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Association of Platelet-Derived Growth Factor-B Chain with Simian Human Immunodeficiency Virus Encephalitis

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Chemokines and cytokines play a critical role in HIV infection, serving both to modulate virus replication and to recruit target cells to the site of infection. Platelet-derived growth factor (PDGF), a mitogen and chemoattractant for a wide variety of cells, is secreted by macrophages. Since macrophages are the target cells for lentiviral infection in the brain and PDGF is a known inducer of macrophage chemoattractant protein-1 (MCP)-1, a potent chemokine closely associated with HIV encephalitis, we investigated the association of PDGF-B chain (PDGF-B) with encephalitis in macaques caused by simian human immunodeficiency virus (SHIV), a chimera of HIV and SIV. Northern blot analysis confirmed elevated expression of PDGF-B chain mRNA in the brains from encephalitic macaques. Validation of these *in vivo* studies was confirmed in rhesus macrophage cultures infected with $SHIV_{KU2}$ in which we demonstrated heightened expression of PDGF-B chain mRNA. Nuclear run-off analysis established transcriptional up-regulation of PDGF-B chain in virus-inoculated macrophage cultures. Reciprocally, addition of exogenous PDGF enhanced virus replication and MCP-1 expression in these cells. Inhibition of virus replication by tyrosine kinase inhibitor, STI-571, and by PDGF-B antisense oligonucleotides confirmed the specificity of the PDGF effect. Relevance of these findings was confirmed by analysis of archival brain tissue from SHIV encephalitic and non-encephalitic macaques for PDGF-B chain expression. PDGF-B chain protein expression was observed in the virus-infected cells in microglial nodules in the brains of SHIV-encephalitic macaques. *(Am J Pathol 2004, 165:815–824)*

HIV-associated dementia (HAD) is a clinical disorder characterized by progressive cognitive, motor, and behavioral abnormalities[1,2] caused by HIV-1 infection. Prominent neurological disease occurs in 15 to 20%[3] of infected individuals and is associated with a marked depletion of $CD4^+$T lymphocytes.[4–6] HIV-1 encephalitis (HIVE), a common pathological manifestation of HAD includes infiltration of macrophages into the brain where they become productively infected with the virus.[7,8] This is accompanied by considerable cytokine[9] and chemokine[10–12] dysregulation in the brain that often culminates into the unique pathological features that characterize this syndrome. Characteristic pathological changes of HIVE include perivascular and parenchymal accumulations of mononuclear cells, formation of microglial nodules and multinucleated giant cells, activation and proliferation of astrocytes, and neuronal dysfunction and loss.[13,14] In the central nervous system (CNS), the majority of the cells that support productive viral replication are monocyte-derived macrophages and microglia.[11,14] HIV invades the CNS early after infection where it later gives rise to cognitive, motor, and behavioral manifestations. A possible explanation of the emergence of productive virus replication in HIV-dementia in the brain is the increased monocytic infiltration across the blood-brain barrier. Although the mechanisms leading to entry of monocytes in the brain still remain unclear, it is speculated that secretory products, including CC chemokines, released by HIV-1-infected cells could be potentially important for recruitment of monocytes from blood to the brain. One such CC chemokine, macrophage chemoattractant protein (MCP)-1, has been shown to be the most potent among a variety of other major chemokines closely associated with AIDS dementia.[15]

Macaques infected with neuropathogenic strains of SIV[12,16,17] and SHIV[12,18,19] develop many of the pathological and behavioral changes observed in HIVE. These non-human primate models have served as excellent

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Table 1. Primers for Semi-Quantitative RT-PCR Analyses of Platelet-Derived Growth Factor

	Primer	Position	Size of the PCR product (bp)
PDGF-A chain	Forward 5′-TTT TCT GCC ATG CCT AAG TGT G-3′ Reverse 5′-GTG GAA AGT CAT TCA TCA CAG GG-3′	1986–2007 2460–2480	494
PDGF-B chain	Forward 5′-GCA CAC GCA TGA CAA GAC GGC-3′ Reverse 5′-AGG CAG GCT ATG CTG AGA GGT CC-3′	1663–1683 2102–2124	461
PDGFRA	Forward 5′-CCT GTA ACC TTA CAC AAC AGT GAG G-3′ Reverse 5′-TTT CTT TGA CCT CCC TGG TAG C-3′	611–635 1022–1043	432
PDGFRB	Forward 5′-ATG TCT ACA GAC TCC AGG TGT C-3′ Reverse 5′-CTT TGA ACC ACA GGA CAG TGG-3′	1089–1110 1512–1532	443

GenBank accession numbers: PDGF-A chain - S 62078; PDGF-B chain - X 02811; PDGFRA - M21574; PDGFRB - BCO32224.

working models to explore the role of various cytokines and chemokines in enhancement of virus replication. Our recent findings aimed at highlighting gene expression profiles that accompany encephalitis demonstrated an up-regulation of platelet-derived growth factor-B chain (PDGF-B).[20] This growth factor has hitherto not been recognized for having a role in the pathogenesis of HIV dementia. Four known PDGF ligands (A-D) have been described that can exert their effect via specific cell membrane receptors designated α and β.[12,18,21,22] PDGF is a known mitogen and chemoattractant for a number of cell types both *in vitro* and *in vivo* and can activate early transcription of a number of otherwise quiescent genes, several of which encode potent cytokines and other proto-oncogenes.[23] In addition to its role in various pathophysiological conditions[24–32]. PDGF is a known inducer of MCP-1,[33] a chemokine that plays a pivotal role in HIVE.[15,34] We therefore hypothesized that regulation of PDGF expression is critical for the development of the syndrome. In this study we therefore sought to explore the association of PDGF-B chain in virus replication in macaque macrophage cultures and in macaques with SHIV-E.

