ASTROCYTE-ENDOTHELIAL INTERACTIONS: IMPLICATIONS FOR HIV-ASSOCIATED NEUROCOGNITIVE DISORDER

BY

Crystal Bethel-Brown

Submitted to the Graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas Medical Center in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Committee:

_____

Shilpa Buch, Ph.D., Co-chairperson

_____

Michael Wolfe, Ph.D., Co-chairperson

_____

Gustavo Blanco, Ph.D

_____

Paul D. Cheney, Ph.D

_____

Kee Jun Kim, Ph.D

_____

Anil Kumar, Ph.D

_____

Steven LeVine, Ph.D

Date defended: _____

The dissertation committee for Crystal Bethel-Brown certifies that this is the approved version of the following dissertation:

ASTROCYTE-ENDOTHELIAL INTERACTIONS: IMPLICATIONS FOR HIV-ASSOCIATED NEUROCOGNITIVE DISORDER

Committee:

_____

Shilpa Buch, Ph.D., Co-chairperson

_____

Michael Wolfe, Ph.D., Co-chairperson

Date approved: _____

Abstract

During the course of HIV-1 disease, virus neuroinvasion occurs as an early event, within weeks following infection. Intriguingly, subsequent central nervous system (CNS) complications manifest only decades after the initial virus exposure. In the current era of combined anti-retroviral therapy (cART), while the virus replication in the periphery is brought under complete control, in the CNS owing to poor drug penetrance, there occurs development of increasing neurocognitive complications. So while the HIV-infected patients are living longer and have decreased incidence of the disease, the prevalence of HIV-associated neurocognitive disorders (HAND) is actually on a rise. Although CNS is commonly regarded as an immune-privileged site, emerging evidence indicates that innate immunity elicited by the CNS glial cells is a critical determinant for the establishment of protective immunity. Sustained expression of the protective immune responses manifested as increased expression of a mediator, such as platelet-derived growth factor (PDGF), however, can be a double-edged sword. While PDGF has been defined as a neuroprotective immune mediator, in concert with other inflammatory mediators (elicited by HIV or its protein products), it can tip the balance from a protective to a toxic state in the CNS. Herein, we present an overview of some of the essential elements of the cerebral innate immunity in HIV neuropathogenesis including the role that platelet-derived growth factor (PDGF) released from astrocytes has in exacerbating HAND.

Out of the various PDGF isoforms, PDGF-BB is critical for astrocytes and hence is the focus of our study. PDGF-BB exposure to astrocytes results in astrogliosis and release of the chemokine monocyte chemotactic protein-1 (MCP-1), the two hallmark

features of HAND. To further understand these observations and explore the role of PDGF-BB regulation in HAND, our central hypothesis is that exposure of astrocytes to HIV transactivating protein Tat results in the release of growth factors such as PDGF-BB, which could ultimately lead to enhanced astrogliosis and blood-brain barrier(BBB) disruption. The aim of this study was to explore the mechanism of PDGF-BB regulation in astrocytes as it relates to HAND pathogenesis.

Dedication

This work is dedicated to my husband, Tavares Brown.

I am most grateful for your encouragement, guidance and loving support throughout this journey. There's no me without you.

Acknowledgements

The number of people who have had an impact on my scientific career are too numerous to mention and I apologize in advance to those whom I may have mistakenly left out.

To my mentor, Dr. Shilpa Buch: Thanks for all of your guidance and support throughout the years. Thank you for taking a chance and believing in me. I am undoubtedly a better scientist and person because of the experiences and obstacles you've walked me through. Thanks for sharing your knowledge and skills pertinent to my scientific development. You have taught me more than I'll ever be able to express, but know that I am forever grateful and blessed to call you my mentor.

To Dr. Paul Cheney, words cannot express my gratitude for all that you have done for me. I will forever be indebted to you for all the time you've taken to listen, advise and encourage me throughout the years. Thanks for being the best department chair a student can possibly wish for.

To my committee members Dr. Gustavo Blanco, Dr. Paul Cheney, Dr. Kee Jun Kim, Dr. Anil Kumar and Dr. Steven LeVine: Thank you the hours sacrificed, the critical analysis of my work and the interesting discussions that have undoubtedly led to more questions. I'll be forever grateful to you all.

To Dr. Lane Christenson: You went beyond what your responsibilities required to help me in so many ways and I'll always be indebted to you for the things that you've done and made happen.

To the physiology department of the University of Kansas Medical Center: I am grateful for your overall support as a graduate student.

To the pharmacology and experimental neuroscience department of the University of Nebraska Medical Center including Dr. Howard Gendelman for taking me under the wings of this department and allowing me to participate in many of the student activities while away from KUMC.

To Dr. Honghong Yao: Thank you for working closely with and consulting me on many of the scientific roadblocks along the way. Your expertise and the lessons I've learnt from you are priceless and I cherish the friendship that we have developed in the process.

To my lab colleagues, Dr. Fuwang Peng, Dr. Guoku Hu, Dr. Ming Duan, Shannon Callen, Yaman Lu and Lu Yang: Thanks for the wonderful working environment each one of you has had a part in creating. Thanks for the thought-provoking scientific discussions we've had as well as the light-hearted things we've shared along the way.

To the past members of the Buch lab including Dr. Navneet Dhillon, Dr. Rachel Williams, Xuhui Zhu, Dr. Yangjing Yang, and Dr. Hongxiu Wen: Thanks for the scientific training and the warm friendships that have become an integral part of who I am.

To Dr. John Stanford: Your expertise in animal behavior work and survival surgeries has definitely strengthened my overall competence as a scientist. Thanks for showing me how to prioritize family and the demands of science successfully.

To my fellow colleagues and friends at KUMC including Sarah Tague, Heather Teply, Argenia Doss, Jill Morris, and Laurie Shannon: Thanks for your friendship and having the wisdom to know when I needed advice and when I simply needed to vent.

To Dr. Ken McCarson and Dr. Sam Enna: I am most grateful for the opportunity given to me through the Summer Undergraduate Research Fellowship (SURF) program. Thanks for exposing me as an undergraduate to scientific research and planting the initial seeds of science that have led to where I am today.

To Dr. Debbie Scheuer: My sincere thanks for your guidance on survival surgeries and for teaching me that there are many other aspects of being a scientist other than the science itself and for always stressing the importance of going were the science leads, even if it doesn't support my initial hypothesis.

To my sister Kayshell Eulin-Petty: You've been there for me through it all. You've guided me and watched over me as a mom would from the time I could remember. Thanks for always having a listening ear and being there for me.

To the members of my family including my dad, Cyril Bethel, step-mother, Shelly Austin, Vernon and Shirley Brown, Portia Brown, Patrice Munroe and family, Omar Lockhart, Sean, Jeffery and Tyrone Brown: Each of you has touched my life in different ways and has played an integral role in my development both as a person. Much thanks.

Finally to my husband Tavares Brown: I can never begin to put into words what you mean to me. Thanks for being there when experiments didn't work and for rejoicing with me when they did. For the late lab nights, your patience, encouragement and love.

Table of Contents

List of Figures

List of Abbreviations

HIV……Human Immunodeficiency Virus

HAND……..HIV-associated Neurocognitive Disorders

HAD……HIV-associated Dementia

HIVE……..……..……HIV Encephalitis

SIV……...Simian Immunodeficiency Virus

SIVE……..……SIV Encephalitis

SHIV……...SIV/HIV

ANI……Asymptomatic Neurocognitive Disorder

MCMD……..…..…Minor Cognitive Motor Disorder

CNS…….…… Central Nervous System

cART……...……..Combined Anti-retroviral Therapy

BBB……..……Blood-Brain Barrier

PDGF……...Platelet-Derived Growth Factor

PDGFR……..… Platelet-Derived Growth Factor Receptor

MCP-1……Monocyte Chemoattractant Protein 1

Tat……..……Transactivator of Transcription

HBMECs……..…..…Human Brain Microvascular Endothelial Cells

Egr-1……..……Early Growth Response 1

MAPK……Mitogen-activated Protein Kinase

JNK……c-Jun N-terminal kinases/stress-activated protein kinase

MTT……Mitochondrial dehydrogenase

DMSO……..……Dimethyl Sulfoxide

DN……Dominant Negative

WT……..…….Wild type

Ct……..……Cycle threshold

ChIP……..…Chromatin Precipitation

PI3K……...…Phosphatidinylinositol-3 Kinase

siRNA……..……short interfering RNA

vWF……..……von Willebrand Factor

GFAP……..……Glial Fibrillary Acidic Protein

HSPG……..…Heparan Sulfate Proteoglycan

LRP……..……...Lipoprotein-related Protein

HTLV……Human T-cell leukemia Virus

LTR……..……Long Terminal Repeat

TAR……Transactivation Responsive Region

P-TEFb…….. Positive Elongation Factor b

CDK9……Cyclin-dependent Kinase 9

kDa……..Kilodalton

AJ……..…….Adherens Junctions

TJ……..…….Tight Junctions

Introduction/Overview

Almost 60 million people have been infected worldwide with HIV-1 since its discovery. This global epidemic leaves 25 million HIV-related deaths in its wake with approximately 33 million people still infected [1]. Combined anti-retroviral therapy (cART) is able to control HIV replication in the periphery, significantly extending the longevity of those infected with HIV-1 [2, 3]. However, while cART is able to control the virus in the periphery, the drugs have inferior penetration across the blood-brain barrier (BBB) [4]. Hence although HIV-1 infected patients enjoy the benefits of longevity, they now have to deal with the long-term central nervous system (CNS) complications associated with HIV-1 infection, termed collectively as HIV-associated neurocognitive disorders (HAND). Additionally, it is also becoming evident that most age-related complications are also an integral part of individuals on cART.

During the course of HIV-1 disease, viral neuroinvasion occurs within weeks of infection [5, 6] via the “Trojan Horse” theory [7]. HIV does not enter the brain directly; rather it gains access into the brain by infecting monocytes in the periphery. These infected monocytes can now pass through BBB into the brain. Intriguingly, subsequent CNS complications manifest decades after the initial infection. Although the CNS was once regarded as an immune-privilege site, emerging evidence suggests that innate immune responses elicited by glial cells are critical for the establishment of protective immunity. Studies have revealed that **the severity of HIV-associated dementia (HAD)/HIV-encephalitis (HIVE), the two extreme forms of HAND, correlates with the presence of activated glial cells rather than with the presence and amount of**

HIV-infected cells or virus replication in the CNS [2, 8, 9]. These immune responses by activated glia include release of protective mediators including growth factors, chemokines and cytokines, which while protective for neurons, may actually have deleterious effects on other types of cells in the CNS. Below is a stepwise introduction of HAND and the various mediators that have been used in our study.

HIV and HIV-associated Neurocognitive Disorders (HAND)

The Human Immunodeficiency Virus (HIV), a member of the lentivirus family, that causes acquired immunodeficiency syndrome (AIDS) was discovered during the 1980s in humans but was thought to have originated from the non-human primates in Sub-Sahara Africa [10-12]. The virus is transmitted sexually, through intravenous (IV) drug use or maternally from mother to child. Once a person becomes infected, the HIV targets T lymphocytes and monocytes by binding to the CD4+ receptors on the surface gaining entry into these cells. Here, the virus hijacks the host cellular machinery ultimately killing the cell and reducing the overall CD4+ T cell population critical in immune defense. The immune system becomes severely suppressed making the individual susceptible to a variety of opportunistic infections, to which they eventually succumb. Due to the pandemic effect that this disease has had worldwide, much scientific research has been undertaken to better understand the pathogenesis of HIV-1. These studies have led to exciting breakthroughs which, in concert have paved the way for discovery of effective anti-retroviral therapeutic drugs including non-nucleoside reverse inhibitors (NNRTIs), nucleoside reverse inhibitors (NRTIs), protease and fusion inhibitors [12, 13]. The advances of cART successfully control viral replication in the

periphery significantly extending the lifespan of those infected with HIV-1. However, despite its efficacy in peripheral viral suppression, these drugs have limited BBB penetration in the brain [4] consequently, the brain now becomes a reservoir for the virus. It has been shown that the virus enters the brain shortly after infection [6], and owing to the limited penetrance of cART in the CNS, the virus can infect microglia and to a lesser, extent astrocytes in this milieu. Infected microglia and astroglia can then become activated producing a plethora of cytokines/chemokines, viral proteins as well as other toxic mediators resulting in the dysfunction/death of surrounding neurons and the development of HAND. Various neurocognitive conditions ranging from Asymptomatic Neurocognitive Impairment (ANI), Minor Cognitive Motor Disorders (MCMD), and HIV-associated dementia (HAD) in the most severe form are included under the umbrella of HAND. It is intriguing that almost **60%** of HIV-infected individuals will develop HAND in their lifetime, affecting their daily functioning and thus quality of life [3, 14]. These patients are diagnosed by changes in behavioral, cognitive and motor abnormalities [14]. HAD, the most severe form of HIV- induced CNS impairment is clinically characterized by motor and behavioral dysfunctions that, in the absence of therapy could lead to seizures, coma and death within six months of onset [9]. Pathological correlates of HAD include astrogliosis, microglial activation, monocyte infiltration and neuronal damage. With the increasing prevalence of HAND which affects the normal functioning of the affected individuals and their quality of life, it is very important to understand the cellular and molecular mechanisms by which HIV-1 infection exerts its detrimental effects in the progression of HAND.

Critical Role of Astrocytes in HAND

Astrocytes, the most abundant cells of the CNS once believed to be involved primarily in providing and maintaining structural support, are now recognized not only as critical players in the overall health of the CNS, but also as a key mediators of pathology. The heterogeneity of astrocytes may, in part, explain their diverse functions which include the uptake of ions, glutamate, glycine, glucose and water from the microenvironment as well as the release of growth factors, energy substrates, cytokines and chemokines. In the context of HIV-1 or other insults, the normal function of astrocytes in providing and maintaining homeostasis and BBB integrity through structural and metabolic support is disrupted, resulting in deleterious implications. Similar to neurons, these cells of neuroectodermal origin were once suggested to be resistant to HIV-1 infection. However, recent studies have shown that astrocytes can indeed become infected under certain conditions [15-18]. Following infection and/or exposure to viral proteins released from neighboring infected cells, astrocytes release cytokines, chemokines and growth factors that are toxic for neurons and other neighboring cells in the CNS, thus providing an important reservoir for the generation of inflammatory mediators. Although astrocytes do not support productive viral replication, early HIV-1 genes such as Tat, Rev and Nef [19, 20] are expressed by these cells. It has been well documented that exposure of astrocytes to HIV-1 Tat protein results in their activation and release of toxic mediators [20] highlighting the critical role these cells play in amplifying neuroinflammation.

Because the severity of HAND correlates with the presence of activated glial cells rather than with the viral load in the brain [21] it is critical to understand the immune capabilities and consequences of immune activation in astrocytes. Once activated by

infection, insult (exposure to toxic proteins) or injury, these cells undergo astrogliosis, an initial protective response characterized by cellular proliferation [22]. Additionally, a number of molecular and functional changes including the release of a myriad of cytokines and chemokines also accompanies activation [23, 24]. Astrogliosis is actually a well-meant protective response by the host to a toxic insult. However, because HIV-1 proteins persist in the CNS, this well-meant acute response becomes sustained and prolonged, thereby manifesting as a double edged sword, leading to chronic cellular activation with the release of pro-inflammatory mediators. This, in turn, leads to increased neuronal injury, thus turning astrogliosis into a pathological feature of HAND. While astrocytes are non-productively infected to a limited extent with HIV, the sheer number of astrocytes in the brain has far-reaching implications in the CNS.

HIV-1 Transactivator of Transcription (Tat)

HIV-1 transactivator of transcription (Tat) protein was identified primarily due to its role in trans activation of the long terminal repeat (LTR) in Human T-cell leukemia Virus (HTLV)-infected cells [25, 26]. Since its discovery, extensive research has been ongoing to understand its activity and function in cells. The basic region of Tat binds a transactivation responsive region (TAR) on the virus, recruiting a positive elongation factor b (P-TEFb) complex, which includes cyclin-dependent kinase 9 (CDK9) and cyclin T1, to the promoter region of the virus LTR resulting in efficient elongation of the viral transcript [27, 28]. Tat is encoded by two exons and due to splicing variation, exists predominantly in two lengths 1-86 and 1-101 *in vivo* [29]. *H*owever, because the first exon 1-72 is sufficient for transactivation *in vitro* [29], Tat 1-72 is also commonly used in

toxicity studies [30-34]. Although this project also included Tat lengths 1-86 and 1-101, Tat 1-72 is the primary form of Tat utilized for cell culture studies.

An interestingly advantageous characteristic of Tat is not only its ability to be secreted from infected cells, but rather the diverse ways in which it can be taken up by neighboring cells to modulate cellular function. The arginine-rich RKKRRQRRR domain of Tat not only allows it to traverse across cell membrane but also contains the nuclear localization signal targeting it to the nucleus to transactivate the HIV-1 transcription genes [35]. In addition to crossing the plasma membrane, Tat can also bind to a number of cell surface proteins including viral co-receptor CXCR4, integrins, cell surface lipoprotein heparin sulfate proteoglycans (HSPG) and low density lipoprotein receptor-related protein (LRP) [35]. The binding of Tat to these cell surface receptors triggers various cell signaling pathways depending upon the cell type and other factors leading to activation of the cell.

In addition to activating transcription, other roles of Tat have now come to light. Several studies have suggested that Tat plays a role in neurotoxicity [36-39]. This direct neurotoxic effect of Tat is further compounded by the ability of Tat to also indirectly cause neuronal dysfunction/death by modulating the release of toxic substances from glial cells. For example, it has been shown that intraventricular injection of Tat results in prominent glial cell activation with subsequent infiltration of blood borne monocytes [40]. Tat exposure can also impact glial cell function by stimulating the production of pro-inflammatory cytokines [41, 42]. In fact, several cell culture studies have suggested that exposure of astrocytes to Tat protein leads to

astrocyte activation with the induction of cytokines and chemokines [20, 43, 44]. Significant among these are tumor necrosis factor-α (TNF-α), monocyte chemoattractant factor-1 (MCP-1)/CCL-2, and CXCL10 [45-48]. These findings have tremendous clinical implications since it is becoming increasingly appreciated that the severity of HAD/HIVE correlates more closely with the presence of activated glial cells rather than with the presence and amount of HIV-infected cells in the brain [2, 8, 9]. Thus HIV-1 Tat by its ability to activate astrocytes, the predominant cell types in the CNS, can actually lead to amplification of toxic responses in the CNS.

Role of Platelet-Derived Growth Factor (PDGF) in HAND

PDGF was originally identified as a potent mitogen in fibroblasts [49] and smooth muscle cells [50] however other functions of the PDGF family are rapidly emerging. PDGFs are a family of proteins comprising of four chains (A-D) encoded by four genes located on different chromosomes, that are highly conserved throughout the animal kingdom [51]. These proteins are usually expressed as dimers; PDGF-A and PDGF-B that can form homodimers or heterodimers, while PDGF-C and D only form homodimers. For the sake of clarity, PDGF (A-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 [52]. Members of this family are disulfide-bonded polypeptides that play critical roles in kidney, cardiac, blood vessel and placental development [51, 53]. The mitogenic and angiogenic properties of PDGF are now being capitalized upon in clinical applications to speed tissue recovery and wound healing and more recently are also

being examined for bone repair [54-57]. Despite their obvious beneficial role in tissue repair, recent studies have shed an alternative light on PDGF function. PDGF signaling has been implicated in a number of pathological disease including liver, lung and cardiac fibrosis, atherosclerosis, restenosis, pulmonary hypertension, cancer, gliomas and stroke [51, 58-62]. Its role in HIVE/HAND however, specifically as it pertains to astrogliosis has not been elucidated.

In a previous study, we had investigated the association of PDGF-B and HIVE in rhesus macaques infected with the chimeric SIV/HIV (SHIV). In this study, PDGF expression was primarily localized in cells around the blood vessels. The functional role of PDGF-BB in HAND however, remains unclear. To this end, this project was undertaken in an attempt to determine the role of PDGF-BB in HAND specifically as it pertained to astrocytic function and BBB breakdown. We hypothesize that excess astrocyte activation leads to astrogliosis and proinflammatory chemokine/cytokine release, hallmark features of HAND.

Role of Monocyte Chemoattractant Protein 1 (MCP-1) in HAND

Monocyte Chemattractant Protein 1 (MCP-1) also known as Chemokine –CC motif ligand 2 (CCL2) is a small 14kDa protein member of the CC chemokine family which plays a key role in mediating leukocyte transmigration across the BBB via binding to its cognate receptor CCR2. A number of studies have implicated MCP-1 in the pathogenesis of HAND [63-65]. The recruitment of inflammatory cells to the site of injury is a critical component of for resolution of disease pathogenesis, however, when there is a sustained level of MCP-1 driving a persistent transmigration of leukocytes into the

brain, glial activation ensues leading to tipping of the balance, from a much needed physiological response to a pathological one. Enhanced MCP-1 levels have been correlated with a number of disease conditions including renal dysfunction, multiple sclerosis (MS), liver disease and AIDS [65-67]. Moreover, MCP-1 has been shown to correlate with increased HIV replication [68] thereby underscoring the functional role that MCP-1 may play in accelerating HIV.

In a previous study, we observed a positive correlation between expression of MCP-1 in the brain and neuropathological changes in rhesus macaques infected with chimeric SHIV [69]. However, the mechanism of induction of MCP-1 in the brains of HIV-infected patients remains poorly characterized. It has been shown that the HIV-1 protein Tat significantly induces the expression of MCP-1 in astrocytes [65]. Moreover, our findings also demonstrate that Tat up-regulates PDGF-BB a known inducer of MCP-1. This finding forms the basis of the second part of our studies. The data presented herein reveal that PDGF-BB exposure of astrocytes resulted in enhanced expression of MCP-1. Based upon previous evidence that MCP-1 alters BBB integrity and recruits monocyte transmigration into the brain, we rationalize that MCP-1 produced in excess by PDGF-BB-exposed astrocytes, can in turn, lead to increased transmigration of monocytes across the BBB, a hallmark feature of HIV-associated neuroinflammation.

Early Growth Response Gene 1 (Egr-1)

The zinc-finger transcription factor early growth response 1 (Egr-1) also known as Krox24, Zif 268, NGFI-A, TIS8 was originally identified as a serum-inducible zinc-finger nuclear protein and predominantly known for its roles in neuronal plasticity and development [70, 71]. Egr-1 belongs to the Immediate Early Gene (IEG) family, which

also includes c-fos, fra1, fra2 and fos B [72] that can be induced without de novo protein synthesis allowing for rapid and transient induction by a number of growth factors, cytokines and stress signals [73]. The physiological role of Egr-1 is unknown, however the rate at which Egr-1 can be induced and its ability to be induced by a number of molecules has led others to suggest a potential function for this factor in the adult CNS [74-76]. Moreover, studies involving the interruption or deficiency of Egr-1 have demonstrated its involvement in learning and memory [76-78].

Although the physiological role of Egr-1 remains enigmatic, the pathological role of Egr-1 is rapidly evolving. Egr-1 binds to specific Egr-response elements (EREs) in the promoter region to modulate the transcription of target genes [79]. Studies suggest that Egr-1 primarily functions as a suppressor [77, 80, 81], however, Egr-1 can activate the transcription of several genes implicated in HIV pathogenesis including PDGF , MCP-1 and ICAM [77, 82-85].

NF-κB signaling

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a factor involved in activating the transcription of target genes and since its discovery research has been undertaken to better understand its activity and regulation. NF-κB exists in a bound state sequestered by an inhibitory protein, Iκ-B. Upon stimulation by a vast number of factors including HIV-1 Tat, Iκ-Bα is rapidly phosphorylated, ubiquinated and degraded releasing the transcriptionally active NF-κB p65 subunit to the nucleus where it can bind the promoter region and activate transcription. Due to the vast number of target genes that can be activated by NF-κB, it has been implicated in a number of

cellular responses particularly immune response and inflammation. Our findings implicate the role of NF-κB in mitigating MCP-1 released from astrocytes exposed to PDGF-BB.

Blood-brain barrier (BBB) disruption in HAND

The integrity of the BBB plays an important role in maintaining a safe neural microenvironment in the brain. BBB normally functions as an interface between the blood and brain parenchyma, acting as a watch guard to inhibit the entry of ions, molecules and infiltrating cells into the CNS. The presence of interendothelial adherens junctions (AJ) and tight junctions (TJ) between endothelial cells (EC) and EC-astrocytes maintain barrier properties [86]. During the normal immune surveillance, lymphocytes and monocytes cross into the CNS with little or no effect on BBB integrity. However, during progressive HIV-1 infection, there is a breach in this barrier [87-90] leading to influx of inflammatory cells into the brain resulting in clinical and pathological abnormalities, ranging from asymptomatic cognitive impairment to frank dementia. Previous studies have demonstrated that HIV-1 infected cells can disrupt an *in vitro* model of the BBB, which was supported by *in vivo* evidence depicting areas of BBB disruption found in regions enriched with activated, infected monocytes/macrophages, thereby leading to the speculation that infected cells themselves could disrupt the BBB.

