in Figure 1B).

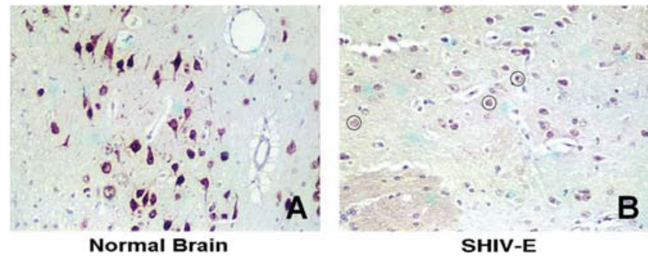

Figure 1 Decrease of PDGF-B chain in neurons of macaque brain with SHIV encephalitis. Representative brain sections from the basal ganglia region of an uninfected (**A**) and SHIV-E (**B**) macaque immunostained with PDGF-B chain antibody. Red color represents PDGF-B chain–positive cells. Degenerated neurons are shown in circle.

gp120 down-regulates PDGF-B chain expression in neurons

We next wanted to explore whether the down regulation of PDGF in neurons was result of direct effect of HIV viral protein, gp120, a known neurotoxic agent (Bansal *et al*, 2000; Meucci and Miller, 1996). To test this, we used a neuroblastoma cell line, SH-SY5Y, differentiated into neurons by retinoic acid for 7 to 8 days followed by exposure to 200 ng/ml of gp120. After 24 h of exposure, cells were collected for RNA and protein extraction to look for expression of PDGF-B chain by semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) and Western blot analysis. As shown in Figure 2A and C, treatment of cells with gp120 resulted in inhibition of PDGF-B chain expression at both RNA and protein levels. Down-regulation of PDGF-B chain by gp120 was further confirmed in fetal rat primary neuronal cultures (Figure 2B).

Protective effect of PDGF-BB against gp120-mediated neurotoxicity

Because PDGF-B chain is produced by healthy neurons (Smits *et al*, 1991) and has neuroprotective

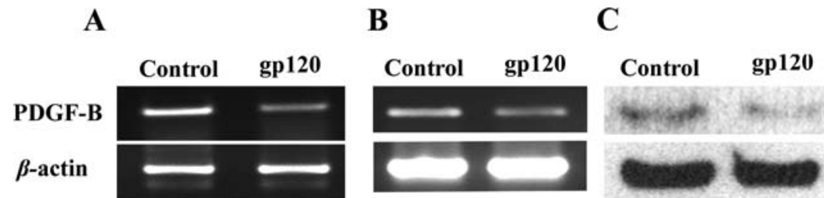

Figure 2 Down-regulation of PDGF-B chain in neurons exposed to gp120. RT-PCR analysis for PDGF-B chain was done on total RNA extracted from SH-SY5Y cells (**A**) or primary rat neurons (**B**) exposed to 200 ng/ml gp120 for 24 h. (**C**) Western blot analysis of PDGF-B chain on cell lysate obtained from SH-SY5Y cells treated with or without gp-120 (200 ng/ml) for 24 h. Figure shown is a representative of three different experiments.

properties (Hynds *et al*, 1997; Mohapel *et al*, 2005; Othberg *et al*, 1995; Sung *et al*, 2001; Tseng and Dichter, 2005), we hypothesized that pretreatment of neurons with PDGF-BB protein could protect against gp120-mediated neurotoxicity.

Treatment of SH-SY5Y cells with gp120 (200 ng/ml) for 5 days resulted in increased cell death compared to untreated cells, as evidenced by rounding of cells with concomitant loss of the neurites (Figure 3A). These findings were consistent with