Materials and Methods

Viruses

We obtained SHIV-4 DNA encoding the *env*, *tat*, *rev*, and *vpu* genes of HIV-1 HXBc2 on a background of SIV_{mac239},[35] from Dr. Joseph Sodroski, Harvard University. Viral DNA was transfected into CEMx174 cells to produce a virus that was used to initiate sequential passages in macaques. Virus isolated from cerebrospinal fluid obtained 8 weeks post-inoculation from pig-tailed macaque PNb in passage four was amplified in a culture of peripheral blood mononuclear cells (PBMC) from a normal macaque and subsequently designated as $SHIV_{kU-1}$[36]. A further passage of this virus in rhesus macaques gave rise to $SHIV_{kU-2}$[37], the virus used in our study.

Macrophage Cultures

PBMCs from rhesus macaques were obtained by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation and suspended at a concentration of 2×10^6 cells/ml in macrophage differentiation medium consisting of RPMI medium supplemented with 20% heat-inactivated human serum, 5 U/ml of M-CSF (PeproTech, Inc, Rocky Hill, NJ), 100 U/ml of GM-CSF (PeproTech), and 5% heat-inactivated rhesus monkey serum. Six-well dishes (Costar, Cambridge, MA) were seeded with 3 ml of medium containing 6 to 10×10^6 cells/per well and incubated overnight at 37°C. Cultures were then rinsed to remove nonadherent cells, re-fed with macrophage differentiation medium, and maintained for 7 days to allow adherent monocytes to differentiate into mature macrophages. Cells in our cultures were exclusively macrophages as greater than 98% of them expressed the CD14 monocyte/macrophage-specific cell surface marker (data not shown). Experiments involving effects of infection on PDGF expression were performed in 6-well plates. The cells were inoculated with cell-free $SHIV_{kU-2,}$ at a multiplicity of 0.1 for 24 hours at 37°C. Cultures were then rinsed three times with RPMI medium and replenished with macrophage medium. All experiments involving treatment of cells with exogenous PDGF-BB protein were conducted under serum-free conditions since serum induces PDGF. Studies were performed on macrophage cultures derived from at least three separate animals and each experiment was performed in triplicate.

RT-PCR Analysis of PDGF mRNA in the Normal Rhesus Macrophage Cultures

Total RNA from macrophage cultures was extracted with Trizol (GIBCO BRL, Carlsbad, CA) and subjected to RT-PCR analysis using macaque-specific PDGF primers (Table 1). For RT reactions, one μg of total RNA from each sample was used in the Titan One-Tube RT-PCR System (Boehringer-Mannheim, Indianapolis, IN). The presence of any DNA contamination in the RNA preparations was tested by duplicate reactions, and subjecting one of them

to 99°C for 2 minutes followed by 3 minutes at 95°C, to inactivate the RT activity. When present, DNA was removed with DNase I (Life Technologies, Gaithersburg, MD), followed by extraction. The reactions were carried out in a Perkin-Elmer DNA Thermal Cycler 480 with a temperature profile of 42°C for 30 minutes, 1 cycle; 94°C for 5 minutes, 1 cycle; 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 45 seconds, 10 cycles; 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 45 seconds, 5 second extension/cycle, 30 cycles; and 68°C for 6 minutes. PCR products were resolved by electrophoresis in 2% agarose gels (SeaKem MR; FMC BioProducts, Rockland, ME) in 0.04 mol/L Tris-acetate (pH 8.5)-0.001 mol/L EDTA containing 0.05 mg of ethidium bromide per ml. The UV fluorescence of cDNA bands was measured with a Kodak Imaging System (Eastman Kodak Company, Rochester, NY).

Determination of c-Fos Induction

Rhesus macrophages grown in chamber slides were rendered quiescent by serum starving for 48 hours and treated for 30 minutes with PDGF-BB (100 ng/ml). Cells were washed with PBS, fixed in Zn-formalin for 20 minutes, and then incubated overnight at room temperature with a rabbit polyclonal serum against c-Fos protein (Oncogene Research Products, San Diego, CA). Biotinylated goat anti-rabbit IgG (Dako Laboratory), peroxidase-conjugated streptavidin (Dako Laboratory), and NovaRed substrate (Vector Laboratories, Burlingame, CA), were used to visualize the reaction, which yields a reddish reaction product.

Slot Blot Analysis of PDGF-B Chain Following Inoculation with UV-Inactivated SHIV

RNA from rhesus macrophages was extracted by Trizol (Life Technologies) and the integrity of the RNA was confirmed by fractionation on 1.2% (wt:vol) agarose-formaldehyde gels and staining the ribosomal bands with ethidium bromide. For slot blot analysis, 0.6 to 5.0 μg aliquots of RNA were applied in a final 200 μl solution of 6.15 mol/L formaldehyde and 10X SSC (1.5 mol/L sodium chloride and 0.15 mol/L sodium citrate, pH 7.0) onto a Hybond-N+ nylon membrane (Amersham, Arlington Heights, IL). Five μg of ribosomal RNA was loaded onto the slot blot and used as a negative control. Total RNA from UV-inactivated SHIV-inoculated rhesus macrophages and control rhesus macrophages was denatured in a loading buffer containing 2.2 mol/L formaldehyde for 5 minutes at 65°C and electrophoresed in 1% (wt:vol) agarose gels containing 0.66 mol/L formaldehyde. The RNAs were transferred to nylon membrane using the capillary transfer technique. After transfer, the blots were air dried and fixed in an UV crosslinker (Stratagene, La Jolla, CA). The probe was labeled with deoxycytdine 5′-[α-^{32}P] triphosphate by a random-primed labeling system (Amersham), with specific activity of 3.0×10^9 counts/min/μg DNA. Pre-hybridization (2 hours) and hybridization (overnight) conditions have been described earlier.[38]

Nuclear Run-Off Assay

Nuclear run-off transcript reactions were performed with isolated nuclei and [α-^{32}P] UTP as described.[39] Briefly, the nuclei from uninoculated and $SHIV_{kU-2}$-inoculated rhesus macrophages were incubated with 10 μl of [α-^{32}P] UTP (760 Ci/mmol, 10mCi/ml) for 30 minutes at 30°C. Labeled RNA was extracted by the phenol-chloroform and hybridization was carried out with equal numbers of labeled RNA counts on filters prepared by slot blotting of 10 μg of linearized plasmid probes. The filters were hybridized in plastic vials at 65°C for 48 hours, washed and exposed to x-ray film for detection of signal.

