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HIV-1-INDUCED AMYLOID BETA ACCUMULATION IN BRAIN ENDOTHELIAL CELLS IS ATTENUATED BY SIMVASTATIN

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Abstract

HIV-1-infected brains are characterized by increased amyloid deposition. To study the influence of HIV-1 on amyloid beta (A β) homeostasis at the blood-brain barrier (BBB) level, we employed a model of brain microvascular endothelial cells exposed to HIV-1 in the presence or absence of A β . HIV-1 markedly increased endogenous A β levels and elevated accumulation of exogenous A β . Simvastatin, the HMG-CoA reductase inhibitor, blocked these effects. We next evaluated the effects of HIV-1 and/or simvastatin on expression of the receptor for lipoprotein related protein (LRP1) and the receptor for advanced glycation end products (RAGE), known to regulate A β transport across the BBB. LRP1 expression was not affected by HIV-1; however, it was increased by simvastatin. Importantly, simvastatin attenuated HIV-1-induced RAGE expression. These results suggest that HIV-1 may directly contribute to A β accumulation at the BBB level. In addition, statins may protect against increased A β levels associated with HIV-1 infection in the brain.

INTRODUCTION

A new and emerging development in HIV-1 epidemiology is an increase in the older population infected with HIV-1. This phenomenon results both from more effective anti-retroviral therapy (ART) and increased infection rate among people over 50 years old. Indeed, the number of people 50 years and older infected with HIV-1 has increased by 77% from 2001 to 2005 according to the Centers for Disease Control (Xu and Ikezu, 2008). Thus, a large population of infected patients has been living with HIV-1 disease for more than 20 years. This is an important fact, because the increasing age of infected persons may have a detrimental effect on their cognitive functions and may facilitate and enhance the development of neurodegenerative diseases in HIV-infected patients (Brew et al., 2009). The effect of aging on cognitive impairment has also been observed in clinical studies. For example, Valcour et al. (2004) reported a significantly higher prevalence of dementia in a cohort of HIV-1-infected individuals aged over 50 as compared to younger patients between 20 and 39 years old (25% vs. 13%). Interestingly, these effects did not appear to be related to plasma viral load. These observations were recently confirmed by Bhaskaran et al. (2008), who reported that older age

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at seroconversion and the duration of infection significantly increased the risk of HIV-1-associated dementia.

Strong evidence indicates increased amyloid deposition in the brain of HIV-1-infected patients (Esiri et al., 1998). A correlation between the years of infection and amyloid deposition in the brain was also demonstrated (Rempel and Pulliam, 2005). There appears to be a prevalence for amyloid deposition in the hippocampus and frontal lobe in HIV-1-infected individuals (Brew et al., 2009). In addition, prominent A β localization was observed in pyramidal neurons and along axonal tracks. Patients with HIV-associated encephalitis (HIVE) had higher levels of intraneuronal A β immunoreactivity compared to HIV-1 patients without HIVE. Furthermore, intracellular deposition of A β correlated with age in the group of patients with HIVE (Achim et al., 2009). HIV-associated neurocognitive disorders (HAND) in older populations have been partially linked to early signs of beta-amyloidosis observed in Alzheimer's disease (AD), further demonstrating the importance of A β deposition for the clinical outcome of HIV-1 infection. However, there are some distinctive differences in A β deposition in AD and HIV-1 brains. While extracellular amyloid plaques are the major amyloid pathology in AD, intraneuronal amyloid accumulation or perivascular diffuse amyloid depositions are more characteristic for HAND (Xu and Ikezu, 2008).

The mechanisms underlying the interactions between A β and HIV-1 infection are not fully understood but several factors and/or pathways are likely to be involved. It has been hypothesized that aging, HIV-1 infection, and the secondary effects of ART may all contribute to brain A β accumulation in neurons and in perivascular space (Green et al., 2005). Several clinical studies support this concept (Rempel and Pulliam, 2005). For example, accumulation of the A β precursor protein was demonstrated in the brain during HIV-1 infection (Giometto et al., 1997). In addition, HIV-1 Tat protein was shown to inhibit the A β degrading enzyme neprilysin, resulting in elevated soluble A β in cell cultures (Rempel and Pulliam, 2005). HIV-1-induced inflammatory mediators, such as CCL2/MCP-1 produced during chronic neuroinflammation, might also contribute to increased levels of A β in the CNS (Pulliam, 2009).

