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HIV-1 Tat-Mediated Induction of Platelet-Derived Growth Factor in Astrocytes: Role of Early Growth Response Gene 1

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HIV-associated neurologic disorders (HAND) are estimated to affect almost 60% of HIV-infected individuals. HIV encephalitis, the pathologic correlate of the most severe form of HAND, is often characterized by glial activation, cytokine-chemokine dysregulation, and neuronal damage and loss. However, the severity of HIV encephalitis correlates better with glial activation rather than viral load. Using the macaque model, it has been demonstrated that SIV encephalitis correlates with increased expression of the mitogen platelet-derived growth factor (PDGF) B chain in the brain. The goal of this study was to explore the role of PDGF-B chain in HIV-associated activation and proliferation of astrocytes. Specifically, the data demonstrate that exposure of rat and human astrocytes to the HIV-1 protein Tat resulted in the induction of PDGF at both the mRNA and protein levels. Furthermore, PDGF-BB induction was regulated by activation of ERK1/2 and JNK signaling pathways and the downstream transcription factor early growth response 1. Chromatin immunoprecipitation assays demonstrated binding of Egr-1 to the PDGF-B promoter. Exposure of astrocytes to PDGF-BB in turn led to increased proliferation and the release of proinflammatory cytokines MCP-1 and IL-1β. Because astrogliosis is linked to disease severity, understanding its regulation by PDGF-BB could aid in the development of therapeutic intervention strategies for HAND. The Journal of Immunology, 2011, 186: 4119–4129.

Human immunodeficiency virus 1 is capable of penetrating the brain shortly after initial infection (1). Although combined antiretroviral therapy is able to control the virus in the periphery, the drugs have inferior penetration across the blood-brain barrier (2). Patients with HIV are living longer, but they have to cope with the long-term effects of having HIV in the brain. Despite the efficacy of combined antiretroviral therapy, HIV-associated neurocognitive disorders (HANDs)—which include HIV-associated dementia, minor cognitive motor disorders, and other HIV-related neuropsychiatric impairments—are estimated to affect almost 60% of patients with HIV (3, 4). A diagnosis is made by changes in behavior and cognitive and motor abnormalities (4). HIV-associated dementia, the most severe form of HIV-induced CNS impairment (5), is clinically characterized by motor and behavioral dysfunction that in the absence of therapy can lead to seizures, coma, and death within 6 mo of onset (6). Pathologic correlates of HIV-associated dementia include astrogliosis, microglial activation, monocyte infiltration, and neuronal damage. With the increasing prevalence of HAND, it is essential to understand the cellular and molecular mechanisms by which HIV exerts its detrimental effects on the CNS.

Astrocytes, the most abundant cells in the CNS, are a type of glial cell capable of releasing toxic mediators after activation by either infection or injury. In addition to activation, these cells can also undergo active proliferation to replace neurons undergoing apoptosis (7). Although astrocytes are not productively infected with HIV, the provirus in these cells is able to make the early HIV-1 genes Tat, Rev, and Nef (8, 9). HIV-1 Tat is expressed in astrocytes and other productively infected cells like microglia, and it can be released from these cells to activate other neighboring cells. Kutsch et al. (2000) has reported that astrocytes activated by Tat have the ability to release toxic mediators (9).

One of the toxic mediators shown to be upregulated in the brains of macaques with SIV encephalitis (SIVE) is the mitogen platelet-derived growth factor (PDGF) (10). PDGFs are a family of proteins comprising of four chains (A–D) encoded by four genes located on different chromosomes, and they are highly conserved throughout the animal kingdom (11). These proteins are usually expressed as dimers; PDGF-A and -B can form homodimers or heterodimers, and PDGF-C and -D form homodimers. For the sake of clarity, PDGF-A to -D refer to the RNA expression, whereas PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD refer to the protein expression of these genes. All four PDGF ligands exert their effects via tyrosine kinase receptors PDGF-Rα and PDGF-Rβ (12). Members of this family are disulfide-bonded polypeptides that have multifunctional roles ranging from embryonic development to wound healing (13–15). PDGF signaling has been implicated in a number of pathologic disease states including, liver and lung cardiac fibrosis, atherosclerosis, restenosis, etc.
**FIGURE 1.** HIV-1–Tat-mediated upregulation of PDGF-BB in astrocytes. 

**A.** HIV-mediated induction of PDGF-BB in human A172 astrocytes. Whole cell lysates from human A172 cells were subjected to Western blot analysis using Abs specific for PDGF-BB. 

