Published in final edited form as:

J Neuroimmune Pharmacol. 2008 September ; 3(3): 173–186. doi:10.1007/s11481-008-9110-x.

HIV-1 infected astrocytes and the microglial proteome

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Abstract

The human immunodeficiency virus (HIV) invades the central nervous system early after viral exposure but causes progressive cognitive, behavior, and motor impairments years later with the onset of immune deficiency. Although in the brain, HIV preferentially replicates productively in cells of mononuclear phagocyte (MP; blood borne macrophage and microglia), astrocytes also can be infected, at low and variable frequency, particularly in patients with encephalitis. Among their many functions, astrocytes network with microglia to provide the first line of defense against microbial infection; however, very little is known about its consequences on MP. Here, we addressed this question using co-culture systems of HIV infected mouse astrocytes and microglia. Pseudotyped vesicular stomatitis virus/HIV was used to circumvent absence of viral receptors and ensure cell genotypic uniformity for studies of intercellular communication. The study demonstrated that infected astrocytes show modest changes in protein elements as compared to uninfected cells. In contrast, infected astrocytes induce robust changes in the proteome of HIV-1 infected microglia. Accelerated cell death and redox proteins, amongst others, were produced in abundance. The observations confirmed the potential of astrocytes to influence the neuropathogenesis of HIV-1 infection by specifically altering the neurotoxic potential of infected microglia and in this manner, disease progression.

Keywords

astrocytes; microglial; human immunodeficiency virus; pseudotyped viral infection; proteomics; cell mobility; neurotoxicity

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“The original publication is available at springerlink.com”

Introduction

Human immunodeficiency virus-1 (HIV-1) associated neurocognitive disorder (HAND) is a complication marked by cognitive, behavioral, and motor dysfunction that develops during the later stages of AIDS (Janssen et al., 1992; Navia et al., 1998; Antinori et al., 2007). The pathological hallmarks of HIV-1 associated dementia, the most severe form of HAND are characterized by microglia cell activation, astrogliosis, decreased synaptic function, leukocyte infiltration, multinucleated giant cells, and selective neuronal loss (Budka, 1991; Masliah et al., 1992; Everall et al., 1993; Everall, 1995). Microglia/macrophages are the most commonly infected cells in the brain and serve as lifelong hosts for HIV (Minagar et al., 2002; Gonzalez-Scarano and Martin-Garcia, 2005; Kramer-Hammerle et al., 2005). Microglial HIV infection and viral replication result in the secretion of neurotoxic pro-inflammatory cytokines, chemokines, and viral proteins that strongly implicate microglia hyper-activation in the progression of HAND (Gendelman et al., 1994; Stevenson and Gendelman, 1994; Strizki et al., 1996; Conant et al., 1998; Gabuzda et al., 1998; Nath and Geiger, 1998; Ryan et al., 2002).

A second cellular target for HIV in the brain is the astrocyte (Conant et al., 1994; Brack-Werner, 1999; Canki et al., 2001). Astrocytes are the most abundant cell type in the brain (Schubert et al., 2001) and perform many essential functions, such as maintenance of a homeostatic environment and bidirectional communication with neurons (reviewed in: Fields and Stevens-Graham, 2002). Astrocytes also contribute to both the guidance and support of neuronal migration during development and perform immune functions within the nervous system. This includes effects on blood brain barrier (BBB) function (Vitkovic and da Cunha, 1995; Yoshioka et al., 1995; Brack-Werner, 1999), control of extracellular glutamate, and regulation of neuronal cell function and neural signaling (Benveniste, 1998; Bezzi and Volterra, 2001; Danbolt, 2001; Fields and Stevens-Graham, 2002). It is clear that changes in any of these functions by HIV could severely impair brain functions and contribute to viral neuropathogenesis.

The biology of HIV interaction with astrocytes differs significantly from the well-known productive viral infection in T cells and macrophages. On one hand, astrocytes, which lack surface CD4, can still bind gp120 and HIV particles throughout the entire cell population (Li et al., 2007). On the other hand, the efficient virus binding leads to infection only in a small fraction of cells *in vitro* and *in vivo* (Saito et al., 1994; Tornatore et al., 1994; Canki et al., 2001; Trillo-Pazos et al., 2003). The inefficient infection of astrocytes by HIV has been recently attributed to a block at virus entry and can be overcome through the use of a VSV-G-pseudotyped HIV or expression of exogenous surface CD4, which can lead to efficient viral replication (Canki et al., 2001; Schweighardt and Atwood, 2001; Li et al., 2007). An increasing body of evidence suggests that noninfectious binding of HIV/gp120 and infection in a fraction of astrocytes can have profound effects on cell physiology and influence HAND. For example, HIV or gp120 binding to astrocytes was shown to induce inhibition of glutamate transport by the cells (Benos et al., 1994c; Benos et al., 1994a; Benos et al., 1994b; Wang et al., 2003), cause broad dysregulation of cellular gene expression (Su et al., 2002; Wang et al., 2004), and alone or as a consequence of neighboring macrophages/microglia induce cell activation with production of cytokines and chemokines (Nebuloni et al., 2000; Yeung et al., 2005; Ronaldson and Bendayan, 2006; Li et al., 2007). Disrupted glutamate transport by astrocytes can damage neurons by excitotoxicity (Gegelashvili et al., 2000; Plachez et al., 2000; Plachez et al., 2004) while excessive immune activation of the cells can contribute to the neuroinflammatory environment in HIV infected brain.