It has been hypothesized that brain vascular dysfunction and the blood-brain barrier (BBB) may affect A β homeostasis and contribute to A β accumulation in the brain (Deane and Zlokovic, 2007). In addition, impairment of the BBB is involved in HIV-1 pathology in the brain (Toborek et al., 2005). Therefore, the aim of the present study was to evaluate the role of brain endothelial cells in A β accumulation in the context of HIV-1-related pathology. Our results indicate that exposure to HIV-1 can increase intracellular accumulation of A β in brain endothelial cells. In addition, treatment with HIV-1 increased expression of the receptor for advanced glycation end products (RAGE) that transports A β into the brain. Interestingly, these effects were attenuated by simvastatin, suggesting that statins may protect the BBB against amyloid beta accumulation in the brain induced by HIV-1 infection.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal anti-A β antibody (with specificity for human A β amino acid residues 17–24) was purchased from Millipore (Temecula, CA). Mouse monoclonal anti-A β antibody (reactive to amino acid residues 1–16 of human A β) was obtained from Covance (Emeryville, CA). Mouse monoclonal anti-LRP1 antibody (5A6) was from Abcam (Cambridge, MA). Goat polyclonal anti-RAGE (N-16) was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti HIV-1 p24 antibody was from DakoCytomation (Carpinteria, CA). Secondary antibodies were purchased from Santa Cruz Biotechnology and Invitrogen (Carlsbad, CA).

Cell cultures and A β treatment

Human brain microvascular endothelial cell line (hCMEC/D3 cells)—hCMEC/D3 cells (a gift from Dr. P-O Couraud, Institut Cochin, Paris, France) represent a stable, well characterized, and differentiated cell line of brain endothelial cells (Weksler et al., 2005). The cells were cultured on collagen type I (BD Biosciences Pharmingen, San Jose, CA) coated dishes in EBM-2 medium (Clonetics, East Rutherford, NJ) supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin, and 2.5% fetal bovine serum (FBS).

U937 cells—U937 cells (a human monocytic cell line; American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂.

Co-culture models—U937 cells were added into the upper chamber of the Transwell system with polycarbonate membranes (pore size 0.4 μ m; Corning Costar, Corning, NY). hCMEC/D3 cells were cultured in the lower chamber of the system.

A β (1–40) and A β (1–40) HiLite were purchased from Anaspec (San Jose, CA) and dissolved in sterile ultra-pure water (ELGA Purelab Classic, Lowell, MA). Freshly solubilized A β solutions without pre-aggregation were used for the experiments. Such a form of A β was demonstrated to induce proinflammatory reactions in isolated rat brain microvessels (Paris et al., 2002).

Cells were treated with A β at the concentration of 1 μ M for 10 min in complete medium. This concentration was selected in dose-dependent experiments on the effects of A β on stimulation of pro-inflammatory responses (Andras et al., 2008). Treatment with similar A β levels was shown to result in an uptake by the BBB (Yamada et al., 2008). In selected experiments, cells were pretreated for 5 h or 24 h with 5 μ M simvastatin followed by co-treatment with A β (1–40) for 10 min. Simvastatin was left in cell culture media for the duration of A β treatment. Our preliminary studies (data not shown) indicated that simvastatin concentrations between 5–20 μ M did not affect cell viability. hCMEC/D3 cells were exposed to HIV-1-infected U937 cells or HIV-1 viral isolate for 24 h.

Generation of HIV-1 stock and infection of U937 cells

HIV-1 stock was generated using human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA). The cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% FBS and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). When the cultures reached ~70% confluence, the cells were transfected with pYK-JRCSF plasmid containing full-length proviral DNA using calcium phosphate as a transfection reagent. Following transfection, cells were incubated in RPMI 1640 medium with 10% FBS and penicillin plus streptomycin (100 U/ml penicillin, and 100 μ g/ml streptomycin) for 24 h. Then, culture supernatants were collected, filtered through 0.45 μ m filters (Millipore, Bedford, MA), and frozen at –80°C. HIV-1 p24 levels in the supernatants were determined by ELISA (ZeptoMetrix, Buffalo, NY) as the marker of HIV-1 infection.