Effect of PDGF-BB on Virus Replication

Serum-starved rhesus macrophage cultures were inoculated with $SHIV_{kU-2}$ for 4 hours at 37°C, following which the inoculated cells were maintained in the presence or absence of 100 ng/ml of recombinant PDGF-BB protein (R&D Systems). The medium was replenished every third day with fresh PDGF-BB. Supernatant fluids were collected at regular intervals for determination of SIV core antigen concentration by p27 ELISA (Coulter, FL, USA) as described by the manufacturer. Studies were performed on macrophage cultures from at least three separate animals and each experiment was performed in triplicate.

Synthetic Oligodeoxynucleotides

Purified oligodeoxynucleotides (ODNs) were synthesized using a 394 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA). The 25-mer ODNs were targeted against the translation initiation site of PDGF-B chain. The antisense (AS) PDGF-B ODN sequence was 5′ TAC-AGC-AAA-TAC-CAT-ATT-AAA-CCC-T 3′, that for sense (S) PDGF-B cDNA was 5′ ATG-TCG-TTT-ATG-GTA-TAA-TTT-GGG-A 3′, and the scrambled (SC) PDGF-B ODN was 5′ TGC-AAT-TGA-GTA-TAA-TTT-GGG-TTT-C 3′. The rhesus macrophages cultures were infected as described above, following which the cultures were treated with ODNs (80 μmol/L/L) in the presence or absence of 100 ng/ml of recombinant PDGF-BB protein (R&D Systems). The medium was replenished every third day with fresh ODNs and PDGF-BB. To rule out non-specific effects of PDGF-B ODN, parallel experiments were carried out with an unrelated cytokine such as IL-8. Supernatant fluids were collected at regular intervals for determination of SIV core antigen concentration by p27 ELISA.

Inhibition of Virus Replication by Tyrosine Kinase Inhibitor, STI-571

Virus-infected rhesus macrophage cultures were incubated with or without (10 μmol/L) STI-571, an inhibitor of PDGF receptor tyrosine kinase (obtained from Novartis, Basel, Switzerland) for 3 hours and then stimulated with PDGF-BB (100 ng/ml). Supernatant fluids were collected

at regular intervals for detection of virus antigen by ELISA.

MCP-1 Induction by PDGF-BB

To examine whether exogenous PDGF-BB could induce MCP-1 expression in rhesus macrophage cultures, both uninfected and infected cultures were maintained in serum-free medium and treated with exogenous PDGF-BB (100 ng/ml). Sequentially collected supernatant fluids were examined for MCP-1 protein using ELISA (R&D Systems). The ELISA detected concentrations as low as 10 pg/ml. Protein measurements were determined by comparison to a standard curve, which was run in duplicate with each assay.

Studies on Archival Brain Tissue

Five rhesus macaque monkeys previously used to define cytokine/chemokine gene expression profiles in the brain were used in this study. The five animals were infected with SHIV89.6P and all developed AIDS-defining illnesses. All five had also developed virus infection in the brain but only three of these animals developed CNS lesions and SHIV-E as demonstrated by histopathology of nine different regions of the brain.[20] These three animals also had CNS-associated diverse opportunistic infections in the brain. The other two macaques died with AIDS but had no CNS lesions. Details of viral inoculation, disease course, processing of tissue samples, and histological analysis of the tissues have been described earlier.[20] Prominent neuropathological changes were present in basal ganglia, motor cortex, and brain stem regions in the encephalitic animals.

Immunohistochemistry

Paraffin sections were used for immunohistochemical analysis. To detect the presence of PDGF-B chain protein or viral antigen, paraffin sections were treated with either a monoclonal antibody, PGF-007, directed against PDGF-B chain (obtained as a gift from Mochida Co, Japan) or FA2 antibody against SIV P27 (AIDS Reagent Center, National Institutes of Health, Bethesda, MD), followed by treatment with biotinylated goat anti-mouse IgG (Dako Laboratory), peroxidase-conjugated streptavidin (Dako Laboratory), and NovaRed substrate (Vector Laboratories), which yields a reddish reaction product.

Northern Blot Analysis of PDGF mRNA in the Brains of SHIV-Infected Rhesus Macaques with and without SHIV-E

Archival brain tissues form SHIV-infected macaques with and without SHIV-E were used in this study. RNA was isolated by homogenizing weighed portions of brain tissues in Trizol (Life Technologies) followed by precipitation with isopropanol. Briefly, total RNA (20 μg) from basal ganglion regions of the brains from SHIV-infected macaques with and without SHIV-E was denatured in a loading buffer containing 2.2 mol/L of formaldehyde for 5 minutes at 65°C and electrophoresed in 1% (w:v) agarose gels containing 0.66 mol/L of formaldehyde. The RNAs were transferred to nylon membrane using the capillary transfer technique. After transfer the blots were air dried and fixed in an UV crosslinker (Stratagene). To obtain PDGF-B chain riboprobe, macaque-specific PDGF-B chain sequences were amplified by PCR from a human PDGF-B chain cDNA (GenBank No. X02811) and subsequently cloned in a pGEM-T easy vector. The probe was labeled with deoxycytidine 5′-[α-^{32}P] triphosphate by a random-primed labeling system (Amersham), with a specific activity of 3.0×10^9 counts/min/μg DNA. Prehybridization (2 hours) and hybridization (overnight) conditions were performed as described.[38]

Statistical Analysis

Two-factor analyses of variance with interaction were run to assess the effect of parameter and time on p27 and MCP-1 levels. When a significant interaction term was found ($P \leq 0.05$), then pair-wise comparisons were performed across parameter at given time points and across time points within a specific parameter to determine exactly where significant differences occurred. For all pairwise comparisons $P \leq 0.01$ was deemed to be significant. Pair-wise comparisons at varying time points across parameters were not of interest and were not performed. Since we have destructive sampling in these experiments at each time point, time was considered a factor and not a repeated measurement.