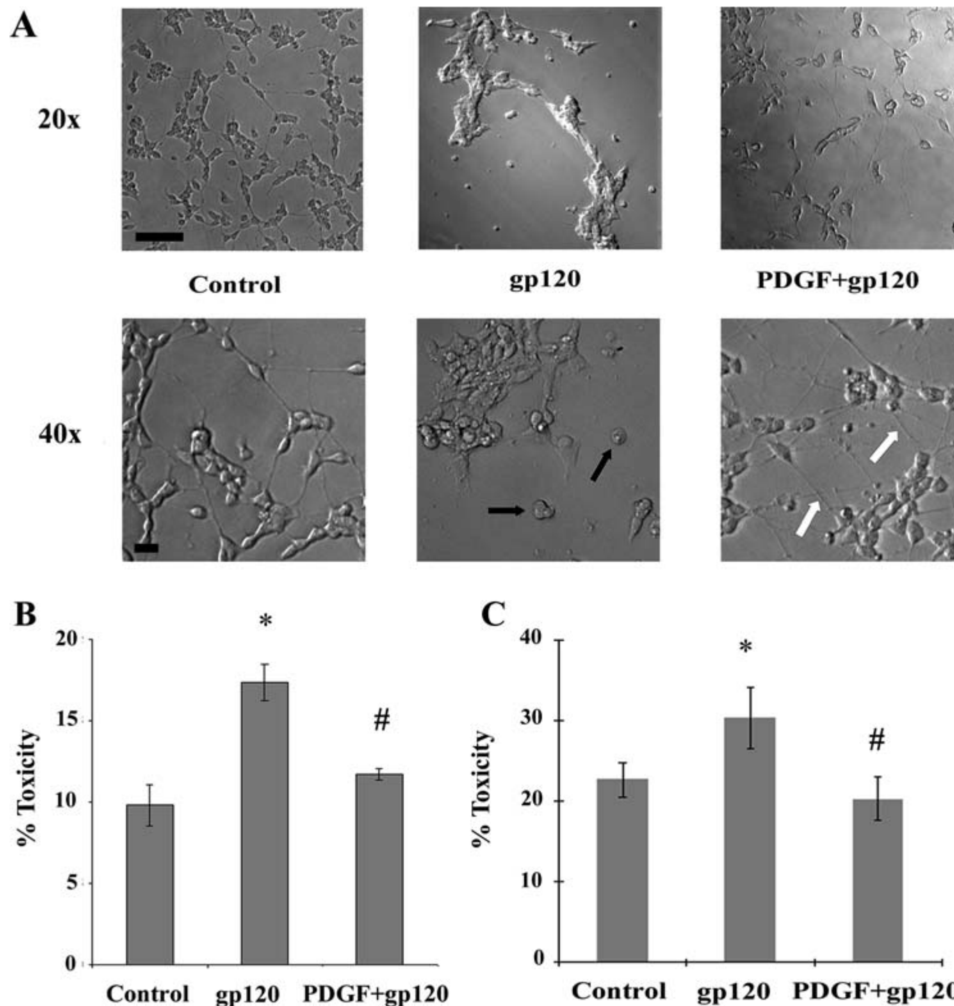

Figure 3 PDGF protects SH-SY5Y cells against gp120-mediated toxicity. (**A**) Phase-contrast microscopy of SH-SY5Y cells treated with gp120 (200 ng/ml) and/or PDGF-BB (20 ng/ml) for 5 days. Cells treated with gp120 alone showed rounding (as shown by black arrows) with shortened neurites. Pretreatment of gp120-treated neurons with PDGF-BB resulted in maintenance of cellular architecture with extended neuronal processes (indicated by white arrows). Scale bars, 50 μm. Neuronal viability in the presence of gp120 and/or PDGF-BB. SH-SY5Y cells (**B**) and rat primary neuronal culture (**C**) were exposed to gp120 (200 ng/ml) with or without pretreatment with PDGF-BB (20 ng/ml). Supernatant fluids and cellular extracts were collected in 24 h for LDH assay. Data are presented as mean ± SEM from three independent experiments. Statistical analysis was performed using Student's t test. $^{*}P < .01$ gp120 versus control; $^{\#}P < .01$ gp120 versus PDGF + gp120.

previous reports demonstrating neurotoxic role of gp120 (Bansal *et al*, 2000; Meucci and Miller, 1996). Pretreatment of neuronal cells with PDGF (20 ng/ml) for 30 min followed by exposure to gp120, however, resulted in protection of cells against gp120 toxicity (Figure 3A).

To further corroborate these findings, the release of the stable cytosolic enzyme lactate dehydrogenase (LDH), used as an indicator of tissue or cell damage, was examined in both SH-SY5Y and in fetal rat primary neuronal cultures treated with gp120 in presence or absence of PDGF-BB. gp120 exposure induced significant damage of SH-SY5Y cells ($P < .05$ versus control), as shown by increase in percentage of cell death compared to untreated cells (Figure 3B). When PDGF was examined for the protective effect against gp120-induced cell death, it was clear that pretreatment of neurons with PDGF-BB was able to suppress the cell damage (Figure 3B). The protection of neurons by PDGF from gp120 toxicity was also observed in fetal rat primary cortical neurons (Figure 3C).

PDGF-BB prevents gp120-mediated apoptosis

Because the primary mechanism by which gp120 induces neuronal death is in large part via the apoptotic pathway (Singh *et al*, 2004; Wright *et al*, 2007), it was hypothesized that PDGF protects neurons from gp120-mediated neurotoxicity by preventing apoptosis. Deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was done on SH-SY5Y cells treated or untreated with gp120 and/or PDGF-BB. As shown in Figure 4, 15% of cells treated with gp120 were TUNEL positive at 18 h and this increase in cell death was significantly reduced in the presence of PDGF-BB. Interestingly, the number of TUNEL-positive cells treated with gp120 in presence of PDGF-BB was even less than the untreated cells (Figure 4).

