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HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) NEUROPATHOGENESIS IN *IN VITRO* MODELS

MUKHTAR, M.

The Dorrance H. Hamilton Laboratories, Center for Human Virology and Biodefense, Division of Infectious Diseases and Environmental Medicine, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107. E-mail: muhammad.mukhtar@jefferson.edu

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Abstract: HIV-1 enters into the brain soon after seroconversion; however the mechanism of viral entry into the brain is elusive. This viral entry is supposed to be mediated by transmigration of HIV-1 infected cells (monocytes/macrophages, leukocytes) or direct infection of blood-brain barrier (BBB) forming cells. Recently, our studies on HIV-1 neuropathogenesis reveal that primary human brain microvascular endothelial cells (MVECs), a major constituent of the human BBB express a variety of chemokine receptors; however, none of them is involved in viral entry/infection. To better understand neuropathogenesis of HIV-1 our laboratory has developed an in vitro BBB which is totally comprised of primary human brain MVECs and astrocytes. This model is being used to study and completely understand viral entry into the brain as well neuroprotective strategies for HIV-1-associated dementia. This article discusses our ongoing work in this area of research.

Key words: Human immunodeficiency virus, Neuropathogenesis

Many individuals infected with HIV-1 develop HIV-associated dementia (HAD) also known as AIDS dementia complex (ADC), which is characterized by cognitive dysfunction, motor neuron disease, coordination abnormalities and other neurological signs and symptoms[1]. The molecular mechanisms involved in these HIV-1-associated dysfunctions of the central nervous system (CNS) are not completely understood and controversial [2]. Of importance, the passage of virus across the blood-brain barrier (BBB) is not a straightforward process, owing to the tight junctions between brain microvascular endothelial cells (MVECs) and the relatively permeable underlying basement membrane [3]. A prominent model for viral entry is the "Trojan Horse" hypothesis, which suggests that HIV-1-infected T-lymphocytes and monocyte/macrophages transmigrate across the BBB [4]. Direct infection of the endothelium and the subsequent transmission of the virus into the CNS and free virions ferrying through the BBB has also been suggested [5,6]. Based on previous findings and the current level of knowledge, several major challenges remain in HAD research. The most prominent among these are the development of antiretrovirals that could cross the BBB to effectively eradicate viral infection or halt the transfer of HIV-1-infected cells into the brain. Similarly, identification of biomarkers that could provide the most reliable evidence for the presence of ongoing infection in CNS also poses a challenge. HIV-1 research in these areas has been hampered by the unavailability of primary human cells constituting the BBB and optimal conditions for their growth as well as the lack of a representative animal model.

The BBB is a specialized structure containing brain capillaries. Anatomically, the BBB is composed of brain MVECs on the lumenal side, and a thin basement membrane, called the basal lamina, that is supported by the foot processes of adjacent astrocytes on the ablumenal side. The astrocytic foot processes are involved in the structural integrity of the BBB [7]. Brain MVECs exhibit unusually impermeable cell-cell connections, called tight junctions that provide a major barrier to the entry of circulating molecules, microorganisms or cells into

the brain. Additional barrier function is provided by the basement membrane and adjacent astrocytes.

The integrity and permeability of the BBB can be perturbed during various pathological conditions. Typically, these pathological changes in the BBB are accompanied by opening of the tight junctions, increased pinocytosis by the brain MVECs, decreased membrane rigidity, and disease/toxicant induced nutrient changes. Weakening of tight junctions has been reported in autoimmune encephalitis, multiple sclerosis, and after exposure to inflammatory mediators such as TNF-α, IL-1ß, arachidonic acid, and reactive oxygen species both *in vitro* and *in vivo* [7]. Enhanced secretion of matrix metalloproteinases, inflammatory cytokines, and various other cytotoxic or pro-apoptotic agents by local immune and non-immune cells have also been proposed to weaken the BBB [7].