**B.** Total RNA isolated from primary rat astrocytes was subjected to real-time PCR analysis using primer sets for PDGF-A, PDGF-B, and PDGF-C primers. Tat mediated induction of PDGF-B mRNA expression, but no changes in PDGF-A and PDGF-C were evident. Heated Tat abolished Tat-mediated induction of PDGF-B. 

**C.** All Tat preparations upregulated PDGF-BB protein expression equally. Human A172 cells were treated with Tat lengths 1–72, 1–86, and 1–101 and PDGF-BB levels were assessed by Western blot analysis. 

**D.** Tat-mediated upregulation of PDGF-BB in human primary astrocytes. Whole cell lysates from human primary astrocytes treated with Tat for 12 h were subjected to Western blot analysis. 

**E.** Time dependence of Tat-mediated induction of PDGF-B mRNA expression in rat primary astrocytes, rat gliomas, and human A172 cells. 

**F.** Whole cell lysates from rat primary astrocytes, rat gliomas, and human A172 cells were subjected to Western blot analysis using Abs specific for PDGF-BB. Time dependence of Tat-mediated induction of PDGF-BB protein expression in rat primary astrocytes, rat gliomas, and human A172 cells. All data are presented as mean ± SD of three individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control group, ****p < 0.001 versus Tat-treated group. 

**G.** Representative image of PDGF-BB staining in rat glioma cells (original magnification ×400). Images were acquired using an ×40 oil-immersion lens and fluorescent microscopy. 

**H.** Representative image of GFAP plus...
pulmonary hypertension, cancer, gliomas, and stroke (11, 16–20); however, its role in HIV encephalitis (HIVE) and HAND, specifically as it pertains to astrogliosis, has not been elucidated.

In this study, we used rat and human astrocytes to study the effect of Tat on rat and human astrocytes. The data demonstrate that exposure of rat and human astrocytes to Tat resulted in the induction of PDGF at both the mRNA and protein levels. We further elucidated that signaling mechanisms involved in PDGF-BB induction were regulated by activation of ERK1/2 and JNK signaling pathways and the downstream transcription factor early growth response gene 1 (Egr-1). Chromatin immunoprecipitation (ChIP) analysis revealed increased binding of Egr-1 to the PDGF-B promoter in rat glioma cells treated with Tat. Exposure of astrocytes to PDGF-BB led to increased proliferation and release of proinflammatory cytokines MCP-1 and IL-1β. Because PDGF is a known cerebrovascular permeant (21), the release of PDGF by astrocytes may have significant consequences, including disruption of the blood-brain barrier. This finding is particularly important because astrocyte end-feet processes are in close contact with the endothelial cells of the blood-brain barrier. Furthermore, PDGF expression could also enhance astrocyte activation. An understanding of PDGF-BB regulation and its inhibition could aid in the development of therapeutic intervention strategies for those suffering from HAND.

Materials and Methods

Materials

Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). Because serum induces PDGF, all experiments involving the treatment of cells with exogenous PDGF-BB were conducted under serum-free conditions. ST-571, an inhibitor of tyrosine kinase receptors, was obtained from Novartis (Basel, Switzerland). The specific phosphatidylinositol-3-kinase (PI3K) inhibitor (LY294002), MEK inhibitor (U0126), and p38 inhibitor (SB203580) were purchased from Promega (Madison, WI). The JNK inhibitor (SP600125) was purchased from Assay Designs (Ann Arbor, MI). The concentrations of these inhibitors were based on the concentration-curve study and our previous reports (22, 23). Tat 1–72, 1–86, and 1–101 (supplied by Philip Ray, University of Kentucky) were used in these studies at a concentration of 200 ng/ml. Details of Tat production and purification have been published previously (24–26). Control treatments included heat inactivated HIV-1 Tat and cells receiving no treatment. The concentration of Tat in the cerebral spinal fluid has been reported at 16 ng/ml (27); however, the Tat concentration used in this in vitro study is generally accepted (28–32). HIV-1 LTR virus propagated in stimulated PBMCs (33) (supplied by Dr. Howard Gendelman, University of Nebraska Medical Center) was used in this study. The rationale for using CXC4R (X4)-tropic virus was based on previous studies demonstrating astrocyte activation in response to X4 viruses (34, 35). Dominant negative and constitutively active constructs of MEK and Egr-1 were provided by Dr. Young Han Lee (Konkuk University, Korea). Chromatin immunoprecipitation (ChIP) assay was purchased from Upstate (Billerica, MA).