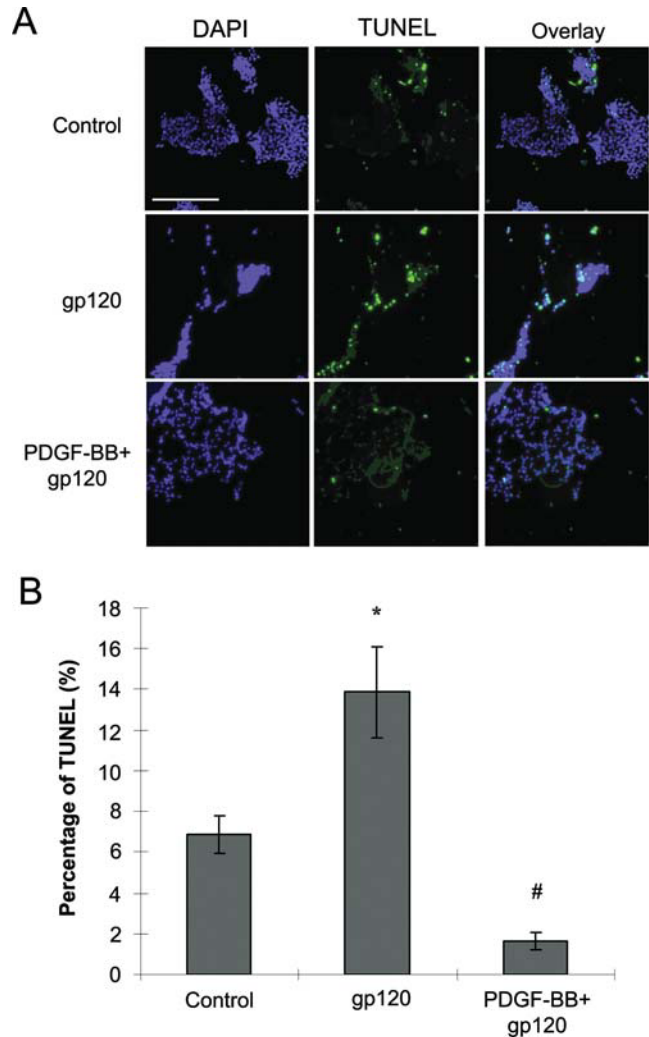

Figure 4 PDGF-BB mediates neuronal protection against gp120. (**A**) SH-SY5Y cells treated with or without gp120 (200 ng/ml) in the presence or absence of PDGF-BB (20 ng/ml) for 18 h were stained for TUNEL. Scale bar, 100 μm. (**B**) Histogram showing the percentage of TUNEL positive SH-SY5Y cells relative to total number of neurons following various treatments. Six to eight images were randomly taken for counting positive signal in each experiment. Values are presented as mean ±SEM. $^*P < .01$ gp120 versus control; $^{\#}P < .001$ gp120 versus PDGF + gp120.

To corroborate the findings that PDGF-BB–mediated protection involved abrogation of the apoptotic pathway, we monitored the activation of caspase-3 and stained the cells treated with gp120 and/or PDGF-BB with cleaved caspase-3, a protease essential for neuronal apoptosis (Wright *et al*, 2007). The activity of caspase-3 was measured by colorimetric assay in cell lysates treated with or without gp120 and/or PDGF-BB for 6 h. In agreement with previous findings (Bansal *et al*, 2000; Meucci and Miller, 1996), gp120 treatment of SH-SY5Y cells resulted in a significant increase in active caspase-3 compared with untreated control cells ($P < .05$ versus control) (Figure 5A). Pretreatment with PDGF-BB, however, was able to abrogate gp120-mediated increase in caspase-3 activation. This was further confirmed by immunostaining for caspase-3. As shown in Figure 5B, cells treated with gp120 IIIB for 6 h demonstrated positive staining for activated caspase-3, whereas pretreatment with PDGF-BB resulted in reduction of gp120-induced caspase-3 activity.

Reduction in neuronal injury in cells pretreated with PDGF-BB was also confirmed by Western blot analyses of the anti and proapoptotic proteins Bcl-xL and Bax, respectively. As shown in Figure 6, neurons challenged with HIV-1 protein gp120 demonstrated a reduced band density ratio of Bcl-xL to Bax. Pretreatment of gp120-exposed neurons to PDGF-BB, however, resulted in an increase in the antiapoptotic gene expression, as demonstrated by an increase in Bcl-xL to Bax ratio.