HIV-1 stock was used to infect U937 cells. Briefly, 5 $\times$ 10⁶ U937 cells were cultured in T-25 flasks (Corning Costar, NY) in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were infected with a viral isolate containing 80 ng HIV-1 p24 and incubated overnight at 37°C and 5% CO₂. The cultures were maintained for up to 14 days with fresh medium added every other day. To determine the levels of HIV infection, p24 antigen levels were assessed by ELISA. In co-culture experiments, confluent hCMEC/D3 cells in 6-well plates were exposed to U937 cells at a ratio of 1:1 and HIV-1 p24 at a level of 230 pg/ml. This p24 level is similar to that in the serum of AIDS patients (Pascale

et al., 1997). Non-infected U937 cells were used as controls. In the majority of the monoculture experiments, confluent hCMEC/D3 cells cultured on collagen I coated chambered glass slides (BD Biosciences, San Jose, CA) were exposed to HIV-1 particles (initial p24 level 30 ng/ml; final p24 level 1 ng/ml).

Real-Time Reverse-Transcription-Polymerase Chain Reaction

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to study the RAGE mRNA level changes. Total RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA, USA) and reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI, USA) with random hexamer primers. Real-time PCR was conducted on the ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using TaqMan Universal PCR Master Mix and pre-developed primer pairs and probes (Applied Biosystems). The following thermocycling conditions were used: 95°C for 10 min, followed by 95°C for 15 sec, and 60°C for 60 sec for 50 cycles. The linear range of the exponential phase of DNA amplification was between 27 and 40 cycles. Expression of mRNA was calculated and analyzed by the comparative CT method. PCR amplification of 18S rRNA and human GAPDH (housekeeping genes) were performed for each sample to normalize mRNA levels of the target genes.

Immunoblotting

Homogenates of cultured hCMEC/D3 cells were prepared in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5% Nonidet P40, 1 mM EDTA, 2.5 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 µg/ml aprotinin). After centrifugation at 15,000 x g for 15 min, supernatants were collected and protein concentrations were determined using the Bradford protein assay. Samples (15–25 µg protein/lane) were separated on SDS-polyacrylamide gel, blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and incubated with the respective antibodies. For visualization of detected proteins, immunoblots were analyzed using an ECL Western blot detection kit (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation

Immunoprecipitation of A β was performed using 100 mg of protein extract. Samples were incubated with 1 µg of mouse monoclonal anti-A β antibody for 4 h at 4°C. Then, 30 µl of Protein A/G PLUS-Agarose immunoprecipitation reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each sample and immunoprecipitation was performed overnight at 4°C. The beads were recovered by centrifugation at 1500 x g for 5 min at 4°C and washed four times with 1 ml of phosphate-buffered saline (PBS). Bound proteins were eluted by boiling in sodium dodecyl sulfate sample buffer for 5 min and analyzed on 4% to 15% sodium dodecyl sulfate polyacrylamide gels. Fractionated proteins were blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with the mouse monoclonal anti-A β antibody. For visualization of detected proteins, immunoblots were analyzed using an ECL Western blot detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence microscopy

hCMEC/D3 cells cultured on collagen I coated chambered glass slides (BD Biosciences, San Jose, CA) were fixed with ethanol for 30 min at 4°C. After washing with PBS and blocking with 3% bovine serum albumin in PBS for 30 min, samples were incubated overnight at 4°C with the primary antibody. Then, excess of the primary antibody was removed, the slides were washed with PBS, and incubated with the Texas Red- or Alexa Fluor⁵⁶⁸-conjugated secondary antibody for 2 h at room temperature. After washing with PBS, slides were mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen,

Carlsbad, CA) to visualize the nuclei. Specimens were covered with coverslips and evaluated under an epifluorescence Nikon Eclipse E600 microscope. The images were captured using a Spot CCD camera system.

Transport of A β (1–40) across hCMEC/D3 monolayers

Confluent hCMEC/D3 cells cultured on Transwell filter inserts (pore size 0.4 μ m, 12-well cell culture plate) were pretreated with simvastatin (5 μ M) for 5h and exposed to 1 μ M A β (1–40) HiLite added to the upper chamber for 20 min in 37°C. The fluorescence signal from A β (1–40) HiLite was measured in the lower chamber at 503 nm (excitation) and 528 nm (emission) as the indicator of transendothelial A β transfer.

Statistical Analysis

Data were analyzed using SigmaStat software (SPSS Inc., Chicago, IL). One- or two-way ANOVA was used to compare responses among treatments. Treatment means were compared using Bonferroni's least significant procedure and $p < 0.05$ was considered significant.