BBB plays a central role in the development of HAND because it serves as the conduit by which free virus and infected immune cells enter the brain from the circulatory system [23-25]. BBB compromise in neuropathogenesis of HIV-1 infection is substantiated by a number of laboratory, animal models and human studies

demonstrating BBB breakdown as a consequence of progressive viral infection and immune compromise [26-30]. Structurally, the BBB is composed of specialized nonfenestrated human brain microvascular endothelial cells (HBMECs) connected by intercellular junctions in an impermeable monolayer devoid of transcellular pores. Intercellular junctions are involved in regulation of integrity of the brain vascular endothelium and the BBB function.

Although the mechanisms of BBB disruption during HIV-1 infection still remain unclear yet, alterations in TJ expression have been reported to contribute, at least in part, to this disruption. Hence down modulation of these tight junction proteins could be considered an indicator of the BBB breach. In brain microvascular endothelial (BMEC) cells it has been shown that Tat treatment resulted in decreased expression and/or distribution of the TJ proteins, claudin-1, claudin-5, and zonula occludens (ZO)-2. Decreased claudin-5 expression was further confirmed *in vivo* in mice that were administered Tat into the right hippocampus [91]. Mechanisms underlying Tat-mediated alteration of TJs involved redox-responsive signal transduction pathways, such as VEGFR-2/Ras/ERK1/2 pathway, PI3K/Akt/NF-κB pathway and calcium-dependent signaling [92]. Tat-mediated altered expression of both claudin and ZO-1, -2 could have a significant effect on vascular permeability since claudins are believed to be essential for the structural and functional maintenance of the TJ of BBB and also since ZO-1 proteins mediate interactions of claudins with the actin cytoskeleton and stabilize the claudins in the membrane.

Our findings demonstrate that exposure of rat and human astrocytes to HIV Tat protein resulted in induction of PDGF-B RNA and protein levels. PDGF-B chains once released can dimerize to form PDGF-BB protein. To determine the functional implications of PDGF-BB production, human astrocyte and HMBECs were exposed to PDGF-BB. Rationale for using HBMECs is based on the premise that astrocytes are known to be present in close proximity to cells of the brain endothelium in the neovascular unit. Our findings suggest that exposure of astrocytes to PDGF-BB results in astrogliosis (increased astrocyte activation) as well as release of the chemokine MCP-1, the two hallmark features of HAND [63-65]. To further understand the implications of these findings and to explore the role of PDGF-BB in HIV neuropathogenesis, our central hypothesis is that exposure of astrocytes to viral protein Tat, results in the release of PDGF-BB, which in turn, leads to enhanced astrogliosis and BBB breakdown. **The long-term *goal of this study is to develop targets that can block Tat-mediated up-regulation of PDGF-BB as a therapeutic strategy for HAND.*** The following specific aims were designed to elucidate our hypothesis:

1. Determine the molecular mechanisms involved in HIV-1 Tat mediated induction of PDGF-B chain in astrocytes.
2. Dissect the PDGF-BB-mediated cell signaling mechanisms involved in astrocyte release of MCP-1.

Significance

There is an estimated 33 million people worldwide living with HIV/AIDS [1]. Studies reveal that 60 percent of those infected have some level of HIV associated neurodegenerative syndrome, termed HAND is clinically characterized by behavioral, cognitive and motor abnormalities [93]. The inability of protease inhibitors to penetrate the BBB raises concerns that while cART may effectively control HIV-1 replication elsewhere in the body, replication could continue uncontrolled in the brain, ultimately fueling neurological disease. Thus, the development of therapeutic interventions for the prevention and treatment of HAND among those infected with HIV is of paramount importance. Astroglial activation and dysfunction are key factors mediating neuroinflammation, thus controlling the cascade of actions contributing to the disruption of neuronal homeostasis. Astrogliosis and MCP-1 expression are major features of HAND and our studies provide evidence that both these factors are a consequence of Tat-mediated induction of PDGF-BB in astrocytes. These findings are of clinical importance as understanding the molecular mechanisms of increased astrogliosis and neuroinflammation via chemokine secretion, can lead to effective development of therapeutic strategies aimed at curbing HAND.

Innovations:

1. HIV-1/Tat-mediated induction of PDGF in astrocytes has never been explored before.
2. These studies will identify mechanisms involved in PDGF-mediated induction of the chemokine MCP-1 critical for neuroinflammation.

3. The proposed studies will examine the potential detrimental effects of PDGF on BBB, leading to increased influx of inflammatory cells in the CNS.

The hypothesis was tested exploring the following questions:

Question 1: What are the molecular mechanisms involved in HIV-1/HIV-1Tat-mediated induction of PDGF-BB in astrocytes? This question is addressed in Chapter 3.

Question 2: What are the functional implications of PDGF-BB up-regulation? This question is addressed in Chapter 4.

Question 3: What are the molecular mechanisms involved in the effect of astrocyte-released PDGF-BB on MCP-1 up-regulation and BBB integrity. This question is addressed in Chapter 5.

Chapter 2

HIV-1 Tat mediated up-regulation of PDGF in astrocytes

Abstract

HIV-associated neurocognitive disorders (HAND) are estimated to affect almost 60% of HIV infected individuals. HIV-encephalitis (HIVE), the pathological 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 HIVE correlates better with glial activation rather than viral load. Using the rhesus macaque model, it has been demonstrated that simian immunodeficiency virus encephalitis (SIVE) correlates with increased expression of the mitogen platelet-derived growth factor-B (PDGF-B) chain in the brain. The data therein demonstrate an up-regulation of PDGF-BB in cells exposed to HIV-1 LAI virus which was abrogated by heat-inactivating the virus. Further studies reveal that HIV-1 Tat up-regulated PDGF-B mRNA chain but changes in PDGF-A and PDGF-C chains were not evident. Furthermore, Tat preparations, Tat 1-72, Tat 1-86 and Tat 1- 101 all up-regulated PDGF-BB. The up-regulation of PDGF-BB by Tat was also validated in human primary astrocytes. In an attempt to ascertain the time course of PDGF-B up-regulation, our results reveal a time dependent increase in RNA and protein PDGF-B levels in rat and human astrocyte cell lines C6B2 and A172 as well as human primary cells. The increase in PDGF-BB was further confirmed *in vitro* by immunostaining. Cumulatively, these data clearly demonstrate HIV-1 Tat-mediated induction of PDGF-BB in human astrocytes and underscores the role that astrocytes play in HAND.

Introduction

In the present work, we utilized rat and human astrocytes to study the effects of Tat on rat and human astrocytes. The data herein demonstrate that exposure of rat and human astrocytes to Tat resulted in the induction of PDGF at both the mRNA and protein levels. Since PDGF is a known cerebrovascular permeant [94], the release of PDGF by astrocytes may have significant consequences including disruption of blood-brain barrier(BBB). This is particularly important since astrocyte endfeet processes are in close contact with the endothelial cells of the BBB. Furthermore, PDGF expression could also enhance astrocyte activation. An understanding of PDGF-BB regulation and its inhibition may thus aid in the development of therapeutic intervention strategies for those suffering from HAND.

Materials and Methods

Materials

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 [95-97]. 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 [98]; however, the Tat concentration used in this in vitro study is generally accepted [99-103]. HIV-1 LAI virus propagated in stimulated PBMCs (supplied by Dr. Howard Gendelman, University of Nebraska Medical Center) was used in this study [104]. The rationale for using CXCR4 (X4)-tropic

virus was based on previous studies demonstrating astrocyte activation in response to X4 viruses [105, 106].

Cell culture and cell lines

The human astrocytic cell line A172 (ATCC no. CRL-1620; American Type Culture Collection) was cultured as described previously [107]. 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 CO_2 incubator at 37℃ for 7 d. The cells were then shaken t o remove loosely attached cells. Attached cells that remained, primarily astroglia, were allowed to grow for 3–4 d and then passaged and seeded. Seven days after plating, cultures consisted of 97% astroglia. C6B2 immortalized cell line and rat primary astrocytes were cultured in DMEM medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% heated-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml). Human primary astrocytes were cultured in DMEM medium (Invitrogen Life Technologies) containing 10% heat-inactivated FBS, 2 mM glutamine, sodium bicarbonate, gentamicin, nonessentialamino acids, and vitamins.

Reverse transcription and real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies); 1 µg of RNA was used for cDNA production

according to manufacturer's instructions (Thermo Scientific, Waltham, MA). Real Time RT_2 qPCR primer sets for rat PDGF-A, PDGF-B, PDGF-C and 18s and human PDGF-B and 18s were obtained from SA Biosciences (Frederick, MD). Quantitative analyses of mRNA were conducted using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Data were normalized using the cycle threshold (Ct) values for 18s or GAPDH in each sample. To calculate relative amounts mRNA, the average Ct values were subtracted from 18s or GAPDH values for each target gene to provide changes in Ct value. Fold change in expression was calculated as log2 relative units.

Western blotting

Treated cells were lysed using the Mammalian Cell Lysis kit (Sigma, St. Louis, MO). Cell lysates were subjected to separation by 12% SDSPAGE electrophoresis (30 mg protein per well) and transferred to polyvinylidene difluoride membranes. The blots were blocked with 5% nonfat dry milk in PBS. Western blots were then probed with Abs recognizing PDGF-BB (1:1000), β-actin (1:5000; Santa Cruz Biotechnology,
Santa Cruz, CA) then incubated with goat–anti-rabbit secondary Ab (1:5000; Invitrogen Life Technologies). Signals were detected by chemiluminescence (Pierce, Rockford, IL). All experiments were repeated four times individually and representative blots are presented in the figures.

Immunofluorescence labeling and image analyses

Immunocytochemical analysis of PDGF-BB were performed on rat glioma cells grown on cover slips. After Tat treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with PBS containing 0.3% Triton X 100. After blocking with PBS containing 10% normal goat serum, cells were incubated at 4℃ overnight with anti-PDGF-BB (1:200) rabbit p olyclonal Abs (Santa Cruz Biotechnology). After washing, cells were incubated with goat anti-rabbit Alexa Fluor 488-conjugated secondary Ab (1:500). For negative controls, cells were treated as described above, but the primary Ab was omitted. Cells were mounted with Prolong Gold containing DAPI to stain nuclei. Images were captured with a 40X objective.

Slide specimens (5 mm thick) of paraffin-embedded, SIV-infected rhesus macaque and HIV-1–infected human basal ganglia (National NeuroAIDS Tissue Consortium) were selected and treated with paired combinations of primary mouse and rabbit Abs anti-PDGF-BB (1:50; Santa Cruz Biotechnologies), PDGF-BB (1:1000; PGF007, Mochida, Tokyo, Japan), anti-von Willebrand factor (vWF; 1:00; Chemicon, Billerica, MA) and anti-glial fibrillary acidic protein (GFAP) (1:50) Abs (Santa Cruz Biotechnologies). Primary Abs were labeled with secondary anti-mouse and anti-rabbit Abs conjugated to the fluorescent probes Alexa Fluor 488 and Alexa Fluor 594, and nuclei were labeled with DAPI. Slides were covered with a coverslip with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA) and allowed to dry for 24 h at room temperature. Images were captured with a 20X objective.

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

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 an multiplicity of infection of 0.1 for 12 h followed by assessment of cell lysate for PDGF-BB expression by Western blot. As shown in Fig. 1 there was HIV-1–mediated up-regulation 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 up-regulation of PDGF-B mRNA (2.15- and 3.2-fold respectively), compared with the untreated cells. As expected, heated Tat had no effect on the induction of PDGF-B mRNA. In contrast to PDGF-B, no significant changes in the mRNA levels of PDGF-A and PDGFC were evident (Fig. 2). Because only PDGF-B chain was up-regulated in response to Tat, further studies were 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. 3A). Validation of

our findings in the human astrocyte cell line was also confirmed in human primary astrocytes (Fig. 3B).

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. 4A, PDGF-B mRNA was up-regulated in a time-dependent manner in all the cell types examined, with a peak expression at 3–6 h. To examine whether the mRNA up-regulation 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. 4B, Tat up-regulated 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. 5). Images were captured with a 40X 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 GFAP. As shown in Fig. 6A, there was up-regulated expression of

PDGF-BB in astrocytes surrounding the blood vessels in brains of macaques with SIVE (lower panels) compared with the infected animals without encephalitis (upper panels). In addition to being a marker for endothelial cells, Liu and colleagues have demonstrated that enhanced vWF immunoreactivity was associated with loss of BBB integrity in the spinal cords of mice with Experimental autoimmune encephalomyelitis (EAE) [108]. These findings, coupled with the fact that gliotic reactions around blood vessels were observed, raise the possibility that vWF immunoreactivity present in HIV and SIV-infected basal ganglia tissues may also reflect a leaky endothelial barrier.

Similar studies were performed on basal ganglia sections from human subjects with HIVE and uninfected controls. As shown in Fig. 6B, there was increased expression of astrocytic PDGF-BB in the sections from HIVE versus uninfected controls. Images were captured with a 20X objective lens and fluorescence microscopy.

Discussion

Anti-retroviral 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 dysfunction/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 up-regulated in the brains of macaques with SIVE [109]. 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-αR and PDGF-βR [51, 60, 109, 110].

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 [37], suggesting that both astrocyte dysfunction and certain factors induced by HIV-1/HIV-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-1 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.

Figure 1

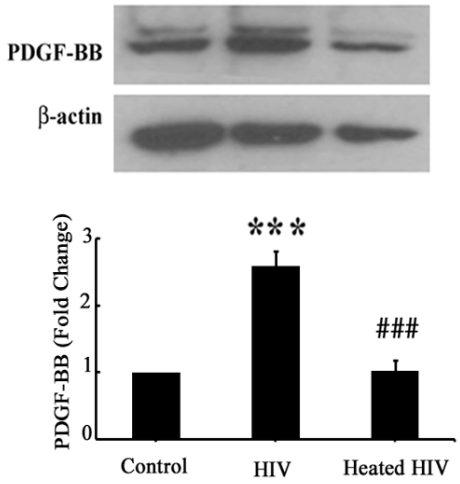


Figure 1: HIV-mediated induction of PDGF-BB in human A172 astrocytes. Cells were either treated with HIV LAI virus or heat inactivated HIV LAI virus at a multiplicity of infection 0.1 for 12 hours. Whole cell lysates were subjected to Western Blott analysis using antibodies specific for PDGF-BB. The data are presented as mean ± SD of three individual experiments. ***p < 0.001 versus control group, ### p < 0.001 versus HIV-treated group.

Figure 2

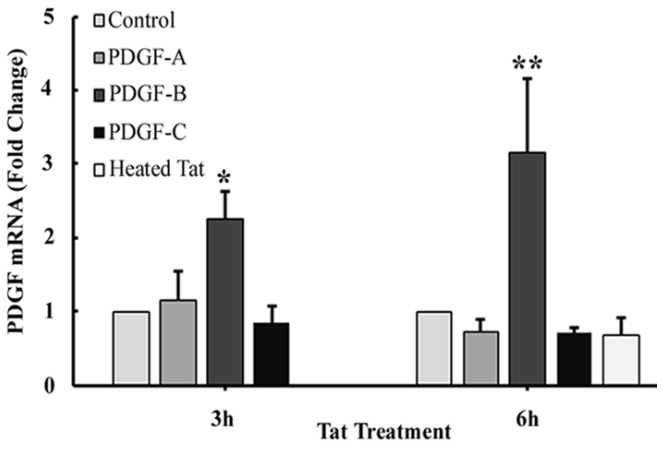


Figure 2: HIV-1 Tat-mediated induction of PDGF-B mRNA in human A172 astrocytes. Cells were either treated with HIV Tat 200ng/ml for 3 and 6hours. Total RNA isolated from primary rat astrocytes was subjected to real-time PCR analysis using primer sets for PDGF-A, PDGF-B, and PDGF-C primers. Tat-mediated 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. The data are presented as mean ± SD of three individual experiments. *$p < 0.05$, **$p < 0.01$ vs untreated control.

Figure 3

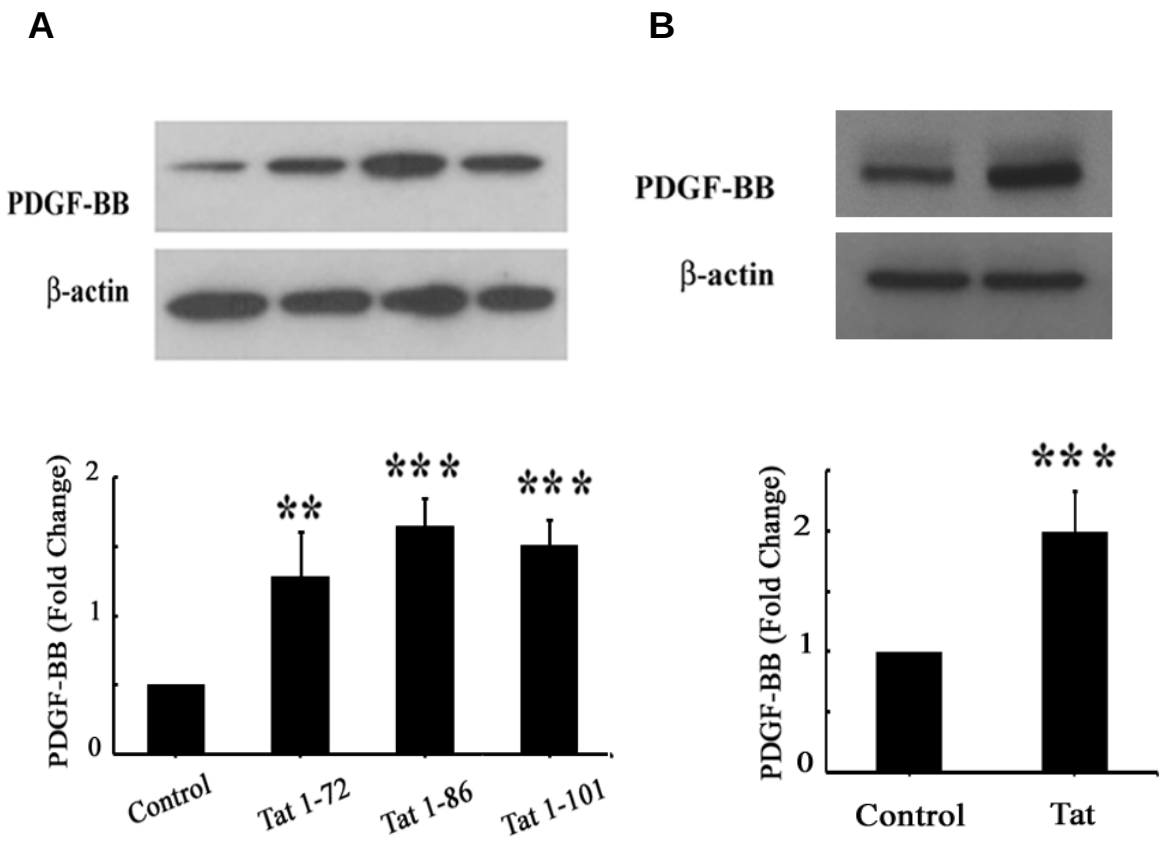


Figure 3: HIV-1 Tat-mediated induction of PDGF-BB protein in human astrocytes. A, Human A172 cells were treated with 200ng/ml of Tat lengths 1–72, 1–86, and 1–101 and PDGF-BB levels were assessed by Western blot analysis. B, Tat-mediated up-regulation of PDGF-BB in human primary astrocytes. Whole cell lysates from human primary astrocytes treated with Tat for 12 h were subjected to Western blot analysis. The data are presented as mean ± SD of three individual experiments. **p <0.01, ***p < 0.001 vs untreated control.

Figure 4

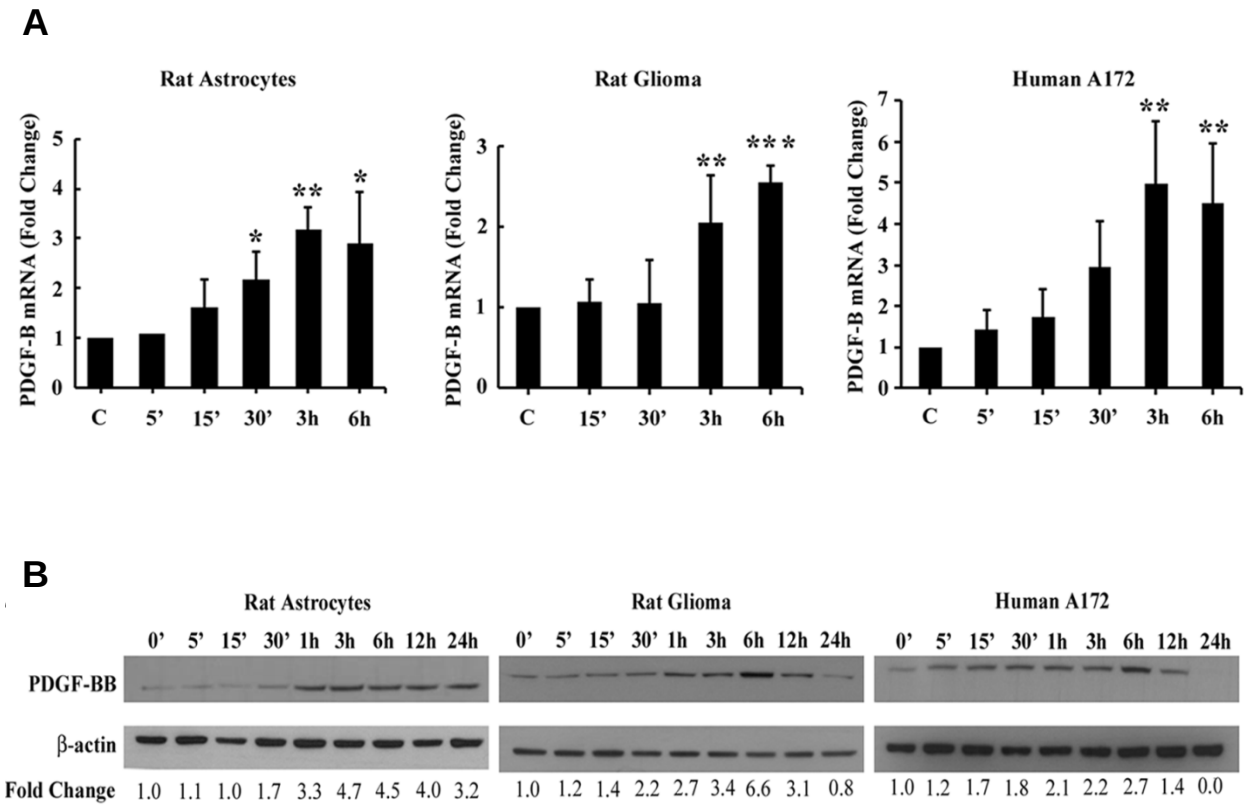


Figure 4: Time dependence of Tat-mediated induction of PDGF-B mRNA and protein expression in rat primary astrocytes, rat gliomas, and human A172 cells. A, Total RNA isolated from rat primary astrocytes, rat gliomas, and human A172 cells were subjected to Real-time PCR analysis using primer sets for PDGF-B mRNA. B, Time dependence of Tat-mediated induction of PDGF-BB protein expression in rat primary astrocytes, rat gliomas, and human A172 cells. 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. The data are presented as mean ± SD of three individual experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs untreated control.