Results

Expression of PDGF and Its Receptors in Rhesus Macrophage Cultures

In our previous studies, we had examined brains from five SHIV-infected macaques that succumbed to AIDS. Three of the five animals had developed CNS lesions and had lentiviral encephalitis associated with opportunistic infections. The two other animals died with AIDS but had no CNS lesions.[20] Microarray analysis for cytokine and chemokine genes in the brains from the two groups of macaques demonstrated a up-regulation of PDGF-B chain in the brains of macaques with SHIV-E compared to brains of infected macaques without encephalitis.[20] To understand whether macrophages, the primary target cells for virus production in the brain, expressed PDGF and its receptors, we assessed the expression of PDGF ligands, A & B chains, and their cognate receptors, α & β, by RT-PCR. As shown in Figure 1 A, these cells expressed RNA for PDGF-B chain and β receptor, however there was negligible detection of PDGF-A chain or its α receptor. Since microarray analysis of brains of macaques with and without SHIV-E revealed up-regulation of only the PDGF-B chain[20] in this report, we focused only on the expression of the PDGF-B chain.

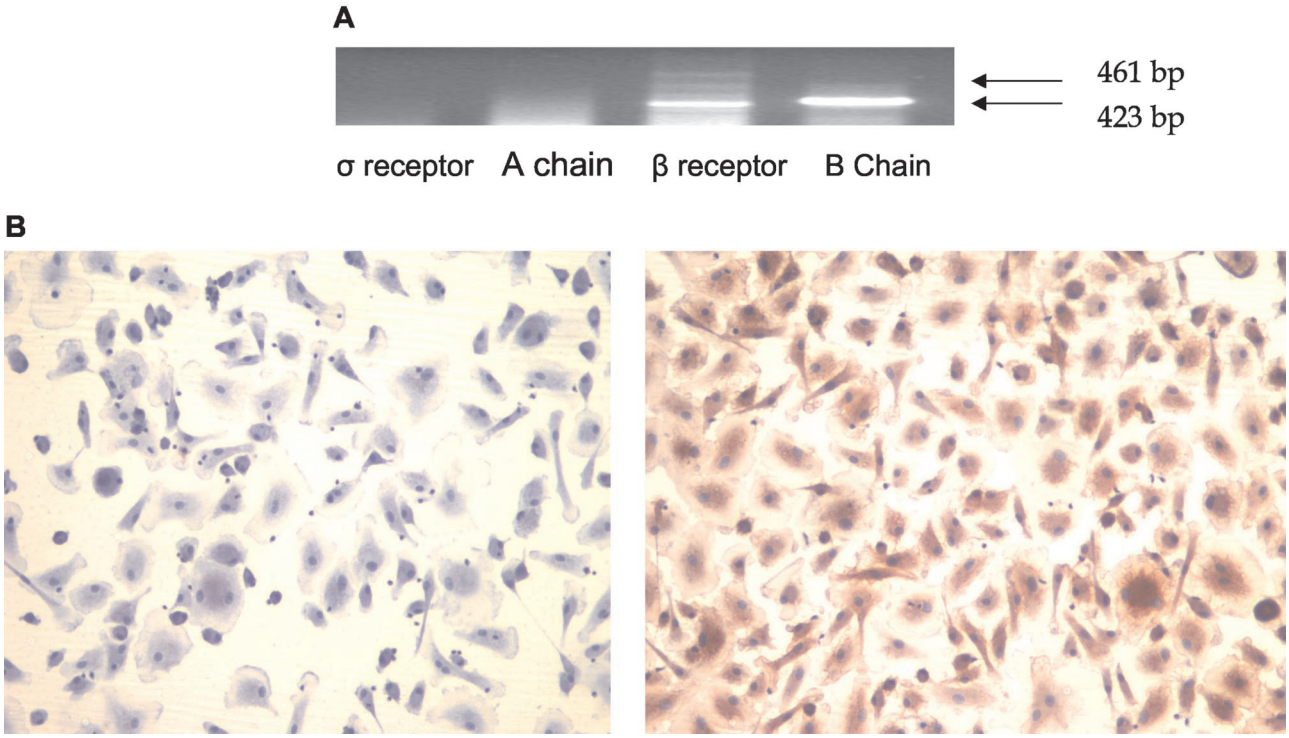

Figure 1. A: Endogenous level of PDGF-A & B chains and PDGF receptors, α & β in rhesus macrophages. RNA from macrophage cultures was subjected to RT-PCR using macaque-specific PDGF primers. Amplified products were electrophoresed on 2% agarose gel. **B:** Nuclear staining of c-fos protein in serum-starved macrophages with (**right**) and without (**left**) PDGF-BB treatment. Rhesus macrophages rendered quiescent by serum starvation for 48 hours followed by stimulation with PDGF-BB (100 ng/ml) stained intensely with antibody directed toward c-fos protein. A reddish reaction product (NovaRed substrate) developed approximately 30 minutes after treatment, in and around the cell nuclei. Control cells which were not stimulated with PDGF-BB did not develop any color.

Cultured Rhesus Macrophages Have Functional PDGF β Receptors

Serum-starved macrophage cultures stimulated with PDGF-BB protein were stained intensely with an antibody directed toward c-Fos protein, whereas unstimulated did not (Figure 1B). Staining appeared approximately 30 minutes post-PDGF-BB treatment and was localized in the cell nuclei.