RESULTS

Characterization of hCMEC/D3 cells exposed to amyloid beta and HIV-1

The present study was performed on confluent hCMEC/D3 cells cultures that feature a characteristic monolayer morphology with fusiform and cobblestone shaped endothelial cells as imaged by the phase-contrast microscopy (Figure 1, left panel). The experimental design required exposure to HIV-1 particles and A β (1–40) HiLite (a fluorescently labeled A β) to visualize accumulation of exogenous A β . Figure 1, right panel, confirms that such an exposure results in positive immunoreactivity for p24 as the marker of HIV-1 infection (marked in red; open arrows) and diffuse intracellular green fluorescence corresponding to intracellular A β levels (closed arrows).

HIV-1 exposure increases intracellular levels of A β in hCMEC/D3 cells

Increased perivascular A β accumulation was observed in HIVE and HAD brains (Xu and Ikezu, 2008) and HIV-1 Tat protein was demonstrated to elevate A β levels in cell culture (Rempel and Pulliam, 2005). In addition, the BBB plays a critical role in regulating A β levels in the brain (Deane and Zlokovic, 2007). Therefore, we hypothesized that exposure to HIV-1 may predispose brain endothelial cells to alterations of A β levels and thus contribute to brain amyloid pathology. Due to the pleiotrophic protective effects of statins in HIV-1-induced pathology (Gilbert et al., 2005), we also evaluated the effects of simvastatin on A β levels in hCMEC/D3 cells exposed to HIV-1.

As illustrated in Figure 2A, endogenous A β immunoreactivity exhibited weak intracellular staining patterns in control cells. However, a 24 h exposure to HIV-1 particles (p24 levels between 250 pg/ml - 1 ng/ml) markedly increased intensity of A β -positive staining.

To confirm the effects of HIV-1 on cellular A β levels, hCMEC/D3 cells were co-cultured with HIV-1-infected or control U937 cells for 24 h. Immunoprecipitation with the antibody specific to the 1–16 fragment of A β revealed several A β -specific bands of molecular weights of ~30, 37, and 65 kDa in hCMEC/D3 cells. These multiple A β immunoreactive bands may be caused by the formation of various A β oligomers as described earlier (Rangachari et al., 2007) and/or by the binding of A β to other cellular proteins. Exposure to HIV-1-infected cells increased all A β -specific bands as compared to control cultures. These changes were statistically significant as quantified by combined densitometric measurements of all A β immunoreactive bands (Figure 2B).

We also determined the effects of simvastatin on HIV-1-mediated elevation of A β immunoreactivity. Pretreatment of hCMEC/D3 cells with simvastatin for 5 h did not affect HIV-1-induced intracellular A β levels. However, a 24 h pretreatment with simvastatin appeared to decrease HIV-1-mediated an increase in A β positive staining (Figure 2C).

Our final studies in this series of experiments were to evaluate the effect of HIV-1 and/or simvastatin on A β transfer across hCMEC/D3 monolayers. Exposure of hCMEC/D3 cells to 1 μ M A β (1–40) HiLite in the presence of HIV-1 in a Transwell model system resulted in significant transendothelial transfer of A β . This effect was significantly attenuated by pretreatment with simvastatin (5 μ M) for 5 h (Figure 2D).

HIV-1-induced accumulation of exogenous A β is inhibited by simvastatin

Blood-borne A β is considered to be the main source of amyloid deposition in the brain (Clifford et al., 2007). Therefore, in the next series of experiments, we studied the combined effects of HIV-1 and exogenous A β on intracellular A β levels in hCMEC/D3 cultures. hCMEC/D3 cells were exposed to HIV-1 particles for 24 h, followed by treatment with fluorescent A β (1–40) HiLite for 10 min. Selected cultures were also pretreated with 5 μ M simvastatin for 5 h and 24 h. As shown in Figure 3A, green fluorescence originating from A β (1–40) HiLite was highly enhanced in the presence of HIV-1 as compared to control cultures treated with A β HiLite alone. Interestingly, pretreatment with simvastatin markedly diminished HIV-1-induced accumulation of fluorescently-labeled A β (1–40).

