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PDGF-mediated protection of SH-SY5Y cells against Tat toxin involves regulation of extracellular glutamate and intracellular calcium

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Abstract

The human immunodeficiency virus (HIV-1) protein Tat has been implicated in mediating neuronal apoptosis, one of the hallmark features of HIV-associated dementia (HAD). Mitigation of the toxic effects of Tat could thus be a potential mechanism for reducing HIV toxicity in the brain. In this study we demonstrated that Tat induced-neurotoxicity was abolished by NMDA antagonist-MK801, suggesting the role of glutamate in this process. Furthermore, we also found that pretreatment of SH-SY5Y cells with PDGF exerted protection against Tat toxicity by decreasing extracellular glutamate levels. We also demonstrated that extracellular calcium chelator EGTA was able to abolish PDGF-mediated neuroprotection, thereby underscoring the role of calcium signaling in PDGF-mediated neuroprotection. We also showed that Erk signaling pathway was critical for PDGF-mediated protection of cells. Additionally, blocking calcium entry with EGTA resulted in suppression of PDGF-induced Erk activation. These findings thus underscore the role of PDGF-mediated calcium signaling and Erk phosphorylation in the protection of cells against HIV Tat toxicity.

Keywords

PDGF; SH-SY5Y cells; Glutamate Ca²⁺; Erk

Introduction

Worldwide there are around 40 million people infected with human immunodeficiency virus (HIV). In the late phase of HIV-1 infection, a subset of patients will go on to develop end-organ diseases including HIV-associated dementia (HAD) (Albright *et al.*, 2003; McArthur *et al.*, 2005). Clinically, the disease is characterized by cognitive impairment that is later

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Conflict of interest statement

All authors state that there are no actual or potential conflicts of interest.

accompanied by motor symptoms such as gait disturbance and tremor (Navia *et al.*, 1986). Pathological manifestation of the syndrome 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 mechanism(s) underlying the pathogenesis of HAD are complex. Multiple pathways have been implicated in the HIV-mediated neuronal apoptosis/death, including cellular and viral factors.

Neurons are rarely infected by HIV-1 however, neuronal cell death is a common feature of HIV neuropathogenesis. It is thus speculated that the cellular and viral toxic products that are released from virus-infected and/or activated cells could be indirectly responsible for neuronal apoptosis (Zauli *et al.*, 2000; Eugenin *et al.*, 2003). One of the potent viral toxins implicated in neuronal injury/death is the virus transactivator protein, HIV-1 Tat that can both be secreted from infected cells and can also be taken up by neighboring non-infected cells, including neurons (Liu *et al.*, 2000; Eugenin *et al.*, 2003). Tat, a mediator of virus replication, was first identified as a neurotoxin by Nath *et al.* (Nath *et al.*, 1996). It was shown to be released by HIV-infected cells and was detected in the cerebrospinal fluid and sera of HIV-infected patients (Hudson *et al.*, 2000; Wiley *et al.*, 1996). Previous studies have also demonstrated that Tat can interact with the lipoprotein related protein receptor and be taken up by the neurons, resulting in a series of cytoplasmic and nuclear events (Liu *et al.*, 2000; Kumar *et al.*, 1999; McManus *et al.*, 2000; Rappaport *et al.*, 1999).

Neuronal homeostasis is maintained by a fine balance between neurotrophic versus neurotoxic factors. Various neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) have been implicated in the protection of neurons against neurotoxins (Almeida *et al.*, 2005; Arthur *et al.*, 2006; Deierborg *et al.*, 2008). 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). Various neurotrophic factors such as BDNF, fibroblast growth factor, and insulin-like growth factor have been shown to exert neuroprotection against HIV-1 toxicity (Bachis and Mocchetti, 2005; Sanders *et al.*, 2000; Kulik *et al.*, 1997). In our previous study, we have also documented the role of PDGF as a neuroprotective factor against gp120-mediated neurotoxicity (Peng *et al.*, 2008; Peng *et al.*, 2008). The focus of this study was to explore the robustness of PDGF-mediated neuroprotection against yet another potent viral toxin, HIV Tat. The current study sheds light on yet another mechanism of PDGF-mediated neuroprotection against HIV Tat neurotoxicity involving regulation of extracellular glutamate and intracellular calcium.