We studied the effect of HIV infection on astrocyte biology (as contrasted to virus binding alone) and in particular how this infection may affect the crosstalk between astrocytes and

microglia that is essential for functional defenses against retroviral infection. Although infected astrocytes constitute less than 1% of the total number of astrocytes in encephalitic brains (Takahashi et al., 1996; Trillo-Pazos et al., 2003), astrocytes through their processes form intercellular networks with tens or hundreds of proximal and distal cells (Bezzi and Volterra, 2001; Fields and Stevens-Graham, 2002). Thus, even low-frequency infection of astrocytes could have significant deleterious effects on the cellular environment in the brain and contribute to disease pathogenesis. To increase the frequency of infected astrocytes, we used a pseudotyped vesicular stomatitis virus/HIV (HIV-1/VSV) to circumvent the viral entry restriction (Spector et al., 1990; Bencheikh et al., 1999; Canki et al., 2001; Nitkiewicz et al., 2004; Dou et al., 2006; Gorantla et al., 2007). Previous studies with astrocytes infected by this method employing either microarray gene expression profiling (Kim et al., 2004) or biochemical methods (Cosenza-Nashat et al., 2006) revealed that uniform productive infection of astrocytes by HIV affects cell cycle dependent pathways and cell proliferation. These works indicated that HIV causes extensive changes in cellular gene expression, particularly in pathways involved in cell cycle, profile as determined by Affymetrics oligonucleotide microarray analysis (Kim et al., 2004). In the current study, we used murine astrocytes and microglia to ensure genetic homogeneity and evaluated the effects of efficient HIV infection on the cellular proteome and biological consequences of astrocyte-microglial crosstalk. Infection of both cell types enabled a thorough analysis of the influence of astrocytes on microglial functions that are linked to disease and viral replication. The results showed that astrocytes serve to accelerate microglial neurotoxicity as well as affect viral growth and compensatory regulatory pathways of the cell. These investigations provide novel insights into the role of astrocytes in microglial functions and open new research directions for neuropathogenesis and developmental therapeutics for HIV-1-mediated neurological disease.

Materials and Methods

Primary mouse microglia, astrocytes and neurons

Fetuses (18-day-old) were harvested from anesthetized pregnant C57/BL6 mice, maintained and bred in full compliance of the University of Nebraska Medical Center and the National Institutes of Health ethical care and treatment of animals. Cerebral cortices were isolated and digested using 0.25% trypsin (Invitrogen, Carlsbad, CA). Isolated neural cells were differentiated into neurons, microglia (MCG), and astrocytes (AST) under different culture conditions. For neuron differentiation, cells were seeded at a density of 1.2×10^5 cells/well on poly-D-lysine coated cover slips, placed in 24-well plates, and cultured for 10 days in neurobasal medium supplemented with 2% B27 (Invitrogen), penicillin/streptomycin, and 0.5 mM L-glutamine (all from Invitrogen). The expression of microtubule-associated protein-2 (MAP-2), a mature neuronal marker, and the lack of expression of glial fibrillary acidic protein (GFAP, an astrocyte marker) determined cell purity. For microglia differentiation, the isolated cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin, and 2 ug/ml macrophage colony stimulating factor (MCSF) (a generous gift of Wyeth Inc., Cambridge, MA) for 7 days when microglial cells were collected by shaking. For astrocyte differentiation, cortical isolates were cultured in DMEM supplemented with F12 (Invitrogen), 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin (Gorantla et al., 2007). After serial 3-time passage of the cells, the purity of the astrocytes was > 85%.

Laboratory transwell culture systems

For single cell-type cultures, microglia were seeded at the concentration of 2×10^6 cells/well in 6-well culture plates, and astrocytes were cultured at 1×10^6 cells/well. For multiple cell-type co-cultures, we used cell culture inserts matched for 6-well culture plates (BD Falcon 353090). Microglia were seeded first in the 6-well culture plates at the concentration of

2×10^6 cells/well and continuously cultured for 3 d, then astrocytes added to the track-etched polyethylene terephthalate membrane inserts at 1×10^6 cells/insert and both cell types placed into the 6-well plates. Importantly, the media used in both single or co-cultures for experiments were the 1:1 ratio mixture of microglial and astrocytes media (Wang et al, submitted). On day 3 after infection, half media was exchanged. On day 7 all the culture supernatants were collected.

Viruses and viral infections

VSV pseudotyped HIV-1 strain YU2 (HIV-1/VSV) was used to circumvent the required viral/cellular receptors necessary to infect mouse cells. all the cells were exposed to HIV-1/VSV at 1 pg HIV-1 p24/cell at same time for 24 h before a complete media exchange. Infected cells were cultured for 7 days. Greater than 98% of microglia and astrocytes expressed HIV-1 p24 by day 7 after infection as determined by immunohistochemical assays (Canki et al., 2001).

TUNEL assays

Apoptotic cells were determined by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and were achieved using the *In Situ* Cell Death Detection Kit, AP (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Briefly, neurons were fixed with 4% paraformaldehyde in phosphate buffered saline [PBS (pH 7.4)] and permeabilized with 0.1% Triton-100 in 0.1% sodium citrate. Cells were subsequently labeled with TUNEL working solution. Apoptotic cells were identified as green fluorescent cells by fluorescence microscopy, counted, and normalized to the total number cells as determined by 4',6'-diamidino-2-phenylindole (DAPI) nuclear stain. TUNEL⁺ cells were scored, and the percentage of apoptotic neurons to total DAPI⁺ neurons were calculated.