For western blot analyses, hCMEC/D3 cells were exposed to HIV-1-infected or uninfected U937 cells, pretreated with 5 μ M simvastatin for 24 h, and treated with exogenous A β (1–40) for 10 min. Consistent with data illustrated in Figure 2B, these analyses revealed several A β -specific bands that corresponded to various A β oligomers and/or the binding of A β to other cellular proteins. Treatment with exogenous A β increased A β -specific immunoreactive bands (Figure 3B). However, this effect was strongly potentiated in cultures exposed to A β in the presence of HIV-1-infected U937 cells and was related to both low and high molecular weight A β forms. The combined intensity of all A β -immunoreactive bands was significantly reduced as the result of simvastatin pretreatment (Figure 3B).

Simvastatin effectively elevates expression of the receptor for lipoprotein related protein 1 (LRP1) and attenuates HIV-1-induced RAGE overexpression

A balance between LRP1 and RAGE in the BBB was proposed to regulate A β levels in the brain (Deane et al., 2004). Therefore, we determined expression of these transporters in brain endothelial cells exposed to HIV-1 and/or simvastatin. HIV-1-mediated effects on LRP1 expression were inconsistent; however, the overall outcome of these experiments did not show significant changes as determined by immunoreactivity or western blotting analyses (Figures 4A–4C). On the other hand, treatment with 5 μ M simvastatin significantly increased LRP1 as compared to control or HIV-1-exposed cells (Figures 4B and 4C).

In the next series of experiments, we focused on the effects of HIV-1 and/or simvastatin on RAGE expression. In control cultures, RAGE immunoreactivity showed a distinct cytoplasmic staining pattern that appeared to imitate an intricate intracellular linear network. A 24 h exposure to HIV-1 particles resulted in a markedly stronger RAGE immunoreactivity with more detailed ramification of this staining pattern. Interestingly, pretreatment with simvastatin, either for 5 or 24 h, attenuated HIV-1-induced RAGE immunoreactivity (Figure 5A). Exposure of hCMEC/D3 cells to HIV-1-infected or control U937 cells also resulted in increased intensity of a double band corresponding to RAGE immunoreactivity as determined by western blotting. Importantly, pretreatment with simvastatin for 5 or 24 h significantly protected against HIV-1-induced overexpression of RAGE (Figures 5B and 5C).

To further evaluate these effects, hCMEC/D3 cells were exposed to HIV-1 particles for 4 h and RAGE mRNA levels were determined by real-time RT-PCR. As illustrated in Figure 5D, treatment with HIV-1 significantly increased RAGE mRNA levels; however, a 5 h pre-treatment with 5 μ M simvastatin protected against this induction.

RAGE is directly involved in HIV-1-induced A β accumulation in hCMEC/D3 cells

The functional role of RAGE in HIV-1-induced A β accumulation in hCMEC/D3 cells was addressed in the final series of experiments. Confluent cultures were exposed to HIV-1 particles for 24 h, followed by treatment with A β (1–40) HiLite for 10 min. In selected cultures, RAGE was inhibited by a specific neutralizing antibody (40 μ g/ml) added for the last 2 h of HIV-1 treatment. Consistent with data presented in Figure 3A, HIV-1 markedly increased A β HiLite fluorescence in cells. Pretreatment with anti-RAGE antibody protected against this effect, confirming the role of RAGE in HIV-1-induced A β accumulation (Figure 6).

DISCUSSION

Novel results of the present study demonstrated for the first time that exposure of hCMEC/D3 cells to HIV-1 or HIV-1-infected monocytic cells resulted in a significant increase in intracellular A β levels and elevated transendothelial transfer of this peptide. Accumulation of A β was potentiated by adding exogenous A β into cell culture media and was observed after a 10 min exposure to A β . Such rapid effects were probably a snapshot of the equilibrium of A β entry, degradation, and removal from endothelial cells.