Figure 5

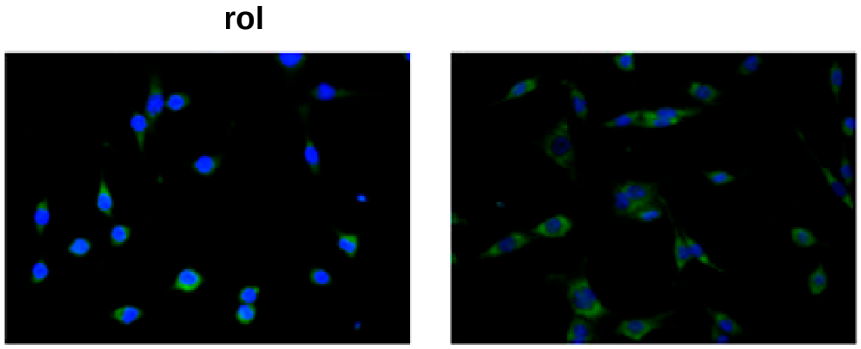
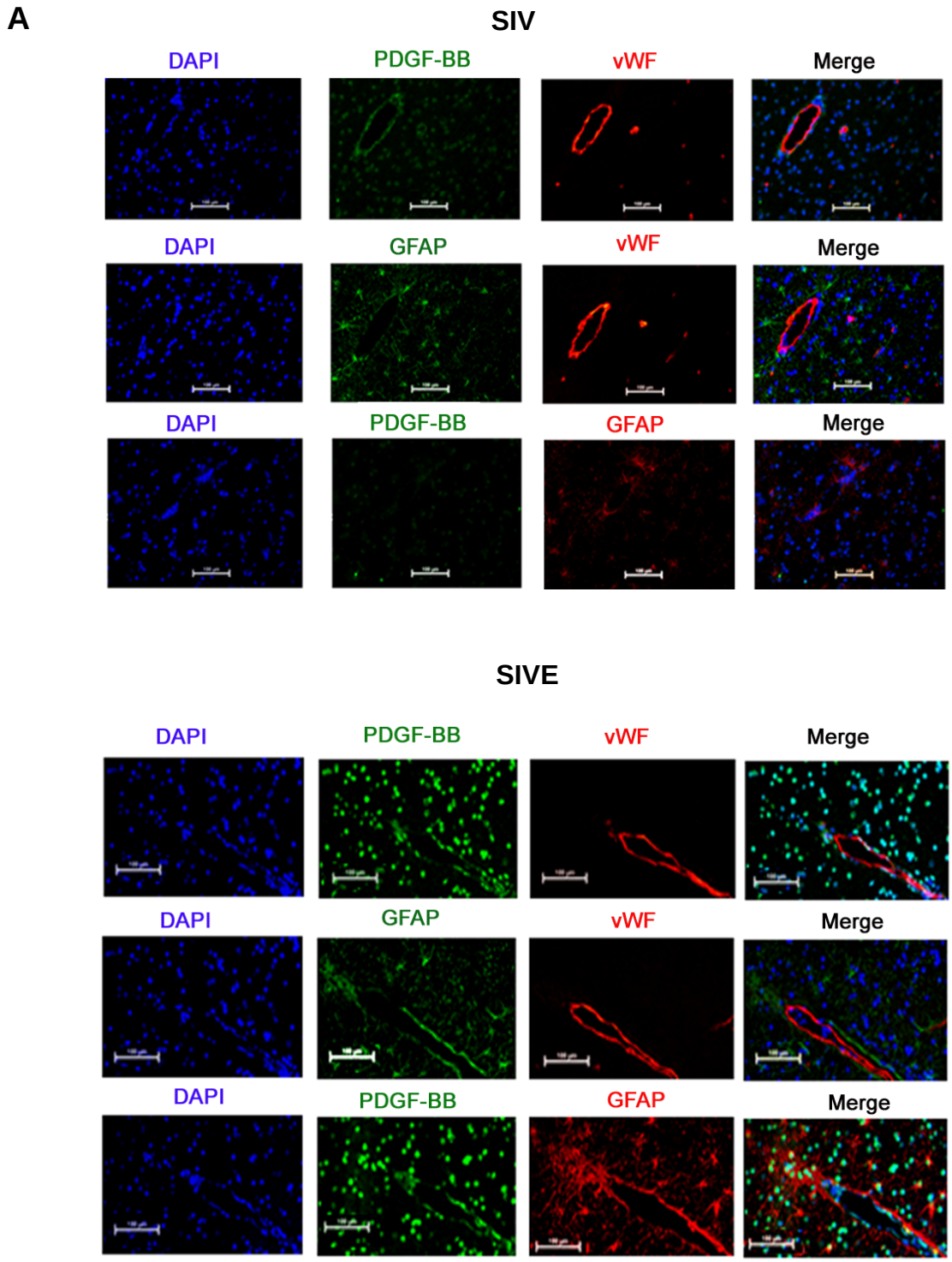


Figure 5: Representative image of PDGF-BB staining in rat glioma cells (original magnification 400X). Rat glioma cells were treated with Tat (200ng/ml) for 6 hours followed by immunostaining using Abs specific for PDGF-BB. Images were acquired using an 40X oil-immersion lens and fluorescent microscopy.

Figure 6


A
SIV
DAPI
PDGF-BB
vWF
Merge
DAPI
GFAP
vWF
Merge
DAPI
PDGF-BB
GFAP
Merge
SIVE
DAPI
PDGF-BB
vWF
Merge
DAPI
GFAP
vWF
Merge
DAPI
PDGF-BB
GFAP
Merge

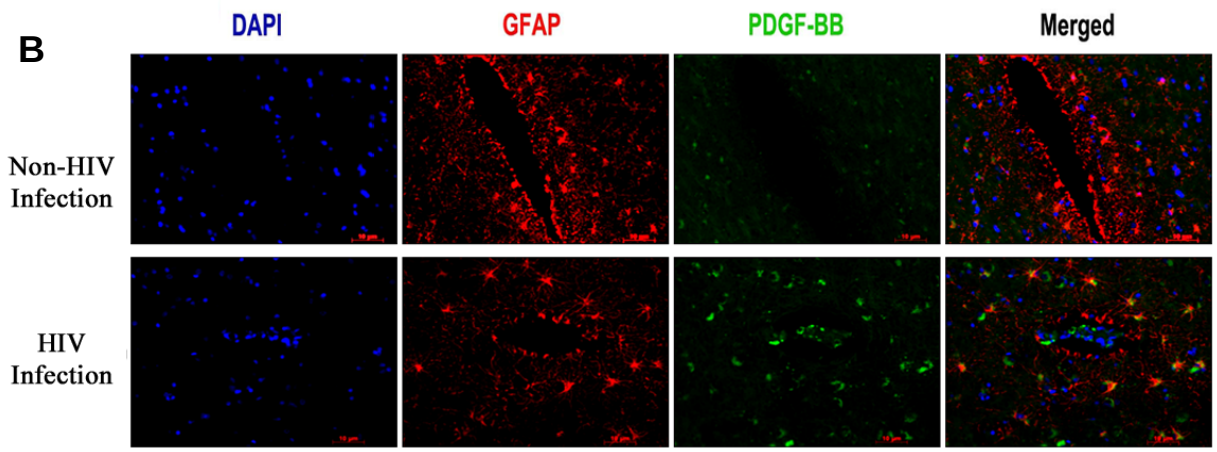


Figure 6: A, Representative image of GFAP plus astrocytes expressing PDGF-BB surrounding the blood vessels (vWF positive) in the basal ganglia of SIV-infected rhesus macaques with or without SIVE (original magnification 200X). Images were acquired using a 20X lens and fluorescent microscopy. B, Representative image of GFAP plus astrocytes expressing PDGF-BB in the basal ganglia sections from humans with HIVE compared with uninfected (HIV-) controls (original magnification 200X). Images were acquired using a 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.

Chapter 3

Signaling Pathways involved in Tat-mediated up-regulation of PDGF in astrocytes

Abstract

We have previously shown that HIV-1 Tat modulates PDGF-BB in astrocytes. The present study was aimed at exploring the mechanisms involved in Tat-mediated PDGF-BB induction observed in astrocytes. Specifically, the data herein demonstrate that HIV-1 Tat activates ERK, JNK and p38 MAPKs as well as PI3k/Akt pathway, however further studies indicate that PDGF-BB induction was regulated by activation of ERK1/2 and JNK signaling pathways, but not P38 MAPK and PI3K/Akt pathways. Moreover, using pharmacological and genetic approaches we demonstrate that JNK and ERK was involved in the activation of the downstream transcription factor, early growth response 1 (Egr-1) in response to HIV-1 Tat. Further studies using PDGF-βR siRNA transfected astrocytes, suggest that Egr-1 plays a critical role in the up-regulation of PDGF-BB in astrocytes exposed to HIV-1 Tat. Chromatin immunoprecipitation (ChIP) assays demonstrated increased binding of Egr-1 to the PDGF-B promoter validating the role of Egr-1. These results map out a detailed pathway in which the exposure of astrocytes to Tat leads to enhanced PDGF-BB expression in astrocytes.

Materials and Methods

Materials

The specific phosphatidinylinositol-39 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). Dominant negative (DN) and constitutively active (CA) constructs of MEK (MAPK) and Egr-1 were provided by Dr. Young Han Lee (Konkuk University, Korea). Chromatin immunoprecipitation (ChIP) assay kit was purchased from Upstate (Billerica, MA).

Reverse transcription and real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies); 1 mg of RNA was used for cDNA production according to manufacturer's instructions (Thermo Scientific, Waltham, MA). Real Time RT_2 qPCR primer sets for and human Egr-1 and 18s or GAPDH were obtained from SA Biosciences (Frederick, MD). Quantitative analyses of mRNA were conducted using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Data were normalized using the cycle threshold (Ct) values for GAPDH in each sample. To calculate relative amounts mRNA, the average Ct values were subtracted from 18s or GAPDH values for each target gene to provide changes in Ct value. Fold change in expression was calculated as $\log_2$ relative units.

Western blotting

Treated cells were lysed using the Mammalian Cell Lysis kit (Sigma, St. Louis, MO), and cell lysates were subjected to separation by 12% SDSPAGE electrophoresis (30 ug protein per well) and transferred to polyvinylidene difluoride membranes. The

blots were blocked with 5% nonfat dry milk in PBS. Western blots were then probed with Abs recognizing Egr-1 (1:500), β-actin (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated forms of ERK1/2, JNK, p38, and Akt (1:200; Cell Signaling, Danvers, MA). Cells were then incubated with goat–anti-rabbit secondary Ab (1:5000; Invitrogen Life Technologies). Signals were detected by chemiluminescence (Pierce, Rockford, IL). All experiments were repeated four times individually and representative blots are presented in the figures.

Immunofluorescence labeling and image analyses

Immunocytochemical analysis of Egr-1 were performed in rat glioma cells grown on cover slips. After Tat treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with PBS containing 0.3% Triton X-100. After blocking with PBS containing 10% normal goat serum, cells were incubated at 4℃ overnight with anti-Egr-1 (1:200) rabbit polyclonal Ab (Santa Cruz Biotechnology). After washing, cells were incubated with goat anti-rabbit Alexa Fluor 488-conjugated secondary Ab (1:500). For negative controls, cells were treated as described above, but omitting the primary Ab. Cells were mounted with Prolong Gold containing DAPI to stain nuclei. Images were captured with a 40X objective.

Transfection with plasmid constructs

A172 cells were transfected with plasmid constructs containing either wild type (WT) or dominant negative (DN) forms of MEK and Egr-1. Knockdown efficiencies were determined by Western blotting.

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 X 10^4 cells per well 1 d prior to transfection. Cell culture medium was replaced with 250 ml prewarmed culture medium. Dharma-FECT 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 mM. The siRNA and the reagent mixture 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℃. 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 1X protease inhibitor mixture. The cell pellet was harvested by spinning at 800 X g at 4℃, and lysis buffer was add ed (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 ml protein A magnetic beads and 5 mg immunopreciptating Abs against Egr-1, acetyl histone H3 (as a positive control), and

normal rabbit IgG (as a negative control) diluted in 450 ml dilution buffer overnight at 4℃. 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℃ for 2 h and purified using DNA purification spin columns following the manufacturer's instructions. Finally, the purified DNA was amplified via PCR 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, 59 GCAGAGGCCTGAGCGCCTGATC- 39; anti-sense, 59-GCAGCGATTCATGCCGACTCCG-39.

Results

Having determined Tat-mediated induction of PDGF-BB, we 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. 7A). 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. 7B).

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. 8A, 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) also did not result in the amelioration of PDGF-BB expression in response to Tat (Fig. 8B). 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. 8C). These findings confirm the involvement of ERK MAPKs, but not p38 and PI3K/Akt cascade in Tat-mediated induction of PDGF-BB in astrocytes.

Egr-1 expression is up-regulated 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 [82], we rationalized the involvement of Egr-1 in Tat-mediated induction of PDGF-BB. 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.

9A, Egr- 1 mRNA was up-regulated in a time-dependent manner in all the cell types examined, with a peak at 15–30 min. To examine whether the mRNA up-regulation translated into increased protein expression, all the cell types were treated as described above, followed by Western blotting. As shown in Fig. 9B, Tat up-regulated 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. 9C). 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. 10A, 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. 10B). 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-induced expression of PDGF-BB in astrocytes

Since 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. 11, 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. 12A, 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 up-regulation of PDGFBB expression (Fig. 12B). 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. 12C). 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. 12D,E).

Discussion

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 [77, 83, 111]. Our findings demonstrated a time-dependent up-regulation 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 [112]. 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. [82], 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. which revealed that Tat enhances Egr-1 by directly transactivating the Egr-1 promoter and that this interaction may be involved in the molecular events that initiate Tat-induced astrocyte dysfunction [113].

We further elucidated that signaling mechanisms involved in PDGF-BB induction was regulated by activation of ERK1/2 and JNK signaling pathways and the downstream transcription factor, Egr-1. ChIP analysis revealed increased binding of Egr-1 to the PDGF-B promoter in rat glioma cells treated with Tat. Based upon our findings we propose molecular pathway of Tat-mediated induction of PDGF-BB schematically illustrated in Figure 13.

Figures

Figure 7

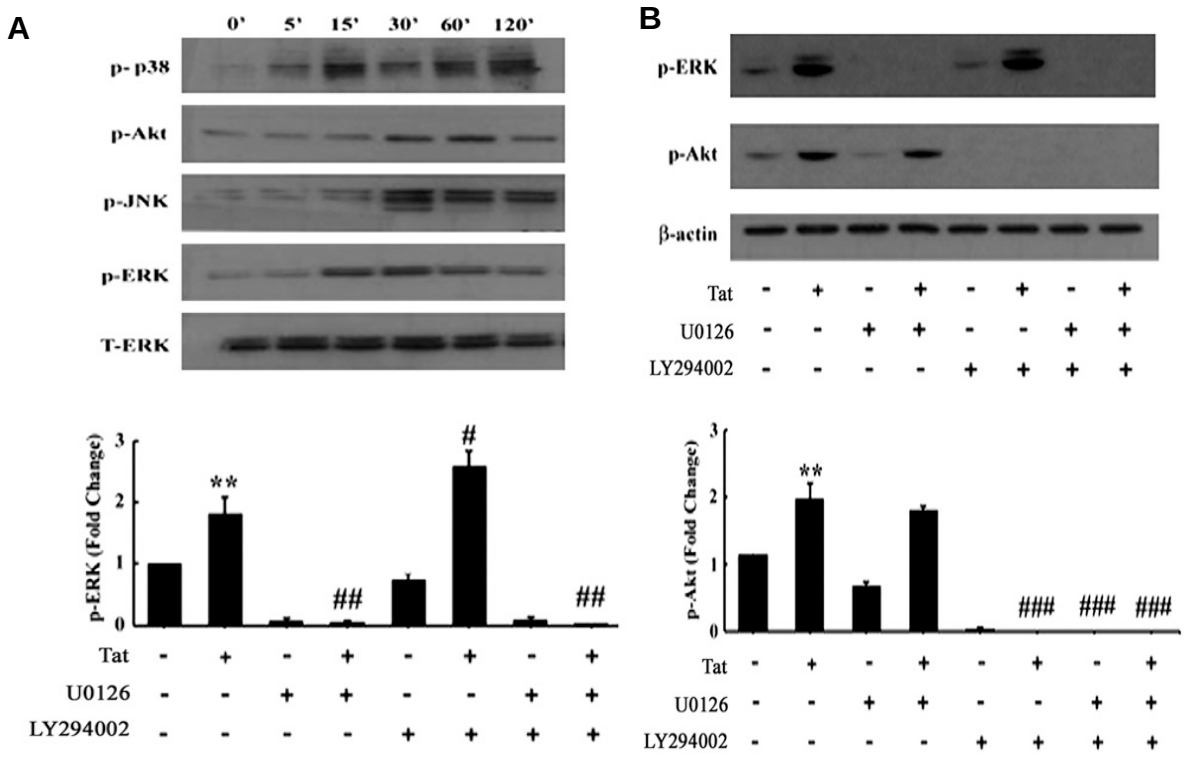


FIGURE 7. Tat up-regulates MAPK and Akt/PI3k pathways. A, Western blot analysis of time-dependent activation ERK1/2, JNK, p38, and Akt by Tat. B, ERK1/2 and Akt pathways are activated independently of each other. Pretreatment of cells with MAPK (MEK) inhibitor (U0126), resulted in abrogation of Tat-induced 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. All data are presented as mean ± SD of three individual experiments. **p < 0.01, versus control group, #p < 0.05, ##p < 0.01, ###p < 0.001 versus Tat-treated group.

Figure 8

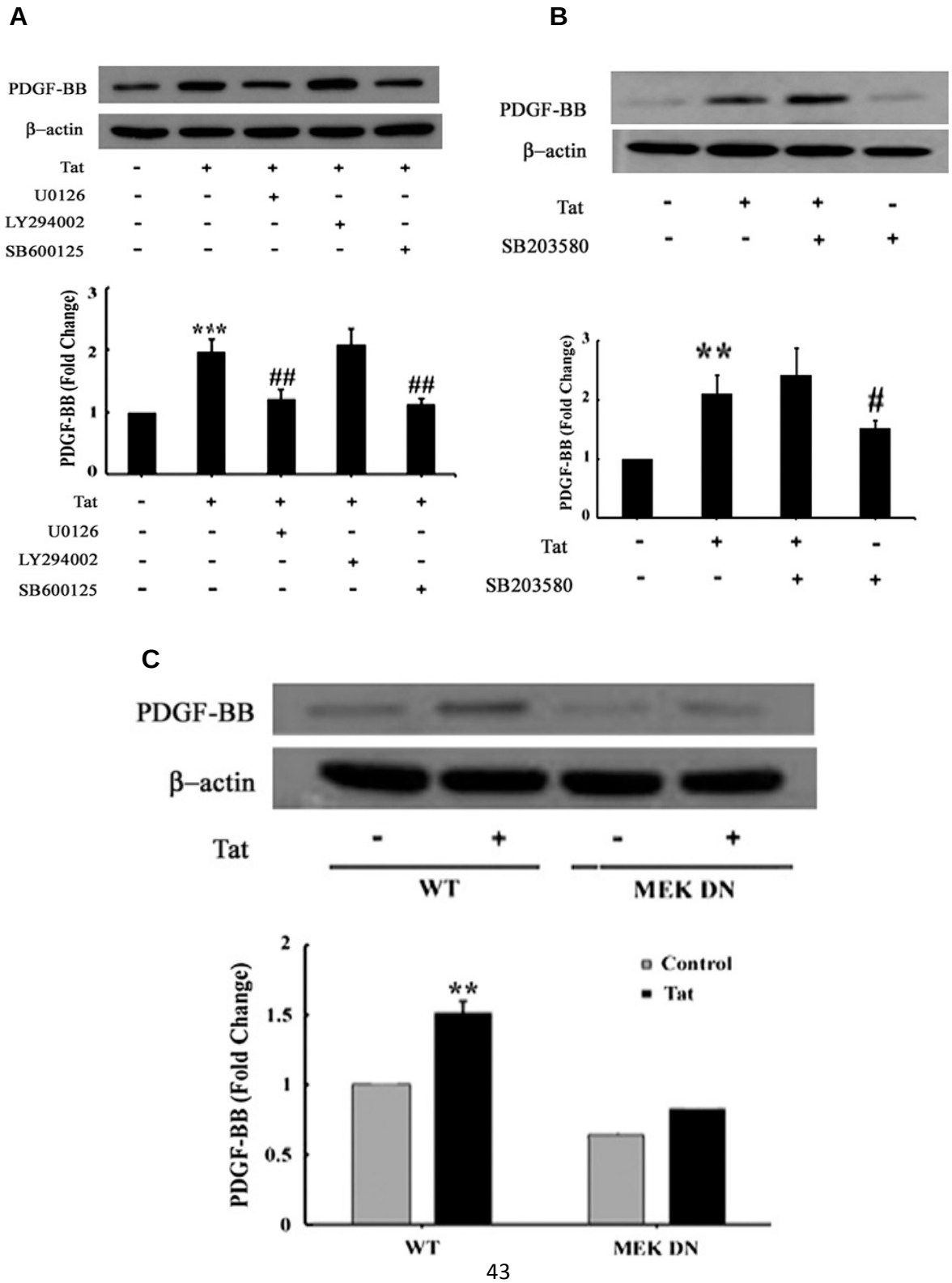

A
PDGF-BB
β–actin
Tat
U0126
LY294002
SB600125
PDGF-BB (Fold Change)
***
##
##
B
PDGF-BB
β–actin
Tat
SB203580
PDGF-BB (Fold Change)
**
#
C
PDGF-BB
β–actin
Tat
WT
MEK DN
Control
Tat
PDGF-BB (Fold Change)
**
WT
MEK DN

Figure 8. ERK1/2 and JNK MAPK but not p38 and PI3K/Akt pathways are involved in Tat-mediated PDGF-BB expression in astrocytes. A, 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. B, Pretreatment of cells with p38 inhibitor (SB203580) did not ameliorate Tat-mediated induction of PDGF-BB. C, 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, #$p < 0.05$, ##$p < 0.01$ versus Tat-treated group.

Figure 9.

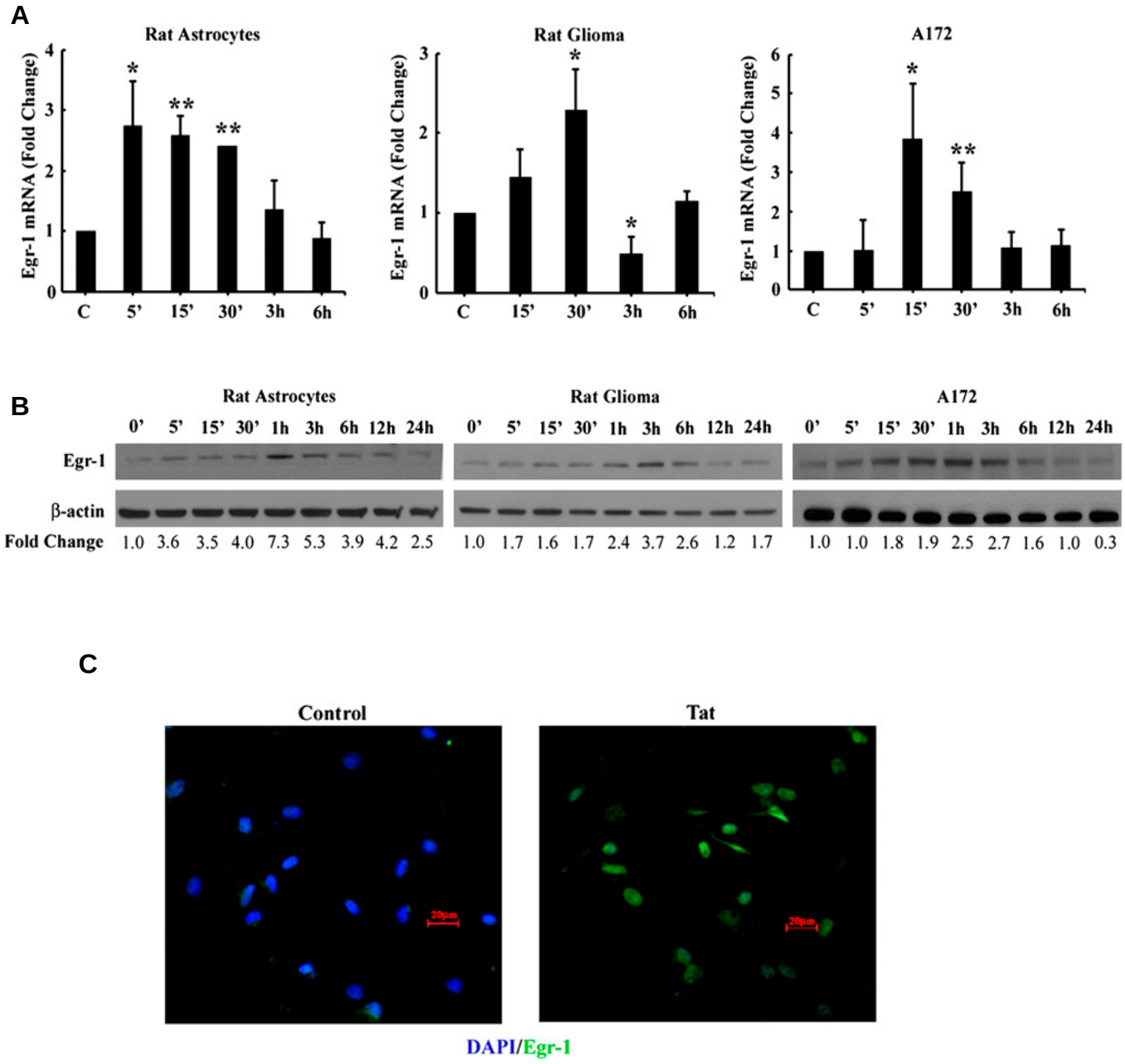


FIGURE 9. Egr-1 expression is up-regulated in astrocytes exposed to Tat. A, Time dependence of Tat-mediated induction of Egr-1 mRNA expression in rat gliomas and

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. Time dependence of Tat-mediated induction of Egr-1 protein expression in rat gliomas and human A172 cell lines and rat primary astrocytes. C, Representative picture of Egr-1 staining in rat gliomas. . *$p < 0.05$, **$p < 0.01$ vs untreated control.