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 (Sanders *et al.*, 2000; Overall *et al.*, 2002). Human recombinant PDGF-BB was purchased from R&D Systems (Minneapolis, MN, USA) and viral Tat protein was obtained from the AIDS Research and Reference Reagent Program of National Institutes of Health.

Cell culture

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% CO₂. Confluent cells were re-plated at a density of 1-5×10⁵ cells/ml for various experiments and differentiated by treatment with 10μM retinoic acid (Sigma, St, Louis, MO) for 7 days with medium changes every 2 days.

Measurement of extracellular glutamate

Media from SH-SY5Y cells after 24 h of treatment or under control conditions was collected, and the concentration of glutamate was determined spectrophotometrically using a commercially-available kit according to the manufacturer's instructions (Sigma). Briefly, glutamate is transformed into aketoglutarate, NH₄ and NADH in the presence of NAD and glutamic dehydrogenase (GLDH). NADH fluorescence is monitored at an excitation wavelength of 340 nm after 40 min of incubation at room temperature. The concentration of glutamate in the media of mixed cultures treated for 24 h with Tat or PDGF plus Tat was determined after generation of a standard curve. All experiments were repeated at least three times

Measurement of free intracellular Calcium

The changes in Ca²⁺ were monitored using fluo-4/AM (Molecular Probes, Eugene, OR) dissolved in dimethylsulfoxide. SH-SY5Y cells cultured in 35-mm culture dishes were rinsed twice with Bath solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Glucose, 5.5 mM HEPES, pH 7.4), then incubated in Bath solution containing 5μM Fluo-4/AM with 5% CO₂, 95% O₂ at 37°C for 40 minutes, rinsed twice with the Bath solution, mounted on a perfusion chamber, and scanned every second using confocal microscopy (X400) (fluoview 300; Olympus). Fluorescence was excited at 488 nm and the emitted light was read at 515 nm. All analyses of Ca²⁺ were processed at a single-cell level. In order to normalize for variations in initial fluorescence values, the values of the Ca²⁺ response were divided by the resting fluorescence value (calculated as the mean of at least three values prior to the application of PDGF-BB). All experiments were repeated at least three times and representative blots are presented in the figures.

MTT Assay

Cell viability was measured by mitochondrial dehydrogenases [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (MTT) (Sigma) assay as described previously (Yao *et al.*, 2005). Briefly, following treatment of cells 20 μl MTT was added to each well and the cells were cultured for additional 1-4h at 37°C in 5% CO₂. Subsequently the medium containing MTT was removed and replaced with 200 μl methanol in each well. The cell culture plate was shaken gently for 10 min at room temperature and read at 560 nm.

Hoechst staining

To quantify apoptotic cells, cells were stained with 5μM Hoechst 33324 (Molecular Probes, Eugene, OR) and fixed for 15 min at room temperature. The morphological features of apoptosis (cell shrinkage, chromatin condensation, and fragmentation) were monitored by fluorescence microscopy (Nikon TE2000E microscope).

Caspase-3 activity assay

Activity of caspase 3 was analyzed using the Caspase 3 Colorimetric Assay Kit Systems (R&D) following the manufacturer's instructions. Briefly, SH-SY5Y cells were plated at 2×10⁵ cells per well in 6-well plates. Following treatment, cells were collected and lysed with 50μl lysis buffer for 10 min on ice. The lysate was centrifuged at 200g for 5min and was incubated with 50μl of 2×reaction buffer containing 0.5μl DTT and 5μl of the caspase-3 colorimetric substrate, DEVD-pNA. Two hour post-incubation at 37°C, caspase-3 protease activity was measured in a spectrophotometer at a wavelength of 405 nm. Absorbance was normalized to the protein

concentration of each lysate, which was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). The changes in caspase-3 activity in treated cells are presented relative to the values obtained from the untreated samples. Each experiment consisted of three replicates and was repeated at least three times.

Western blotting

Treated cells were lysed using the Mammalian Cell Lysis kit (Sigma). Western blots were then probed with antibodies recognizing the phosphorylated forms of Erk (Cell Signaling, Danvers, MA 1:500), and β -actin (sigma, 1:1000). Secondary antibodies included alkaline phosphatase conjugated to goat anti mouse/rabbit IgG (1:5000). Signals were detected by chemiluminescence (Pierce). All of the Western blot experiments were repeated three times individually and representative blots are presented in the figures.