Cell culture and cell lines

The human astrocytic cell line A172 (ATCC no. CRL-1620; American Type Culture Collection) was cultured as described previously (36). Rat glioma C6B2 cells were obtained from Dr. Myron Toews (University of Nebraska Medical Center). Human primary astrocytes were obtained from the Congenital Defects Lab (University of Washington). Rat primary astrocytes were isolated from embryonic day 18–19 Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA). Cortical tissues were dissected in HBSS and trypsinized. Cells were pelleted, plated, and sustained in CO2 incubator at 37°C for 7 d. The cells were then shaken to remove loosely attached cells. Attached cells that remained, primarily astrocytes expressing PDGF-BB surrounding the blood vessels (vWF positive) in the basal ganglia of SIV-infected rhesus macaques with or without SIVE (original magnification ×200). Images were acquired using an ×20 lens and fluorescent microscopy. l, Representative image of GFAP plus astrocytes expressing PDGF-BB in the basal ganglia sections from humans with HIV compared with uninfected (HIV−) controls (original magnification ×200). Images were acquired using an ×20 lens and fluorescent microscopy. GFAP immunoreactivity was stained in red, PDGF-BB immunoreactivity was stained in green, and DAPI staining was performed to visualize the nuclei (blue). n = 3 per group.
**Short interfering RNA transfection**

Short interfering RNA (siRNA) targeting against Egr-1 were obtained from Dharmacon (Boulder, CO). Human A172 cells were plated in 24-well plates at a density of $4 \times 10^4$ cells per well 1 d prior to transfection. Cell culture medium was replaced with 250 µl prewarmed culture medium. DharmaFECT 1 transfection reagent (Dharmacon) was then combined with serum-free DMEM medium (Invitrogen Life Technologies) for 5 min at room temperature. The Egr-1 siRNA was then added into the mixture described above to a final concentration of 5 µM. The siRNA and the reagent mixture

**FIGURE 2.** ERK1/2 and JNK MAPK but not p38 and PI3K/Akt pathways are involved in Tat-mediated PDGF-BB expression in astrocytes. A, Western blot analysis of time-dependent activation ERK1/2, JNK, p38, and Akt by Tat. B, ERK1/2 and Akt pathways are activated independently of each other. Pretreatment of cells with MAPK (MEK) inhibitor (U0126), resulted in abrogation of Tat-induced phosphorylation of ERK1/2. However, pretreatment of cells with PI3K inhibitor (LY294002) failed to inhibit ERK1/2 phosphorylation. Conversely, treatment of cells with PI3K inhibitor resulted in the inhibition of Tat-induced activation of Akt, but not ERK1/2. C, Pretreatment of cells with MEK inhibitor (U1026) and JNK inhibitor (SB600125) resulted in the amelioration of Tat-mediated PDGF-BB expression. Conversely, pretreatment with PI3K inhibitor (LY294002) failed to inhibit Tat-mediated PDGF-BB expression. D, Pretreatment of cells with p38 inhibitor (SB203580) did not ameliorate Tat-mediated induction of PDGF-BB. E, Transfection with DN-MEK and not WT-MEK resulted in abrogation of Tat-mediated induction of PDGF-BB. All data are presented as mean ± SD of three individual experiments. **p < 0.01, ***p < 0.001 versus control group, *p < 0.05, ##p < 0.01, ###p < 0.001 versus Tat-treated group.
were incubated for 20 min at room temperature, after which the combined mixture was added to the cells. The cell culture plate was shaken gently for 5 s and incubated for 48 h at 37°C. Knockdown efficiencies were determined by Western blotting.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer’s instructions (Upstate) with slight modifications. After treatment of the cells, 18.5% fresh formaldehyde was added directly into the medium at a final concentration of 1% formaldehyde and incubated for 10 min at room temperature, followed by quenching with 125 mM glycine. The cells were then scraped using 2 ml prechilled PBS containing 1× protease inhibitor mixture. The cell pellet was harvested by spinning at 800 × g at 4°C, and lysis buffer was added (provided in the kit) to harvest nuclei. DNA was then sheared by sonication. Fifty microliters of the sheared cross-linked chromatin was then mixed with 20 μl protein A magnetic beads and 5 μg immunoprecipitating Abs against Egr-1, acetyl histone H3 (as a positive control), and normal rabbit IgG (as a negative control) diluted in 450 μl dilution buffer overnight at 4°C. The magnetic beads binding Ab–chromatin complex was then washed with 0.5 ml each of a series of cold wash buffers in the order of low salt buffer, high salt buffer, LiCl buffer, and Tris-EDTA buffer. The cross-linking of protein–DNA complexes were reversed to free DNA by incubation at 62°C for 2 h and purified using DNA purification spin columns following the manufacturer's instructions. Finally, the purified DNA was amplified via PCR.