Protein sample preparation

Cells were washed in PBS, harvested, and lysed in 200 μ l cell lysis buffer pH 8.5 containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 1 $\times$ protease inhibitor cocktail (Biovision, Mountain View, CA). Cell lysates were sonicated at 25 W for three 3-sec pulses with a Sonicator W-225 (Heat Systems-Ultrasonics, Inc., Farmingdale, NY). To remove impurities and concentrate each sample, cell lysates were treated with the 2D Clean Up Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. To preclude possible interfering substances in the lysates, protein concentrations were determined with the 2D Quant Kit (GE Healthcare) (Ricardo-Dukelow et al., 2007).

Difference gel electrophoresis (DiGE) 2D-Page and image analysis

Fifty micrograms of protein from uninfected and HIV-1 infected microglial lysates were each labeled with 400 pmol of N-hydroxy-succinimidyl ester of Cy3 or 1-(5-carboxypentyl)-1-methylindodi-carbocyanine halide (Cy5) dyes (CyDye Minimum Labeling kit, GE Healthcare), respectively. Twenty-five micrograms of protein from uninfected and 25 μ g from HIV-1 infected microglia were mixed and labeled with 1-(5-carboxypentyl)-1-propylindocarbocyanine halide (Cy2) to serve as an internal standard. The resulting pools of proteins (Cy2-, Cy3-, and Cy5-labeled) were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 1% Pharmalyte (pH 3–10NL) and applied to gel strips with immobilized pH gradient (24 cm; pH 3–10 NL). For first dimension separation, electrophoresis of gel strips was achieved in an IPGphor II apparatus (GE Healthcare) at 500 V for 1 h, 1000 V for 1 h, and 8000 V for 3 h. Electrophoresed strips were treated with equilibration solution [6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.8] containing 100 mM DTT for 10 min. Equilibrated strips were alkylated with 100

mM iodoacetamide (Sigma) in equilibration solution for 10 min. Immediately after alkylation, second dimension separation was achieved by electrophoresis through a 10-20% gradient polyacrylamide gel at constant currents of 5 mA for the initial 30 min and 12 mA for 14 h. Labeled proteins were visualized on a 2D Master Imager (GE Healthcare) at excitation wavelengths of 488 nm, 520 nm, and 620 nm and emission wavelengths of 520 nm, 590 nm, and 680 nm to detect Cy2-, Cy3-, and Cy5-labeled proteins, respectively. The relative amount of protein was determined by digital quantification using DeCyder-DIA software (GE Healthcare). Protein amounts exhibiting greater than 1.5-fold changes above or below relative amounts after normalization were considered candidates for further analyses (Ricardo-Dukelow et al., 2007).

Spot picks and in-gel tryptic digestion

Protein spots within 2 mm² fragments were robotically picked from the gel using the Ettan™ Spot Picker (GE Healthcare) and destained for 1 h at room temperature using 100 µl of 50% acetonitrile (ACN)/50 mM NH₄HCO₃. Gel fragments were dried and incubated with 0.25% trypsin/10 mM NH₄HCO₃ (Promega, Madison, WI) overnight at 37°C. Peptides were extracted by washing gel pieces twice with 0.1% trifluoroacetic acid (TFA) and 60% ACN (Ciborowski et al., 2007).

Mass spectrometry

All samples were purified using ZipTip® (Millipore, Sunnyvale, CA) prior to mass spectrometric analysis. Peptide samples were resuspended in 0.1% formic acid/HPLC-grade water and analyzed by liquid chromatography dual mass spectroscopy (LC/MS/MS, LCQ DECA XPPlus, ThermoElectron, Inc. Waltham, MA) and Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF/TOF, ABI 4800, Applied Biosystems, Foster City, CA). Proteins were identified from the NCBI database interfaced with BioWorks 3.1SR software (ThermoElectron). Protein identifications scored greater than 3000 by the Unified Score scale, and greater than 50% on ion score were considered for further analyses (Ciborowski et al., 2007). For MALDI-TOF/TOF, peptide samples in L-cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich) matrix were spotted on to Opti-TOF® 384 well MALDI plate inserts (Applied Biosystems). The mass profile analyzed by the MALDI-TOF/TOF mass spectrometer was searched in Mascot database assisted by GPS Explorer software (Applied Biosystems) and only significant protein identifications (P<0.05) were considered for further analyses.

Ingenuity pathway analysis

A data set containing protein identifiers and corresponding expression values was uploaded into the application Ingenuity Pathways Knowledge Base. Each protein identifier was mapped to its corresponding gene in the database, and networks of these focused genes were algorithmically generated based on their connectivity. The Functional Analysis module identified the biological functions and/or diseases that were algorithmically significant to the data set. The graphical representation of the molecular relationships between genes/gene products was represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge database (Ingenuity® Systems, www.ingenuity.com) (Liu et al., 2008).

Immunocytochemistry

Cells, adhered to cover slips, were fixed and permeabilized with acetone and methanol (ratio 1:1, at -20 °C) for 10 min, and nonspecific activity was blocked by incubation of fixed cells in 6% bovine serum albumin/phosphate buffered saline (BSA/PBS). Blocked samples were

incubated with 250 μ l of primary antibody that included mouse anti-NeuN (1/100; Chemicon, Temecula, CA), rabbit anti-MAP-2 (1/1000; Chemicon), rabbit anti-tubulin- α (1:1000; Novus, Littleton, CO), rabbit anti-Iba-1 (1:500, Wako, Richmond, VA), and mouse-anti-vimentin (1:1000; Dako, Carpinteria, CA). F-actin was detected with rhodamine-conjugated phalloidin (Invitrogen). Primary antibody treated cells were incubated with 250 μ l of the appropriate diluted goat anti-mouse or goat anti-rabbit Abs secondary antibodies (1:250; Invitrogen) conjugated to Alexa-488 or Alexa-568. Cells were mounted using anti-fade Pro-Long Gold mounting reagent (Invitrogen) and examined with a Zeiss LSM 510 META NLO microscope (Zeiss MicroImaging, Inc., Thornwood, NY) (Gorantla et al., 2007).