Human *in vitro* blood-brain barrier:

To study human neurovirological disorders, we have developed a sophisticated cellular model of the BBB *in vitro*, using primary cultures of brain-derived MVECs, macrophages, neuronal cells, and astrocytes [3]. Unlike many other models, this BBB model uses only human CNS cell types, a key requirement for studies of HIV-1. Furthermore, unlike CNS explants, this BBB model system can be used to selectively investigate the involvement of each individual cell type in HIV-1-induced CNS cytopathology. To produce this model, primary human brain MVECs are obtained from the brain (Cell Systems Corporation, Kirkland, WA, USA) and verified to be endothelial cells by >95% staining for von Willebrand factor. For studies of the passage of virions, the cells are then cultured to 100% confluency on the upper side of 0.45-µm polyethylene tetraphthalate (PET) membrane inserts coated with attachment factor. Expression of zona occludens 1 (ZO-1), a protein specifically associated with tight junctions and the intercellular sealing of adjacent brain MVECs, is verified by immunocytochemistry (Fig. 1, Panel B). For cellular transmigration studies the cell-culture inserts with a pore size of > 8.0 µm are used. The integrity of the BBB formed on the insert membrane is also demonstrated by transendothelial electrical resistance (TEER) [3]. After the formation of a complete brain MVECs monolayer, a two compartment system is constructed by placing the cell culture insert into a 35-mm culture well (part of a 6-well culture plate) as demonstrated in figure 2. In the 35-mm well are also placed different CNS cells, *i.e.,* human astrocytes, immature neurons, and mature neurons. For ease of later isolation, the astrocytes can be placed onto an additional membrane that floats in the 6-well plate and eventually adheres to the insert membrane. The cell culture inserts and membranes are in close juxtaposition to the surface of the 35-mm well, thereby allowing intimate communication between the different cell types, separated by the PET membranes. We have used this system to study the movement of different HIV-1 strains across brain MVECs [3] as well as cellular transmigration [8].

HIV-1-infectivity-associated receptors on brain microvascular endothelial cells:

The presence of HIV-1 in cerebrospinal fluid (CSF) and various CNS-based cells suggest that these cells are potential targets for HIV-1/SIV infection as well as transfer virus to other CNS elements. It has become established that the chemokine receptors, CXCR4 and CCR5, act as major co-receptors together with CD4 for HIV-1 entry into diverse target cells [9]. CD4-independent, and chemokine (CXCR4, CCR5)-dependent infection of various cells suggest the capability of certain HIV-1 isolates to infect cells devoid of CD4 but with functional chemokine co-receptors [10,11]. The BBB forming MVECs are the first line of defense against viral entry into brain [2]. Of note, previous reports suggest that HIV-1 infection/passage of human brain MVECs and astrocytes via CD4-independent mechanisms [10,11]. We examined primary isolated human brain MVECs for the presence of the chemokine receptors CCR3, CXCR4, CCR5 and APJ mRNAs. We also explored whether DC-SIGN and L-SIGN mRNA exist in human brain MVECs.

We observed that primary isolated human brain MVECs do not express CD4, the major cellular receptor for HIV-1 attachment and viral entry. However, primary brain MVECs express the chemokine receptors APJ, CCR3, CXCR4, and CCR5 [12]. These findings suggest that HIV-1 attachment and entry into human brain MVECs possibly involves a CD4-independent mechanism, utilizing either one or multiple chemokine receptors.

To explore the functional role of each chemokine receptor in lentiviral binding onto the surface of human brain MVECs, we blocked each receptor with inhibitory concentrations of corresponding chemokines