$SHIV_{kU-2}$ Inoculation Enhanced PDGF-B Chain Expression in Rhesus Macrophage Cultures

To determine whether our earlier *in vivo* microarray analysis[20] of PDGF-B chain up-regulation in the brains of macaques with SHIV-E could be reproduced in cell culture, we inquired whether infection of macrophages with $SHIV_{kU-2}$, could lead to an altered expression of PDGF-B chain RNA. Following inoculation with $SHIV_{kU-2}$, rhesus macrophage cultures were harvested at various times post-inoculation and the RNA was assessed for expression of PDGF-B chain by slot blot analysis using ^{32}P-labeled macaque-specific PDGF-B ribroprobe. As shown in Figure 2A, there was an increase in PDGF-B chain RNA at 30 minutes post-inoculation following which the levels returned to baseline values by 4 hours. A similar kinetic response was observed following inoculation of the cultures with UV-inactivated virus (data not shown). This indicated that transient virus-cell interaction was sufficient for induction of PDGF-B chain RNA, and that this process did not require infection or virus replication.

To determine whether the increase in PDGF-B chain expression following viral inoculation was mediated at the

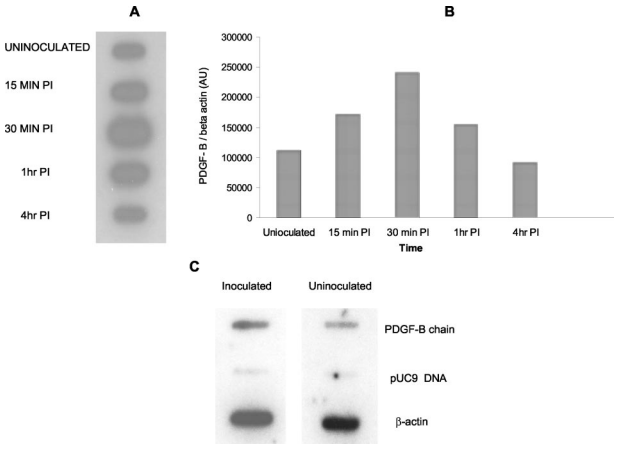

Figure 2. A: Slot blot analysis of PDGF-B chain mRNA following treatment with UV-inactivated SHIV. Macrophages cultured in serum-free medium that were either uninoculated or treated with UV-inactivated $SHIV_{kU-2}$ were processed at varying times post-inoculation for RNA extraction. RNA was then spotted onto a slot blot and hybridized with [α-^{32}P] PDGF-B chain riboprobe. The autoradiogram is representative of two separate experiments. **B:** Laser densitometric scanning of the autoradiogram from uninoculated and inoculated macrophages. **C:** Nuclear run-off assay was performed with nuclei isolated from either uninfected or $SHIV_{kU-2}$-treated macaque macrophages. Isolated nuclei were labeled with [α-^{32}P] UTP and hybridized to specific cDNAS. PDGF-B chain intensity was normalized to that of β-actin. The figure is representative of two sets of experiments.

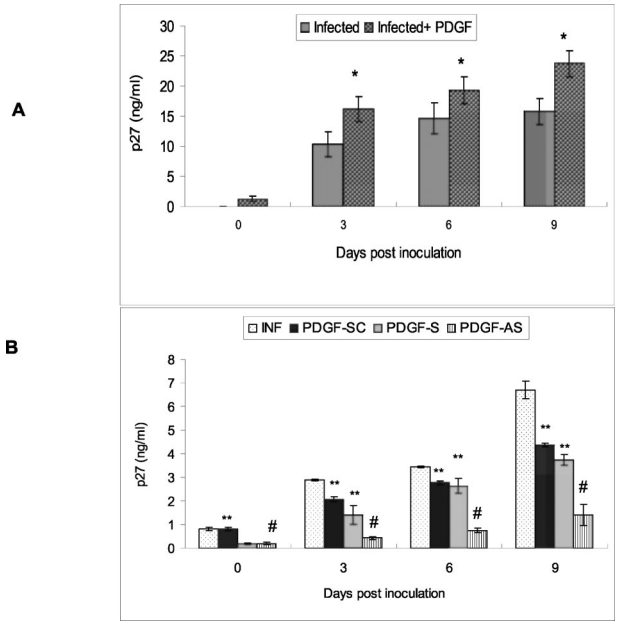

Figure 3. A: Effect of exogenous PDGF-BB on $SHIV_{kU-2}$ virus replication. Supernatants of SHIV-infected rhesus macrophage cultures treated or untreated with recombinant PDGF-BB (100 ng/ml) were analyzed for p27 ELISA. **B:** Effect of PDGF-antisense ODNs on viral replication. p27 levels in supernatants of SHIV-infected rhesus macrophage cultures treated with PDGF-antisense, sense, and scrambled ODNs. All data represent the mean ± SEM for three values at each time point. The R^2 value for the two-factor analysis of variance with interaction was 0.9606 (**A**) and 0 0.9863 (**B**), respectively. *, $P < 0.01$ infection *versus* infection plus PDGF; #, $P < 0.0001$ infection *versus* infection plus PDGF-antisense; **, $P < 0.0001$ PDGF-scrambled and sense *versus* PDGF-antisense.

level of transcription, we performed nuclear run-off assays. The number of radioactive transcripts for PDGF-B chain synthesized in nuclear run-off assays by nuclei from uninfected and $SHIV_{kU-2}$-inoculated macrophages (30 minutes post-inoculation) was assessed by hybridization of the transcripts to immobilized cDNAs for PDGF-B chain and β actin followed by autoradiography (Figure 2C). An increased rate of transcription for PDGF-B chain (1.6-fold) was noted in $SHIV_{kU-2}$-inoculated macrophages as compared to uninoculated cells.

PDGF Enhances Virus Replication in Macrophage Cultures

Since rhesus macrophage cultures express PDGF-B chain and its receptor, we inquired whether this factor had a role in regulating virus replication in these cells. $SHIV_{kU-2}$-infected macrophage cultures were treated with 100 ng/ml of PDGF-BB in serum-free medium and the supernatant fluids collected periodically for assay of viral p27 protein by ELISA. As shown in Figure 3A, addition of PDGF led to increased viral p27 antigen production in the culture supernatants. To investigate whether the effect of PDGF-BB in modulating virus replication was specific, we tested the effect of antisense, sense, and scrambled PDGF ODNs on virus replication. As shown in Figure 3B, the inhibition of virus replication by PDGF-antisense ODN was highly significant ($P < 0.0001$) compared with that caused by sense and scrambled PDGF ODNs. This effect was specific for PDGF-BB since an unrelated cytokine, IL-8, had no effect on virus replication (data not shown).