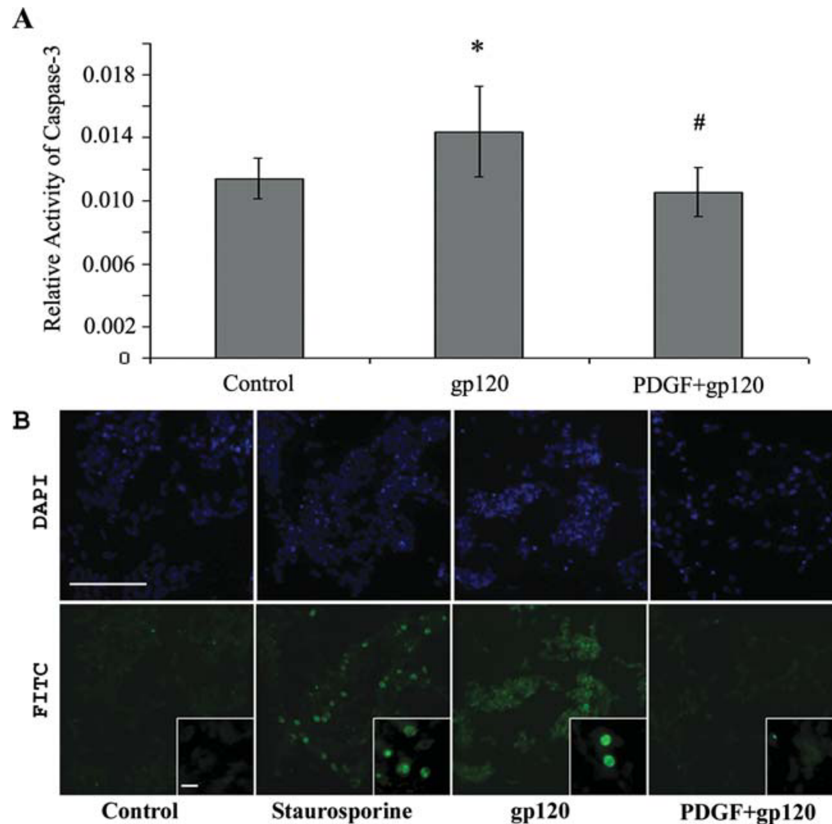

Figure 5 Neuronal apoptosis in SH-SY5Y cells exposed to gp120 with or without PDGF-BB pretreatment. Cells treated with or without gp120 IIIB in presence or absence of PDGF-BB pretreatment were monitored for (**A**) caspase-3 activity assay in cell lysates and (**B**) active caspase-3 staining by immunocytochemistry using the anti-cleaved caspase-3 antibody. The immunochemistry images showing nuclear (upper panel) and active caspase-3 staining (*lower panel*) were captured on Nikon inverted fluorescence microscope TE2000-E with 20 × and 100 × (*inset*) objectives (scale bar, 100 μm). Data for caspase-3 activity represents the mean ± SEM from seven independent experiments. * $P < .05$ gp120 versus control; # $P < .01$ gp120 versus PDGF + gp120.

Role of PI3K/Akt pathway in PDGF-BB–mediated protection of neurons against gp120 toxicity

Phosphoinositide 3-kinase (PI3K)/Akt pathway has been implicated in regulating several important cellular processes, including survival, proliferation, and metabolism (Hanai *et al*, 2006; Romashkova and Makarov, 1999; Simakajornboon *et al*, 2001; Zhang *et al*, 2003). PI3K pathway plays a critical role in PDGF signaling upstream of 3-phosphoinositide-dependent protein kinase 1/2 (PDK1/2) and Akt/protein kinase B (PKB) kinases (Ullrich and Schlessinger, 1990). Therefore it was hypothesized that activation of PI3K/Akt pathway by PDGF-BB may be involved in neuronal protection against gp120 toxicity. In order to confirm this, SH-SY5Y cells were pretreated with or without the inhibitor for PI3K for 1 h prior to exposure of cells with PDGF-BB and/or gp120 as described above, followed by analysis of cell death by LDH assay. As shown in Figure 7, pretreatment of cells with PI3K inhibitor partially abrogated PDGF-mediated protection against gp120 toxicity (Figure 7A; $P < .05$) compared with cells treated with PDGF-BB plus gp120. These findings led to the suggestion that PI3K plays a role in PDGF-mediated protection of neurons against gp120 toxicity.

Because Akt lies downstream of PI3Kinase, the next set of experiments were undertaken to examine whether the protection mediated by PDGF also involves Akt activation. Using Western blot analyses, it was demonstrated that SH-SY5Y cells pretreated with the PI3K inhibitor followed by exposure to PDGF and/or gp120 had decreased activation of Akt compared with cells not treated with the inhibitor (Figure 7B).

Another downstream pathway employed by PDGF during wound healing and mitotic activity (Rubinfeld and Seger, 2005) is the extracellular regulated kinase 1/2 (Erk1/2) mitogen-activated protein (MAP) kinase. This kinase can be activated by PDGF both via PI3K-dependent pathway and by the Ras/Raf MEK pathway. To examine the role of Erk1/2 in PDGF-mediated protection against gp120 toxicity, cell lysates from SH-SY5Y cells pretreated with PDGF followed by gp120 were assessed for phosphorylated Erk1/2 by Western blot analysis. As shown in Figure 7B, PDGF pretreatment of gp120-treated