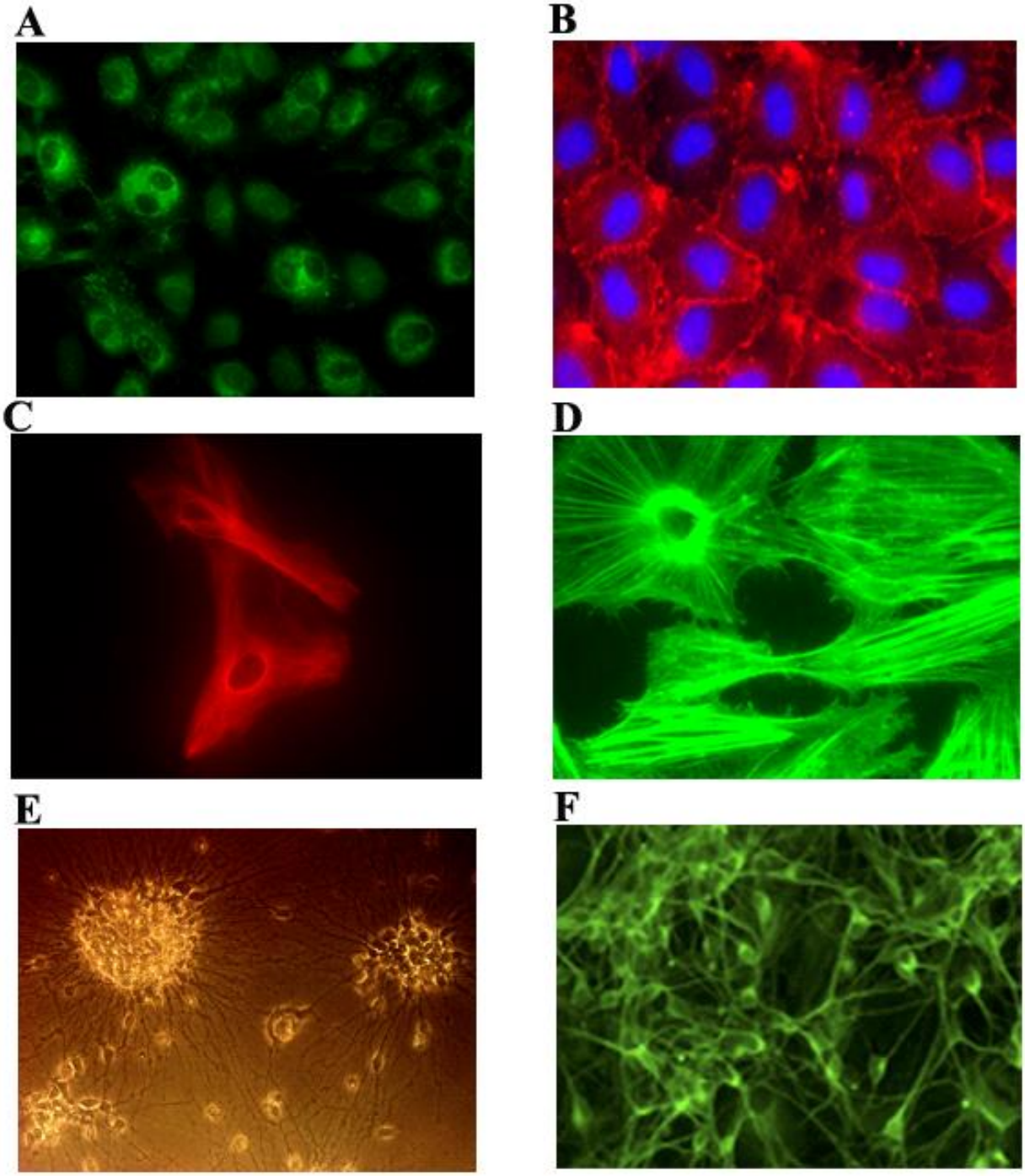

Fig. 1: Cellular elements of the *in vitro* system to study neuropathogenesis of HIV-1 infection: (A) Primary human brain MVECs expressing von Willebrand Factor, (B) Primary human brain MVECs expressing ZO-1, a tight junctions-associated protein, (C) Primary human brain astrocytes expressing glial fibrillary acidic protein (GFAP), (D) Primary human astrocytes stained for cytoskeletal structural proteins, (E) Phase-contrast photomicrograph of postmitotically differentiated neurons and (F) Postmitotically differentiated neurons immunostained for MAP-2, a neuronal characterization marker.