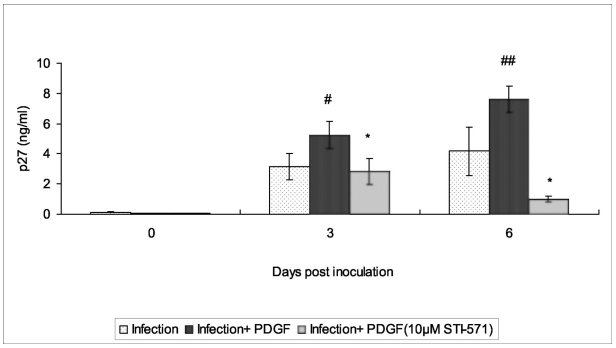

Figure 4. Effect of STI-571 on viral p27 in PDGF-BB-treated SHIV-infected cultures. Supernatant fluids from SHIV-infected macrophage cultures treated with PDGF-BB or PDGF-BB plus STI-571 were monitored for viral p27 by ELISA. All data represent the mean ± SEM for three values at each time point. The R^2 value for the two-factor analysis of variance with interaction was 0.9371. #, $P < 0.005$; ##, $P < 0.0001$, infection *versus* infection plus PDGF; *, $P < 0.001$ infection with PDGF *versus* infection with PDGF plus STI-571.

STI-571, a Tyrosine Kinase Inhibitor, Inhibits PDGF-BB-Mediated Increase in $SHIV_{kU-2}$ Replication in Macrophages

To further examine the specificity of PDGF-BB in enhancing virus replication, the effect of STI-571 (a selective inhibitor of PDGF receptor tyrosine kinase) on viral replication was monitored in rhesus macrophage cultures. As shown in Figure 4, $SHIV_{kU-2}$-infected macrophages, when stimulated with PDGF-BB, showed enhanced virus replication with respect to time as compared to cells treated with virus alone. Treatment of virus-infected cells with the inhibitor, STI-571, followed by PDGF-BB treatment resulted in significant inhibition of PDGF-mediated increase in virus replication. These results clearly demonstrated that a PDGF-BB-mediated increase in viral replication occurs via activation and binding of PDGF to the PDGF-β receptor tyrosine kinase.

PDGF Induces MCP-1 in Rhesus Macrophages

In addition to its role as a mitogen and chemoattractant,[40,41] PDGF is also known to induce MCP-1[42] a potent chemokine whose levels are elevated in AIDS-dementia and whose function is thought to be the recruitment of monocytes from the blood to the brain.[15,34] Since PDGF-B chain and MCP-1 were both up-regulated in SHIV-E,[20,43] it was of interest to assess whether treatment of rhesus macrophages with PDGF-BB would also lead to induction of MCP-1 in rhesus macrophage cultures. In these studies serum-starved rhesus macrophage cultures were maintained in the presence or absence of PDGF-BB following which cells were harvested for MCP-1 RNA and the supernatant fluids collected for assay of MCP-1 protein by ELISA. As shown in Figure 5, treatment of macrophage cultures with exogenous PDGF-BB for 12 hours enhanced the expression of MCP-1 RNA (Figure

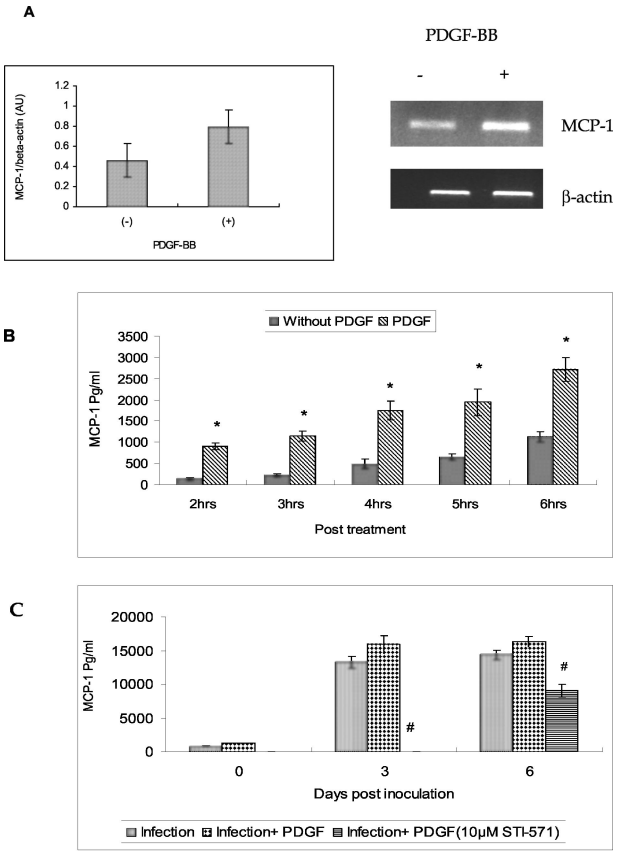

Figure 5. A: Induction of MCP-1 mRNA following PDGF treatment. Laser densitometric scanning of MCP-1 RNA in macrophage cultures treated with and without exogenous PDGF-BB. RNA from cultures with and without the treatment was subjected to RT-PCR analysis using macaque-specific MCP-1 primers. **B:** Induction of MCP-1 protein following PDGF-BB stimulation. MCP-1 protein levels as measured by ELISA, in the culture supernatants of rhesus macrophage cultures that were treated with or without recombinant PDGF- BB at various time intervals. **C:** Inhibition of MCP-1 by STI-571. MCP-1 levels in the culture supernatants of SHIV-infected macrophage cultures that were treated either with recombinant PDGF-BB or PDGF-BB plus STI-571. All data represent the mean ± SEM for three values at each time point. The R^2 value for the two-factor analysis of variance with interaction was 0.9695 (**B**) and 0.9993 (**C**), respectively. *, $P < 0.0001$ PDGF treated *versus* without PDGF treatment; #, $P < 0.0001$ infection with PDGF *versus* infection with PDGF plus STI-571.