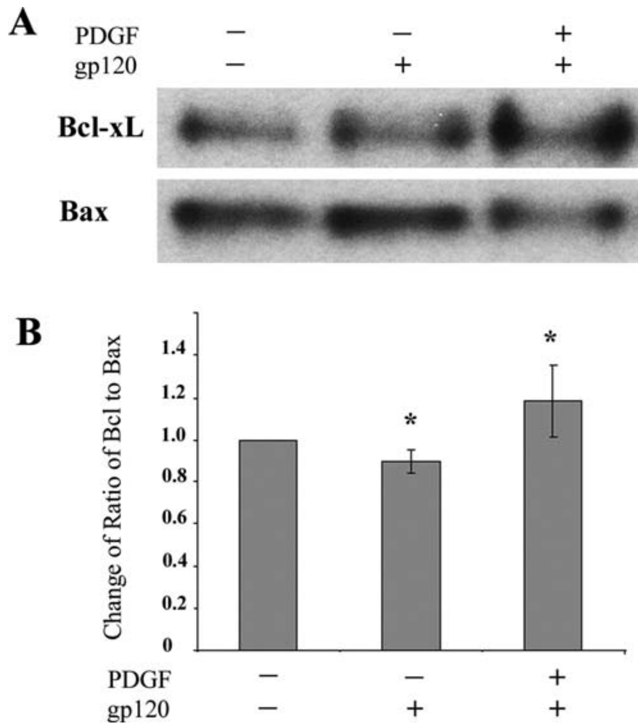

Figure 6 Ratio of anti- and proapoptotic markers in PDGF-mediated neuroprotection of SH-SY5Y cells. (**A**) Cells treated with or without gp120 in the absence or presence of PDGF-BB pretreatment were lysed and assayed for levels of anti- and proapoptotic markers Bcl-xL and Bax, respectively, by Western blot analysis in the same membrane. (**B**) Densitometry scan of the ratio of band intensities of Bcl-xL/Bax from three different Western blot experiments. $^*P < .05$, treatment versus control.

SH-SY5Y cells resulted in increased activation of Erk1/2 compared with cells not treated with PDGF. To confirm that PDGF-mediated activation of Erk1/2 is via the PI3K pathway, pharmacological inhibitor of PI3K was used to pretreat cells prior to PDGF and/or gp120 exposure. Cells treated with the inhibitor demonstrated a decrease in Erk1/2 activation (Figure 7B). Despite the reduction in Erk1/2 activation by PI3K inhibitor, the ability of PDGF to mediate neuroprotection against gp120 remained unchanged in the presence of PI3K inhibitor (Figure 7A), thus suggesting that Erk1/2 activity is not involved in the PDGF-mediated neuroprotection against gp120 toxicity.

Discussion

HIV-associated dementia (HAD) is the most severe form of HIV-related neuropsychiatric impairment. The syndrome is characterized by motor and behavioral dysfunctions leading to seizures, coma, and death within months (Navia *et al*, 1986) and is associated with neuropathology involving HIV-1 proteins and activation of proinflammatory cytokine circuits. Although the incidence of HAD has decreased considerably in the era of antiretroviral therapy (ART), its prevalence is on the rise with the emergence of a more subtle form of minor cognitive motor disorder (McArthur *et al*, 2003). HIVE, the pathologic correlate of HAD reveals a broad spectrum of pathological changes, including multifocal and subacute encephalitis, focal accumulation of macrophages and multinucleated giant cells, widespread reactive astrogliosis, cerebral cortical atrophy, loss of specific neuronal subpopulations, and diffuse white matter pallor (Bell, 1998; Everall *et al*, 1991; Gendelman *et al*, 1994; Nath, 1999; Price *et al*, 1988). Neuronal dysfunction/loss associated with HAD is often exemplified by loss of synapses, shortening of neuritis, and appearance of dendritic abnormalities (McArthur *et al*, 2005). Interestingly, unlike most other viral encephalopathies, neuronal loss associated with HIVE is not due to direct viral infection of the neurons. One broad explanation frequently advocated to explain this indirect killing of neurons is attributed to the toxicity mediated by the viral proteins, including gp120, that are released from virus-infected cells in the CNS (Adamson *et al*, 1996; Brenneman *et al*, 1988; Chen *et al*, 2000; Dreyer *et al*, 1990; Hof *et al*, 1998; Kruman *et al*, 1998; New *et al*, 1997; Patel *et al*, 2000).

The HIV envelope glycoprotein gp120 has been demonstrated to have neurotoxic effects both *in vitro* and *in vivo* (Bachis and Mocchetti, 2006; Bansal *et al*, 2000; Kaul *et al*, 2001; Lipton *et al*, 1991; Meucci and Miller, 1996). gp120 is able to induce apoptosis of neurons in cell culture, both in the presence and in the absence of microglia. Transgenic mice expressing gp120 in the brain manifest neuropathological features similar to those observed in AIDS patients (Mucke *et al*, 1995). Hence, it is both direct and indirect mechanisms that are responsible for the neuronal death associated with HIV-1 infection.