and then exposed them to X4, R5 and dual-tropic HIV-1 isolates. The APJ ligand, Apelin-36, CCR3 ligand eotaxin, CXCR4 ligand SDF-1α, and a mixture of CCR5 ligands MIP-1α, MIP-1β and Rantes, were used for blocking respective chemokine receptors. For DC-SIGN, blocking polyclonal anti-DC-SIGN antiserum was used, whereas a soluble CD4 was utilized for blocking CD4-mediated entry. Even though primary brain MVECs are devoid of CD4 receptor, we also used soluble CD4 to analyze the effects on virion binding on the surface of these cells. Compared with the controls we observed a higher amount of virus attached to soluble CD4-treated cells [12].

In continuation of our efforts to understand the mechanisms involved with HIV-1 entry into the brain, we also analyzed the role of lipids rafts in HIV-1 entry into primary human brain MVECs, which represent

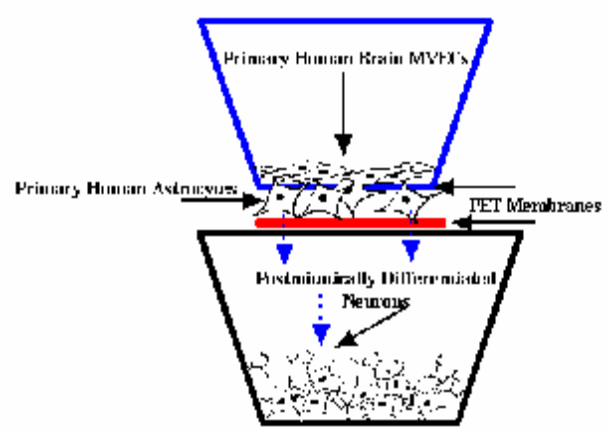

Fig. 2: Schematic representation of an *in vitro* blood-brain barrier model.

an important HIV-1 CNS-based cell reservoir, and a portal for neuroinvasion. Cellular cholesterol was depleted by exposure to ß-cyclodextrins (BCDs) and HMG-CoA reductase inhibitors (statins), the loss of cholesterol was quantitated, and disruption of membrane rafts was verified by immunofluorescence. Nevertheless, these treatments did not affect binding of several strains of HIV-1 virions to brain MVECs at 4°C, nor their infectivity at 37°C [5]. In contrast, we confirmed that cholesterol depletion and raft disruption strongly inhibited HIV-1 binding and infection of JURKAT T-cells. Enzymatic digestion of cell-associated heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) on human brain MVECs dramatically inhibited HIV-1 infection. These findings, in combination with our earlier work showing that human brain MVECs lack CD4 [12], indicate that the molecular mechanisms for HIV-1 entry into brain MVECs are fundamentally different from viral entry into T-cells, where lipid rafts, CD4, and probably HSPGs/CSPG may play important roles.

HIV-1 proteins and HIV-1 associated dementia:

Our studies have demonstrated that extracellular, recombinant HIV-1 Vpr protein is highly neurotoxic in the microenvironment of differentiated mature human neurons and undifferentiated neuronal precursors [13]. Although most of the direct neurotoxic effects of HIV-1 have been attributed previously to the envelope gene product, gp120, and the Tat regulatory protein, it was demonstrated that the levels of apoptosis caused by Vpr protein is comparable to that induced by gp120 protein in a dose-dependent manner in the neuronal system [13]. Our laboratory is also interested in dissecting the role of various other specific HIV-1 proteins on BBB forming cellular elements particularly in the presence of various other cofactors. We observed that alcohol augment the apoptosis inducing capability of various HIV-1 proteins [14]. Having observed the neurocytopathic effects of extracellular Vpr protein previously, the effects of virally-expressed Vpr on non-dividing, terminally-differentiated human neurons were investigated by our group. An HIV-1-based three-plasmid expression vector system was utilized to study the effects of intracellularly expressed Vpr. These virion preparations were then used to transduce neurons generated from the human neuronal precursor NT2 cell-line.