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 $p < 0.05$ by analysis of variance.

Results

Tat toxin-mediated cytotoxicity of SH-SY5Y cells was reversed by the NMDA receptor antagonist ($\pm$)-MK-801

It is well-known that HIV-1 protein Tat is toxic to the cells (Nath *et al.*, 1996). In order to imitate the neurotoxicity induced by Tat in SH-SY5Y neuroblastoma cells, differentiated SH-SY5Y cells were exposed to varying doses of HIV-1 Tat (50 to 200ng/ml), heat-inactivated Tat or mutant Tat (200ng/ml). Following exposure of SH-SY5Y cells to Tat for 24 h, MTT assay was conducted to analyze the cell viability. As shown in figure 1, 100ng/ml Tat was enough to induce significant cell death ($p < 0.001$ in 100ng and 200ng treatment group). Treatment with heat inactivated or mutant Tat (200ng/ml) did not result in a significant change in cell survival compared with the untreated group.

Pretreatment of SH-SY5Y cells with the NMDA receptor antagonist ($\pm$)-MK-801 (10 μ M) for 60 min, however, increased cell viability by 22% compared with the Tat-treated group, thereby suggesting the role of glutamate-mediated excitotoxicity in Tat induced cytotoxic effects (Fig. 2).

PDGF-BB ameliorated Tat-mediated neurotoxicity

PDGF-B chain is produced by healthy cells (Smits *et al.*, 1991) and has been shown to have neuroprotective properties (Hynds *et al.*, 1997)(Mohapel *et al.*, 2005). It has also been shown to protect cells against HIV-1 coat protein gp120-mediated cytotoxicity (Peng. *et al.*, 2008). We thus hypothesized that PDGF-BB was robust enough to protect cells against yet another HIV protein (Tat) toxicity. To examine the protection of PDGF-BB against Tat-induced neurotoxicity differentiated SH-SY5Y cells were pretreated with 20ng/ml PDGF-BB, followed by exposure to 100ng/ml Tat. Compared with Tat treated cells, pretreatment of cells with PDGF-BB resulted in remarkable reversal of neurotoxicity induced by Tat (Figure 3A) [$p < 0.001$ PDGF plus Tat versus Tat alone]. To corroborate these initial cell viability findings, we also monitored morphological changes in the nuclei of Tat and/or PDGF treated cells using the Hoechst staining. As shown in Figure 3B, Tat-treated SH-SY5Y cells displayed increased nuclear condensation and DNA fragmentation, while pretreatment with PDGF-BB prevented appearance of Tat-induced disintegrated nuclei.

Since there is documented evidence demonstrating apoptosis as a major contributor of neuronal death in HIV-1 infected patients (Gray *et al.*, 2000), we next sought to determine whether

PDGF-pretreatment could lead to reversal of apoptosis mediated by Tat in the differentiated SH-SY5Y cells. As shown in Figure 3C and as expected, exposure of SH-SY5Y cells to Tat resulted in significantly increased activation of caspase-3 compared with the control untreated cells ($p<0.05$). This effect was reversed in cells pretreated with PDGF-BB. PDGF was thus able to reverse Tat-mediated neuronal apoptosis.

PDGF-BB decreased extracellular glutamate levels induced by Tat in SH-SY5Y cells

In order to determine whether the protective role of PDGF against Tat-induced SH-SY5Y cell death was related to reversal of excitotoxicity we monitored levels of extracellular glutamate in cells cultured in the presence of Tat and/or PDGF. As shown in Fig.4 and as expected Tat significantly increased the extracellular glutamate concentration by 84.2% compared with the untreated cells. Pre-treatment of cells with PDGF-BB prior to Tat exposure, however, was able to mitigate Tat-induced increase in extracellular glutamate. Treatment with PDGF-BB alone did not affect the extracellular glutamate levels (data not shown).

PDGF-mediated neuroprotection involves calcium influx

Since Ca^{2+} is known to mediate cell survival, it is likely that Ca^{2+} influx in the cells is a prerequisite for neuroprotection mediated by PDGF-BB in SH-SY5Y cells. In order to test this hypothesis, cells were pre-treated with the extracellular calcium chelator EGTA and subsequently assessed for PDGF-mediated protection against Tat toxicity. As shown in Figure 5 in SH-SY5Y cells pre-treated with EGTA, PDGF-mediated neuroprotection against Tat toxicity was inhibited compared with cells not pretreated with EGTA. These findings underscore the role of calcium influx in PDGF-mediated neuroprotection.