**FIGURE 3.** Egr-1 expression is upregulated in astrocytes exposed to Tat. A, Time dependence of Tat-mediated induction of Egr-1 mRNA expression in rat gliomas and human A172 cell lines and rat primary astrocytes. B, Whole cell lysates from rat gliomas and human A172 cell lines and rat primary astrocytes were subjected to Western blot analysis using Abs specific for Egr-1. C, Representative picture of Egr-1 staining in rat gliomas (original magnification ×400). Images were acquired using fluorescent microscopy. D, Inhibition of the ERK1/2 and Akt pathways by MEK (U0126) and PI3K inhibitor (LY294002) resulted in amelioration of Tat-mediated Egr-1 expression. E, Transfection with DN-MEK and not WT-MEK resulted in abrogation of Tat-mediated induction of Egr-1. All data are presented as mean ± SD of three individual experiments. *p < 0.05, **p < 0.01 versus control group, ###p < 0.001 versus Tat-treated group.
to identify the promoter region containing Egr-1 binding site “GCG GGG GCG.” The sequence of the primers used to identify the PDGF-B promoter bound to Egr-1 were as follows: sense, 5'-GCAGAGGCGCTGGAGCGCCTGATC-3'; anti-sense, 5'-GCAGCGATTGACGGCGACTCCG-3'.

Assay for cell viability

Cell viability was measured by mitochondrial dehydrogenases (i.e., MTT) method. Human A172 and rat glioma cells were seeded in 96-well plates at a density of 10^4 cells/cm^2 and were exposed to PDGF-BB for 4 d. After incubation, 20 μl MTT salt dissolved in HBSS at a final concentration of 5 mg/ml was added to each well for 4 h. The medium was aspirated from each well, and 200 μl DMSO was added to dissolve the formazan crystals. The absorbance of each well was obtained using a Synergy Mx (BioTek) plate counter at test and reference wavelengths of 570 and 630 nm, respectively.

Statistical analysis

Statistical analysis was performed using one-way ANOVA with a post hoc Student t test. Results were judged statistically significant if p < 0.05 by ANOVA.

Results

HIV-1/Tat-mediated upregulation of PDGF in astrocytes

Because astrocytes in the CNS are exposed to HIV-1, we first sought to examine the modulation of PDGF-BB by HIV-1 exposure in astrocytes. Serum-starved human astrocyte cell line A172 was infected with HIV LAI at a multiplicity of infection of 0.1 for 12 h followed by assessment of cell lysate for PDGF-BB

FIGURE 4. Involvement of Egr-1 in Tat-induced expression of PDGF-BB in astrocytes. A, Kinetic profiles of Egr-1 and PDGF-B in rat primary astrocytes, rat gliomas and human A172 cells. B, Whole-cell lysates from A172 cells transfected with either Egr-1 or Nonsense (Non) siRNAs were subject to Western blot analysis using Abs specific Egr-1. C, Egr-1 siRNA, but not nonspecific siRNA, inhibited HIV-1 Tat-mediated induction of PDGF-BB expression. D, Whole-cell lysates from A172 cells transfected with either WT or DN forms of MEK were subjected to Western blot analysis using Abs specific Egr-1. DN-MEK, but not WT-MEK, inhibited Tat-mediated induction of PDGF-BB expression. E, Schematic illustration of Egr-1 binding consensus sequence on the PDGF-B promoter region. F, ChIP assay demonstrating Tat-mediated binding of Egr-1 to the PDGF-B promoter. All data are presented as mean ± SD of three individual experiments. **p < 0.01, *** p < 0.001 versus control group.
expression by Western blot. As shown in Fig. 1A, there was HIV-1–mediated upregulation of PDGF-BB in cells exposed to the virus, and heat inactivated virus failed to mediate induction of PDGF-BB.