Western blot assays

For Western blots, 10–40 μ g of protein from each total cell lysate were separated on (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Roche). Membranes were incubated overnight at 4°C with primary antibodies including mouse anti-caldesmon (1:500; Cell signaling, Danvers, MA), mouse anti-GFAP (1:500; Dako), rabbit anti-peroxiredoxin-1 (1:1000; Abcam, Cambridge, MA), rabbit galectin-3 (1:5000; Abcam), rabbit anti-enolase- α (1:1000; Santa Cruz, Santa Cruz, CA), mouse anti-calmodulin (1:1000; Abcam), and mouse anti-HIV-1 p24 (1:500; Dako). Detection of reacted primary antibodies was achieved with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000) or goat anti-rabbit IgG (1:10,000), (Jackson Immunoresearch, West Grove, PA.) HRP activity was visualized by enhanced chemiluminescence (Pierce, Rockford, IL) (Rozek et al., 2007).

Results

HIV-1 infected astrocyte-microglial neurotoxicity

To assess the affects of HIV-1 infected astrocytes on neurotoxicity of infected microglia secretions, we assessed the morphology and apoptotic state of mouse cortical neurons cultured for 24 h in conditioned media (CM) from uninfected or infected microglia, astrocytes, or microglia-astrocyte co-cultures. Neurons stained for expression of MAP-2 (green) and neuronal nuclei (NeuN, red) demonstrated that >98% of the cells were indeed neurons (Fig. 1). We next evaluated percentages of apoptotic neurons (green) compared to total DAPI-stained neuronal nuclei (blue). Neurons cultured in 20% CM from uninfected astrocytes, microglia, or microglia-astrocyte co-cultures respectively showed 3.6%, 3.0%, and 3.9% apoptotic (TUNEL⁺) neurons. In contrast, increased percentages of apoptotic neurons were observed after culture in CM from infected astrocytes (9.5%) or microglia (21.8%) and were significantly increased to 33.8% after culture in CM from infected microglia-astrocyte co-cultures compared to CM from single cell types.

HIV-1 infected astrocytes modulate the virus-infected microglial proteome

To evaluate cellular changes in viral infected microglia and astrocytes, we compared the proteomes from lysates of uninfected (green) and HIV-1/VSV infected (red) microglia or astrocytes by 2D DiGE and digital image analyses. For astrocytes, a total of 1743 spots were digitally detected (Fig. 2a). Lysates from infected astrocytes compared to uninfected controls showed 9 (0.6%) up-regulated proteins, while 40 (2.5%) proteins were down-regulated. For microglia, 2116 were detected, and 39 (1.9%) proteins were up-regulated, while 24 (1.2%) spots were down-regulated (Fig. 2b).

We next assessed the effect of infected astrocytes on protein expression of HIV-1/VSV infected microglia by analyzing the proteome from those microglia acquired after transwell co-culture with infected astrocytes. Uninfected microglia co-cultured in astrocyte containing transwells served as comparative controls for DiGE. From a total of 1974 proteins, we found that virus-

infected microglia co-cultured with infected astrocytes up-regulated 161 (8.3%) proteins and down-regulated 87 (4.5%) proteins when compared to uninfected replicate cells (Fig 2C). This represented a marked increase in the level of regulated proteins compared to levels obtained with microglia or astrocytes separately cultured.

Protein identification and validation

From the 2D DiGE of astrocyte lysates (Fig. 2a), 49 protein spots were robotically picked and analyzed by LC MS/MS. Twenty-one proteins were identified and functionally grouped as structural, regulatory, or enzyme according to the description provided by the ExPASy Proteomics Server Classification (<http://ca.expasy.org/>) (Table 1). Structural proteins, such as vimentin, capping protein, and proliferating cell nuclear antigen (PCNA) were up-regulated after viral infection, while the energy-associated protein, voltage-dependent anion-selective channel protein 1 (VDAC-1) was down-regulated.

From 220 proteins picked from DIGE of microglia co-cultured with infected astrocytes (Fig. 2c) and analyzed by LC/MS/MS, 29 proteins were identified (Table 2). The structural protein fibronectin-1 (FN1), myristylated alanine-rich protein kinase C substrate (MARCKS), and vimentin were up-regulated. After infection, redox and virus replication related proteins, such as serine/threonine protein phosphatase 2A (PP2A or PPP4R2), caldesmon, ferritin, and peroxiredoxin were up-regulated, whereas cell death associated enzymes proteins, such as enolase- α , transketolase, glutamine synthetase, and lactate dehydrogenase B were down-regulated. The relative expression of regulated proteins identified by mass spectrometry, including, caldesmon, glial fibrillary acidic protein (GFAP), enolase- α , peroxiredoxin, galectin-3, ferritin, and calmodulin were validated by Western blot analysis (Fig. 3). Results from triplicate Western blot assays were consistent with DiGE and digital image analyses.

Ingenuity pathway analysis

To examine the biological networks of microglial proteins affected by astrocytes and viral infection, we uploaded the 29 regulated proteins (Table 2) and their corresponding relative changes into the Ingenuity Pathway Analysis (IPA) database (Ingenuity® Systems, www.ingenuity.com). Dynamic pathway modeling of these proteins/genes by IPA identified interactions through cell death, DNA replication, recombination, and repair networks (Fig. 4). The IPA network assay pointed out three proteins with changing trends related to p53 pathway. They were nucleosome assembly protein 1-like 1 (NAP1L1), PPP4R2, and heat shock protein 70 (HSPA9) (Fig. 4). Modeling with the effect-on-function IPA module indicated that (a) down-regulation of enolase- α is associated with increased activation of protein binding sites and increased DNA replication; (b) up-regulation of NAP1L1 is associated with increased activation of genes associated with DNA replication; and (c) up-regulation of peroxiredoxin 6 results in increased DNA degradation and metabolism.