The balance between the LRP1 and RAGE transport activities at the BBB level emerged as one of the primary mechanisms that regulates A β levels in the brain (Figure 7) (Deane et al., 2004). LRP1 binds A β at the abluminal side of the BBB and initiates amyloid clearance from the brain to the circulation via transcytosis across the BBB. The binding reaction is mediated by clusters II and IV, the two major binding domains present on LRP (Sagare et al., 2007). Interestingly, the binding of LRP to A β (1–40) is stronger than A β (1–42). Thus, it is important to note that the experiments presented in the current study were performed using A β (1–40); i.e., the form of A β that is mainly present in the circulation and in the cerebral vasculature. In contrast, A β (1–42) is found more frequently in senile plaques (Seubert et al., 1992). In addition to clearance from the brain, A β can be transported from the blood into the brain by the receptor for advanced glycation end products (RAGE) (Deane et al., 2003) (Donahue et al., 2006). RAGE expression in brain microvasculature is increased in AD parallel to advanced stages of the disease (Miller et al., 2008). A decreased expression of microvascular LRP1 and elevated RAGE immunoreactivity were shown in human AD brains (Donahue et al., 2006). Thus, LRP1 and RAGE were suggested as new therapeutic targets in treatment of AD (Zlokovic, 2008) (Chen et al., 2007). Soluble forms of both LRP and RAGE (sLRP and sRAGE, respectively) were shown to be highly effective in decreasing amyloid levels in the brain by binding and removing A β from the circulation (Sagare et al., 2007; Yan et al., 2000).

While the role of LRP1 and RAGE in controlling brain amyloid levels in the context of AD has been described (Zlokovic, 2008), the effects of HIV-1 on expression of these transporters constitute the novelty of the present study. Our important results demonstrate that exposure of brain endothelial cells to HIV-1 results in markedly elevated RAGE expression. RAGE is able to bind both A β monomers and fibrils (Yan et al., 1996). Thus, by stimulation of the A β transport from the circulation into the brain, HIV-1-induced upregulation of RAGE may directly contribute to increased A β levels as observed in HIV-1-infected patients. In addition, activation of RAGE was demonstrated to initiate inflammatory responses via NF- κ B activation (Schmidt et al., 1996) which may further contribute to amyloid accumulation in the brain. One of the most significant results of the present manuscript demonstrates that blocking RAGE by

a specific neutralizing antibody can protect against accumulation of exogenous A β in HIV-1-exposed hCMEC/D3 cells.

In contrast to alterations of RAGE expression, exposure to HIV-1 particles or HIV-1-infected monocytic U937 cells did not affect LRP1 levels in hCMEC/D3 cells. However, it has been described that protease inhibitors commonly used in ART can reduce LRP levels due to a 63% homology between the HIV-1 protease and the LRP (Tran et al., 2003). This fact may constitute an important mechanism involved in amyloid accumulation in HIV-1-infected patients on ART. In addition, increased brain amyloid levels may be caused by its deficient enzymatic degradation (Zlokovic et al., 2000). For instance, reduced A β degrading activity in the cerebral vessels may lead to cerebral amyloid angiopathy (Miners et al., 2008). Therefore, it is important to note that protease inhibitors used in ART may inhibit A β degradation (Hamel et al., 2006) and thus contribute to increased amyloid deposition in the brain. An earlier report also indicated that HIV-1 Tat protein can inhibit the A β degrading enzyme neprilysin resulting in elevated soluble A β in cell culture (Rempel and Pulliam, 2005). The importance of this mechanism in regulation of amyloid levels was recently confirmed by the observation that peripherally expressed neprilysin can reduce brain amyloid burden by reducing soluble brain A β peptide levels by ~30% and lowering the accumulation of A β peptides by 50–60% (Guan et al., 2009).

Neuroinflammation is another factor that can facilitate amyloid accumulation in the brain (Pulliam, 2009). This fact is important because HIV-1-related brain pathology is associated with chronic inflammatory responses. HIV-1 specific proteins, such as Tat or gp120, can also promote proinflammatory responses at the BBB level (Toborek et al., 2005) and A β by itself is a pro-inflammatory factor which can trigger chronic neuroinflammatory responses (Rogers et al., 2007) and disrupt endothelial integrity (Blanc et al., 1997). Thus, HIV-1-induced chronic inflammation and A β accumulation may cross-influence each other. It is possible that despite different pathologies, neurodegeneration and dementia in AD and HIV-associated brain disease may have common molecular mechanisms that can be linked to the induction of chronic neuroinflammatory responses.

Previous studies from our laboratory indicate that simvastatin can inhibit inflammatory responses induced by HIV-1 and A β in brain endothelial cells (András et al., 2008). The mechanisms of these effects have been linked to affecting prenylation of the small GTPases and/or altering membrane lipid raft microdomains that are crucial for cell signaling (Greenwood and Mason, 2007). In addition, direct inhibitory effects of statins on HIV-1 replication were suggested (Gilbert et al., 2005). Therefore, we evaluated the effects of simvastatin on LRP1 and RAGE expression and HIV-1-induced A β accumulation in brain endothelial cells. In the performed experiments, hCMEC/D3 cells were pretreated with simvastatin at the dose of 5 μ M for 5 or 24 h. This level of simvastatin is consistent with that reported in clinical studies. For example, administration of 15 mg/kg simvastatin to patients with multiple myeloma for 1 week resulted in the peak serum values of simvastatin ~2.7 μ M (Sondergaard et al., 2009).