Having determined the effect of whole virus on inducing PDGF-BB expression, the next step was to assess whether viral transactivator protein Tat, which is produced by both infected astrocytes and released from neighboring infected CNS cells, could also mediate this effect. Rat primary astrocytes were serum starved overnight followed by treatment with recombinant Tat (200 ng/ml) for 3 or 6 h. As an initial screen to identify which PDGF chains were expressed in response to Tat, mRNA levels of PDGF-A, PDGF-B, and PDGF-C chains were determined by real-time PCR. After exposure of rat astrocytes to Tat for 3 and 6 h, there was upregulation of PDGF-B mRNA (2.15- and 3.2-fold, respectively), compared with the untreated controls. As expected, heated Tat had no effect on the induction of PDGF-B mRNA. In contrast, no significant changes in the mRNA levels of PDGF-A and PDGF-C were evident (Fig. 1B). Because only PDGF-B chain was upregulated in response to Tat, further study was focused only on the PDGF-B chain. Similar to Tat 1–72, both Tat 1–86 and the full length Tat 1–101 also mediated induction of PDGF-BB in human A172 cells (Fig. 1C). Validation of our findings in the human astrocyte cell line was also confirmed in human primary astrocytes (Fig. 1D).

To assess the time course of Tat-mediated induction of PDGF-B, rat and human astrocyte cell lines (C6B2 and A172) as well as rat primary astrocytes were treated with Tat (200 ng/ml) for varying times (5 min to 6 h), followed by RNA extraction and assessment of PDGF-B chain mRNA levels by real-time PCR. As shown in Fig. 1E, PDGF-B mRNA was upregulated in a time-dependent manner in all the cell types examined, with a peak expression at 3–6 h. To examine whether the mRNA upregulation translated into increased protein expression, all the cell types were treated as described above, followed by cell lysis and Western blotting. Because PDGF-BB is an early response protein, we also chose earlier time points for detection. As shown in Fig. 1F, Tat upregulated PDGF-BB protein expression in a time-dependent manner with a peak expression at 6 h and a decline thereafter. PDGF-BB expression in rat astrocytes appeared to peak earlier (3 h) compared with its expression in the cell lines (6 h). Confirmation of these findings by immunostaining also revealed increased PDGF-BB expression in Tat-treated rat glioma cells at 6 h after treatment (Fig. 1G). Images were captured with an ×40 objective lens and fluorescence microscopy. Cumulatively, these data clearly demonstrate HIV-1–Tat-mediated induction of PDGF-BB protein in rat and human astrocytes.

To validate PDGF-BB expression in SIV or HIV infection, paraffin-embedded sections of basal ganglia from SIV-infected rhesus macaques with (SIVE) and without encephalitis were stained for PDGF-BB, an endothelial cell marker, von Willebrand Factor (vWF), and glial fibrillary acidic protein. As shown in Fig. 1H, there was upregulated expression of PDGF-BB in astrocytes surrounding the blood vessels in brains of macaques with SIVE (right panels) compared with the infected animals without encephalitis (left panels). Similar studies were performed on basal ganglia sections from human subjects with HIV/E and uninfected controls. As shown in Fig. 1H, there was increased expression of astrocytic PDGF-BB in the sections from HIV/E versus uninfected controls. Images were captured with a ×20 objective lens and fluorescence microscopy.

FIGURE 5. PDGF-BB induces cell proliferation and proinflammatory cytokines expression in astrocytes. A, A representative RT-PCR gel of PDGF-BB mRNA expression in human A172, rat primary astrocytes, and gliomas. B, Rat gliomas and human A172 cell lines incubated with PDGF-BB showed increased proliferation by MTT assay. C, mRNA isolated from human A172 cells was subjected to real-time PCR analysis using MCP-1 and IL-1β primers. PDGF-BB induction of MCP-1 and IL-1β mRNA expression in human A172 cell line. All data are presented as mean ± SD of at least three individual experiments. *p < 0.05, *** p < 0.001 versus control group.

Having determined Tat-mediated induction of PDGF-BB, we next sought to elucidate the signaling pathways involved in this process. Because Tat signaling involves MAPK pathways, we examined the involvement of ERK1/2, JNK, and p38 kinases in Tat-mediated induction of PDGF-BB. Treatment of rat glioma cells with HIV-1 Tat resulted in a time-dependent increase in phosphorylation of ERK1/2, JNK, p38, and Akt, with maximal activation at 30 min after treatment (Fig. 2A). Specificity of these signaling pathways was subsequently assessed using a pharmacologic approach. Pretreatment of cells with MAPK (MEK) inhibitor U0126 resulted in abrogation of Tat-induced phosphorylation of ERK as expected, because MEK lies upstream of ERK1/2. However, pretreatment of cells with PI3K inhibitor LY294002 failed to inhibit ERK phosphorylation. Conversely, treatment of cells with PI3K inhibitor resulted in the inhibition of Tat-induced activation of Akt, but not ERK1/2 (Fig. 2B).