HIV-1 p24 processing qualification

Reproductive HIV-1 infection requires virus DNA synthesis, recombination and replication; pathways involving networks of DNA replication, recombination, and repair as demonstrated by pathway modeling (IPA) to be altered by co-culture with infected astrocytes (Fig. 4). Thus, we reasoned that astrocytes could affect HIV-1 replication in microglia. To assess this, we harvested microglia (in plates) and astrocytes (in cell inserts) separately, and analyzed infected microglia co-cultured in the presence of astrocytes by Western blot for intracellular HIV-1 p24 and its precursor, HIV-1 p55. Single culture microglia infected with HIV-1/VSV showed both p55 and p24 bands and indicating a normal protein processing from p55 to p24 proteins (Cordelier et al., 2003) (Fig. 5 lane a). The levels of the viral proteins p24 and p55 were produced in abundance in microglia with/out coculture. Viral protein processing was markedly affected by astrocytes (lane b and c). Co-culture with uninfected astrocytes showed a notable

increase in p55 protein with a concomitant decrease in p24 (lane c), which is consistent with inhibition of p55/p24 conversion. However, compared to uninfected astrocytes, co-culture in the presence of infected astrocytes showed notable diminution of p55 with concomitant increase in p24 (lane b) suggesting the reversion of the inhibited p55/p24 processing. Of interest, HIV-1 infected astrocytes cultured alone showed HIV-1 p55, p41, and p24 cleavage products (lane d).

Discussion

In a previous report, we demonstrated that astrocytes attenuate HIV-1/VSV infected microglial neurotoxic activities (Wang et al., submitted). Currently, we extended our prior analyses by using proteomic approaches to investigate profiles associated with intercellular interactions of viral-infected astrocytes and microglia. Surprisingly once infected, the ability of astrocytes to modulate the microglial phenotype was changed dramatically from beneficial to destructive. We now demonstrate that HIV-1 infected astrocytes enhance the neurotoxicity caused by HIV-1 infected microglia. The proteome of HIV-1-infected microglia was changed by virus-infected astrocytes, particularly in networks associated with cell death and DNA replication, recombination, and repair. Crosstalk with HIV-1 infected astrocytes enhanced HIV-1 p24 processing in virus-infected microglia. Alteration of the proteome and HIV-1 p24 processing in microglia resulted in augmentation of neurotoxicity. Negative outcomes of HIV-1 infected microglia induced by infected astrocytes represents an important mechanism for the onset of neurodegeneration related to the dynamics of permissive and productive HIV-1 infected astrocytes within the CNS.

Permissive infection of astrocytes by HIV has been reported and is based on CD4-independent mechanisms, including viral entry induced by chemokine (C-C motif) receptor 5 (CCR5), CXCR4 (Reeves et al., 1999), DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Deiva et al., 2006), and human mannose receptor (Liu et al., 2004; Lopez-Herrera et al., 2005). Astrocytes suffer dysregulation by neighboring cells leading to viral transmission and productive replication in adjoining microglia, which contribute to neuropathogenesis. Meantime, astrocytes are exposed continuously to HIV-1 particles, viral proteins (such as Nef and Tat), cytokines, and neurotoxic substances secreted by HIV-1 infected microglia or macrophages (Lipton, 1994; Vesce et al., 1997; Brennehan et al., 2000; Krebs et al., 2000; Dou et al., 2006; Jayadev et al., 2007). In this study, we employed VSV pseudotyped M-tropical HIV-1 YU2 strain to circumvent astrocytic receptor restrictions and trigger the intracellular entry of virus RNA. We demonstrated that HIV-1/VSV infected astrocytes accelerated and enhanced HIV-1/VSV infected microglia to express HIV-1 p55 and p24. Similar reports from other independent groups indicate astrocytes, lymphocytes, and macrophages are susceptible to productive infection by VSV pseudotyped HIV-1 NL4 strain as assessed by detection of p24, Tat, viral protease-mediated processing of Gag, appropriately spliced viral RNA, and infectious progeny virus (Canki et al., 2001; Nitkiewicz et al., 2004).

More importantly, proteomic analysis clearly showed that HIV-1 infected astrocytes dramatically affected proteomic profiles of HIV-1 infected microglial compared with infected microglia cultured alone. Most of the differentially expressed proteins were mainly related to microglial activation and cytoskeletal changes including calponin, vimentin, GFAP, heat shock protein (HSP)-70, HSP-40, PP2A, caldesmon, and MARCKS. Calponin and caldesmon are regulatory proteins, which function in the induction of actin polymerization and depolymerization, and maintain cytoskeletal stability during hypertrophy (el-Mezgueldi, 1996; Huber, 1997), while caldesmon prevents actin reorganization (Castellino et al., 1995). In this study, calponin and caldesmon expression were enhanced in both infected microglia and astrocytes and have been shown to block actin reorganization, induce actin aggregation, and destabilize cytoskeletal integrity (Winder et al., 1993; Lu et al., 1995; Koganehira et al.,