Figure 10.

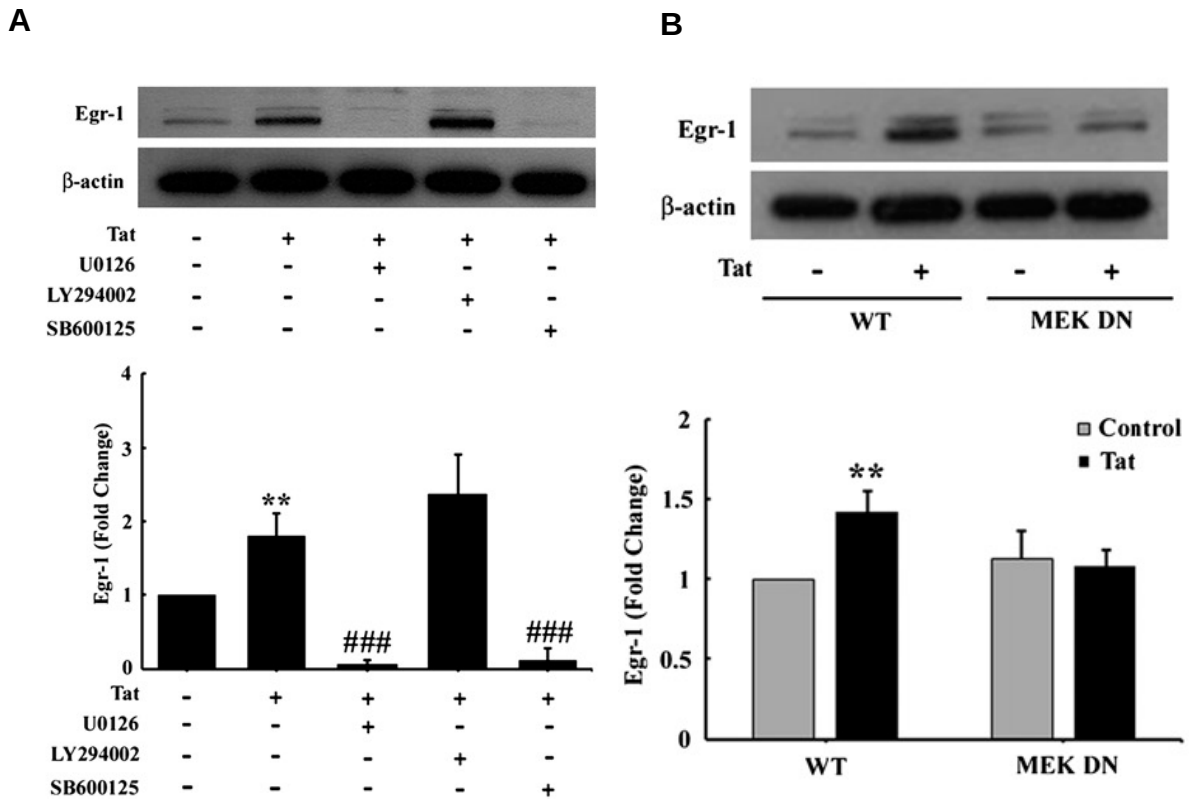


Figure 10. MAPK but not Akt/PI3k is involved in Tat-mediated induction of Egr-1. A, Inhibition of the ERK1/2 and Akt pathways by MEK (U0126) and PI3K inhibitor (LY294002) resulted in amelioration of Tat-mediated Egr-1 expression. B, Transfection with DNMEK and not WT-MEK resulted in abrogation of Tat-mediated induction of Egr-1. **p <0.01 vs untreated control, ###p < 0.001 versus Tat-treated group.

Figure 11.

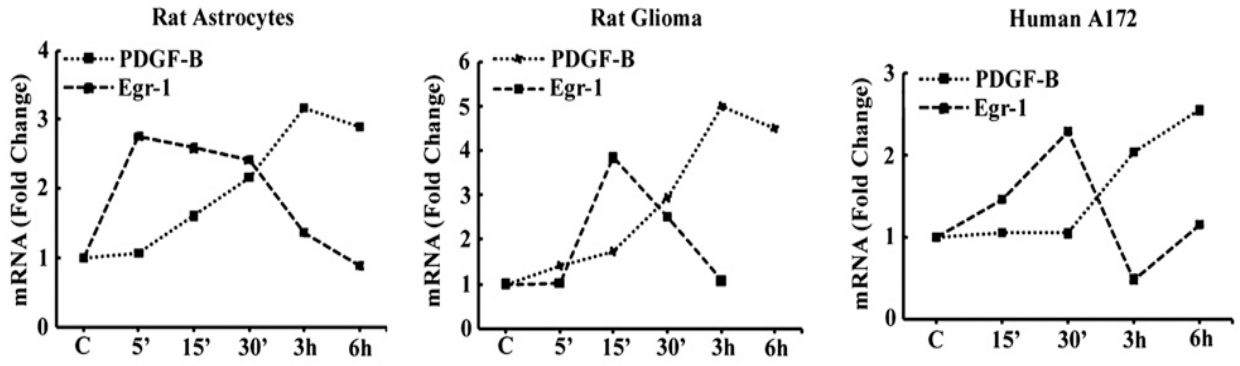


Figure 11. Kinetic profiles of Egr-1 and PDGF-B mRNA levels in rat primary astrocytes, rat gliomas and human A172 cells. Egr-1 expression preceeds PDGF-B expression suggesting the involvement of Egr-1 in Tat-induced expression of PDGF-BB in astrocytes.

Figure 12

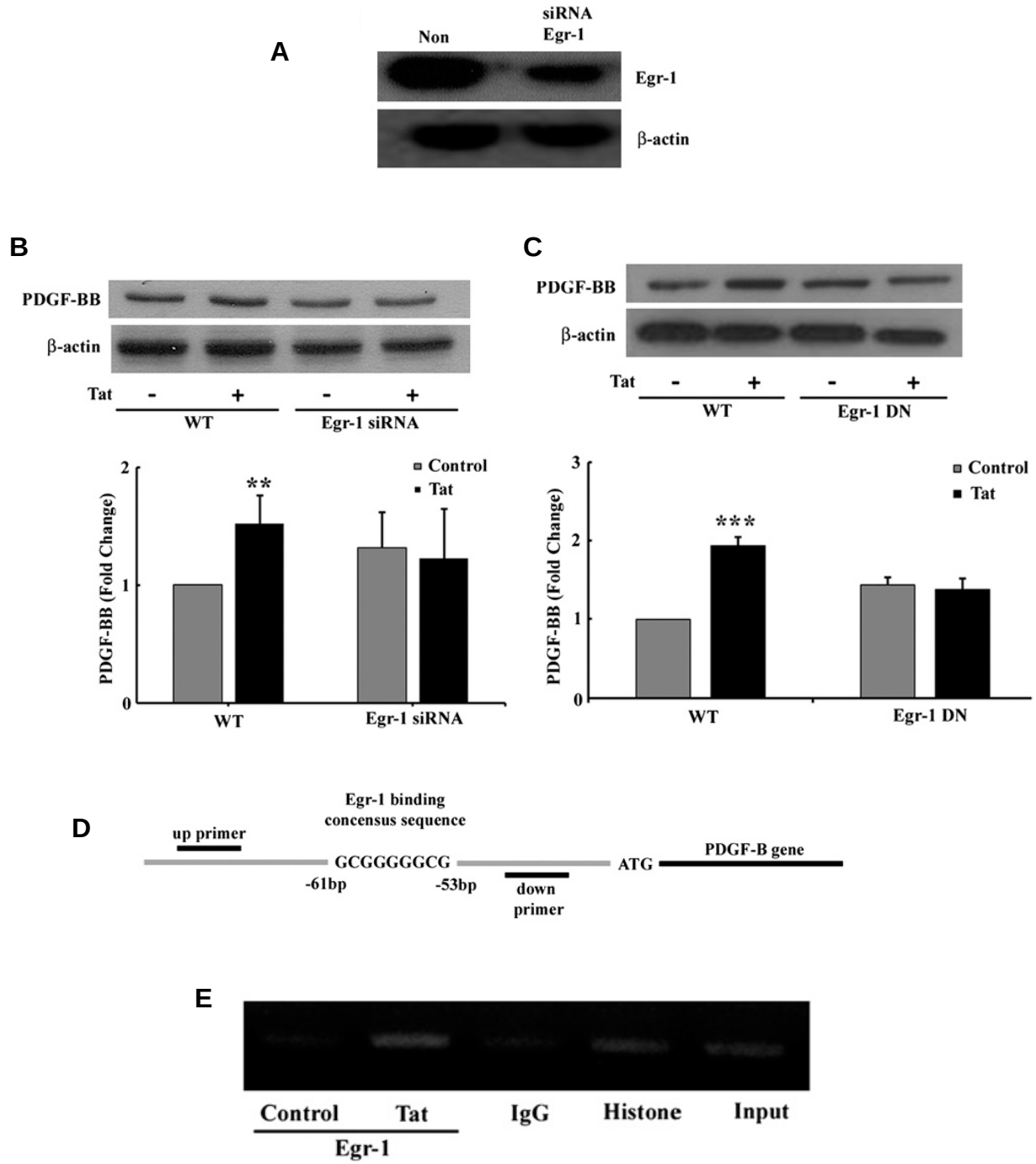

A
Non
siRNA Egr-1
Egr-1
β-actin
B
PDGF-BB
β-actin
Tat - + - +
WT
Egr-1 siRNA
PDGF-BB (Fold Change)
Control
Tat
**
WT
Egr-1 siRNA
C
PDGF-BB
β-actin
Tat - + - +
WT
Egr-1 DN
PDGF-BB (Fold Change)
Control
Tat
***
WT
Egr-1 DN
D
Egr-1 binding concensus sequence
up primer
GCGGGGGCG
ATG
PDGF-B gene
-61bp
-53bp
down primer
E
Control
Tat
IgG
Histone
Input
Egr-1

Figure 12. Involvement of Egr-1 in Tat-induced expression of PDGF-BB in Astrocytes. A, 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. B, Egr-1 siRNA, but not nonspecific siRNA, inhibited HIV-1 Tat-mediated induction of PDGF-BB expression. C, 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. D, Schematic illustration of Egr-1 binding consensus sequence on the PDGF-B promoter region. E, 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.

Figure 13

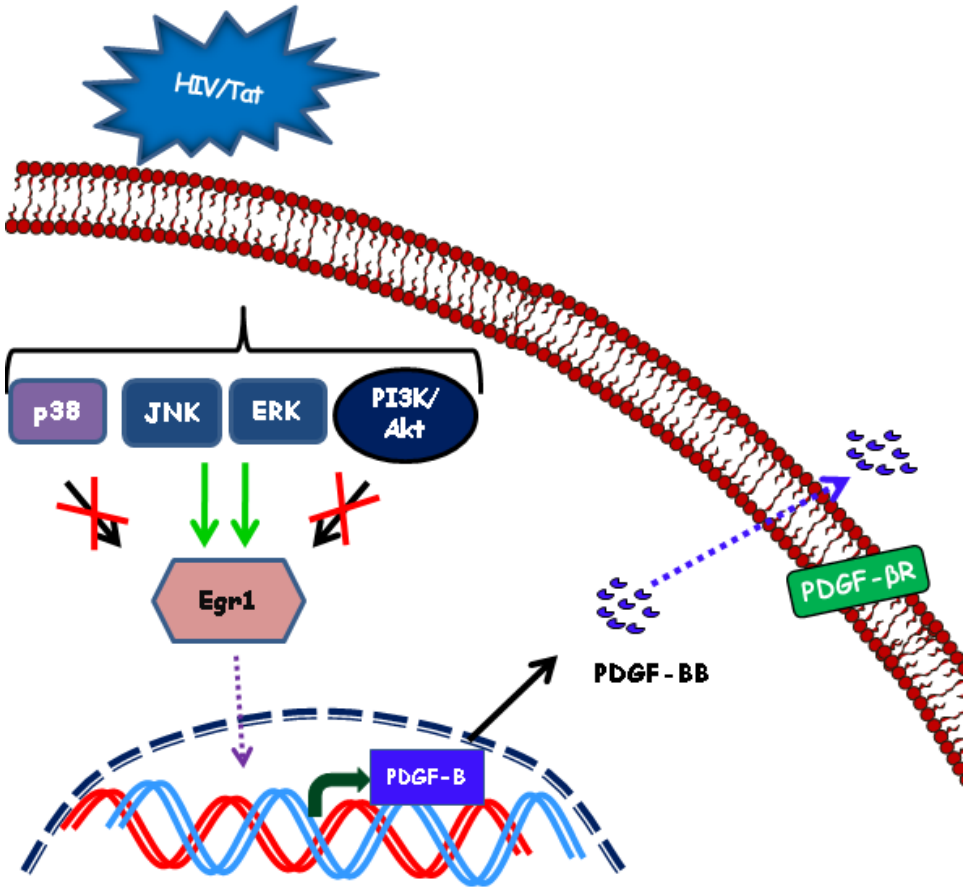


Figure 13. Schematic of the signaling pathways involved in the increased induction of PDGF-BB in astrocytes stimulated with Tat. Tat-mediated activation of ERK1/2, JNK, p38 MAPKs, and PI3K/Akt signaling pathways. ERK1/2 and JNK MAPKs but not and PI3K/Akt and p38 signaling resulted in the subsequent activation of the downstream transcription factor, Egr-1. Activation of Egr-1 in turn leads to enhanced PDGF-BB expression as shown in Figure 13.

Chapter 4: Functional Implications of PDGF-BB up-regulation

Abstract

We have previously shown that HIV-1 Tat modulates PDGF-BB in astrocytes. The present study was aimed at exploring the role of PDGF-B chain in HIV-associated activation and proliferation of astrocytes.

PDGF-BB, released from Tat-treated astrocytes resulted in both increased proliferation and release of pro-inflammatory cytokines MCP-1 and IL-1β in astrocytes. Since astrogliosis is linked to disease severity, understanding its regulation by PDGF-BB and its relationship with MCP-1 could aid in the development of therapeutic intervention strategies for HAND.

Materials and Methods

Reverse transcription and real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies); 1 mg of RNAwas used for cDNA production according to manufacturer's instructions (Thermo Scientific, Waltham, MA). Real Time RT2 qPCR primer sets for rat PDGF-βR and GAPDH and human PDGF-βR, MCP-1, IL-1β 18s and GAPDH were obtained from SA Biosciences (Frederick, MD). Data were normalized using the 18s or GAPDH amounts in each sample. To calculate relative amounts mRNA, the average Ct values were subtracted from 18s or GAPDH values for each target gene to provide changes in Ct value. Fold change in expression was calculated as $\log_2$ relative units.

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 $1X10^5$ 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.

Results

PDGF-BB induces cell proliferation and proinflammatory cytokine expression in astrocytes

Having determined the induction of PDGF-BB by Tat, this study was undertaken to explore the functional relevance of this up-regulation. 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-βR. As shown in Fig. 14A, rat glioma and human astrocyte cell lines (C6B2 and A172) as well as rat primary astrocytes cells expressed PDGF-βR 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. 14B).

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 [114], we next examined the effect of PDGF-BB on the induction of MCP-1 and the pro-inflammatory cytokine IL-1β in A172 cells. As shown in Fig. 14C, treatment of human A172 cells with PDGF-BB resulted in a dramatic up-regulation of MCP-1 and a significant induction of IL-1β as measured by real-time PCR.

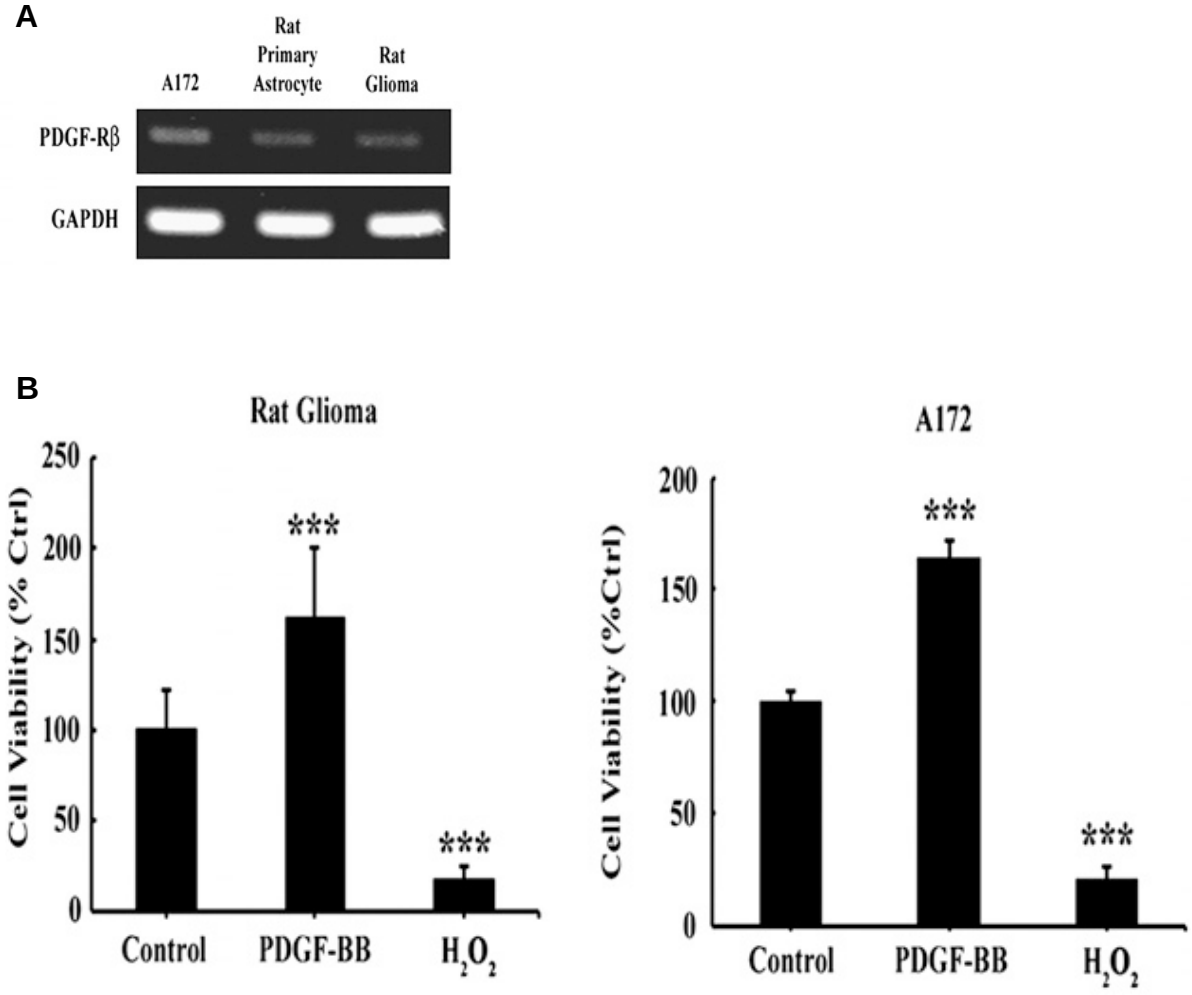
Discussion

Because our previous findings had demonstrated the induction of PDGF-BB by HIV-1 Tat, 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 [115, 116]. MCP-1 is a known biomarker of HIV neuropathogenesis [117, 118], and PDGF-mediated up-regulation 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 [65, 114] and whose function includes the recruitment of monocytes from the blood to the brain [119]. 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. Exposure of astrocytes to PDGF-BB, in turn, led to both increased proliferation and release of pro-inflammatory cytokines MCP-1 and IL-1β.

Figures

Figure 14



C

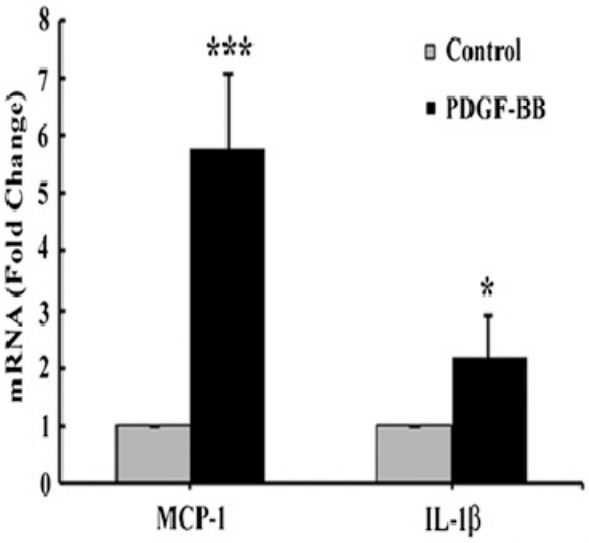


Figure 14. PDGF-BB induces cell proliferation and proinflammatory cytokines expression in astrocytes. A, Representative RT-PCR gel of PDGF-βR mRNA expression in human A172, rat primary astrocytes, and gliomas. B, Rat gliomas and human A172 cell lines incubated with PDGFBB showed increased proliferation 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.

Chapter 5: Molecular Mechanisms of PDGF-BB-mediated MCP-1 up-regulation

Abstract

Chemokine (C-C motif) ligand 2, also known as MCP1 is an important factor in HAND. The mechanisms, in part revolve around its neuroinflammatory processes and the recruitment of monocytes into the CNS via the disrupted BBB. We have previously demonstrated that HIV-1/HIV-1 Tat up-regulates PDGF-BB, a known cerebrovascular permeant and a potent inducer of MCP-1 in astrocytes. MCP-1 alone has been shown to disrupt the endothelial cell barrier providing a rationale of further endothelial disruption by PDGF-BB. Moreover, we hypothesize that PDGF-BB-mediated disruption of endothelial cells is due to MCP-1. The present study was aimed at exploring the regulation of MCP-1 by PDGF-BB in astrocytes with implications in HAND. Specifically, the data herein demonstrated that exposure of human astrocytes to human recombinant PDGF-BB protein significantly increased the production and release of MCP-1 at both the RNA and protein levels. Furthermore, MCP-1 induction was regulated by the activation of ERK1/2, JNK and p38 MAP kinases and PI3K/Akt pathways and the downstream transcription factor, NFκB. Chromatin Immunoprecipitation (ChIP) assays demonstrated increased binding of NF-κB to the human MCP-1 promoter with PDGF-BB exposure. Conditioned media from PDGF-BB-treated astrocytes increased permeability of HBMECS, an effect that was blocked by STI-571, a tyrosine kinase inhibitor (PDGF-R blocker). PDGF-BB-mediated release of MCP-1, was the mediator of increased permeability in a BBB *in vitro* model as evidenced by the blocking antibody assays. Since MCP-1 is linked to disease severity, understanding its modulation by

PDGF-BB could aid in understanding the proinflammatory responses in HAND. These results suggest that astrocyte activation exaggerates monocyte recruitment into the brain via MCP-1 and underscores the critical role astrocytes play in HAND.

Introduction

HAND remains a common complication of HIV infection affecting up to 60% of infected individuals despite the use of anti-retroviral therapy (ART) [120]. With the advancement of ART the prevalence of HAND has actually increased partly due to increased survival rates of HIV infected individuals. Among the factors involved in HAND pathogenesis, BBB disruption remains a critical aspect in accelerating this disease. HIV infected monocyte traverse the BBB into the brain triggering a cascade of events including astrocyte activation resulting eventually in increased neuropathogenesis and neuroinflammation. It has been well established that MCP-1, otherwise known as CCL2, is a major factor in the recruitment of monocytes into the brain that contributes to neuroinflammation and BBB disruption [44, 121]. MCP-1 is a chemokine that has been well studied and is expressed by a number of cell types including astrocytes, microglia and neurons [122, 123]. Elevated expression of MCP-1 has been demonstrated in various diseases including multiple sclerosis, amyloid lateral sclerosis, lupus nephritis, peripheral neuropathy and Alzheimer's disease [66, 124-130]. The role of MCP-1 in contributing to disease is unclear however, enhanced MCP-1 levels have been shown to correlate with disease progression [64, 65, 131]. Understanding the molecular mechanisms modulating MCP-1 may thus provide insights into development of therapeutic targets for many disease including HAND.