In order to study the effects of PDGF on the intracellular Ca^{2+} transients ($[\text{Ca}^{2+}]_i$) in SH-SY5Y cells, we next measured PDGF-induced intracellular $[\text{Ca}^{2+}]_i$ release using the calcium sensitive Fluo-4 AM imaging technique. As shown in Figures 6A & B, exposure of cells to PDGF-BB triggered a transient but substantial increase in $[\text{Ca}^{2+}]_i$ in SH-SY5Y cells.

Calcium influx is required for PDGF-mediated activation of Erk but not for the Akt pathway

Erk/mitogen-activated protein kinase (MAPK) pathway has been demonstrated to play a crucial role in anti-apoptotic mechanisms. It was therefore of interest to examine whether PDGF-BB mediated neuroprotection involved the Erk activation in SH-SY5Y cells. As shown in Figure 6A exposure of cells to PDGF-BB resulted in a sustained and time-dependent activation of both Erk.

In order to link the PDGF-BB-mediated calcium influx with Erk phosphorylation, SH-SY5Y cells were pretreated with EGTA followed by assessment of PDGF-induced Erk phosphorylation. As shown in Figure 6A, EGTA treatment of cells resulted in decreased activation of Erk. These findings thus underpin the roles of calcium influx and Erk phosphorylation in PDGF-BB mediated neuroprotection.

Discussion

HAD or HIV-associated encephalitis (HIVE) is the most severe form of HIV-related neuropsychiatric impairment found in almost 20-30% of HIV-1 infected patients during late stage AIDS (Kolson and Gonzalez-Scarano, 2000). The histopathology of HAD is characterized by neuronal dysfunction, apoptotic cell death and abnormalities in dendritic processes (Masliah *et al.*, 1997)(Petito and Roberts, 1995). Most strikingly, the neurons of patients with HAD do not appear to be infected with HIV-1, although there is significant neuronal apoptosis. HIV Tat, a virus encoded protein, has been confirmed to be an important pathologic factor present in the brains of HIV-1 infected patients and has been identified a

potent neurotoxin both *in vitro* and *in vivo* model systems (Bonavia *et al.*, 2001)(Perez *et al.*, 2001).

Although our previous findings have reported the protective function of PDGF-BB against gp120 toxicity in SH-SY5Y cells, the present study demonstrated that PDGF can also exert its protection against yet another potent HIV protein-Tat. In this study, we demonstrated PDGF-BB exerted neuroprotection against tat toxicity through regulation of both extracellular glutamate and intracellular calcium.

In the present study we examined Tat-mediated toxicity in a human neuronal model system-differentiated SH-SY5Y cells. Tat exposure of SH-SY5Y cells demonstrated concentration-dependent neurotoxicity as shown in Figure 1. NMDA antagonist MK-801 abolished the neurotoxicity induced by Tat, suggesting that glutamate plays an important role in this process.. Glutamate is a major excitatory neurotransmitter acting on a variety of synapses in the nervous system. It is believed to contribute to neuronal damage by glutamate-calcium overload, based on pharmacological and electrophysiological studies (Eugenin *et al.*, 2003). HIV-Tat can also interact with the cell membrane and enter the cytoplasm and nucleus of cells, resulting in potentiation of N-methyl-D-aspartate (NMDA) activation of currents and increased intracellular calcium ($[Ca^{2+}]_i$) in cells (Reiter and Maible, 2003). Furthermore, HIV Tat is known to increase the release of glutamate through a Ca^{2+} -dependent mechanism (Reiter and Maible, 2003). Similar to findings by Eugenin (Eugenin *et al.*, 2003) and others on the involvement of glutamate excitotoxicity in Tat-mediated apoptosis of primary human cells, our findings on SH-SY5Y cells also demonstrate the role of glutamate in toxicity mediated by Tat. Pretreatment of cells with MK-801 (10 μ M) resulted in significant alleviation of Tat cytotoxicity, thus underscoring the role of glutamate-mediated excitotoxicity in this process. To confirm the role of PDGF in this process, we next monitored the level of extracellular glutamate in cells treated with Tat and/or PDGF. It was found that PDGF pretreatment resulted in decrease in extracellular glutamate levels, thus validating its role in protecting cells against Tat toxicity.