We next wanted to address the functional role of MAPK and PI3K/Akt in the PDGF-BB expression induced by Tat. Human A172 cells were pretreated with inhibitors specific for the respective signaling pathways prior to stimulation with Tat and
assessed for expression of PDGF-BB. As shown in Fig. 2C, pretreatment of cells with MEK (U0126, 20 μM), JNK (SP600125, 20 μM), but not PI3K (LY294002, 10 μM) inhibitor, resulted in the amelioration of Tat-mediated induction of PDGF-BB. Pretreatment of A172 astrocytes with p38 inhibitor (SB203580, 10 μM), however, did not result in the amelioration of PDGF-BB expression in response to Tat (Fig. 2D). Further validation of the involvement of ERK pathway in this process was confirmed by transfecting cells with either the WT or DN constructs of MEK followed by treatment with Tat. Tat-mediated induction of PDGF-BB was attenuated by DN-MEK, but not by WT-MEK construct (Fig. 2E). These findings confirm the involvement of MEK MAPKs, but not p38 and PI3K/Akt cascade in Tat-mediated induction of PDGF-BB in astrocytes.

Egr-1 expression is upregulated in astrocytes exposed to Tat

Having determined the involvement of ERK1/2 and JNK MAPKs in Tat-mediated PDGF-BB expression and from published reports suggesting the binding of Egr-1 to PDGF-B promoter, we rationalized the involvement of Egr-1 in Tat-mediated induction of PDGF-BB (37). Exposure of astrocytes to Tat resulted in a time-dependent increase of Egr-1 expression both at the mRNA and protein levels in rat and human astrocyte cell lines (C6B2 and A172) as well as rat primary astrocytes. As shown in Fig. 3A, Egr-1 mRNA was upregulated in a time-dependent manner in all the cell types examined, with a peak at 15–30 min. To examine whether the mRNA upregulation translated into increased protein expression, all the cell types were treated as described above, followed by Western blotting. As shown in Fig. 3B, Tat upregulated Egr-1 expression in a time-dependent manner with a peak expression between 1 and 3 h and a decline thereafter. Confirmation of these findings by immunostaining also revealed increased Egr-1 expression in Tat treated human A172 cells at 1 h after treatment (Fig. 3C). Cumulatively, these data clearly demonstrate that Tat mediated the induction of Egr-1 expression in rat and human astrocytes.

The next logical step was to examine whether there was a link that could tie together the activation of ERK1/2, JNK MAPKs, and PI3K/Akt with Egr-1. Similar to our studies on signaling molecules described above, astrocytes were pretreated with MEK, JNK, or PI3K inhibitors followed by Tat treatment. As shown in Fig. 3D, MEK and JNK inhibitors but not PI3K inhibitor ameliorated the Tat-mediated activation of Egr-1. These findings thus linked Tat-mediated activation of ERK1/2 and JNK to the downstream activation of Egr-1.

Further validation of the involvement of ERK1/2 pathway in this process was confirmed by transfecting cells with either the WT or DN constructs of MEK followed by treatment with Tat. Tat-mediated induction of Egr-1 was attenuated by DN-MEK, but not by the WT-MEK construct (Fig. 3E). These findings underpin the involvement of MEK MAPKs, but not the PI3K/Akt cascade in Tat-mediated induction of Egr-1 in astrocytes.

Involvement of Egr-1 in Tat-mediated expression of PDGF-BB in astrocytes

Because Egr-1 is a transcription factor implicated in the induction of PDGF-BB, we next wanted to examine the expression kinetics of Egr-1 and PDGF-B mRNAs. As shown in Fig. 4A, in both the cell lines and primary astrocytes, Egr-1 expression (peaking at 15–30 min) preceded that of the PDGF-B chain expression (peaking at 3–6 h).