NF-κB, is a transcription factor known to be involved in the induction of a number of pro-inflammatory molecules including MCP-1 [132-134]. Under basal conditions NF-κB exists in a bound state sequestered by an inhibitory protein, IκB. However, following

stimulation by a vast number of factors, IκBα is rapidly degraded with a concomitant release of NF-κB p65 subunit, that is released and translocates to the nucleus, where it binds the promoter region to activate the transcription of specific target genes. Due to the vast number of target genes that can be activated by NF-κB, it has been implicated in a number of cellular responses particularly immune response and inflammation.

PDGF is a well-known potent inducer of MCP-1. PDGF is a family of proteins very closely related to the VEGF family and is highly conserved throughout the animal kingdom [51]. Many studies on PDGF have focused primarily on its mitogenic effects [49, 50, 135], however, divergent effects of PDGF are rapidly emerging. For example, recent studies by Lawrence *et a*l have demonstrated PDGF to be a cerebrovascular permeant that can disrupt BBB integrity during ischemia stroke conditions [94]. Along similar lines, it has been shown that PDGF-BB can disrupt BBB via modulation of molecules important in maintaining tight junctions like ZO-1 and adhesion molecules [136].

Since astrocytes are a major source of MCP-1 in the brain and PDGF-BB has been shown to be an inducer of MCP-1, the purpose of this study was to explore the modulation of MCP-1 by PDGF-BB (released from Tat-treated astrocytes). Human astrocyte A172 cells were treated with PDGF-BB and MCP-1 expression was monitored. Utilizing pharmacological and genetic approaches, we demonstrated the involvement of ERK1/2, JNK and p38 MAPKs, PI3K/Akt pathways and the transcription factor NF-κB in PDGF-BB-mediated induction of MCP-1 in astrocytes. Furthermore, MCP-1 released from astrocytes results in increased permeability of HBMECs. These

data highlight the role of PDGF-BB in astrocytic dysfunction and BBB disruption via MCP-1 release and underscore PDGF signaling as a potential therapeutic target of HAND.

Materials and Methods

Materials

Recombinant human PDGF-BB was purchased from R&D Systems. All experiments involving the treatment of cells with exogenous PDGF-BB were conducted under serum-free conditions because serum induces PDGF. STI-571, an inhibitor of tyrosine kinase receptors, was obtained from Novartis. The specific phosphatidinylinositol-3' kinase (PI3K) inhibitor LY294002, MAP kinase kinase (MEK) inhibitor U0126 and Janus kinase (JNK) inhibitor SP600125 and p38 mitogen activated kinase (p38) inhibitor SB203558 were purchased from Calbiochem. MCP-1 neutralizing antibody was obtained from eBioscience. The CCR2 antagonist, RS 102895 was purchased from Sigma. Chromatin immunoprecipitation (ChIP) assay kit was purchased from Upstate.

Cell culture and cell lines

The human astrocytic cell line A172 (ATCC #CRL-1620; American Type Culture Collection) were cultured as described previously and maintained in DMEM high glucose medium containing 10% heated-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), essential amino acids and vitamins. In this study, A172 cells were used within 30 passages. Human primary astrocytes were obtained from the Congenital Defects Lab (University of Washington)

and were cultured in DMEM medium (Invitrogen Life Technologies) containing 10% heat-inactivated FBS, 2 mM glutamine, sodium bicarbonate, gentamicin, nonessential amino acids and vitamins. Primary human brain microvascular endothelial cells (HBMECs) were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 µg/ml), essential amino acids and vitamins.

MCP-1 protein analysis by ELISA

MCP-1 levels were examined using a MCP-1 enzyme-linked immunosorbent assay (ELISA) kit purchased from R&D Systems. Samples were analyzed for MCP-1 protein according to the manufacturer's instructions in at least triplicate determined in three independent experiments.

Reverse transcription and Real-Time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies). 1 µg of RNA was used for cDNA production according to manufacturer's instructions (Thermo Scientific). The sequences of primers used for MCP-1 are as follows: sense:CAGCAGGTGTCCCAAAGAAGCTGT antisense: CCATTCCTTATTGGGGTCAGCACAGA. The sequences of primers used for GAPDH were are follows: sense: TGCACCACCAACTGCTTAGC; antisense: GGCATGGACTGTGGTCATGAG. The sequences of primers used for PDGF-βR were are follows: sense: CAGAGCTGCCCATGAACGA; antisense: TTCTGCATGATCTCATAGATCTCGT. The primers for CCR2 used in the first round

are as followed: sense: TCTGGAGACCTCAACCAAATG; antisense: GGAAATGCGTCCTTGTTCAA. The primers used in the second round are as followed: sense: CCCTGTATCTCCGCCTTCACT; antisense: TTCAGCTTGTGGCTTGTCTCA. Quantitative Analyses of mRNA were conducted using ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Data were normalized using Ct values for GAPDH in each sample. To calculate relative amounts mRNA, the average Ct values were subtracted from GAPDH values for each target gene to provide changes in Ct value. Fold change in expression was calculated as $\log_2$ relative units.

Western Blotting

PDGF-BB treated astrocytes were lysed using the Mammalian Cell Lysis kit (Sigma) and the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce) per manufacturer's instructions. Cell lysates were subjected to the separation by 12% SDS-PAGE electrophoresis (30 µg protein per well) and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% nonfat dry milk in PBS. Western blots were then probed with antibodies recognizing phosphorylated forms of ERK1/2, JNK, p38 and Akt and total forms of ERK1/2, JNK, and AkT (Cell Signaling, 1:500); NFκB-p65 and pIκB Cell Signalling, (1:1000); Histone (1:1000) and β-actin antibodies (Santa Cruz, 1:5000). Signals were detected by chemiluminescence (Pierce).

Transduction with adenoviral constructs

A172 cells were transduced with adenoviral constructs containing either wildtype (WT) or dominant-negative (DN) forms of Akt. In addition, cells were also transduced

with recombinant adenoviral vectors expressing full-length p65/RelA or p65/RelA mutant (RelA 1-300) used at a multiplicity of infection (MOI) of 50 as previously described [137].

Transfection with plasmid constructs

A172 cells were transfected with plasmid constructs containing either WT or DN forms of MEK.

Short interfering RNA (siRNA) transfection

Short interfering RNA (siRNA) targeting against PDGF-βR was obtained from Dharmacon. Human A172 cells were plated in 24-well plates at a density of 4×10^4 cells per well 1 d prior to transfection. Cell culture medium was replaced with 250 ml pre-warmed OPTI-MEM I culture medium. Lipofectamine-2000 reagent (Invitrogen) was then combined with serum-free medium for 5 min at room temperature. The PDGF-βR siRNA was then added into the mixture described above to a final concentration of 5 μM. The siRNA and the reagent mixture 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 24 h at 37℃. Knockdown efficiency was determined by RT-PCR.

Chromatin immunoprecipitation (ChIP) 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 1X protease inhibitor mixture. The cell pellet was harvested by spinning at 800 x g at 4℃, 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 ml protein A magnetic beads and 5 mg immunopreciptating Abs against NF-KB p65, acetyl histone H3 (as a positive control), and normal rabbit IgG (as a negative control) diluted in 450 ml dilution buffer overnight at 4℃. 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℃ for 2 h and purified using DNA purification spin columns following the manufacturer's instructions. Finally, the purified DNA was amplified via PCR to identify the promoter region contained NF-κB binding site "GGGCCTTTCC". The sequence of the primers used to identify the MCP-1 promoter bound to NF-κB were as follows: sense: GCATCAGAGCATTGACCCTCA; anti-sense: AGGTCAGTGCTGGCGTGAGA.

Monocyte isolation and transmigration

Monocytes were obtained from HIV-1, HIV-2 and hepatitis B seronegative donor leukopacks, and separated by countercurrent centrifugal elutriation as previously described [138]. Monocytes were washed with PBS and fluorescently labeled with 10 µM Cell tracker green (Molecular Probes) for 10 min at room temperature. Labelled cells ($2X10^5$ cells) were added to the upper compartments of transwell inserts and allowed to transmigrate at 37ºC in a humid atmosphere of 5% CO2 for 24 h.

Transmigrated monocytes were quantified using florescent plate reader (494nm / 517nm excitation/emission).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance with a post hoc Student's *t* test. Results were judged statistically significant if $p < 0.05$ by analysis of variance.

Results

PDGF-BB-mediated up-regulation of MCP-1 in astrocytes

All experiments involving the treatment of cells with exogenous PDGF-BB protein were conducted under serum-free conditions because serum induces PDGF [139]. To investigate the role of PDGF on MCP-1 expression, A172 cells were serum starved in medium containing 0.1% FBS overnight followed by treatment with recombinant PDGF-BB (20 ng/ml) for various times and mRNA levels were assessed by RT-PCR. The concentration of PDGF-BB used was based on previous studies [111, 140]. Following exposure of A172 astrocytes to PDGF-BB, there was a time-dependent up-regulation of MCP-1 mRNA compared with untreated cells (Fig. 15A) with a peak induction at 6 h post treatment (4-fold) and a subsequent down-regulation at 12 h.

To determine whether the increased MCP-1 mRNA expression observed correlate with increased protein release in astrocytes, we next examined the MCP-1 protein levels. Cells were serum-starved as described and treated with PDGF-BB for 0-96 h. The supernatants were collected and MCP-1 protein levels were assessed via ELISA. As shown in Fig. 15B, PDGF-BB up-regulated MCP-1 protein levels in a time-dependent manner with a continual increase that plateaued at 48 h post-treatment. Cumulatively, these data clearly demonstrate that PDGF-BB treatment mediated the induction of MCP-1 RNA and protein in astrocytes.

Engagement of PDGF-βR is critical for PDGF-induced MCP-1 expression in astrocytes

Because PDGF-BB mediates its effects via binding to its cognate receptor PDGF-βR, our next step was to examine the role of this receptor in PDGF-BB-induced MCP-1 release by astrocytes. However, before proceeding with this, it was important to first examine whether astrocytes indeed expressed the PDGF-βR. As shown in Fig 16A, both human astrocyte cell line A172 as well as well as human primary astrocytes expressed PDGF-βR as demonstrated by RT-PCR.

To confirm the role of PDGF-βR played in PDGF-BB-mediated MCP-1 released from astrocytes, human A172 astrocytes and human primary astrocytes were pretreated with STI-571, PDGF-βR blocker, followed by PDGF-BB treatment. As shown in Fig. 16B, STI-571 inhibited PDGF-BB-mediated induction of MCP-1 mRNA in both human A172 astrocytes as well as human primary astrocytes. This was further confirmed by examining MCP-1 protein released from these astrocytes by ELISA. As shown in Fig. 16C, pretreatment of astrocytes with STI-571 also abrogated PDGF-BB mediated release of MCP-1 protein as demonstrated by ELISA. Further validation of the involvement of PDGF-βR in PDGF-BB-mediated MCP-1 release was confirmed by knocking down the expression of PDGF-βR using the siRNA approach and then assessing the effect on PDGF-BB-mediated induction of MCP-1. As shown in Fig. 16D, transfection of human A172 cells with PDGF-βR siRNA resulted in efficient knockdown of PDGF-βR as demonstrated by RT-PCR. Cells were transfected with either PDGF-βR siRNA or non-specific (Non) siRNA control followed by treatment with PDGF-BB for 24 h. PDGF-BB-mediated induction of MCP-1 mRNA was attenuated in the cells transfected with PDGF-βR siRNA but not in the Non siRNA controls cells (Fig. 16E). These results were further confirmed by examining PDGF-BB-mediated induction of

MCP-1 protein levels. As shown in Figure 16F, PDGF-BB enhanced release of MCP-1 protein in non siRNA transfected cells but not in cells transfected with PDGF-βR siRNA. Taken together, these findings confirm the involvement of PDGF-βR in PDGF-BB-mediated induction of MCP-1 in astrocytes.

PDGF-BB-mediated induction of MCP-1 involves MAPK and PI3K/Akt cell signaling pathways.

Having determined PDGF-BB-mediated induction of MCP-1, we next sought to elucidate the signaling pathways involved in this process. Since PDGF-BB is a mitogen and a pro-survival protein, we examined the involvement of mitogen- activated protein kinase (MAPK) and phosphoinositide-3-kinase/ Akt pathways. Human A172 cells were exposed to PDGF-BB and phosphorylation of MAPK and PI3K/Akt signaling molecules were assessed by western blotting. Treatment of astrocytes with PDGF-BB resulted in a time-dependent increase in phosphorylation of ERK1/2, JNK, p38 and Akt, with maximal activation at 30 min following treatment (Fig. 17A). To address the functional role of MAPK and PI3K/Akt in PDGF-mediated induction of MCP-1 expression, A172 cells were pretreated with inhibitors specific for the respective signaling pathways followed by PDGF-BB treatment and subsequently assessed for expression of MCP-1. As shown in Fig. 17B, treatment of cells with MEK (U0126, 1 μM), JNK (SP600125, 10 μM), P38 (SB20358, 1 μM) and PI3K (LY294002, 1 μM) inhibitors resulted in amelioration of PDGF-BB-mediated induction of MCP-1 protein levels.

Further validation of the involvement of MAPK pathway in this process was confirmed by transfecting cells with either the WT or DN constructs of MEK followed by

treatment with PDGF-BB. PDGF-BB-mediated induction of MCP-1 was attenuated by DN-MEK, but not by WT-MEK construct (Fig. 17C). To confirm the role of PI3K/Akt in PDGF-BB-mediated MCP-1, A172 cells were transduced with adenoviral constructs containing either WT or DN forms of Akt. As shown in Fig. 17D, cells transduced with DN Akt construct failed to up-regulate MCP-1 unlike the cells transduced with the WT Akt construct. Taken together, these findings confirm the involvement of both MAPK and PI3K/Akt cascades in PDGF-BB-mediated induction of MCP-1 in astrocytes.

Involvement of PDGF-βR in the regulation of MAPKs and PI3K/Akt cell signaling pathways.

Since PDGF-BB acts through its cognate receptor, the next logical step was to link the MAPK and PI3K/Akt pathways to PDGF-βR. To achieve this, activation of the MAPK and Akt pathways by PDGF-BB was validated using the PDGF-βR blocker – STI-571. A172 cells were pretreated with STI-571 (30 min) prior to PDGF-BB treatment for 1h and assessed for activation of phosphorylated forms of ERK, JNK, P38 and Akt using western blotting. As shown in Fig. 18A, STI-571 pretreatment attenuated activation of ERK, JNK, p38 and Akt induced by PDGF-BB. These results were also confirmed by knocking down the expression of PDGF-βR using the siRNA approach and assessing the ERK, JNK, p38, JNK and Akt phosphorylation. Briefly, A172 cells were transfected with PDGF-βR siRNA for 24 h followed by treatment with PDGF-BB for 1h. Cells transfected with PDGF-βR siRNA failed to demonstrate PDGF-BB-mediated activation of ERK, JNK, p38 and Akt (Fig.18B). Collectively, these results underpin the role of PDGF-βR in PDGF-BB- mediated activation of MAPK and P13K/Akt pathways.

Involvement of NF-κB in PDGF-BB-induced expression of MCP-1 in astrocytes

Having determined the role of MAPK and PI3K/Akt in PDGF-BB-mediated induction of MCP-1 expression, we rationalized the involvement of NF-κB in this process since this transcription factor is downstream of the aforementioned signaling mediators [132, 134, 141]. Exposure of astrocytes to PDGF-BB resulted in a time-dependent increase of p65 subunit of NF-κB in the nucleus with a concomitant increase in cytosolic pIκB (Fig.19A). Validation of the role of NF-κB was further determined by pretreating astrocytes with the IκB pathway inhibitor (SC514), followed by PDGF-BB treatment. As shown in Fig. 19B, SC514 inhibited PDGF-BB-mediated induction of MCP-1, thereby underscoring the role of NF-κB in this process.

To further confirm the role of NF-κB in PDGF-B-mediated induction of MCP-1, A172 cells were transduced with WT or DN adenoviral constructs of NFκB. As shown in Fig. 19C, transduction with DN form of NF-κB resulted in inhibition of PDGF-BB-mediated induction of MCP-1. Transduction with the WT-NF-κB construct, on the other hand, as expected did not inhibit PDGF-BB-mediated induction of MCP-1. Together, these findings underpin the role of NF-κB in PDGF-BB-mediated induction of MCP-1 in astrocytes.

To validate the role of NF-κB , we next sought to confirm the binding of NF-κB with MCP-1 promoter in its natural chromatin context by chromatin immunoprecipitation to reveal active sites accessible to NFκB. A172 cells were treated with PDGF-BB for 1h followed by RNA extraction and processed using a ChIP Analysis kit. These

experiments revealed increased binding of NF-κB to the MCP-1 promoter in A172 cells treated with PDGF-BB (Fig. 19D,E).

MCP-1 released from astrocytes increases monocyte transmigration across human brain microvascular endothelial cells.

Having determined the induction of MCP-1 by PDGF-BB, the next step was to explore the functional relevance of this up-regulation. Since MCP-1 is a known chemoattractant we hypothesized that PDGF-BB-induced MCP-1 released from the astrocytes could, in fact, act on neighboring endothelial cells altering its function. Endothelial cells of the BBB play a vital role in the development of HAND because it serves as the conduit by which infected immune cells migrate across the BBB into the brain in response to increased MCP-1 concentration in the brain thereby disrupting the BBB. Compromise of BBB in HAND has been substantiated by a number of laboratory animal models and human studies, demonstrating BBB breakdown as a consequence of progressive viral infection and immune compromise [142-144]. We thus set out to examine the effect of MCP-1 released from astrocytes on BBB function. However, before proceeding with this, it was important to first examine whether HBMECs (paracrine action) as well as astrocytes (autocrine action) indeed expressed the MCP-1 receptor, CCR2. As shown in Fig. 20A, both astrocytes as well as HBMECs expressed CCR2 mRNA as demonstrated by RT-PCR.

To determine the functional relevance of increased MCP-1 induced by PDGF-BB in this study, A172 cells were treated with or without PDGF-BB for 2 h then replaced with fresh media and incubated for 24h. Endothelial cells were grown on the upper

compartment of transwell plates and spent media was added to the lower compartment overnight. Labeled human monocytes were added to the upper compartment for 24 h and monocyte transmigration was assessed. As shown in Fig. 20B, conditioned-media collected from PDGF-BB treated astrocytes resulted in a significant increase in permeability. PDGF-βR blocker STI-571 was able to block the increased permeability mediated by conditioned media from PDGF-BB treated astrocytes.

To investigate whether this increased in monocyte transmigration was indeed mediated by MCP-1, conditioned media was treated with MCP-1 neutralizing antibody 1 h prior to treatment with the cells. As shown in Fig. 20B, conditioned media from A172 cells treated with PDGF-BB resulted in a dramatic increase in permeability of endothelial cells which was ameliorated by MCP-1 neutralizing antibody. These findings thus confirm that MCP-1 was involved in PDGF-BB mediated disruption of the endothelial barrier permeability. These results not only underpin the role of MCP-1 in BBB breakdown, but reveal a vital role that Tat-mediated PDGF-BB may play in BBB disruption.

Discussion

Anti-retroviral therapies have proven 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 BBB 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 of 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 barrage of inflammatory mediators as well as factors such as PDGF-BB. PDGF-BB has been implicated in a variety of pathological conditions, however, its role in HIV pathogenesis remains poorly defined.

In the present study we demonstrated that PDGF-BB treatment of human astrocyte cell line and primary cultures resulted in induction of MCP-1 and this process was mediated via the binding of PDGF-BB to its cognate PDGF-βR. These findings are consistent with the reports on PDGF-mediated induction of MCP-1 in other cell types such as the fibroblast cell line and smooth muscle cells [115, 116]. In our efforts to dissect the upstream signaling events mediating MCP-1 release in astrocytes, we demonstrate the role of MAPK and PI3K/Akt in meditating MCP-1 release from astrocytes using both the pharmacological as well as genetic approaches. The involvement of MAPK and PI3K/Akt pathways in the induction of MCP-1 expression are in agreement with the role these pathways play in induction of this chemokine in other cell types including osteoblasts, mesangial cells and endothelial cells [145-147]. The

transcription factor, NF-κB is known to play a key role in PDGF-BB signaling and also in the expression of proinflammatory cytokines/chemokines including MCP-1 [132-134]. Consistent with other reports, our studies also revealed that PDGF-BB-mediated induction of MCP-1 involved NF-κB activation and binding to the MCP-1 promoter.

It was next of interest to explore the functional relevance of PDGF-BB mediated induction of MCP-1. Based on the proximity of astrocytes to the endothelial barrier, we rationalized that induced expression of MCP-1 in PDGF-BB treated astrocytes could play a role in barrier integrity. Intriguingly, conditioned media from PDGF-BB treated astrocytes did indeed increase monocyte transmigration and this effect was attributable to MCP-1 as demonstrated in the blocking antibody experiments. This role of MCP-1 is in agreement with the findings reported by Eugenin et al who demonstrated that HIV infected leukocytes transmigration across a tissue culture model of human BBB was due to MCP-1 [117]. In addition to disrupting the barrier permeability, MCP-1 is a known biomarker of HIV neuropathogenesis [117, 118], owing to its role as a chemoattractant. The function of MCP-1 demostrated in this study can have ramification in the pathogenesis of HAND. Based on the proximity of astrocytes to the endothelium and their ability to secrete both PDGF-BB and the chemokine MCP-1 and their abundance in the CNS, it can be argued that during HIV-1 infection, viral proteins can initiate a toxic cascade that can be self-perpetuating. HIV-1 Tat can trigger increased expression of PDGF-BB, which in turn, can lead to increased MCP-1 expression that can manifest as an amplified influx of monocytes into the CNS. PDGF-BB has already been shown to disrupt the BBB [136] and a similar function has been demonstrated for MCP-1 [117]. Therefore the release of both these mediators can independently disrupt the endothelial

barrier while enhancing neuroinflammation [117, 136] which can have serious ramifications in HAND.

In summary, our studies have mapped out a detailed molecular pathway of PDGF-BB-mediated MCP-1 expression in astrocytes involving ERK1/2, JNK MAPK activation, with the subsequent activation of NF-κB resulting in increased MCP-1 expression, ultimately leading to monocytes transmigration and increased permeability in the brains of individuals infected with HIV illustrated schematically in Figure 21.