In addition to the regulation of extracellular glutamate, another novel finding of this study is the identification of the role of calcium influx in PDGF-mediated neuroprotection against Tat toxicity. These findings lend credence to previous reports indicating the involvement of calcium signaling in neuroprotection (Bollimuntha *et al.*, 2005; Zheng *et al.*, 2007). PDGF is known to trigger $[Ca^{2+}]_i$ transients in neuronal precursor cells (Cuddon *et al.*, 2008), however, whether PDGF regulates $[Ca^{2+}]_i$ in SH-SY5Y cells remains unclear. Herein we report that PDGF-BB induces $[Ca^{2+}]_i$ transients in SH-SY5Y cells, and this is critical for its neuroprotective effect. PDGF-mediated calcium influx from extracellular source was confirmed by treating cells with EGTA, an extracellular Ca^{2+} chelator. Influx of Ca^{2+} is known to regulate numerous physiological processes through a wide range of target proteins such as Erk (Bosch *et al.*, 1998; Fukuchi *et al.*, 2005). In the present study, $[Ca^{2+}]_i$ elevations triggered by PDGF-BB activated Erk since EGTA significantly decreased Erk phosphorylation induced by PDGF.

Taken together our findings demonstrate the robustness of PDGF-BB against toxicity by not only HIV gp120 but also the Tat protein. These findings are crucial in view of the development of PDGF as an adjunct therapeutic agent for the treatment of HAD.

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Figure 1. Tat is toxic for SH-SY5Y cells

Effects of varying concentrations of Tat on the cell survival of SH-SY5Y cells using the MTT assay. * $p < 0.05$ vs control group.

Figure 2. NMDA antagonist MK-801 alleviated the cytotoxicity of Tat on SH-SY5Y cells
SH-SY5Y cells were pretreated with MK801 for 60 min followed by exposure to Tat for 24 hrs and neuronal viability was measured using the MTT assay. ** $p < 0.01$ vs control group. # $p < 0.05$ vs Tat (200 ng/ml) group.

Figure 3. PDGF exerts neuroprotection against Tat toxicity

PDGF protects SH-SY5Y cells against Tat neurotoxicity as shown by MTT assay (A), Hoechst 33342 staining monitored by fluorescence microscopy (B) and caspase-3 activity assay (C).

All the data in these figures are presented as mean $\pm$ SEM of four individual experiments.

* $p < 0.05$; *** $p < 0.001$ vs control group; # $p < 0.05$ vs Tat (200 ng/ml) group.

Figure 4. Effect of PDGF on the extracellular glutamate levels in SH-SY5Y cell exposed to Tat. PDGF significantly decreased extracellular glutamate in SH-SY5Y cells. All the data in these figures are presented as mean $\pm$ SEM of four individual experiments. * p <0.05 vs control group; # p <0.05 vs Tat (200 ng/ml) group.

Figure 5. Involvement of calcium influx in PDGF-mediated neuroprotection

Cell viability of SH-SY5Y cells exposed to Tat &/or PDGF in the absence or presence of EGTA (1 mM) was measured by MTT assay. All the data in these figures are presented as mean $\pm$ SEM of four individual experiments. * $p < 0.05$ vs control group; # $p < 0.05$ vs Tat-treated group; + $p < 0.05$ vs both PDGF & Tat treated group.

Figure 6. PDGF induced intracellular Ca^{2+} elevations in SH-SY5Y cells
 (A) SH-SY5Y cells loaded with Fluo-4 [Ca^{2+}]_i sensitive fluorophores before and after PDGF treatment were recorded within a single field using a Fluoview 300 confocal microscope. Numbers in the panels indicate time in seconds. (B) Changes in intracellular [Ca^{2+}]_i levels in cells following PDGF treatment were measured using the Fluo-4/Fura Red ratio, and the change in ratio is illustrated from a typical neuron. All the experiments were repeated at least four times.

Figure 7. PDGF-induced Erk phosphorylation involves calcium influx

(A) SH-SY5Y cells exposed to PDGF in the presence of absence of EGTA were monitored for Erk activation. Densitometric analysis of prk/ β -actin from a representative immunoblot is presented. Data are presented as mean $\pm$ SEM of four individual experiments. All of experiments were repeated at least for three times. * $p < 0.05$ vs PDGF alone group.