To confirm the role of Egr-1 in Tat-mediated induction of PDGF-BB, knocking down the expression of Egr-1 using the siRNA approach was used. As shown in Fig. 4B, transfection of human A172 cells with Egr-1 siRNA resulted in efficient knockdown of Egr-1 protein using Western blot assay. Furthermore, Egr-1 siRNA also significantly abrogated Tat-mediated upregulation of PDGF-BB expression (Fig. 4C). To further validate the involvement of the Egr-1 in Tat-induced regulation of PDGF-BB, cells were transfected with either WT or DN constructs of Egr-1, followed by treatment with Tat. Tat-mediated induction of PDGF-BB was attenuated by the DN–Egr-1 construct, but not by the WT–Egr-1 construct (Fig. 4D). Collectively, these findings thus underscore the role of Egr-1 in Tat-mediated induction of PDGF-BB.

To further confirm the binding of Egr-1 with PDGF-B promoter in its natural chromatin context, we resorted to chromatin immunoprecipitation to reveal active sites accessible to Egr-1. Rat glioma cells were treated with Tat for 3 h followed by DNA extraction and processed using a ChIP analysis kit. These experiments revealed increased binding of Egr-1 to the PDGF-B promoter in rat glioma cells treated with Tat (Fig. 4E, 4F).

FIGURE 6. Schematic of the signaling pathways involved in the increased induction of PDGF-BB in astrocytes stimulated with Tat. Tat-mediated activation of ERK1/2, JNK MAPKs, and PI3K/Akt signaling pathways. ERK1/2 and JNK MAPKs but not and PI3K/Akt signaling resulted in the subsequent activation of the downstream transcription factor, Egr-1. Activation of Egr-1 in turn leads to enhanced PDGF-BB expression.
**PDGF-BB induces cell proliferation and proinflammatory cytokine expression in astrocytes**

Having determined the induction of PDGF-BB by Tat, the next step was to explore the functional relevance of this upregulation. Because PDGF-BB is a known mitogen for various cell types, we hypothesized that Tat-induced PDGF-BB released from the astrocytes could act on the astrocytes themselves via an autocrine loop. We set out to examine the effect of PDGF-BB on both proliferation and expression of proinflammatory cytokines in these cells; however, before proceeding with this, it was important to first examine whether astrocytes indeed expressed the PDGF-β receptor (PDGF-Rβ). As shown in Fig. 5A, rat glioma and human astrocyte cell lines (C6B2 and A172) as well as rat primary astrocytes cells expressed PDGF-Rβ mRNA as demonstrated by RT-PCR.

The effect of PDGF-BB on astrocyte proliferation demonstrated a significant increase in cell proliferation, as evidenced by MTT assays in both rat glioma and human A172 cells (Fig. 5B). In addition to proliferation, astrocytes also respond to activation by releasing a plethora of cytokines and chemokines. To further elucidate the role of PDGF-BB in this process, and because it is a known inducer of the chemokine MCP-1 (38), we next examined the effect of PDGF-BB on the induction of MCP-1 and the proinflammatory cytokine IL-1β in A172 cells. As shown in Fig. 5C, treatment of human A172 cells with PDGF-BB resulted in a dramatic upregulation of MCP-1 and a significant induction of IL-1β as measured by real-time PCR.

The data in this article details a molecular mechanism of Tat-mediated PDGF-BB expression in astrocytes (Fig. 6). Tat activation of the ERK1/2 and JNK MAPKs resulted in activation of the downstream transcription factor, Egr-1. Upon activation, Egr-1 translocates to the nucleus and binds to the PDGFβ promoter region activating its transcription.

**Discussion**

Antiretroviral therapies have proved highly effective in controlling systemic viral infection, thus leading to increased longevity in patients with AIDS. The inability of some of these drugs to cross the blood-brain barrier results in slow and smoldering infection in the CNS. Subsequently, the brain becomes a sanctuary of virus-induced toxicity leading to increased prevalence of HAND in HIV-infected individuals. One of the hallmark features of HAND is increased astrogliosis comprising increased numbers of activated astrocytes, culminating ultimately into increased neuronal degeneration. It is well recognized that activation of astrocytes leads to the release of a plethora of inflammatory cytokines and chemokines as well as factors such as PDGF-BB. PDGF-BB has been implicated in a variety of pathologic conditions; however, its role in HIV pathogenesis remains poorly defined.

PDGF-BB has been shown to be upregulated in the brains of macaques with SIVE (10). It belongs to a family of five dimeric ligands (PDGF-AA, -AB, -BB, -CC, and -DD) assembled from four gene products (PDGF-A, -B, -C, and -D) that act through two classical receptor-tyrosine kinases, PDGF-Ra and PDGF-Rβ (10, 11, 18, 39).