Figures

Figure 15

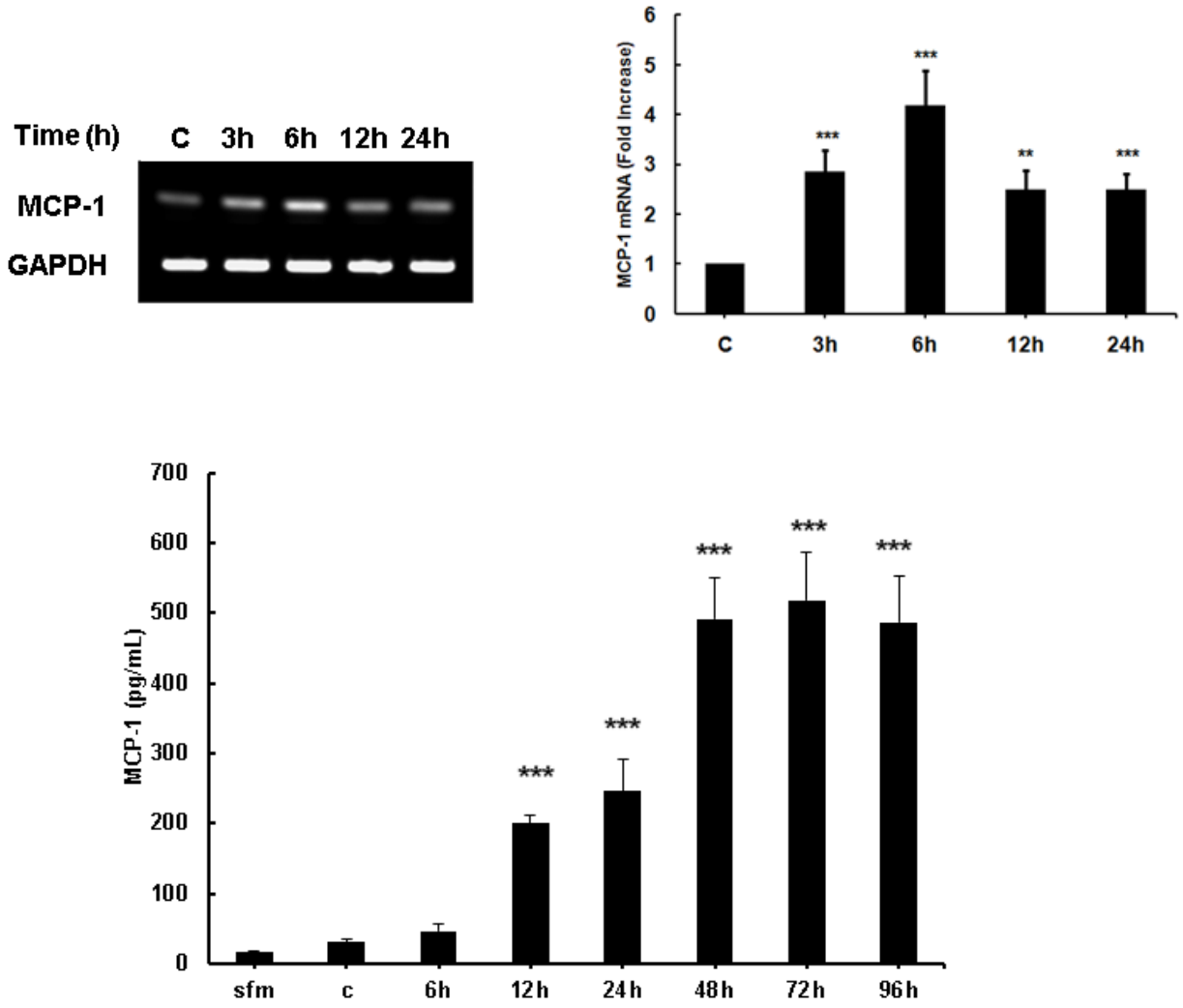
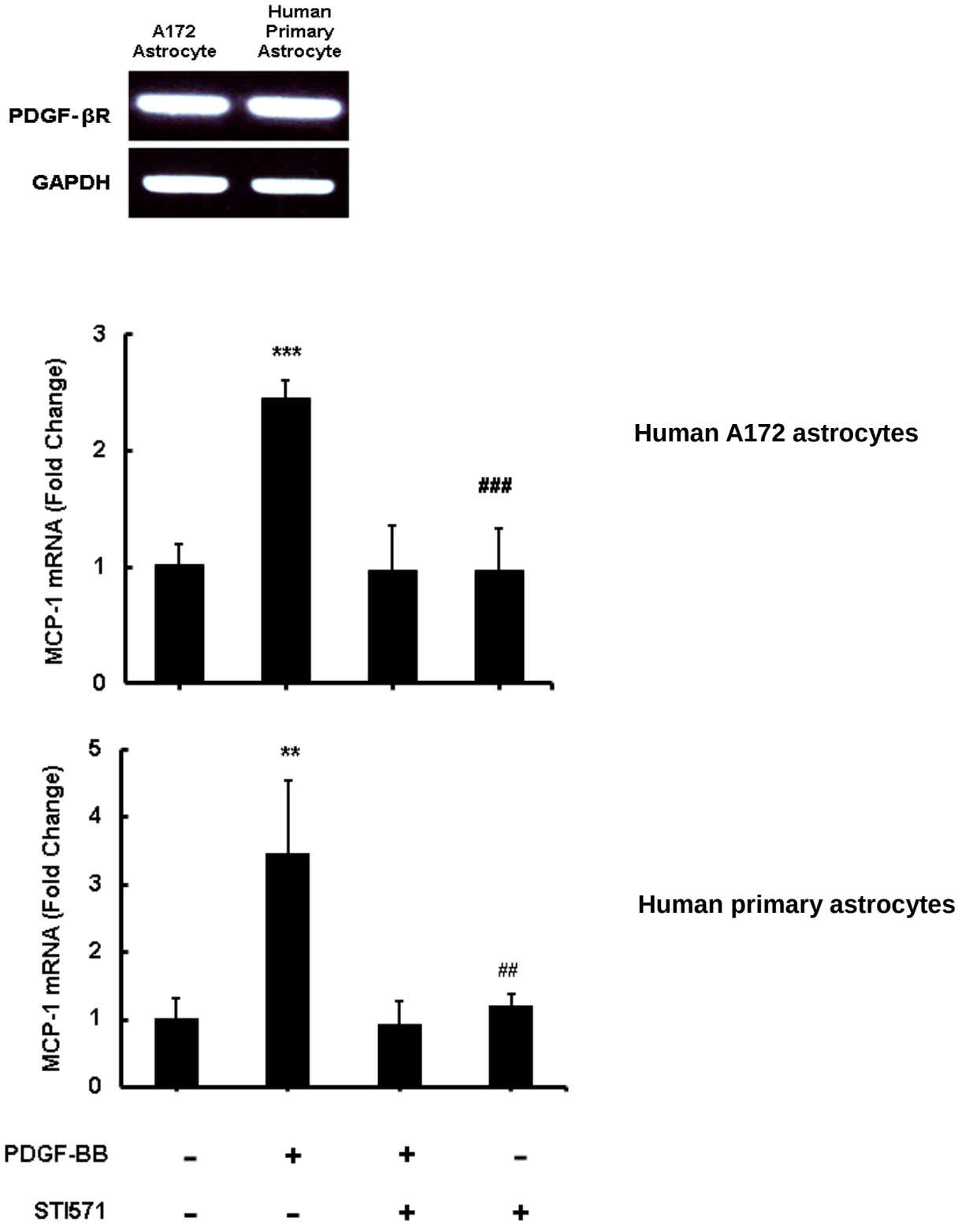
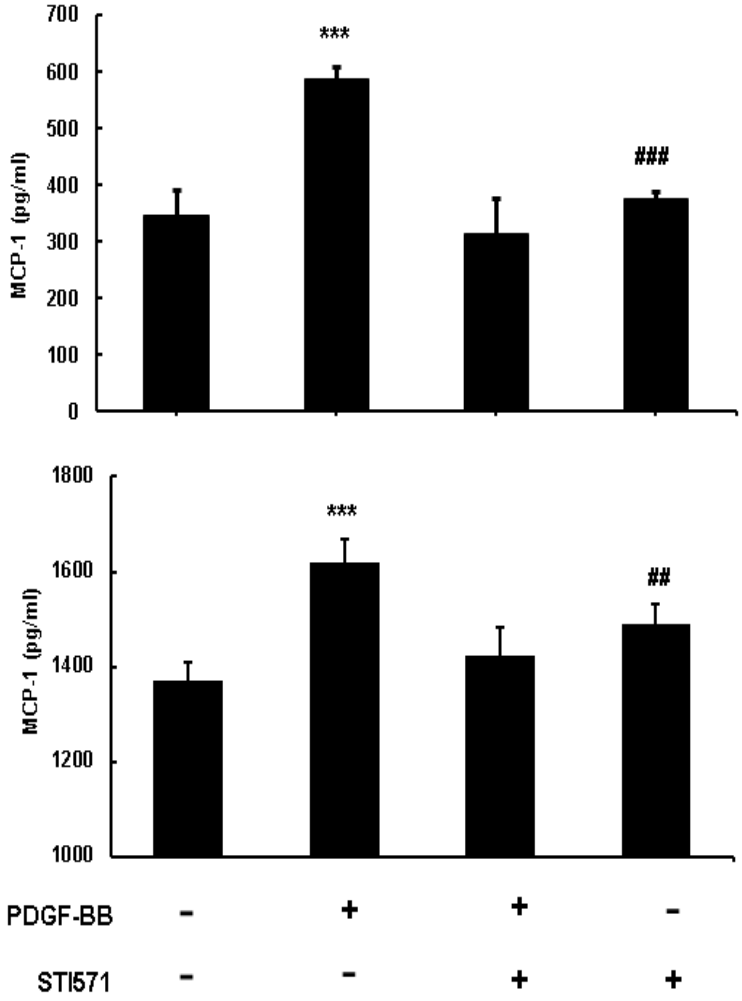


Figure 15. PDGF-BB induces MCP-1 mRNA and protein expression in human astrocytes. A, Time-dependence of PDGF-BB-mediated induction of MCP-1 mRNA expression in human A172 astrocytes. Total RNA isolated from human A172 astrocytes was subjected to Reverse Transcription- PCR analysis using primer s for human MCP-1 and GAPDH. PDGF-BB mediated induction of MCP-1 mRNA expression peaked at 6h

and decline thereafter. B, Time-dependence of PDGF-BB-mediated induction of MCP-1 protein expression in human A172 astrocytes. Supernatant fluid from human A172 cells treated with PDGF-BB for various time points were assessed for release of chemokine MCP-1 using the human MCP-1ELISA array. PDGF-BB treatment resulted in induction of MCP-1 expression. All the data are presented as means ± of 3 individual experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group.

Figure 16


A172 Astrocyte
Human Primary Astrocyte
PDGF-βR
GAPDH
MCP-1 mRNA (Fold Change)
***
###
Human A172 astrocytes
MCP-1 mRNA (Fold Change)
**
##
Human primary astrocytes
PDGF-BB
STI571



Human A172 astrocytes

Human primary astrocytes

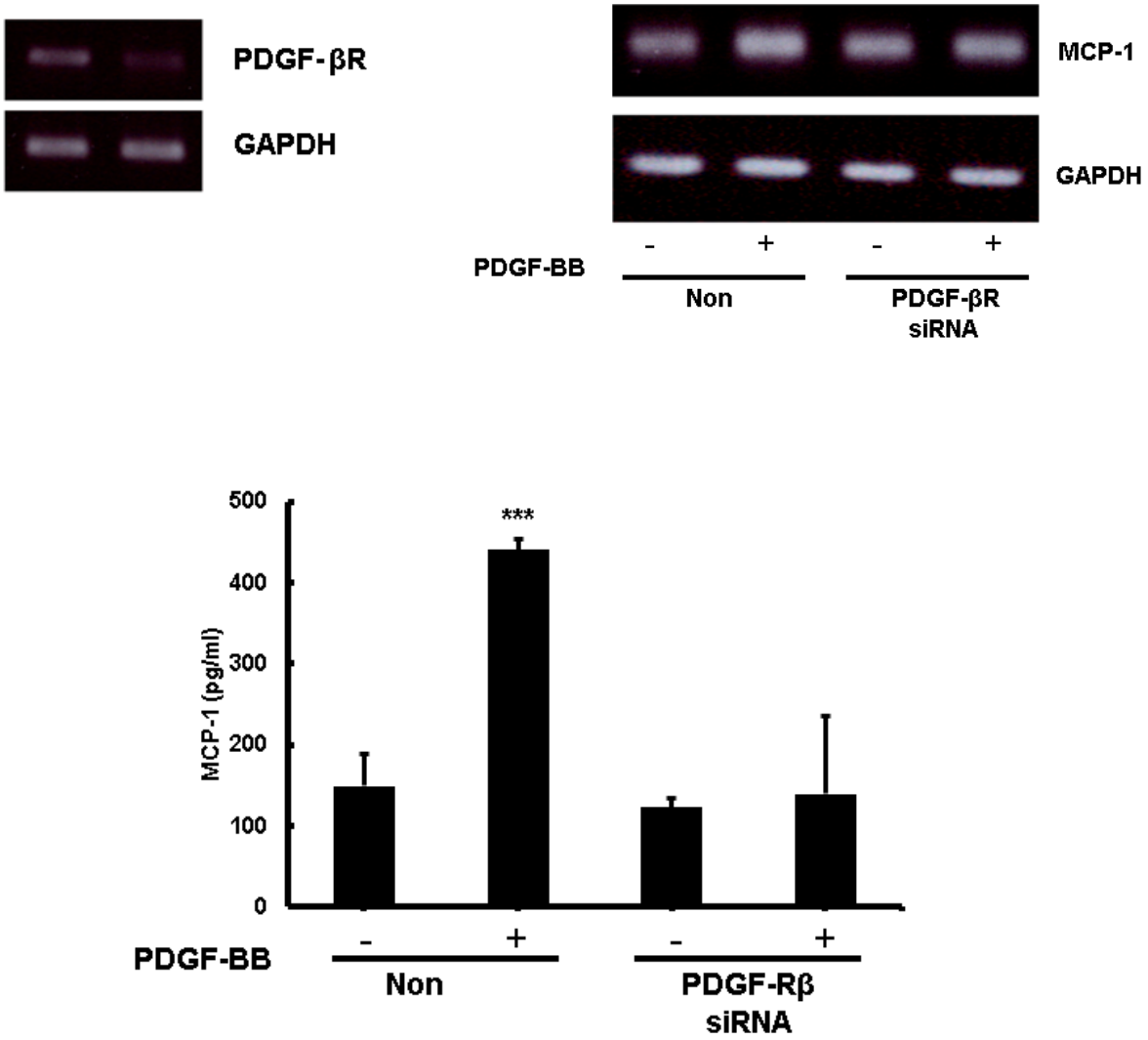
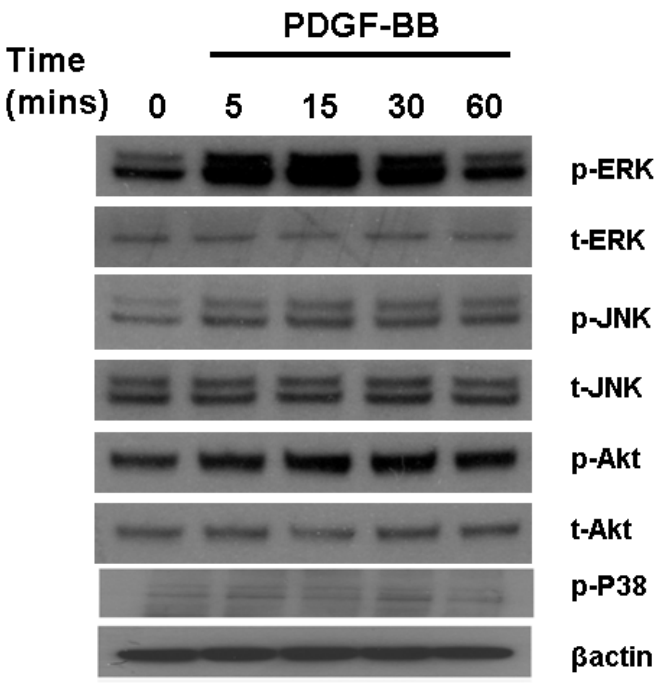
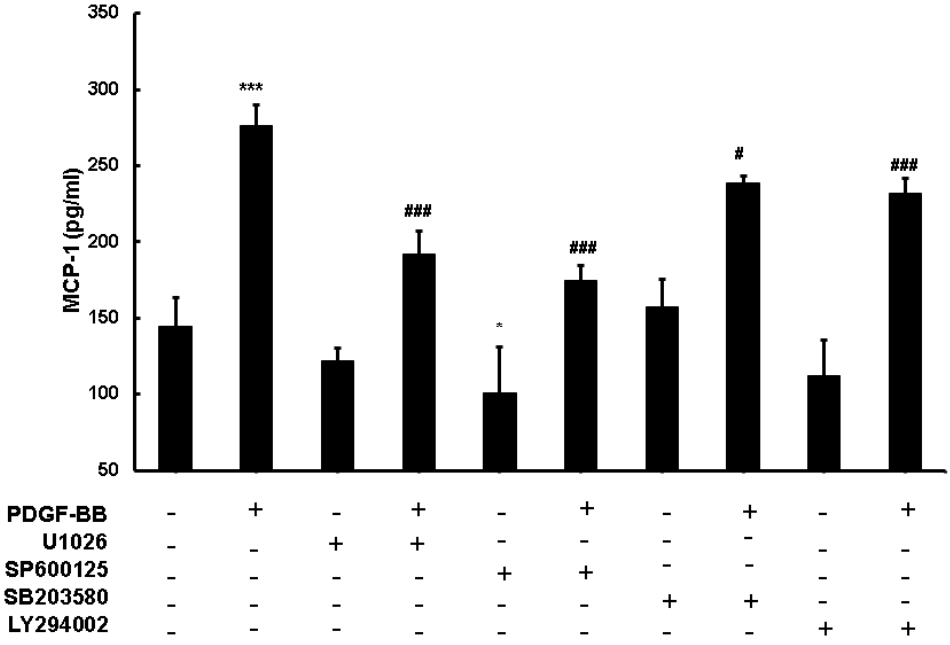


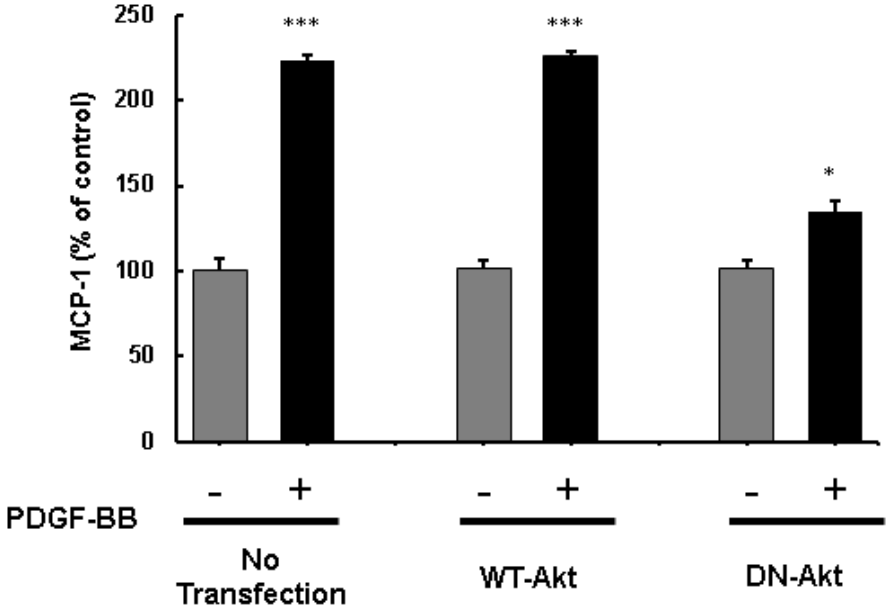
Figure 16. Engagement of PDGF-Rβ is critical for PDGF-BB induced MCP-1 expression in astrocytes. A, Human A172 primary astrocytes express PDGF-Rβ. RNA isolated from human A172 astrocytes and human primary astrocytes were subjected to RT-PCR analysis using PDGF-Rβ and GAPDH primers. B, Pretreatment of human A172 and primary astrocytes with PDGF-Rβ antagonist STI-571 abrogated PDGF-BB-mediated induction of MCP-1 mRNA and protein (C) expression. D. Total RNA from A172 cells transfected with either nonsense (Non) or PDGF-Rβ siRNAs were subjected

to RT-PCR analysis using primers for PDGF-Rβ. E, PDGF-Rβ siRNA, but not Non siRNA inhibited PDGF-BB-mediated induction of MCP-1 RNA. F, Supernatants from A172 cells transfected with either nonsense (Non) or PDGF-Rβ siRNAs were subjected to ELISA assay specific for MCP-1. PDGF-Rβ siRNA, but not Non siRNA inhibited PDGF-BB-mediated induction of MCP-1 protein. **p ≤ 0.01, ***p ≤ 0.001 versus control group, #p ≤ 0.05, ###p ≤ 0.001 versus PDGF-BB-treated group.

Figure 17


PDGF-BB
Time
(mins)
0
5
15
30
60
p-ERK
t-ERK
p-JNK
t-JNK
p-Akt
t-Akt
p-P38
βactin


MCP-1 (pg/ml)
350
300
250
200
150
100
50
***
###
*
###
#
###
PDGF-BB - + - + - + - + - +
U1026 - - + + - - - - - -
SP600125 - - - - + + - - - -
SB203580 - - - - - - + + - -
LY294002 - - - - - - - - + +


250
200
150
100
50
0
MCP-1 (% of control)
***
***
*
- + - + - +
PDGF-BB
No Transfection
WT-Akt
DN-Akt

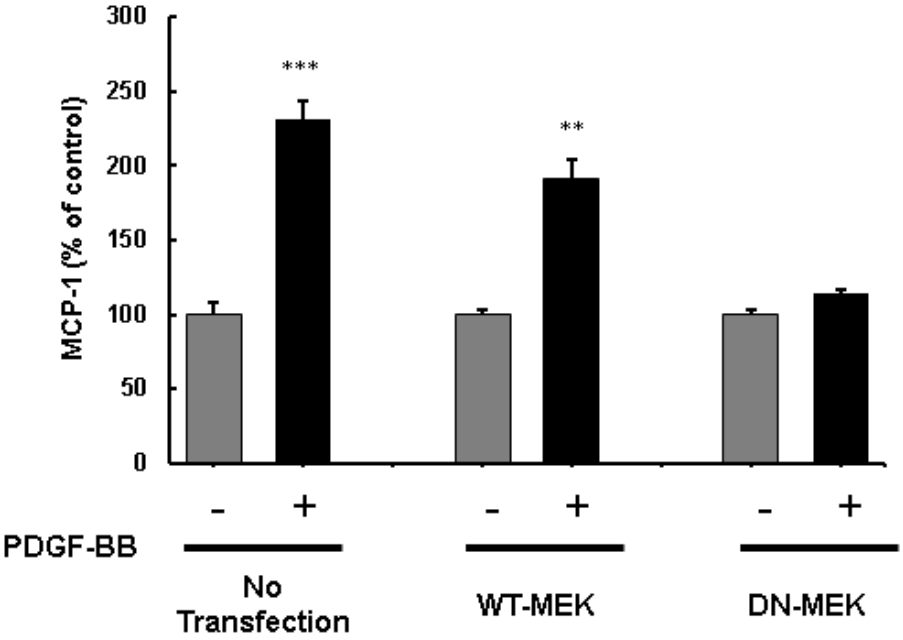

300
250
200
150
100
50
0
MCP-1 (% of control)
***
**
- + - + - +
PDGF-BB
No Transfection
WT-MEK
DN-MEK

Figure 17. PDGF-BB-mediated induction of MCP-1 expression involves MAPKs and PI3K/Akt cell signaling pathways. A, WB analysis of time-dependent activation of ERK, JNK, P38 and Akt by PDGF-BB. B, Inhibition of the ERK, JNK, p38 and Akt pathways by MEK1/2 (U0126), JNK (SP600125), p38 (SB20358) and PI3K (LY294002) inhibitors resulted in amelioration of PDGF-BB-mediated induction of MCP-1 expression in astrocytes. C, Transfection with DN-MEK and not WT-MEK resulted in abrogation of PDGF-BB-mediated induction of MCP-1. D, Transduction with DN-Akt and not WT-Akt also resulted in abrogation of PDGF-BB-mediated induction of MCP-1. All the data are presented as means ± of 3 individual experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus control group, ## $p \leq 0.01$, ### $p \leq 0.001$ versus PDGF-BB-treated group.