While it was already reported that statins can upregulate LRP1 expression in the BBB (Zlokovic et al., 2000), novel data presented in our study demonstrate that simvastatin can elevate LRP1 levels in HIV-1-exposed hCMEC/D3 cells, suggesting that simvastatin may stimulate brain amyloid clearance across the BBB. In addition, pretreatment with simvastatin reduced RAGE expression and protected against accumulation of extracellularly administered A β in hCMEC/D3 cells exposed to HIV-1 or HIV-1-infected monocytic U937 cells. These results indicate beneficial effects of simvastatin on A β homeostasis at the BBB level. Thus, simvastatin may protect the BBB and ultimately the CNS against increased amyloid deposition associated with HIV-1 infection, chronic inflammation, and/or treatment with anti-retroviral drugs. By

decreasing RAGE levels, simvastatin may not only reduce A β transport into the brain but may also inhibit inflammatory responses induced by amyloid and RAGE (Chen et al., 2007). Several RAGE-based A β blockers are already being evaluated in clinical trials in AD (Zlokovic, 2008). In addition to pharmacological intervention, exercise was demonstrated to reduce RAGE and increase LRP1 at the BBB in an animal model of AD (Herring et al., 2008). However, it also should be noted that a recent report failed to show RAGE involvement in A β uptake in rat brain endothelial cell cultures (Yamada et al., 2008).

In summary, novel results of the present study are the first demonstration that accumulation of A β is enhanced in hCMEC/D3 cells in response to HIV-1 exposure. These alterations are associated with increased RAGE expression. Treatment with simvastatin increases LRP1, diminishes RAGE expression, and protects against HIV-induced A β accumulation. These findings contribute to a better understanding of possible mechanisms involved in amyloid accumulation in the CNS during HIV-1 infection. They also provide evidence that statins can exert beneficial effects by reducing amyloid burden in HIV-1-associated brain disease.

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Abbreviations

A β	amyloid beta
AD	Alzheimer's disease
ART	antiretroviral therapy
BBB	blood-brain barrier
CCL-2	CC chemokine ligand-2 (also called monocyte chemoattractant protein-1 [MCP-1])
CNS	central nervous system
HAND	HIV-associated neurocognitive disorders
HIV-1	human immunodeficiency virus type 1
HIVE	HIV-associated encephalitis
LRP1	receptor for lipoprotein related protein 1
NF- κ B	nuclear factor- κ B
RAGE	receptor for advanced glycation end products
TNF- α	tumor necrosis factor α

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Figure 1. Characterization of hCMEC/D3 cells exposed to amyloid beta ($A\beta$) and HIV-1
 The left panel represents a phase-contrast micrograph of a confluent hCMEC/D3 monolayer with the characteristic morphology of fusiform and cobblestone shaped endothelial cells. The right panel confirms that hCMEC/D3 cells exposed to HIV-1 and fluorescent $A\beta(1-40)$ HiLite exhibit immunoreactivity for p24 and diffuse intracellular green fluorescence corresponding to $A\beta$.