The broad spectrum of behavioral and neuropathological abnormalities observed in patients with HAD suggests a sensitive balance between neuroprotective and neurotoxic factors in the CNS. In the CNS, neurotrophic factors are critical for protection of neurons during cellular injury/stress. PDGF, an important neurotrophic factor is known to attenuate excitotoxic death in cultured hippocampal neurons (Tseng and Dichter, 2005) and induce striatal neurogenesis in adult rats with 6-hydroxydopamine lesions (Mohapel *et al*, 2005). In this present study, we report that PDGF neurotrophic activity can also be extended to neuroprotection against the HIV envelope gp120.

Dramatically reduced expression of PDGF-B chain was observed in neurons in the basal ganglia region of infected macaques with encephalitis compared with similar brain regions of macaques without the disease. In the diseased brains, not only was there a reduction in the expression of PDGF-B chain by neurons, but there was also a concomitant alteration in

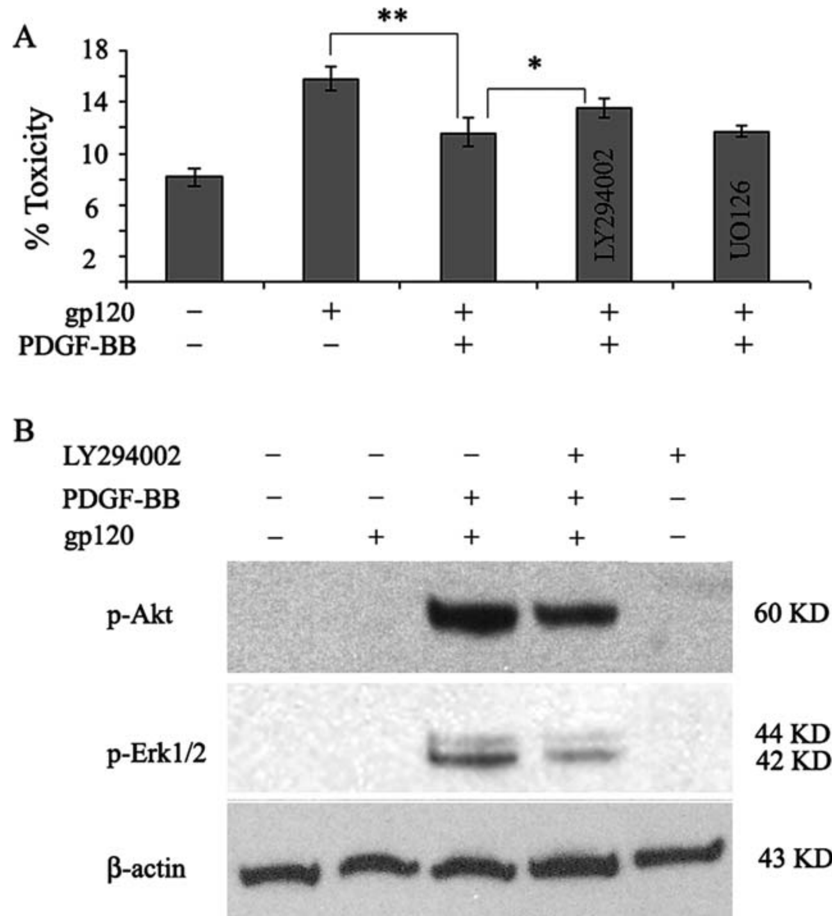

Figure 7 Involvement of PI3k/Akt pathway in PDGF-BB–mediated protection against gp120 toxicity. (**A**) LDH assay of SH-SY5Y cells pretreated with or without PI3K inhibitor (LY294002) or MEK1/2 inhibitor (U0126) followed by PDGF and/or gp120 exposure. Pretreatment of cells with PI3K inhibitor resulted in significant ($P < .05$ PDGF + gp120 + LY versus PDGF + gp120) reduction of PDGF-mediated protection. Pretreatment with Erk1/2 inhibitor did not reverse the protective effect of PDGF. Data represent mean ±SEM from three independent experiments. (**B**) Western blot analysis of lysates of phosphorylated Akt and Erk1/2 in SH-SY5Y cells pretreated with or without PI3K inhibitor (10 μM LY294002) prior to PDGF and/or gp120 exposure. Blots were reprobed with antibody specific for β-actin for normalization.

the neuronal morphology that was visible as rounded cells with retracted neurites.

Treatment of SH-SY5Y with viral neurotoxin gp120 resulted in decrease in PDGF-B chain expression, with corresponding changes in cell morphology involving rounding of cells and loss of neurites. Interestingly, pretreatment of neuronal cells with PDGF-BB abrogated neuronal retraction and resulted in reduced cell death compared with cells treated with gp120 alone. PDGF is known to mediate its neuroprotective effect through the induction of neurite outgrowth, as demonstrated in a variety of *in vitro* neuronal systems, including fetal rat and human dopaminergic neurons (Othberg *et al*, 1995), SH-SY5Y cells (Hynds *et al*, 1997) and hippocampal HiB5 cells (Sung *et al*, 2001). Other neurotrophic factors such as FGF and BDNF have earlier been documented to play a role in rescuing cells against gp120-mediated toxicity (Bachis and Mocchetti, 2005; Hashimoto *et al*, 2002).