2003; Takiguchi and Matsumura, 2005; Perez-Montiel et al., 2006). Additionally, interaction of HIV-infected astrocytes markedly enhanced expression of structural proteins, vimentin and GFAP, by HIV-1 infected microglia, which have been shown to inhibit neurotrophin secretion (Schwartz and Mishler, 1990) and down-regulate production of TNF- α (Hetier et al., 1991). Moreover, up-regulation of FN1 and MARCKS, as demonstrated in these studies by the intercellular interactions of infected astrocytes on microglia, are closely related to increased morphogenesis of cells, formation of cell filaments, and outgrowth of plasma membrane projections (Wiederkehr et al., 1997; Xie et al., 1998; Eliasson et al., 1999). One group of protein changes related to immunological disorders was associated with up-regulation of caldesmon (Scaife et al., 2004), glutamine synthetase (Nakamura et al., 2006), lactate dehydrogenase B (Zheng et al., 2001; Nakamura et al., 2006), peroxiredoxin 6 (Fajardo et al., 2004; Jin et al., 2005), transketolase (Fajardo et al., 2004), and vimentin (Nakamura et al., 2006), with concomitant down-regulation of enolase (Fajardo et al., 2004; Nakamura et al., 2006). Taken together, these findings suggest that HIV-1 infected microglia exhibit an enhanced activated phenotype upon interaction with HIV-1 infected astrocytes, which may serve as one mechanism by which HIV-1 microglia and macrophages exert and augment neurotoxicity during HIV-1 infection of the CNS and HAND.

In this study, proteomic analysis and pathway modeling of HIV-1 infected microglia co-cultured with HIV-1 infected astrocytes, supported the involvement of DNA replication, recombination, and repair networks including tumor protein p53 (p53), a transcription factor regulating cell cycle and apoptosis. Three cell-signaling proteins, PPP4R2, HSPA9, and NAP1L1, were observed which interact with p53 and were up-regulated by astrocyte interactions with infected microglia. Among them, PPP4R2 up-regulation is associated with p53 activation (Harris and Levine, 2005), which in turn down-regulates p53 expression by dephosphorylation to suppress apoptosis (Kong et al., 2004; Arroyo and Hahn, 2005; Shouse et al., 2008). HSPA9 is a mediator of p53 and plays important roles for p53 transcriptional activity (Agoff et al., 1993; Tsutsumi-Ishii et al., 1995; Fourie et al., 1997; Zyllicz et al., 2001). NAP1L1 is known to directly interact with p53 and activate binding of sequence-specific DNA binding proteins, which may be an important step contributing to the activation of p53 transcription (Rehtanz et al., 2004). These findings suggest that increased PPP4R2, HSPA9 and NAP1L1 proteins regulate neuronal apoptosis and viral DNA replication through a p53-mediated mechanism that is regulated by intercellular communication between infected astrocytes and microglia. That p53 may regulate viral replication is supported by interactions of p53 and HIV-1 proteins (Garden and Morrison, 2005). We reported here that supernatant collected from HIV infected astrocytes-microglia co-cultures enhance neuronal death. This would be consistent with the possibility that supernatants activate p53 signaling pathway and accelerate neuronal apoptosis. First evidence supporting this first includes p53 involvement in HIV-1 gp120 induction of microglia, astrocyte, and neuron apoptosis, and the activation of p53 mediated pathways in the glia of HAND patients. This may contribute to the neuroinflammatory processes that promote neurodegeneration by inhibiting glial proliferation and/or promoting glial cell dysfunction (Jayadev et al., 2007). Second, HIV-1 viral protein R (Vpr) cooperates with p53 in regulating viral gene transcription (Sawaya et al., 1998; Jayadev et al., 2007). Third, crosstalk between HIV-1 Tat and p53 has been linked with cellular transformation by HIV-1 infection or activation of HIV-1 replication (Li et al., 1995; Ariumi et al., 2001; Ryu et al., 2004). Interestingly, we demonstrated calmodulin and ferritin were down-regulated in HIV-1 infected microglia co-cultured with infected astrocytes. Calmodulin is reported to directly bind with HIV-1 Nef and regulate virus replication (Brigino et al., 1997; Fackler et al., 1999; Hayashi et al., 2002; Lehmann et al., 2006). Viruses with life cycles involving a DNA phase require chelatable iron for optimum replication, and ferritin is involved in the control of cell growth and DNA synthesis; its downregulation may be implicated in cell toxicity and DNA abnormalities that accompany HIV infection (Ameglio et al., 1993; Savarino et al., 1999). Hori et al. reported that human astrocytes have the capacity to increase or decrease

HIV-1 expression in monocyte-derived macrophages. Thus, imbalance between the positive and negative effects of astrocytes on infected microglia or macrophages may contribute viral expression and production in the brain and the development of HAND (Hori et al., 1999). This is consistent with findings in this study, which demonstrated reduced HIV-1 p24 expression by HIV-1 infected microglia that are co-cultured with uninfected astrocyte and reversal of p24 diminution expression in microglia cultured with HIV-1 infected astrocytes.

In conclusion, we have demonstrated for the first time that HIV-1 infected astrocytes increase the neurotoxicity of HIV-1 infected microglia, which is related to heightened phenotypic levels of microglial activation and alteration of networks associated with HIV-1 p55 expression, processing, and p24 virion maturation. Our findings suggest that the dynamics of permissive and productive HIV-1 infection in microglia is regulated by intercellular interactions with infected astrocytes, and provide an important switch mechanism for onset of HIV-1 related neuroinflammation and neurodegeneration.