5A). This increase was also evident at the protein level (Figure 5B) in cells treated with PDGF-BB.

Since SHIV infection is also known to enhance MCP-1 expression,[43] we also wanted to explore the effect of PDGF-BB and its inhibitor STI-571 on MCP-1 induction in infected macrophage cultures. MCP-1 protein expression was estimated, by ELISA, from the supernatant fluids collected sequentially on different days post-infection. As shown in Figure 5C, and similar to our previously published observations,[43] SHIV infection was accompanied by enhanced MCP-1 protein expression in these cells even after 6 days post-infection. Treatment of infected macrophage cultures with PDGF-BB did not cause a significant increase in MCP-1 expression. Tyrosine kinase inhibitor, on the other hand, inhibited MCP-1 expression to levels lower than those cultures with infection alone indicating the role of PDGF in virus-mediated MCP-1 up-regulation.

Specificity of the PDGF-BB response in induction of MCP-1 was further confirmed by treatment of macrophage cultures with PDGF-B ODN and monitoring the inhibition of MCP-1 expression. Exposure of cells to antisense PDGF-B ODN resulted in almost 50% inhibition of MCP-1 protein production (data not shown). In contrast, the sense and scrambled PDGF-ODNs exhibited no such effect.

PDGF-B Chain Is Expressed in Encephalitic Brains of Rhesus Macaques Infected with CXCR4-Using Viruses

Induction of PDGF-B chain RNA following inoculation with SHIV prompted us to explore the possible implication of this phenomenon *in vivo*. Brain tissue from SHIV-infected macaques with and without encephalitis was assessed for expression of PDGF-B chain by Northern analysis. Equal amounts of RNA from brain samples from the two groups of animals were subjected Northern blotting using the labeled PDGF-B chain riboprobe. As shown in Figure 6A, the abundance of the 3.5-kb transcript of PDGF-B chain RNA was higher in the brains of encephalitic animals as compared to non-encephalitic animals. The localization of PDGF-B chain in the brains of encephalitic animals was further confirmed by immunohistochemical analysis. Brain sections from encephalitic animals demonstrated PDGF-B chain protein both in the perivascular macrophages and in the microglial nodules (Figure 6, left and middle panels). However, not all of the cells in the nodule stained positively for PDGF- B chain protein. On the other hand, the majority of the cells in these nodules comprised of virus-infected cells (Figure 6, right panel).

Discussion

Overexpression of the classical PDGFs, PDGF-A and PDGF-B, have been linked to several diseases, including cancer, fibrotic lung diseases, and atherosclerosis.[24–28,44] Although two other novel PDGF ligands, PDGF-C and PDGF-D, have recently been discovered[21,22] it has not yet been established whether these are linked to disease processes. PDGFs regulate a number of physiological and pathophysiological processes in many cell types via two tyrosine kinase receptors, PDGF α and β. While PDGF has been implicated in the pathogenesis of HIV-associated pulmonary hypertension[45,46] and in AIDS-associated Kaposi's sarcoma,[47,48] its role in lentiviral encephalopathy has never been investigated. The impetus for the current study stems from the observation that in addition to its role in various disease phenotypes, PDGF-B is also known to induce the expression of MCP-1, a potent chemokine that is selectively accumulated in the brains and CSF of patients with AIDS dementia.[15,34] Perhaps, the major role of MCP-1 in AIDS dementia is its ability to initiate the transmigration of monocytes across the blood-brain barrier.

Our preliminary findings on microarray analysis of brains of macaques with SHIV-E revealed an enhance-