gp120-mediated toxicity of neurons involves apoptotic cell death (Singh *et al*, 2004). Neuronal cells pretreated with PDGF-BB exhibited reduced apoptosis as was evident by TUNEL assay. Further corroboration of this phenomenon was also reflected in caspase-3 activity in cells treated with the neurotrophic factor prior to exposure to gp120. Our findings confirmed that gp120 activates caspase-3 and that PDGF-BB abrogated this effect, thus rescuing neurons from the apoptotic effects of gp120. These finds were further confirmed by assessing the relative levels of the anti- (Bcl-xL) and pro- (Bax) apoptotic gene products.

Many studies have implied the involvement of PDGF-mediated PI3K/Akt signaling pathway in cell survival (Simakajornboon *et al*, 2001; Wang *et al*, 2005; Zhang *et al*, 2003, 2005). Using pharmacological inhibitors, our findings also suggest that PDGF exerts its protection against gp120 toxicity by the PI3K/Akt pathway. Erk1/2 that lies downstream of

PI3K did not seem to be involved in the neuroprotection function of PDGF.

Further studies aimed at exploring the detailed signaling pathways and their downstream transcription factors that are involved in the neuroprotective role of PDGF are warranted. Delivery of PDGF in the CNS could have implications in the development of therapeutic strategies against HAD.

Materials and methods

Materials
Human neuroblastoma cells (SH-SY5Y) were purchased from American Type Culture Collection (Manassas, VA). The rationale for choosing these cells was based on their ability to mimic the pathways involved in the neurodegenerative process observed in HIVE (Everall *et al*, 2002; Sanders *et al*, 2000). Human recombinant PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA) and viral gp120 (IIIB strain) was obtained from the AIDS Research and Reference Reagent Program of National Institutes of Health.

Cell culture and treatments
SH-SY5Y cells were plated at a density of 1×10^5/ml and cultured in a 1:1 mixture of Eagle's minimum essential medium containing nonessential amino acids (Gibco, Gaithersburg, MD) and F12 Medium (Gibco) supplemented with heat-inactivated fetal bovine serum (10% v/v), 2 mM glutamine at 37°C in 5% CO_2. Confluent cells were replated at $1–5 \times 10^5$ cells/ml for different experiments and differentiated by treatment with 10 μM retinoic acid (Sigma, St. Louis, MO) for 7 days with medium changes every 2 days. For all of the experiments, cells were serum starved for 24 h in the presence of 10 μM retinoic acid (Sigma) prior to treatment with PDGF-BB (20 ng/ml; predetermined dose) for 30 min, followed by addition of gp120 IIIB (200 ng/ml). In the experiment involving the phosphoinositide 3-OH kinase (PI3K) inhibitor (LY294002; Calbiochem, San Diego, CA) or MEK1/2 inhibitor (U0126; Calbiochem), SH-SY5Y cells were pretreated for 1 h with 10 μM inhibitor, followed by treatment with PDGF-BB and/or gp120 as described above.

Rat cortical neuronal cultures
Primary cultures of rat cortical neurons were prepared from 18-day-old fetuses of Sprague-Dawley rats as previously described (Brewer *et al*, 1993). Briefly, rat brain cortices were harvested by removal of brainstem and hippocampi followed by mechanical trituration. After suspending in Neurobasal medium (Gibco) supplemented with 2 mM glutamax, 2% B-27 supplement, and 1% antibiotic.

The cells were seeded at a density of 40,000 cells per well in 96-well plate or 5×10^5 cells per well in 6-well plate (all plates were precoated with poly-D-lysine) and maintained at 37°C with 5% CO_2. At day 3, half the medium was changed and the cells were cultured for 3 more days. Cells were characterized with immunocytochemistry and were >95% pure.

Immunocyto/histochemistry
Immunohistochemical analysis of PDGF-B chain was carried out on archival paraffin-fixed tissue sections of brain from uninfected or simian human immunodeficiency virus (SHIV)-infected macaques with encephalitis, as described earlier (Sui *et al*, 2004). Sections from basal ganglia region of macaques were treated with rabbit polyclonal PDGF-B antibody (Santa Cruz, CA), followed by treatment with biotinylated goat anti-rabbit secondary antibody and peroxidase-conjugated streptavidin (Dako, Carpinteria, CA) and Nova Red substrate (Vector Laboratories, Burlingame, CA).

For immunocytochemical analysis of caspase-3, cells were treated with various agents as described above followed by fixation with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were then treated with 1:1000 diluted anti-cleaved caspase-3 antibody (Cell signaling, Danvers, MA) for 2 h, followed by treatment with Alexa Fluor 488–conjugated anti-rabbit secondary antibody (1:500; Invitrogen, Carlsbad, CA) for 30 min at room temperature and mounted in Slow Fade anti-fade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). Images were captured using a inverted fluorescence microscope TE2000-E (Nikon, Tokyo, JAPAN).