Acknowledgements

We thank Dr. Ron Cerny, Nebraska Center for Mass Spectrometry, University of Nebraska-Lincoln, for his support for LC/MS/MS protein identification. We thank James Talaska and Janice Taylor of the Confocal Laser Scanning Microscope Core Facility at the University of Nebraska Medical Center (UNMC) for providing assistance with confocal microscopy, and Dr. R. Lee Mosley and Robin Taylor at UNMC for outstanding editorial support and critical reading of the manuscript. This work was supported by the Frances and Louis Blumkin Foundation, the Community Neuroscience Pride of Nebraska Research Initiative, the Alan Baer Charitable Trust, and National Institutes of Health grants 5P01NS31492 and DA17618 (to D.J.V.) and 2R37 NS36126, 2R01 NS034239, P20RR15635, U54NS43011, P01MH64570, and P01 NS43985 (to H.E.G.)

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Fig. 1. Neurotoxic activities of culture fluids from HIV-1/VSV infected astrocytes, microglia and microglia-astrocyte co-cultures

Mouse cortical neurons were cultured for 24 h with 20% CM from uninfected (-) or HIV-1/VSV infected (+) astrocytes (AST), microglial (MCG), or microglia-astrocyte co-cultures (MCG-AST). Neurons were immunostained for expression of MAP-2 (green) and NeuN (red) (upper row). From serial sections, total nuclei were stained with DAPI (blue) (middle row) or by TUNEL (green) (bottom row). Percentages of apoptotic neurons were calculated from the numbers of TUNEL⁺ (green) neurons to DAPI⁺ (blue) cells, and are depicted as a mean percentage ± SEM for n = 3 experiments with 3 replicates/experiment. Differences in means were determined by one-way ANOVA analysis and Tukey's multiple post-hoc comparison. Note: *When performing HIV-1 infection in MCG+AST co-cultivation group, both cell types were infected. Bars for MAP-2/NeuN, 20μm; original magnification, ×63. Bars for DAPI and TUNEL, 50μm, original magnification, ×40.

Fig. 2. 2D-DIGE analyses of lysates from astrocytes, microglia and microglia-astrocyte co-cultures
Cell lysates from uninfected (Cy-3 labeled, green) and HIV-1-infected (Cy-5 labeled, red) were assessed by isoelectric focusing (1st dimension) and reducing PAGE through a 10%-20% gel (2nd dimension). Protein separations of cell lysates obtained by 2D-DIGE gels are shown for separately cultured astrocytes (a), microglia (b), and microglia-astrocyte co-cultures (c). Proteins were quantitated for each DIGE gel by digital image analysis by DeCyder™ 6.5 software, which compared proteins from uninfected controls (green) and infected cells (red). Thus proteins exhibiting no changes appear yellow, while fluorescence for down-regulated proteins are green and up-regulated proteins are red with the fluorescence levels depending on each protein's relative abundance. Histograms (right panels) of each DIGE gel demonstrate the

distribution as a function of relative concentration for increased (red), decreased (green), and similar (yellow) protein spots, as well as a detection summary.

Fig. 3. Validation of differential expression of proteins selected from 2D-DIGE of HIV-1/VSV infected microglia from microglia-astrocyte co-cultures

Lysates of uninfected (-) and HIV-1/VSV infected (+) microglia co-cultured with infected astrocytes were electrophoresed and transferred to PVDF membranes. Based on differential expression by digital image analysis and identification by LC/MS/MS (Table 2), selected proteins were stained for analysis by Western blot and included caldesmon (1), GFAP (2), enolase- α (3), peroxiredoxin (4), galectin-3 (5), ferritin (6) and calmodulin (7). Digital image analysis of each protein spot and software (DeCyder) interpretation are compared for uninfected (left) and infected (right) microglia for each of the 7 proteins. Corresponding Western blots for each protein is indicated under each DeCyder paired images.

Proteins from infected microglia co-cultured with astrocytes, separated by DIGE and identified by LC/MS/MS (Table 2) were analyzed by dynamic pathway modeling. The pathways showed significant correlations with networks associated with cell death and DNA replication, recombination and repair. Line types show the interactions between proteins: Lines with arrows stand for direct acting and lines without arrows represent protein binding only. Solid lines show direct interactions, while broken lines indicate indirect interactions. Shapes and colors represent functional classification nodes. Functions are indicated by shapes: diamonds for enzymes, squares for cytokines, rectangles for ligand-dependant nuclear factors, triangles for kinases, ovals for transcription regulators, trapezoids for transporters, and circles for miscellaneous interactions. Nodes of red color represent microglial proteins, which are up-regulated upon HIV-1 infection, while green nodes represent down-regulated proteins. Nodes without color represent proteins not input by user, but interpreted by the database as high probable interactions within the network. The abbreviations for the up- and down-regulated proteins are: (Red) CKB, creatine kinase B; GLUL: glutamine synthetase; GM2A, GM2 ganglioside activator protein; HSPA9, HSP70 protein (heat shock 70 kDa protein 9); LDHB, L-lactate dehydrogenase B chain; NAP1L1, nucleosome assembly protein 1-like 1; PPP4R2, protein phosphatase 4 regulatory subunit 2; PRDX6, peroxiredoxin 6; TKT, transketolase. (Green) CNBP, cellular nucleic-acid binding protein; ENO1, enolase-alpha; FTL1, ferritin light chain 1; PKM2, pyruvate kinase isozyme M2; RPLP2, 60S acidic ribosomal protein P2.