HIV Tat protein that is released from HIV-infected cells is often taken up by the neighboring cells in the CNS. It has been previously reported that Tat-expressing astrocytes caused astrocytosis, astrocyte dysfunction, and subsequent neuronal death (40), suggesting that both astrocyte dysfunction and certain factors induced by HIV-1/Tat may contribute to neurotoxicity. Because astrocytes, the most abundant cells within the CNS play a key role in the pathogenesis of HAND via the release of proinflammatory cytokines, chemokines, and other toxic mediators, this study was undertaken to explore the role of yet another mediator PDGF-BB that is released by astrocytes in response to HIV-1 or HIV-Tat. We demonstrate that exposure of HIV-1/Tat to rat and human astrocyte cell lines as well as rat primary astrocytes resulted in the induction of PDGF at both the transcriptional and translational levels.

In our efforts to dissect Tat-mediated downstream signaling events, we demonstrated the activation of ERK1/2, JNK, p38 MAPK, and PI3K/Akt pathways by Tat. Further dissection of the signaling pathways involved in Tat-mediated induction of PDGF-BB using both the pharmacologic and genetic approaches revealed activation of ERK1/2, JNK, p38 MAPKs, and PI3K/Akt pathways. Despite the activation of all these pathways, Tat-mediated induction of PDGF-BB involved only the ERK1/2 and JNK, but not p38 and PI3K/Akt signaling.

The transcription factor Egr-1 has emerged as a major regulatory transcription factor for a number of genes including growth factors such as PDGF (41–43). Our findings demonstrated a time-dependent upregulation of Egr-1 again at both the transcriptional and translational levels in rat and human astrocytes. Further dissection of Egr-1 regulation using both the pharmacologic and genetic approaches revealed the activation of upstream ERK1/2 and JNK MAPK pathways in the activation of Egr-1. In agreement with our findings, requirement of ERK1/2 and JNK activation for Egr-1 expression has been reported in FGF2-treated astrocytes (44).

Considering that Egr-1 is an early response gene that regulates a number of other target genes, it was of interest to examine its expression profile compared with that of PDGF-B expression. Interestingly, Egr-1 expression preceded that of PDGF-B, leading us to speculate their interaction. In an effort to determine a link between Egr-1 and PDGF-B expression, we demonstrated increase Egr-1 binding to the PDGF-B promoter in astrocytes treated with Tat, lending credence to the role of Egr-1 in PDGF-BB expression. Further support of Egr-1 involvement in Tat-mediated PDGF-BB induction was also demonstrated using both the siRNA and genetic approaches. Our findings are in agreement with the report by Khachigan et al. (37), which demonstrated that Egr-1 interacts with the PDGF-B promoter in arterial endothelial cells. The role of Egr-1 in Tat-mediated dysfunction of astrocytes has recently been elegantly demonstrated by Fan et al. (52).

Because Tat induced PDGF-BB, it was critical to understand what role released PDGF-BB played in these cells. Based on the mitogenic function of PDGF-BB, we rationalized that PDGF-BB might be involved in increasing astrocyte proliferation. As expected, exogenous PDGF-BB increased astrocyte proliferation and led to the release of the chemokine MCP-1. These findings are consistent with the reports on PDGF mediated induction of MCP-1 in fibroblast cell line and in smooth muscle cells (45, 46). MCP-1 is a known biomarker of HIV neuropathogenesis (47, 48), and PDGF-mediated upregulation of this chemokine can have ramifications for accelerated CNS disease in the context of HIV-1 infection. MCP-1 is a potent chemokine whose elevated levels are closely associated with the progression of HAND (49, 50) and whose function includes the recruitment of monocytes from the blood to the brain (51). Interestingly, in addition to MCP-1, PDGF-BB also induced IL-1β expression in astrocytes, which can have further implications in the amplification of toxic responses in the CNS.

In summary, our findings have mapped a detailed molecular pathway of Tat-mediated PDGF-BB expression represented in Fig. 6. Tat induction of PDGF-BB in astrocytes involves ERK1/2 and JNK MAPK activation, with the subsequent activation of Egr-1.
resulting in increased PDGF-BB expression, ultimately leading to increased astrogliaesis and proinflammatory responses in the brains of individuals infected with HIV.

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Disclosures
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References