Lactate Dehydrogenase (LDH) Assay
The LDH release assay was performed using a CytoTox96 nonradioactive cytotoxicity assay (Promega, Madison, MI). LDH released into the culture medium and total LDH of lysed cells were evaluated by this assay. Briefly, the SH-SY5Y cells were seeded at a density of 5×10^4 cells per well on a 96-well microplate. Following treatment with PDGF-BB and/or gp120, 50 μl of medium were moved to a new multiwell plate to evaluate the LDH released from cells into the medium, whereas the remaining medium was replaced with fresh serum-free medium. The cells were lysed by adding 15 μl of a lysis solution (9% Triton X-100 in water) and incubated for 45 min at 37°C. After incubation, the plate was centrifuged ($250 \times g$ for 4 min) and then 50 μl of the supernatant were transferred to another plate. Thereafter, 50 μl of Substrate Mix solution was added to each well (both supernatant and lysed cells) and incubated at room temperature for 30 min. The reaction was stopped with 50 μl of stop solution (1 M acetic acid) and the plates read at 490 nm. The ratio, released LDH:total LDH, was then calculated as a measure of cell death.

TUNEL staining
SH-SY5Y cells were plated at a density of 1×10^5 cells per well in a 24-well plate with cover slips

for TUNEL staining. Following serum-starvation for 24 h and pretreatment with PDGF-BB and/or gp120 for 16 h at 37°C, cells were washed with phosphate-buffered saline (PBS) and fixed for 30 min with 4% paraformaldehyde at room temperature. The fixed cells were permeabilized with 1% Triton X100 for 30 min, followed by staining with TUNEL reaction mixture for 60 min, according to the manufacturer's instruction (Roche, Palo Alto, CA). Coverslips were mounted using Vectashield Mounting medium with DAPI (Invitrogen) as counterstaining and the slides were visualized under dark field using a fluorescence microscope. Six to eight images per treatment group were analyzed. Blind image analysis of tunnel positive cells in various treatments was done using Image J software (version 1.37; NIH, Bethesda, MD). Images from each slide were captured at 20 × using a Nikon TE2000E microscope with a digital camera (Photometrics, Tucson, AZ). Threshold intensity for DAPI labeling was set to allow DAPI signals to be counted while eliminating false positive background staining. The number of DAPI-positive cells was then quantified for all images. Similarly, threshold intensity for TUNEL labeling was set to allow TUNEL-positive cells to be counted while eliminating false-positive background staining. After quantifying the number of TUNEL-positive cells for all the images, the percentage of TUNEL-positive cells to the total number of DAPI-positive cells was determined. The mean percentage (±SEM) of all images from each treatment group was reported.

Caspase-3 activity assay
Activity of caspase-3 was analyzed using the Caspase 3 Colorimetric Assay Kit from R&D Systems following manufacturer's instructions. Briefly, SH-SY5Y cells were plated at 2×10^6 cells per well in 6-well plates. Following 6 h of treatment with PDGF and/or gp120, cells were lysed with 50 μl lysis buffer for 10 min on ice. Following centrifugation ($200 \times g$ for 3 to 4 min), 50 μl of lysate was incubated with 50 μl of 2 × reaction buffer containing 0.5 μl dithiothreitol (DTT) and 5 μl of the caspase-3 colorimetric substrate, DEVD-pNA. Following 2 h of incubation at 37°C, caspase-3 protease activity was measured in a spectrophotometer at wavelength of 405 nm. Absorbance was normalized to the protein concentration of each lysate, which was determined using the BCA Protein Assay Reagent from Pierce Chemical (Rockford, IL). Fold increase in caspase-3 activity in treated cells was calculated relative to the absorbance value obtained from the lysate of untreated cells. Each experiment consisted of seven replicates.

Western blot analyses
SH-SY5Y cells untreated or treated with PDGF and/or gp120 were lysed in lysis buffer (Sigma) containing protease inhibitors after 24 h post treatment. Protein estimation in these samples was measured using the micro-BCA method (Pierce Chemical) protein assay kit. Western blot analyses were performed using primary antibodies against anti and pro-apoptotic proteins 1:200 Bcl-xL and Bax (Cell Signaling) on the same membrane, respectively. Western blots were also probed with antibodies recognizing phosphorylated forms of Akt (1:500; Cell Signaling), Erk1/2 (1:200; Cell Signaling), and β-actin (1:4000; Sigma). The secondary antibodies used were horseradish peroxidase–conjugated anti-rabbit (1:5000; Pierce Chemical) and detection was performed using the enhanced chemiluminescence system (Pierce Chemical). The ratio of Bcl-xL to Bax was then calculated following densitometric analyses of the intensity of bands.

Statistical analysis
Statistical analysis was performed using one-way analysis of variance with a *post hoc* Student *t* test. Results were judged statistically significant if $P < .05$ by analysis of variance.

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