Fig. 5. HIV-1 p24 protein processing

Lysates from uninfected or HIV-1/VSV infected microglia (MCG) cultured with uninfected or infected astrocytes (AST) in transwells were evaluated by Western blot analysis with anti-p24 antibody for expression of the HIV-1 Gag precursor protein, p55 and its p41 and p24 cleavage products. Lanes were loaded with lysates from (a) HIV-1 infected microglia cultured separately; (b) infected microglia co-cultured with infected astrocytes; (c) infected microglia co-cultured with uninfected astrocytes; and (d) infected astrocytes cultured separately. Gag proteins are not detected in lanes loaded with lysates from uninfected cells. Blots were stripped and stained for expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as sample loading control. Western blot presented is representative from triplicate determinations.

HIV-1/HSV infected astrocyte proteome

Table 1

Regulation and Protein Class	Protein Name	Swissprot Access No.	Fold change ^a	Mass kDa	pI ^b	Peptides ^c
Up-regulated Structural	40S ribosomal protein SA	P14206	1.9	32.7	4.74	3
	Acidic ribosomal phosphoprotein P0	Q5FWB6	1.5	34.2	5.91	3
	Vimentin	P20152	1.6	51.5	5.06	2
	Proliferating cell nuclear antigen	P17918	1.5	28.7	4.66	2
	Tropomyosin-1 alpha chain	P58771	1.5	32.7	4.69	3
Regulatory	Capping protein	O82631	2.1	31.4	4.58	3
	Gelsolin	P13020	1.3	85.9	5.83	6
	Calponin-2	Q08093	1.5	28.7	7.53	3
	Endoplasmic reticulum protein ERp29 [Precursor]	P57759	2.7	28.8	5.90	5
	6-phosphogluconolactonase	Q9CQ60	1.9	27.3	5.55	7
Enzymes Down-regulated Structural	Filamin-B	Q80X90	1.6	277.7	5.44	2
	Dynactin 2	Q99KJ8	2.5	44.1	5.14	2
	Heat shock 70 kDa protein 4	Q61316	1.5	94.1	5.15	4
	Heat shock 47kDa protein	Q5U4D0	1.9	46.5	8.88	5
	Prohibitin	P67778	1.7	29.8	5.57	5
Regulatory	14-3-3 zeta	P63101	1.9	27.7	4.73	3
	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	Q60932	2.8	32.3	8.55	3
	Transitional endoplasmic reticulum ATPase	Q01853	1.7	89.3	5.14	2
	Proteasome 26S non-ATPase subunit 9	Q9CR00	2.4	24.7	6.00	4
	NADH dehydrogenase (ubiquinone) flavoprotein 2	Q8K2L0	1.7	27.3	7.00	2
Enzymes	Phosphatidylethanolamine binding protein 1	P70296	1.5	20.8		2

^aCompared with uninfected astrocytes.

^bTheoretical isoelectric point calculated by Swissprot database at <http://ca.expasy.org/sprot/>.

^cNumber of peptides detected by mass spectrometry for each identified protein.

HIV-1/HSV infected astrocytes affect the HIV-1/HSV infected microglial proteome

Table 2

Regulation and Protein Class	Protein Name	Swissprot Access No.	Fold change ^a	Mass Da	pI ^b	Peptides ^c
Up-regulated Structural	Fibronectin 1	Q3UZF9	1.8	115822	5.54	2
	Lamin A	Q3U733	2.2	72427	6.54	5
	Vimentin.	P20152	2.5	55524	5.06	5
	Tubulin alpha chain	P68369	2.5	50104	4.94	5
Regulatory	Glia fibrillary acidic protein	P03995	2.0	49878	5.36	44
	Caldesmon 1	Q8VQC8	1.9	60417	6.98	8
	MARCKS (myristylated alanine-rich protein kinase C substrate)	P26645	2.3	29644	4.34	15
	HSP70 protein, mitochondrial [Precursor]	P38647	1.8	73483	5.91	32
Enzymes	Galectin-3	P16110	1.5	27366	8.47	14
	Nucleosome assembly protein 1-like 1	P28656	2.5	47332	4.63	2
	Mitochondrial fission 1 protein	Q9CQ92	4.2	16998	8.56	4
	GM2 ganglioside activator protein	Q5FI28	1.6	20811	5.63	3
	Protein phosphatase, regulatory subunit 2	Q4G0D4	2.6	46261	4.53	5
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform (PP2A)	Q76MZ3	2.5	65150	5	4
	Creatine kinase (EC 2.7.3.2) B	O04447	2.2	42713	5.4	23
	Glutamine synthetase	Q91VC6	2.1	42092	6.64	11
	Transketolase	Q3UK62	1.6	67489	6.97	10
	L-lactate dehydrogenase B chain	P16125	2.1	36418	5.64	22
Down-regulated Structural Regulatory	Glutathione S-transferase Mu 1	A2AE89	1.5	25822	8.33	17
	Dihydropyrimidinase-like 3	Q3TT92	2.2	61741	6.04	11
	Peroxiredoxin 6	Q6GT24	1.6	24811	5.98	18
	F-actin capping protein beta subunit (CapZ beta)	P47757	2.2	31195	5.47	2
Enzymes	60S acidic ribosomal protein P2	Q3TKY3	3.4	11644	4.42	6
	Probable protein BRICK1	Q3TLB8	1.6	8813	5.09	2
	Calmodulin	P62204	2.7	16838	4.09	7
	Ferritin light chain 1	P29391	1.8	20802	5.66	6
	Cellular nucleic acid-binding protein	P53996	1.7	19591	8	2
	Enolase-alpha	P17182	2.1	47140	6.37	6
	Pyruvate kinase isozyme M2	P52480	1.7	57719	7.42	3

^a Compared with uninfected microglia.
^b Theoretical isoelectric point calculated by Swissprot database at <http://ca.expasy.org/sprot/>.
^c Number of peptides detected by mass spectrometry for each identified protein.