Intracellularly expressed Vpr induced apoptosis within terminally differentiated neurons, as demonstrated by TUNEL assays [15]. Additionally, virions lacking Vpr expression did not significantly induce apoptosis within these neurons. These results suggest that HIV-1 Vpr may also be directly involved with selective neurotoxicity through intracellular expression. Furthermore, human apoptosis gene microarray of Vpr-transduced neuronal cells comparisons exhibited an up-regulation in the expression of Bcl-2-associated death promoter (Bad) mRNA, as well as other apoptosis genes involved in the mitochondrial apoptotic pathway, when compared to Vpr-negative controls. Thus, Vpr delivered intracellularly, as well as extracellularly, is involved in the induction of significant neuronal apoptosis and may be one of the molecular mechanisms in HIV-1-induced encephalopathy [15].

The use of Afftymetrix GeneChip analyses to study the effects of HIV-1 envelope protein gp120 on postmitotically differentiated neurons has provided us with important clues on the molecular mechanisms involved in HIV-1-induced apoptosis in neuronal cells. Our data suggest that HIV-1 gp120 mediated induction of apoptosis in human neurons is via both the death receptor and NMDA receptor pathways [16].

We have recently completed a very extensive study to describe the molecular mechanism involved with the interactions of HIV-1 accessory protein Nef on the BBB forming brain MVECs. An initial report describing these findings suggests that Nef protein adversely affects the brain MVECs [17].

Neuroprotection and gene therapy :

In a study on the use of molecular therapeutics against HIV-1 in human CNS-based cells *in vitro* [6] we utilized an HIV-1-based vector to transduce human CNS MVECs, astrocytes, macrophages and NT2 cells

(undifferentiated and differentiated), with a trans-dominant negative Rev moiety, RevM10, a previously characterized anti-HIV-1 agent [18]. In these experiments, HIV-1 replication was significantly reduced in relevant human CNS cell-types. We have also shown that down-modulation of chemokine receptor CXCR4 inhibit HIV-1 infectivity in immune as well as neuronal cells [19,20]. Such approaches are being extended to an *in vivo* model in the development of a targeted gene delivery [21].

Novel neuroprotective strategies for HIV-1 associated dementia:

Of particular interest in the area of HIV-1 related neurovirological disorders are our recent studies on utilization of cholesterol-depleting drugs statins as anti-HIV-1 and neuroprotective agents. The author of this article was awarded the Atorvastatin Research Award 2002 from Pfizer pharmaceuticals to initiate an exploratory work. This work provided initial proof that treatment of infected primary human monocytes and T-cells with clinically available inhibitors of cholesterol biosynthesis (statins), particularly atorvastatin, potently inhibits HIV-1-induced transmigration [8]. Also of interest are recent studies suggesting that lovastatin, which is another member of the statin family, inhibits HIV-1 infection and replication in primary cells *in vitro* as well as *in vivo* [22,23]. Similar observations have been made from a small group of chronically infected HIV-1 individuals upon lovastatin administration [22].

Identification of statins with potent antiretroviral effect may provide a novel, safe, and most importantly, affordable antiretroviral to combat the HIV/AIDS pandemic. The capability of statins to halt enhanced transmigration of HIV-1 infected primary monocytes and T-cells, the major target of viral infection, is a promising finding. If this class of drugs can inhibit systemic viral replication, as well as inhibit transmigration of infected immune cells into sequestered areas of the human body, we will have a new opportunity to combat this disease. The author's work at Thomas Jefferson University, Philadelphia, PA, USA has led to several interesting research avenues. A series of manuscripts in HIV-1 neuropathogenesis has been published in neurovirological research [24-35]. In summary, this article has briefly described HIV-1-associated neurological disorders related research at the Division of Infectious Diseases and Environmental Medicine.

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