Figure 18

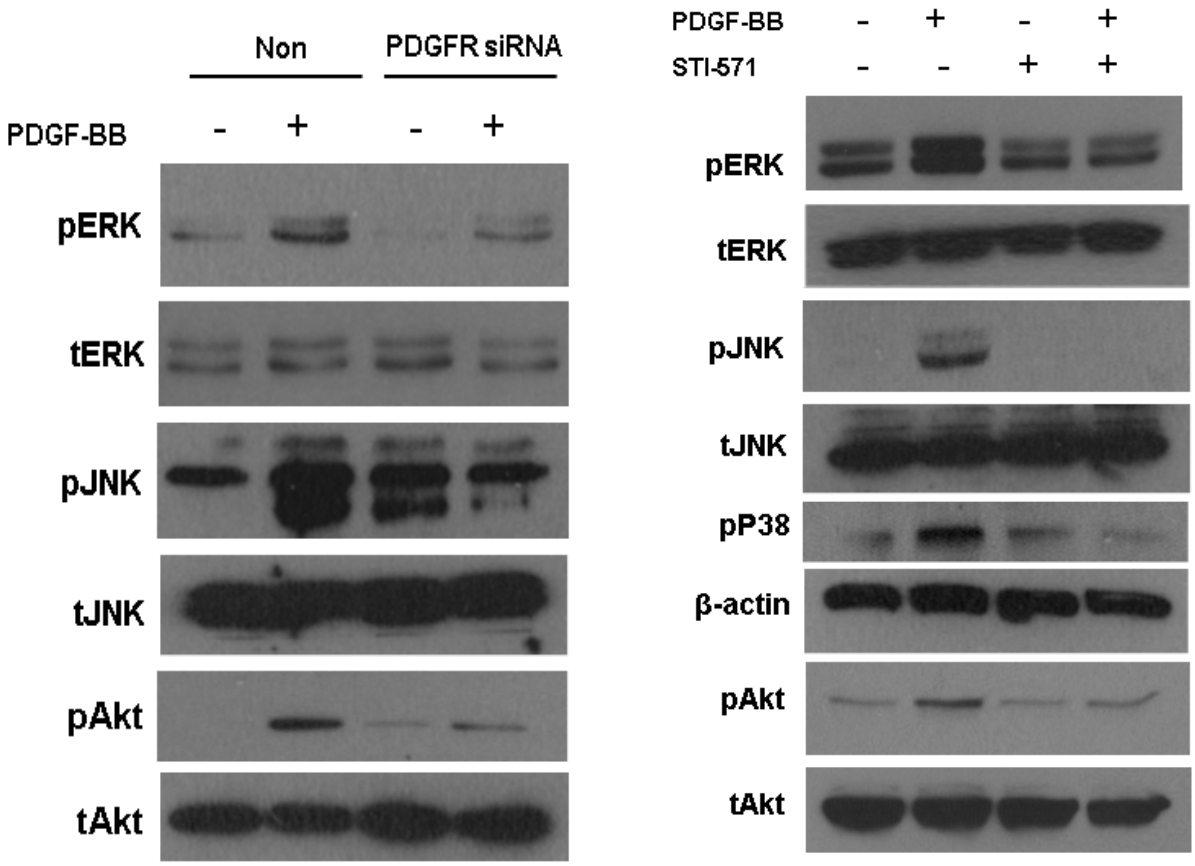
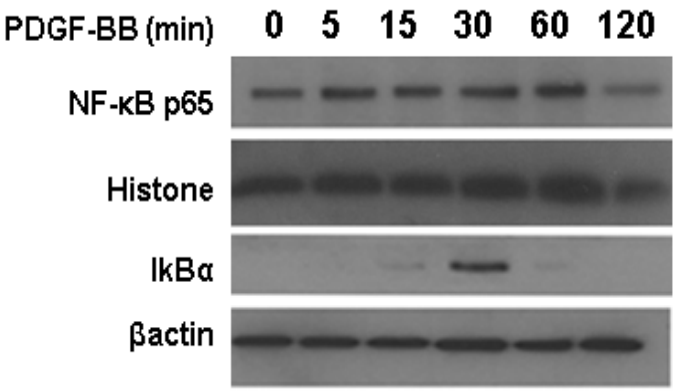
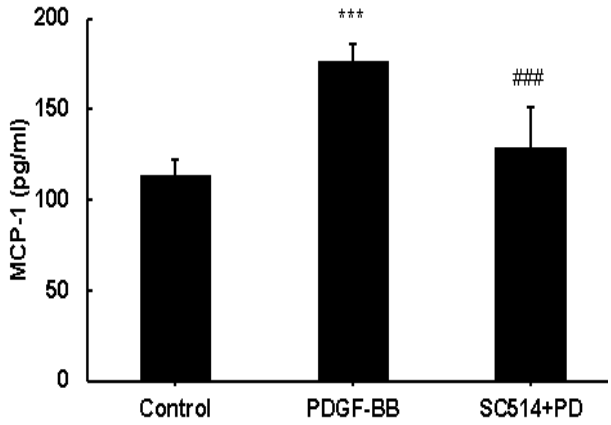
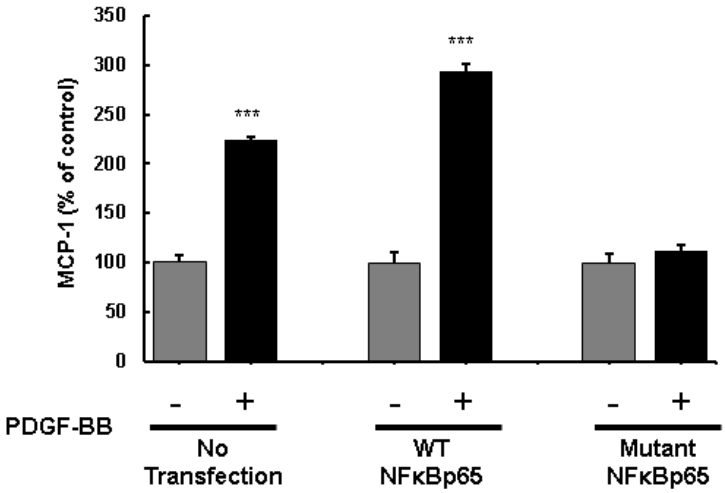


Figure 18. Involvement of PDGF-βR in the regulation of MAPKs and PI3K/Akt cell signaling pathways. A, Pretreatment of A172 cells with PDGF-βR antagonist STI-571 resulted in inhibition of PDGF-BB-mediated phosphorylation of ERK, JNK, p38 and Akt pathways. Representative immunoblots of pERK/tERK, pJNK/JNK,p38/β-actin and pAkt/tAkt. B, Whole cell lysates from A172 cells transfected with either nonsense (Non) or PDGF-βR siRNAs were subjected to immunoblot analysis using antibodies MAPKs and PI3K/Akt cell signaling pathways. PDGF-βR siRNA, but not Non siRNA inhibited PDGF-BB-mediated phosphorylation of ERK, JNK, p38 and Akt pathways.

Figure 19


PDGF-BB (min)
0
5
15
30
60
120
NF-κB p65
Histone
IkBα
βactin


MCP-1 (pg/ml)
200
150
100
50
0
***
###
Control
PDGF-BB
SC514+PD


MCP-1 (% of control)
350
300
250
200
150
100
50
0
***
***
PDGF-BB
-
+
-
+
-
+
No Transfection
WT NFκBp65
Mutant NFκBp65

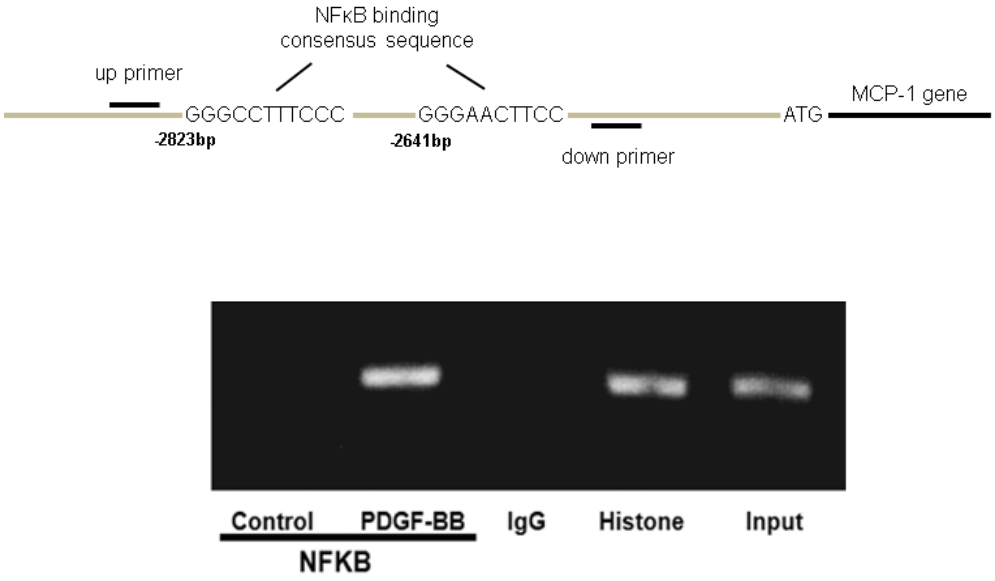


Figure 19. PDGF-BB mediated induction of MCP-1 expression involves NF-κB activation. A, Exposure of A172 cells to PDGF-BB resulted in a time-dependent increase in phosphorylation of the p65 subunit of NF-κB in the nuclear cellular fraction. Reciprocally, PDGF-BB exposure resulted in a time-dependent increase in IκBα phosphorylation in the cytosolic cellular fraction of A172 astrocytes. B, Pretreatment with the IκBα inhibitor, SC514 resulted in inhibition of PDGF-BB-mediated induction of MCP-1. C, Transduction with Mutant-NF-κB and not WT-NF-κB resulted in abrogation of PDGF-BB-mediated induction of MCP-1. All the data are presented as means ± of 3 individual experiments., ***$p < 0.001$ versus control group, ###$p < 0.001$ versus PDGF-BB-treated group. D, Schematic illustration of NF-κB binding consensus sequence on the promoter region of MCP-1. F, ChIP assay demonstrating PDGF-BB-mediated binding of p65NF-κB to the MCP-1 promoter.

F**igure 20**

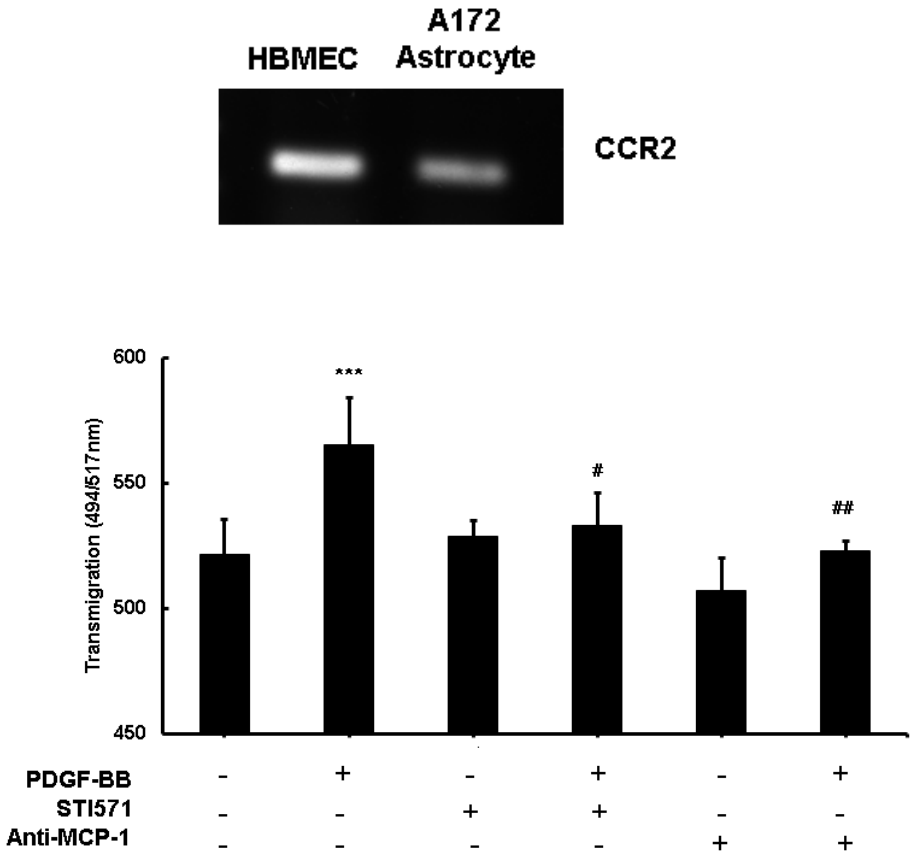


Figure 20. PDGF-BB-induced monocyte transmigration is mediated by MCP-1. A, RNA isolated from untreated HBMECs and human A172 astrocytes were subjected to RT-PCR analysis using CCR2 primers. B, HBMECs were exposed for 24h to supernatants from A172 cells treated with PDGF-BB with or without STI-571 for 2h then media was replaced for 24h. MCP-1 neutralizing antibody was added to PDGF-BB-treated media prior to HBMEC exposure for 24h. Human monocytes were added to the top transwell for 24 h and transmigration assessed. Exposure of HBMECs to supernatants from A172 cells treated with PDGF-BB, resulted in increased permeability which was abrogated by MCP-1 neutralizing antibody.

Figure 21

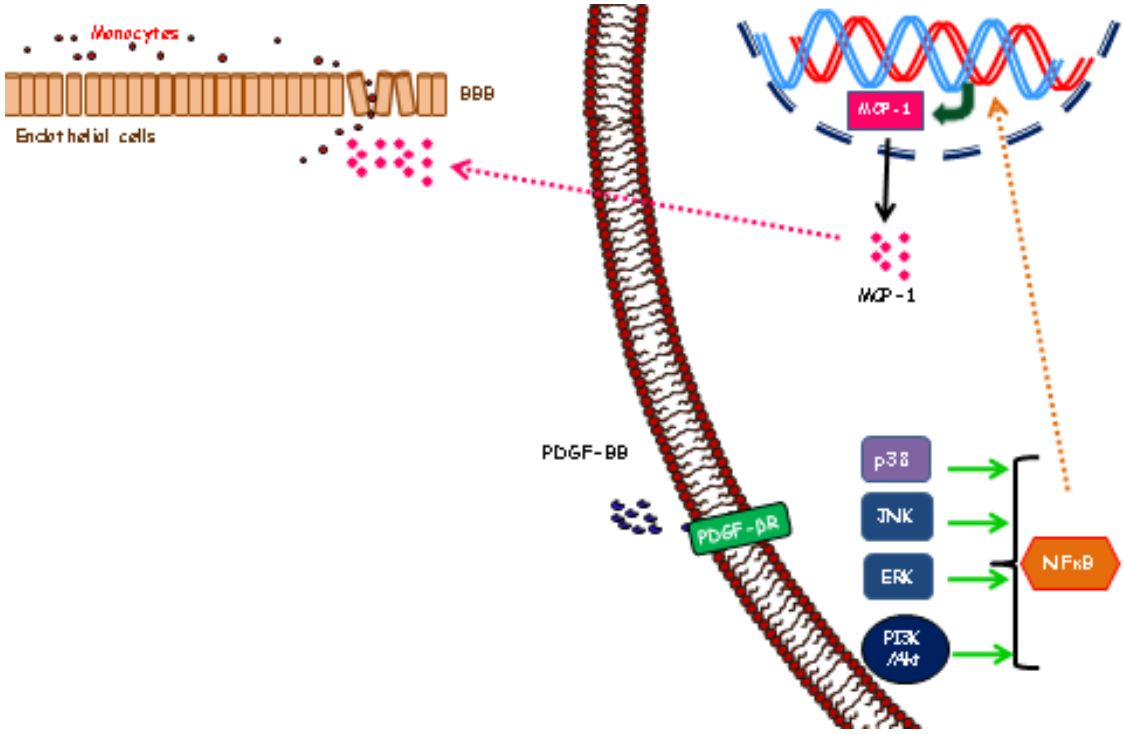


Figure 21. Schematic of the signaling pathways involved in the increased induction of MCP-1 release in astrocytes stimulated with PDGF-BB. PDGF-BB-mediated activation of ERK1/2, JNK p38 MAPKs, and PI3K/Akt signaling pathways. ERK1/2, JNK p38 MAPKs, and PI3K/Akt signaling resulted in the subsequent activation of the downstream transcription factor, NFκB. Activation of NF-κB in turn leads to enhanced MCP-1 release and increased endothelial cell permeability as shown in Figure 21.

Chapter 6: Conclusions

CNS disease remains a serious complication in individuals infected with HIV-1. The early viral protein, HIV-1 Tat has been shown to be a critical determinant for both viral replication and survival. However, in the infected host release of Tat from the infected cells can have serious consequences, as it exerts potent toxicity on various cell types in the brain. In the CNS it can activate monocytes, astrocytes and microglia, which, in turn, leads to a “cytokine/chemokine storm” in the CNS. HIV-1 Tat not only exerts direct toxicity on the neurons, but can also indirectly lead to neuronal apoptosis, via the mediators released from other neighboring cells. These complex cascades of events could be self-propelling, thereby perpetuating a continuum of inflammatory responses in the brain of HIV-1 infected individuals. These are important issues even in the current era of anti-retrovirals, since most of the therapeutic drugs do not cross the BBB. HIV Tat can also disrupt the BBB integrity, allowing for increased numbers of inflammatory cells into the CNS. Furthermore, Tat can also co-operate with various drugs of abuse to potentiate toxicity thereby amplifying untoward inflammatory responses in the CNS. HIV-1 Tat thus acts at multiple steps within the CNS to exacerbate disease pathogenesis and understanding its contributions at various stages of the disease process is crucial for developing strategies that could interfere with disease induction and/or progression. Our findings have demonstrated that in addition to the numerous ways in which HIV-1 Tat causes the release of toxic mediators, it can also utilizes PDGF-BB a well-meant protective response of the astrocyte to drive cellular activation via PDGF-BB-mediated increased expression of MCP-1 as illustrated in figure 21. MCP-1 has been shown to be both neuroprotective, however, due to its strong

chemoattractant property, is detrimental to the CNS as it plays a key role in recruiting infected monocytes into the brain. Even in circumstances where peripheral viral loads are minimal, the enhanced recruitment of monocytes into the brain poses a problem as it disrupts the BBB allowing easier access of toxic products into the CNS.

Figure 22

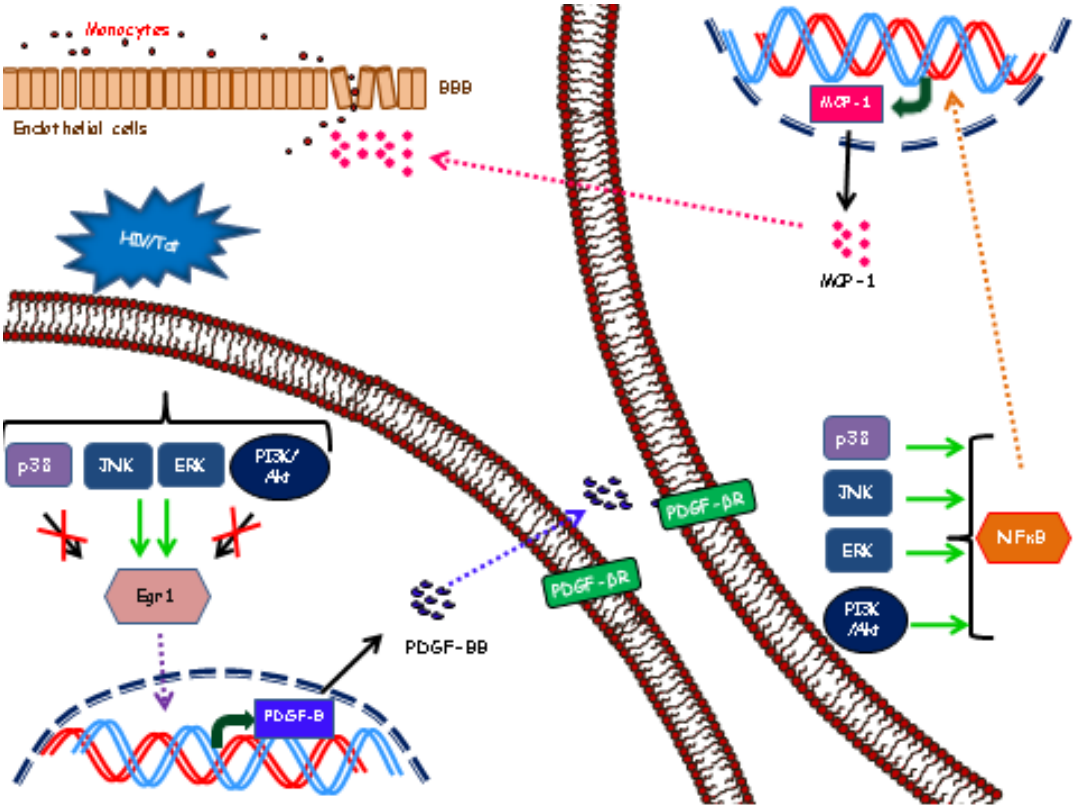


Figure 22. Schematic of the Tat-mediated effects astrocyte-endothelial interations via PDGF-BB and MCP-1. Tat-mediated activation of ERK1/2 and JNK MAPKs but not and PI3K/Akt and p38 signaling resulted in the activation of the downstream transcription factor, Egr-1. Activation of Egr-1 in turn leads to enhanced PDGF-BB expression in astrocytes. PDGF-BB released from astrocytes can act in an autocrine manner to further activate astrocytes. PDGF-BB-mediated activation of ERK1/2, JNK, p38 MAPKs, and PI3K/Akt signaling resulted in the subsequent activation of the downstream transcription factor, NFκB. Activation of NF-κB in turn leads to enhanced MCP-1 release and disruption of endothelial cells as shown in Figure 22.

Chapter 7: Future Directions

Due to the mounting evidence of glial activation on HIV neuropathogensis, it is critical to understand the cellular events culminating in neuronal dysfunction/toxicity. GIial activation remains a critical player in the production of toxic mediators released leading not only to neuronal dysfunction and enhanced glial activation. The increased activation subsequently releases more proinflammatory cytokines/chemokines and other toxic mediators providing an amplification loop of exaggerated and maintained glial activation. Our findings have just scratched the surface of how glial activation can actually aid viral CNS infection and have revealed many other potential questions that should be addressed in order to better understand the role of glial activation in HIV neuropathogesis. Albeit, a critical factor in HIV pathogenesis, MCP-1 is one of a number of cytokines that can be released from activated astrocytes. Other cytokines including CXCL10, IL-6 and IL-1β have also been shown to be enhanced with glial activation [33, 47, 48, 148-151]. Future directions may address the role of these pro-inflammatory cytokines on the CNS.

Because of the close proximity of astrocytes to the endothelial cells of the BBB, it is critical to shed some light on the detrimental effects that astrocytic modulation plays on BBB integrity and the recruitment of monocytes into the brain. How astrocytes affect the normal functioning of cells that make up the neurovascular unit, the migration endothelial cells and the expression of tight junction proteins are paramount to designing methods of curtailing the invasion of monocytes into the brain observe in HIV CNS infection.

Chapter 8: References

1. UNAIDs, *UNAIDS annual report 2009:uniting the world against AIDS.* 2010.
2. Glass, J.D., et al., *Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia.* Ann Neurol, 1995. 38(5): p. 755-62.
3. Ozdener, H., *Molecular mechanisms of HIV-1 associated neurodegeneration.* J Biosci, 2005. 30(3): p. 391-405.
4. Gilgun-Sherki, Y., E. Melamed, and D. Offen, *Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier.* Neuropharmacology, 2001. 40(8): p. 959-75.
5. Johnson, R.T., *Virus in the nervous system.* Ann Neurol, 1988. 23 Suppl: p. S210.
6. Kaul, M. and S.A. Lipton, *Mechanisms of neuronal injury and death in HIV-1 associated dementia.* Curr HIV Res, 2006. 4(3): p. 307-18.
7. Peluso, R., et al., *A Trojan Horse mechanism for the spread of visna virus in monocytes.* Virology, 1985. 147(1): p. 231-6.
8. Gonzalez, D., et al., *Changes of parvalbumin immunoreactive neurons and GFAP immunoreactive astrocytes in the rat lateral geniculate nucleus following monocular enucleation.* Neurosci Lett, 2006. 395(2): p. 149-54.
9. Navia, B.A., et al., *The AIDS dementia complex: II. Neuropathology.* Ann Neurol, 1986. 19(6): p. 525-35.
10. Salemi, M., et al., *Dating the common ancestor of SIVcpz and HIV-1 group M and the origin of HIV-1 subtypes using a new method to uncover clock-like molecular evolution.* FASEB J, 2001. 15(2): p. 276-8.
11. Korber, B., et al., *Timing the ancestor of the HIV-1 pandemic strains.* Science, 2000. 288(5472): p. 1789-96.
12. Wilkin, T.J., et al., *Advances in antiretroviral therapy.* Top HIV Med, 2010. 18(2): p. 66-92.
13. Spaulding, A., G.W. Rutherford, and N. Siegfried, *Tenofovir or zidovudine in three-drug combination therapy with one nucleoside reverse transcriptase inhibitor and one non-nucleoside reverse transcriptase inhibitor for initial treatment of HIV infection in antiretroviral-naive individuals.* Cochrane Database Syst Rev, 2010(10): p. CD008740.
14. Giunta, B., et al., *EGCG mitigates neurotoxicity mediated by HIV-1 proteins gp120 and Tat in the presence of IFN-gamma: role of JAK/STAT1 signaling and implications for HIV-associated dementia.* Brain Res, 2006. 1123(1): p. 216-25.
15. Churchill, M.J., et al., *Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia.* Ann Neurol, 2009. 66(2): p. 253-8.
16. Trillo-Pazos, G., et al., *Detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection.* Brain Pathol, 2003. 13(2): p. 144-54.
17. Liu, Y., et al., *CD4-independent infection of astrocytes by human immunodeficiency virus type 1: requirement for the human mannose receptor.* J Virol, 2004. 78(8): p. 4120-33.
18. Carroll-Anzinger, D. and L. Al-Harthi, *Gamma interferon primes productive human immunodeficiency virus infection in astrocytes.* J Virol, 2006. 80(1): p. 541-4.
19. van Marle, G., et al., *Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10.* Virology, 2004. 329(2): p. 302-18.
20. Kutsch, O., et al., *Induction of the chemokines interleukin-8 and IP-10 by human immunodeficiency virus type 1 tat in astrocytes.* J Virol, 2000. 74(19): p. 9214-21.