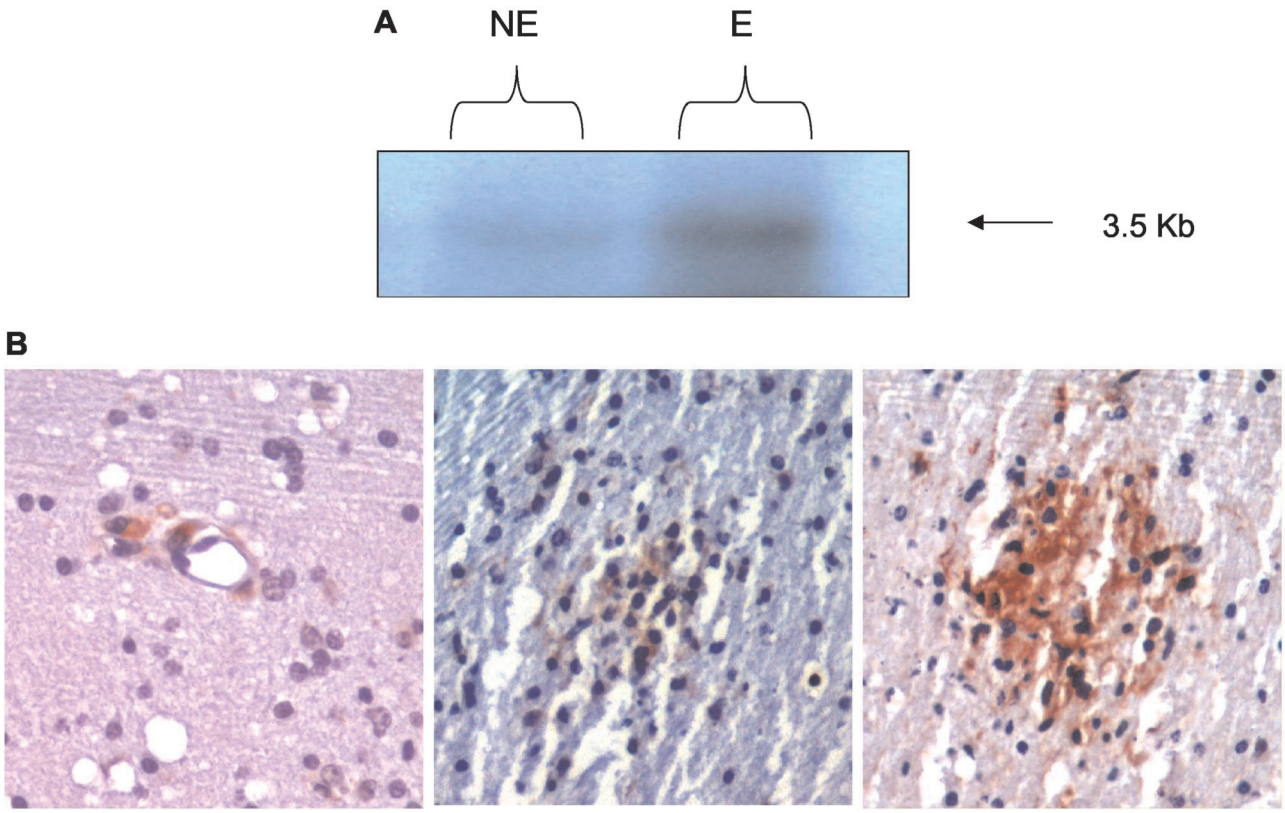

Figure 6. A: Northern blot analysis of PDGF-B chain mRNA in the basal ganglion region of the brains of SHIV-infected macaques with (E) and without (NE) SHIV-E. The autoradiogram is representative of two separate experiments. **B:** Immunohistochemistry showing localization of PDGF-B protein in the perviascular macrophages (**left**) and in the microglial nodule (**middle**) in the basal ganglia region of the brains of macaques with SHIV-encephalitis. Immunohistochemistry for viral protein (**right**) in a serial section of the same brain region of the macaque showing viral-positive cells in the microglial nodule. Positive cells are indicated by a reddish reaction product. Original magnification, ×100 (**right**, **middle,** and **left**).

ment of PDGF-B chain RNA in infected macaques compared to animals without encephalitis.[20] It was therefore of interest to first examine the endogenous levels of PDGF-A and B chains and their cognate receptors in cultured macrophages, the major cellular target for virus replication in the CNS.[49] We demonstrated that rhesus macrophages express detectable levels of PDGF-B chain and β receptor RNA while the levels of the corresponding PDGF-A chain and α receptor were negligible. This suggested that the functional isoform in these cells is very likely PDGF-BB that could be functioning via the PDGF-β receptor.

Having determined that rhesus monocyte-derived macrophages express PDGF-B chain and its β receptor, it was of interest to explore whether virus infection could modulate changes in expression of these factors. Since PDGF is an early gene, we monitored its expression soon after virus attachment and observed that PDGF-B RNA was transiently up-regulated 30 minutes post-inoculation. Virus replication was not required for this induction since UV-inactivated virus had a similar effect. This suggested that the binding of the virus to its receptor on the macrophages transduced expression of PDGF gene expression. The mechanism of regulation of PDGF-B induction on virus binding occurred at the transcriptional levels as determined by nuclear run-off assays performed in infected *versus* uninfected cells.

Since PDGF is secreted by a variety of cells, it is possible that PDGF secreted by neighboring cells could affect virus replication. To simulate this effect, we tested whether treatment of SHIV-infected macrophage cultures with exogenous PDGF-BB could result in modulation of virus replication. We demonstrated that exogenous PDGF-BB enhanced virus replication in rhesus macrophage cultures. This effect was specific for PDGF as antisense PDGF-B ODN abolished this increase in virus replication. Furthermore, sense and scrambled PDGF-B ODNs exerted no inhibitory effect. Further corroboration of these findings was also demonstrated in experiments involving the tyrosine kinase inhibitor STI-571.[44,50–53] Since PDGF exerts its effect via the tyrosine kinase receptors, blocking of the enzyme activity by STI-571 further confirmed the specificity of PDGF response.

In addition to its diverse functions in various pathophysiological states, PDGF is also known to stimulate expression of the immediate early gene set within target cells. A well-characterized example of a fast immediate early gene, such as c-fos, reaches peak levels within 30 minutes and returns to baseline within 2 hours.[54,55]

In contrast to the rapid but transient response exhibited by c-fos, slow immediate-early genes like c-*myc* and MCP-1 display a 60- to 90-minute lag time for initiation.[56,57] While induction of MCP-1 by PDGF has been demonstrated in a variety of cell systems,[58,59] its induc-

tion in monocyte-derived macrophages, the target cells for HIV replication in the CNS, has hithereto not been reported. Since virus infection leads to an increase in PDGF in the macrophages as shown above and because virus infection also leads to increased MCP-1 expression,[43] it was essential to determine whether exogenous PDGF could, by itself, induce MCP-1 expression in rhesus macrophage cultures, irrespective of infection. Similar to other reports on induction of MCP-1, we found increased expression of both MCP-1 RNA and protein in macrophages treated with the growth factor. This effect was specific for PDGF-B since antisense PDGF-B ODN inhibited this induction. Corroborating the cell culture results, we found expression of PDGF-B chain RNA and protein were also up-regulated in the brains of macaques with SHIV encephalitis. Decreased expression was observed in infected macaques without encephalitis. Since the encephalitic lesions in the brains of macaques primarily comprise of cells of the macrophage lineage, it is speculated that virus-infected macrophages up-regulate PDGF-B chain and this in turn, induces MCP-1 that recruits more cells into the CNS. It was interesting that intense PDGF-B chain staining was observed in perivascular macrophages. In the microglial nodules that are comprised primarily of virus-infected cells, not all cells stained positively for PDGF-B chain. Furthermore, not all of the microglial nodules in the brain sections of encephalitic animals stained positively for PDGF-B chain protein. One possible explanation for this could be that macrophages exist in varying phenotypic states in the brain, and PDGF-B chain expression could be associated with macrophages with specific cell phenotype. It is also likely that the transient expression of PDGF could be responsible for this sporadic expression in only certain nodules.

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