21. Gonzalez-Scarano, F. and J. Martin-Garcia, *The neuropathogenesis of AIDS.* Nat Rev Immunol, 2005. 5(1): p. 69-81.
22. Fawcett, J.W. and R.A. Asher, *The glial scar and central nervous system repair.* Brain Res Bull, 1999. 49(6): p. 377-91.
23. Sofroniew, M.V. and H.V. Vinters, *Astrocytes: biology and pathology.* Acta Neuropathol, 2010. 119(1): p. 7-35.
24. Tyor, W.R., et al., *Cytokine expression in the brain during the acquired immunodeficiency syndrome.* Ann Neurol, 1992. 31(4): p. 349-60.
25. Sodroski, J., et al., *Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat.* Science, 1985. 227(4683): p. 171-3.
26. Sodroski, J.G., et al., *trans-Activation of the human T-cell leukemia virus long terminal repeat correlates with expression of the x-lor protein.* J Virol, 1985. 55(3): p. 831-5.
27. Barboric, M., et al., *Binding of Tat to TAR and recruitment of positive transcription elongation factor b occur independently in bovine immunodeficiency virus.* J Virol, 2000. 74(13): p. 6039-44.
28. Huigen, M.C., W. Kamp, and H.S. Nottet, *Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection.* Eur J Clin Invest, 2004. 34(1): p. 57-66.
29. Campbell, G.R. and E.P. Loret, *What does the structure-function relationship of the HIV-1 Tat protein teach us about developing an AIDS vaccine?* Retrovirology, 2009. 6: p. 50.
30. Turchan-Cholewo, J., et al., *NADPH oxidase drives cytokine and neurotoxin release from microglia and macrophages in response to HIV-Tat.* Antioxid Redox Signal, 2009. 11(2): p. 193-204.
31. Nath, A., et al., *Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon.* J Biol Chem, 1999. 274(24): p. 17098-102.
32. El-Hage, N., et al., *Morphine exacerbates HIV-1 Tat-induced cytokine production in astrocytes through convergent effects on [Ca(2+)](i), NF-kappaB trafficking and transcription.* PLoS One, 2008. 3(12): p. e4093.
33. Williams, R., et al., *HIV-1 Tat co-operates with IFN-gamma and TNF-alpha to increase CXCL10 in human astrocytes.* PLoS One, 2009. 4(5): p. e5709.
34. Perry, S.W., et al., *Human immunodeficiency virus-1 Tat activates calpain proteases via the ryanodine receptor to enhance surface dopamine transporter levels and increase transporter-specific uptake and Vmax.* J Neurosci, 2010. 30(42): p. 14153-64.
35. Peruzzi, F., *The multiple functions of HIV-1 Tat: proliferation versus apoptosis.* Front Biosci, 2006. 11: p. 708-17.
36. Yao, H., et al., *Involvement of TRPC channels in CCL2-mediated neuroprotection against tat toxicity.* J Neurosci, 2009. 29(6): p. 1657-69.
37. Zou, W., et al., *Protection against human immunodeficiency virus type 1 Tat neurotoxicity by Ginkgo biloba extract EGb 761 involving glial fibrillary acidic protein.* Am J Pathol, 2007. 171(6): p. 1923-35.
38. Sui, Z., et al., *Inhibition of mixed lineage kinase 3 prevents HIV-1 Tat-mediated neurotoxicity and monocyte activation.* J Immunol, 2006. 177(1): p. 702-11.
39. Eugenin, E.A., et al., *MCP-1 (CCL2) protects human neurons and astrocytes from NMDA or HIV-tat-induced apoptosis.* J Neurochem, 2003. 85(5): p. 1299-311.
40. Jones, M., et al., *Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement.* J Neuropathol Exp Neurol, 1998. 57(6): p. 563-70.

41. Chen, P., et al., *The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases.* J Biol Chem, 1997. 272(36): p. 22385-8.
42. Pulliam, L., et al., *Intranasal tat alters gene expression in the mouse brain.* J Neuroimmune Pharmacol, 2007. 2(1): p. 87-92.
43. D'Aversa, T.G., K.O. Yu, and J.W. Berman, *Expression of chemokines by human fetal microglia after treatment with the human immunodeficiency virus type 1 protein Tat.* J Neurovirol, 2004. 10(2): p. 86-97.
44. Weiss, J.M., et al., *HIV-1 Tat induces monocyte chemoattractant protein-1-mediated monocyte transmigration across a model of the human blood-brain barrier and up-regulates CCR5 expression on human monocytes.* J Immunol, 1999. 163(5): p. 2953-9.
45. Eugenin, E.A., et al., *HIV-1 tat protein induces a migratory phenotype in human fetal microglia by a CCL2 (MCP-1)-dependent mechanism: possible role in NeuroAIDS.* Glia, 2005. 49(4): p. 501-10.
46. McManus, C.M., et al., *Chemokine and chemokine-receptor expression in human glial elements: induction by the HIV protein, Tat, and chemokine autoregulation.* Am J Pathol, 2000. 156(4): p. 1441-53.
47. Williams, R., et al., *Proinflammatory cytokines and HIV-1 synergistically enhance CXCL10 expression in human astrocytes.* Glia, 2009. 57(7): p. 734-43.
48. Williams, R., et al., *Cooperative induction of CXCL10 involves NADPH oxidase: Implications for HIV dementia.* Glia, 2010. 58(5): p. 611-21.
49. Kohler, N. and A. Lipton, *Platelets as a source of fibroblast growth-promoting activity.* Exp Cell Res, 1974. 87(2): p. 297-301.
50. Ross, R., et al., *A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro.* Proc Natl Acad Sci U S A, 1974. 71(4): p. 1207-10.
51. Andrae, J., R. Gallini, and C. Betsholtz, *Role of platelet-derived growth factors in physiology and medicine.* Genes Dev, 2008. 22(10): p. 1276-312.
52. Seifert, R.A., et al., *Two different subunits associate to create isoform-specific platelet-derived growth factor receptors.* J Biol Chem, 1989. 264(15): p. 8771-8.
53. Betsholtz, C., L. Karlsson, and P. Lindahl, *Developmental roles of platelet-derived growth factors.* Bioessays, 2001. 23(6): p. 494-507.
54. Brown, R.L., M.P. Breeden, and D.G. Greenhalgh, *PDGF and TGF-alpha act synergistically to improve wound healing in the genetically diabetic mouse.* J Surg Res, 1994. 56(6): p. 562-70.
55. Lepisto, J., et al., *Effects of heterodimeric isoform of platelet-derived growth factor PDGF-AB on wound healing in the rat.* Eur Surg Res, 1994. 26(5): p. 267-72.
56. Graham, S., et al., *Investigating the role of PDGF as a potential drug therapy in bone formation and fracture healing.* Expert Opin Investig Drugs, 2009. 18(11): p. 1633-54.
57. Hollinger, J.O., et al., *Recombinant human platelet-derived growth factor: biology and clinical applications.* J Bone Joint Surg Am, 2008. 90 Suppl 1: p. 48-54.
58. Sturzl, M., et al., *Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi sarcoma in vivo suggests paracrine and autocrine mechanisms of tumor maintenance.* Proc Natl Acad Sci U S A, 1992. 89(15): p. 7046-50.
59. Cao, Y., R. Cao, and E.M. Hedlund, *R Regulation of tumor angiogenesis and metastasis by FGF and PDGF signaling pathways.* J Mol Med, 2008. 86(7): p. 785-9.
60. Claesson-Welsh, L., *Mechanism of action of platelet-derived growth factor.* Int J Biochem Cell Biol, 1996. 28(4): p. 373-85.

61. Hede, S.M., et al., *GFAP promoter driven transgenic expression of PDGFB in the mouse brain leads to glioblastoma in a Trp53 null background.* Glia, 2009. 57(11): p. 1143-53.
62. Nunnari, G., J.A. Smith, and R. Daniel, *HIV-1 Tat and AIDS-associated cancer: targeting the cellular anti-cancer barrier?* J Exp Clin Cancer Res, 2008. 27: p. 3.
63. Bernasconi, S., et al., *Selective elevation of monocyte chemotactic protein-1 in the cerebrospinal fluid of AIDS patients with cytomegalovirus encephalitis.* J Infect Dis, 1996. 174(5): p. 1098-101.
64. Cinque, P., et al., *Elevated cerebrospinal fluid levels of monocyte chemotactic protein-1 correlate with HIV-1 encephalitis and local viral replication.* AIDS, 1998. 12(11): p. 1327-32.
65. Conant, K., et al., *Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia.* Proc Natl Acad Sci U S A, 1998. 95(6): p. 3117-21.
66. Van Der Voorn, P., et al., *Expression of MCP-1 by reactive astrocytes in demyelinating multiple sclerosis lesions.* Am J Pathol, 1999. 154(1): p. 45-51.
67. Czaja, M.J., et al., *Monocyte chemoattractant protein 1 (MCP-1) expression occurs in toxic rat liver injury and human liver disease.* J Leukoc Biol, 1994. 55(1): p. 120-6.
68. Sozzani, S., et al., *MCP-1 and CCR2 in HIV infection: regulation of agonist and receptor expression.* J Leukoc Biol, 1997. 62(1): p. 30-3.
69. Iantomasi, T., et al., *Glutathione transport system in human small intestine epithelial cells.* Biochim Biophys Acta, 1997. 1330(2): p. 274-83.
70. Gashler, A. and V.P. Sukhatme, *Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors.* Prog Nucleic Acid Res Mol Biol, 1995. 50: p. 191-224.
71. Humblot, N., et al., *5-Hydroxytryptamine induces TIS8/egr-1 and c-fos expression in PC12 cells. Involvement of tyrosine protein phosphorylation.* Eur J Neurosci, 1997. 9(1): p. 84-92.
72. Vilpoux, C., et al., *Ethanol-sensitive brain regions in rat and mouse: a cartographic review, using immediate early gene expression.* Alcohol Clin Exp Res, 2009. 33(6): p. 945-69.
73. Gitenay, D. and V.T. Baron, *Is EGR1 a potential target for prostate cancer therapy?* Future Oncol, 2009. 5(7): p. 993-1003.
74. Christy, B.A., L.F. Lau, and D. Nathans, *A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences.* Proc Natl Acad Sci U S A, 1988. 85(21): p. 7857-61.
75. Lemaire, P., et al., *Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells.* Proc Natl Acad Sci U S A, 1988. 85(13): p. 4691-5.
76. Knapska, E. and L. Kaczmarek, *A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK?* Prog Neurobiol, 2004. 74(4): p. 183-211.
77. James, A.B., A.M. Conway, and B.J. Morris, *Genomic profiling of the neuronal target genes of the plasticity-related transcription factor -- Zif268.* J Neurochem, 2005. 95(3): p. 796-810.
78. Bozon, B., S. Davis, and S. Laroche, *Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation.* Hippocampus, 2002. 12(5): p. 570-7.
79. Milbrandt, J., *A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor.* Science, 1987. 238(4828): p. 797-9.
80. Cao, X., et al., *Detection and characterization of cellular EGR-1 binding to its recognition site.* J Biol Chem, 1993. 268(23): p. 16949-57.
81. Beckmann, A.M. and P.A. Wilce, *Egr transcription factors in the nervous system.* Neurochem Int, 1997. 31(4): p. 477-510; discussion 517-6.

82. Khachigian, L.M., et al., *Egr-1-induced endothelial gene expression: a common theme in vascular injury.* Science, 1996. 271(5254): p. 1427-31.
83. Silverman, E.S. and T. Collins, *Pathways of Egr-1-mediated gene transcription in vascular biology.* Am J Pathol, 1999. 154(3): p. 665-70.
84. Loussert-Ajaka, I., et al., *HIV-1 detection in cervicovaginal secretions during pregnancy.* AIDS, 1997. 11(13): p. 1575-81.
85. La Vecchia, L., et al., *Rescue PTCA for a totally occluded left main coronary artery in acute myocardial infarction with cardiogenic shock: technical success and long-term survival.* Cardiology, 1997. 88(5): p. 482-5.
86. Goldstein, G.W., *Endothelial cell-astrocyte interactions. A cellular model of the blood-brain barrier.* Ann N Y Acad Sci, 1988. 529: p. 31-9.
87. Burger, D.M., et al., *HIV-1 RNA levels in the cerebrospinal fluid may increase owing to damage to the blood-brain barrier.* Antivir Ther, 1997. 2(2): p. 113-7.
88. Nottet, H.S., et al., *Mechanisms for the transendothelial migration of HIV-1-infected monocytes into brain.* J Immunol, 1996. 156(3): p. 1284-95.
89. Dallasta, L.M., et al., *Blood-brain barrier tight junction disruption in human immunodeficiency virus-1 encephalitis.* Am J Pathol, 1999. 155(6): p. 1915-27.
90. Avison, M.J., et al., *Inflammatory changes and breakdown of microvascular integrity in early human immunodeficiency virus dementia.* J Neurovirol, 2004. 10(4): p. 223-32.
91. Andras, I.E., et al., *HIV-1 Tat protein alters tight junction protein expression and distribution in cultured brain endothelial cells.* J Neurosci Res, 2003. 74(2): p. 255-65.
92. Andras, I.E., et al., *Signaling mechanisms of HIV-1 Tat-induced alterations of claudin-5 expression in brain endothelial cells.* J Cereb Blood Flow Metab, 2005. 25(9): p. 1159-70.
93. Gendelman, H.E., et al., *The neuropathogenesis of HIV-1 infection.* J Leukoc Biol, 1994. 56(3): p. 389-98.
94. Su, E.J., et al., *Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke.* Nat Med, 2008. 14(7): p. 731-7.
95. Turchan, J., et al., *Estrogen protects against the synergistic toxicity by HIV proteins, methamphetamine and cocaine.* BMC Neurosci, 2001. 2: p. 3.
96. Hollman, A.M., et al., *Selective isolation and purification of tat protein via affinity membrane separation.* Biotechnol Prog, 2005. 21(2): p. 451-9.
97. Ma, M. and A. Nath, *Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells.* J Virol, 1997. 71(3): p. 2495-9.
98. Westendorp, M.O., et al., *Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120.* Nature, 1995. 375(6531): p. 497-500.
99. Bonavia, R., et al., *HIV-1 Tat causes apoptotic death and calcium homeostasis alterations in rat neurons.* Biochem Biophys Res Commun, 2001. 288(2): p. 301-8.
100. Hayashi, K., et al., *HIV-TAT protein upregulates expression of multidrug resistance protein 1 in the blood-brain barrier.* J Cereb Blood Flow Metab, 2006. 26(8): p. 1052-65.
101. Oshima, T., et al., *HIV-1 Tat increases endothelial solute permeability through tyrosine kinase and mitogen-activated protein kinase-dependent pathways.* AIDS, 2000. 14(5): p. 475-82.
102. Prendergast, M.A., et al., *Neurotoxic effects of the human immunodeficiency virus type-1 transcription factor Tat require function of a polyamine sensitive-site on the N-methyl-D-aspartate receptor.* Brain Res, 2002. 954(2): p. 300-7.
103. Speth, C., et al., *Mechanism of human immunodeficiency virus-induced complement expression in astrocytes and neurons.* J Virol, 2002. 76(7): p. 3179-88.

104. Gorantla, S., et al., *Human dendritic cells transduced with herpes simplex virus amplicons encoding human immunodeficiency virus type 1 (HIV-1) gp120 elicit adaptive immune responses from human cells engrafted into NOD/SCID mice and confer partial protection against HIV-1 challenge.* J Virol, 2005. 79(4): p. 2124-32.
105. Bezzi, P., et al., *CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity.* Nat Neurosci, 2001. 4(7): p. 702-10.
106. Kaul, M., G.A. Garden, and S.A. Lipton, *Pathways to neuronal injury and apoptosis in HIV-associated dementia.* Nature, 2001. 410(6831): p. 988-94.
107. Davis, R.L., J. Dertien, and P.J. Syapin, *Ethanol-induced modulation of inducible nitric-oxide synthase activity in human A172 astrocytoma cells.* Alcohol Clin Exp Res, 2002. 26(9): p. 1404-11.
108. Liu, L., et al., *Severe disease, unaltered leukocyte migration, and reduced IFN-gamma production in CXCR3-/- mice with experimental autoimmune encephalomyelitis.* J Immunol, 2006. 176(7): p. 4399-409.
109. Potula, R., et al., *Association of platelet-derived growth factor-B chain with simian human immunodeficiency virus encephalitis.* Am J Pathol, 2004. 165(3): p. 815-24.
110. Luo, J. and M.W. Miller, *Platelet-derived growth factor-mediated signal transduction underlying astrocyte proliferation: site of ethanol action.* J Neurosci, 1999. 19(22): p. 10014-25.
111. Peng, F., et al., *Platelet-derived growth factor-mediated induction of the synaptic plasticity gene Arc/Arg3.1.* J Biol Chem, 2010. 285(28): p. 21615-24.
112. Vincenzino, J.V., *Trends in medical care cost--revisited.* Stat Bull Metrop Insur Co, 1997. 78(3): p. 10-6.
113. Fan, Y., et al., *Activation of Egr-1 expression in astrocytes by HIV-1 Tat: new insights into astrocyte-mediated Tat neurotoxicity.* J Neuroimmune Pharmacol, 2011. 6(1): p. 121-9.
114. Sanders, V.J., et al., *Chemokines and receptors in HIV encephalitis.* AIDS, 1998. 12(9): p. 1021-6.
115. Freter, R.R., et al., *Platelet-derived growth factor induction of the immediate-early gene MCP-1 is mediated by NF-kappaB and a 90-kDa phosphoprotein coactivator.* J Biol Chem, 1996. 271(29): p. 17417-24.
116. Poon, M., et al., *Secretion of monocyte chemotactic activity by cultured rat aortic smooth muscle cells in response to PDGF is due predominantly to the induction of JE/MCP-1.* Am J Pathol, 1996. 149(1): p. 307-17.
117. Eugenin, E.A., et al., *CCL2/monocyte chemoattractant protein-1 mediates enhanced transmigration of human immunodeficiency virus (HIV)-infected leukocytes across the blood-brain barrier: a potential mechanism of HIV-CNS invasion and NeuroAIDS.* J Neurosci, 2006. 26(4): p. 1098-106.
118. Dhillon, N.K., et al., *Roles of MCP-1 in development of HIV-dementia.* Front Biosci, 2008. 13: p. 3913-8.
119. Bell, J.E., *The neuropathology of adult HIV infection.* Rev Neurol (Paris), 1998. 154(12): p. 816-29.
120. Heaton, R.K., et al., *HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study.* Neurology, 2010. 75(23): p. 2087-96.
121. Fuentes, M.E., et al., *Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1.* J Immunol, 1995. 155(12): p. 5769-76.

122. Lee, S.C., et al., *Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta.* J Immunol, 1993. 150(7): p. 2659-67.
123. Rock, R.B., et al., *Morphine stimulates CCL2 production by human neurons.* J Neuroinflammation, 2006. 3: p. 32.
124. Ransohoff, R.M., et al., *Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis.* FASEB J, 1993. 7(6): p. 592-600.
125. Mahad, D.J. and R.M. Ransohoff, *The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE).* Semin Immunol, 2003. 15(1): p. 23-32.
126. Tanuma, N., et al., *Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis.* Acta Neuropathol, 2006. 112(2): p. 195-204.
127. Berman, J.W., et al., *Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat.* J Immunol, 1996. 156(8): p. 3017-23.
128. Yamamoto, M., et al., *Overexpression of monocyte chemotactic protein-1/CCL2 in beta-amyloid precursor protein transgenic mice show accelerated diffuse beta-amyloid deposition.* Am J Pathol, 2005. 166(5): p. 1475-85.
129. Marks, S.D., et al., *Urinary monocyte chemoattractant protein-1 correlates with disease activity in lupus nephritis.* Pediatr Nephrol, 2010. 25(11): p. 2283-8.
130. Groh, J., et al., *Attenuation of MCP-1/CCL2 expression ameliorates neuropathy in a mouse model for Charcot-Marie-Tooth 1X.* Hum Mol Genet, 2010. 19(18): p. 3530-43.
131. Juffermans, N.P., et al., *Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan.* Infect Immun, 1999. 67(8): p. 4295-7.
132. Panicker, S.R., et al., *Quercetin attenuates Monocyte Chemoattractant Protein-1 gene expression in glucose primed aortic endothelial cells through NF-kappaB and AP-1.* Pharmacol Res, 2010. 62(4): p. 328-36.
133. Remppis, A., et al., *Rhizoma Coptidis inhibits LPS-induced MCP-1/CCL2 production in murine macrophages via an AP-1 and NFkappaB-dependent pathway.* Mediators Inflamm, 2010. 2010: p. 194896.
134. Masai, N., et al., *Indoxyl sulfate stimulates monocyte chemoattractant protein-1 expression in human umbilical vein endothelial cells by inducing oxidative stress through activation of the NADPH oxidase-nuclear factor-kappaB pathway.* Circ J, 2010. 74(10): p. 2216-24.
135. Bowen-Pope, D.F., et al., *Platelet-derived growth factor in vivo: levels, activity, and rate of clearance.* Blood, 1984. 64(2): p. 458-69.
136. Yao, H., M. Duan, and S. Buch, *Cocaine-mediated induction of platelet-derived growth factor: implication for increased vascular permeability.* Blood, 2011. 117(8): p. 2538-47.
137. Kaul, M., et al., *HIV-1 coreceptors CCR5 and CXCR4 both mediate neuronal cell death but CCR5 paradoxically can also contribute to protection.* Cell Death Differ, 2007. 14(2): p. 296-305.
138. Gendelman, H.E., et al., *Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes.* J Exp Med, 1988. 167(4): p. 1428-41.
139. Dhillon, N.K., et al., *PDGF synergistically enhances IFN-gamma-induced expression of CXCL10 in blood-derived macrophages: implications for HIV dementia.* J Immunol, 2007. 179(5): p. 2722-30.
140. Bethel-Brown, C., et al., *HIV-1 Tat-mediated induction of platelet-derived growth factor in astrocytes: role of early growth response gene 1.* J Immunol, 2011. 186(7): p. 4119-29.

141. **Ueda, A., et al.,** ***NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene.*** **J Immunol, 1994. 153(5): p. 2052-63.**
142. **Andersson, L.M., et al.,** ***Increased blood-brain barrier permeability in neuro-asymptomatic HIV-1-infected individuals--correlation with cerebrospinal fluid HIV-1 RNA and neopterin levels.*** **J Neurovirol, 2001. 7(6): p. 542-7.**
143. **Berger, J.R. and M. Avison,** ***The blood brain barrier in HIV infection.*** **Front Biosci, 2004. 9: p. 2680-5.**
144. **Bussolino, F., et al.,** ***Interactions between endothelial cells and HIV-1.*** **Int J Biochem Cell Biol, 2001. 33(4): p. 371-90.**
145. **Han, N., et al.,** ***Calcitonin gene-related peptide induces proliferation and monocyte chemoattractant protein-1 expression via extracellular signal-regulated kinase activation in rat osteoblasts.*** **Chin Med J (Engl), 2010. 123(13): p. 1748-53.**
146. **Ding, G.X., et al.,** ***SP600125, an inhibitor of c-Jun NH2-terminal kinase, blocks expression of angiotensin II-induced monocyte chemoattractant protein-1 in human mesangial cells.*** **World J Pediatr, 2010. 6(2): p. 169-76.**
147. **Shimada, H. and L.E. Rajagopalan,** ***Rho-kinase mediates lysophosphatidic acid-induced IL-8 and MCP-1 production via p38 and JNK pathways in human endothelial cells.*** **FEBS Lett, 2010. 584(13): p. 2827-32.**
148. **Van Wagoner, N.J. and E.N. Benveniste,** ***Interleukin-6 expression and regulation in astrocytes.*** **J Neuroimmunol, 1999. 100(1-2): p. 124-39.**
149. **Van Wagoner, N.J., et al.,** ***Interleukin-6 (IL-6) production by astrocytes: autocrine regulation by IL-6 and the soluble IL-6 receptor.*** **J Neurosci, 1999. 19(13): p. 5236-44.**
150. **Sappington, R.M. and D.J. Calkins,** ***Pressure-induced regulation of IL-6 in retinal glial cells: involvement of the ubiquitin/proteasome pathway and NFkappaB.*** **Invest Ophthalmol Vis Sci, 2006. 47(9): p. 3860-9.**
151. **Lau, L.T. and A.C. Yu,** ***Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury.*** **J Neurotrauma, 2001. 18(3): p. 351-9.**