Figure 2. HIV-1 exposure increases accumulation of A β in hCMEC/D3 cells

(A) hCMEC/D3 cells cultured in chambered slides were exposed for 24 h to HIV-1 particles at the indicated p24 levels. A β levels (red staining) were assessed by immunofluorescence microscopy and DAPI staining was performed to visualize the nuclei (blue staining). (B) Immunoprecipitation followed by western blotting was performed in lysates of hCMEC/D3 cells co-cultured with HIV-1-infected or control U937 cells for 24 h. Mouse antibody against the 1–16 fragment of A β was used in these analyses. The blot is the representative image from three independent experiments. The graph represents the combined densitometric measurements of all A β immunoreactive bands. (C) hCMEC/D3 cultures were exposed for 24 h to HIV-1 particles at p24 level of 1 ng/ml. Selected cultures were pre-treated with 5 μ M simvastatin (Sim) for 5 or 24 h. A β and DAPI staining was performed as in (A). In control cultures, endogenous A β immunoreactivity exhibited a weak cytoplasmic pattern. A 24h exposure to HIV-1 resulted in a marked increase of A β immunoreactivity. Pretreatment with simvastatin for 24 h but not for 5 h appeared to attenuate HIV-1-induced A β positive staining. (D) Simvastatin (Sim) inhibits HIV-1-induced transendothelial transfer of A β . Confluent hCMEC/D3 cells cultured on Transwell filter inserts were exposed to 1 μ M A β (1–40) HiLite for 20 min in the presence of HIV-1 (p24 levels, 1 ng/ml) in the upper chamber, modeling the blood site of the BBB. A fluorescence signal of A β (1–40) HiLite was measured in the lower chamber, which corresponds to the brain side of the BBB. Selected cultures were pre-exposed to 5 μ M simvastatin for 5 h. Values are mean $\pm$ SEM, n=3–6. *Significantly different as

compared to the control group. †Values in the HIV-1+Sim group are significantly different as compared to cells exposed to HIV-1 alone.

Figure 3. Simvastatin protects against HIV-1-induced accumulation of exogenous Aβ
(A) hCMEC/D3 cells were exposed to HIV-1 particles at p24 levels of 1 ng/ml for 24 h, followed by treatment with 1 μ M fluorescently labeled A β (1–40) HiLite for the last 10 min of HIV-1 exposure. In addition, selected cultures were pre-exposed to 5 μ M simvastatin (Sim) for 5 h or 24 h. Green fluorescence corresponding to A β (1–40) HiLite accumulation was assessed by fluorescence microscopy. DAPI staining was performed to visualize the nuclei (blue staining). **(B)** hCMEC/D3 cells were exposed to HIV-1-infected or control U937 cells for 24 h and/or treated with 1 μ M A β (1–40) for 10 min. Selected cultures were also exposed to 5 μ M simvastatin for 24 h. Cellular levels of A β were analyzed by western blotting in whole cell lysates. The blot is the representative image from three independent experiments. The

graph represents the combined densitometric measurements of all A β immunoreactive bands. Values are mean $\pm$ SEM, n=3–4. *Significantly different as compared to the corresponding control group incubated with uninfected monocytes. [†]Values in the group with added simvastatin are statistically different from those without added simvastatin.

Figure 4. Simvastatin increases LRP1 expression

(A) hCMEC/D3 cultures were exposed to HIV-1 and/or simvastatin (Sim) as described in the legend to Figure 2A and LRP1 immunoreactivity was analyzed by immunofluorescence microscopy. In addition, DAPI staining was performed to visualize the nuclei (blue staining). For western blotting, hCMEC/D3 cells were exposed to HIV-1-infected or control U937 cells for 24 h and/or to simvastatin for 5 h (B) or 24 h (C). The blot is the representative image from four independent experiments. The graph represents the quantified combined data from these experiments. Values are mean $\pm$ SEM, $n=4-7$. *Significantly different as compared to the control group incubated with uninfected monocytes.

Figure 5. Simvastatin attenuates HIV-1-induced RAGE expression

(A) hCMEC/D3 cultures were exposed to HIV-1 and/or simvastatin (Sim) as described in the legend to Figure 2A and RAGE immunoreactivity was analyzed by immunofluorescence microscopy. In addition, DAPI staining was performed to visualize the nuclei (blue staining). For western blotting, hCMEC/D3 cells were exposed to HIV-1-infected or control U937 cells for 24 h and/or to simvastatin for 5 h (B) or 24 h (C). The blot is the representative image from four independent experiments. The graph represents the quantified combined data from these experiments. (D) hCMEC/D3 cells were pre-treated with 5 μM simvastatin for 5 h and exposed to HIV-1 particles for 4 h, followed by the determination of RAGE mRNA levels by real-time RT-PCR. Values are mean ± SEM, n=4–7. *Significantly different as compared to the control group incubated with uninfected monocytes. †Values in the group with added simvastatin are statistically different from those without added simvastatin.

Figure 6. RAGE is involved in HIV-1-induced Aβ accumulation

hCMEC/D3 cells cultured in chambered slides were exposed to HIV-1 particles for 24 h, followed by treatment with 1 μ M fluorescently labeled Aβ(1–40) HiLite for the last 10 min of HIV-1 exposure. In addition, RAGE neutralizing antibody (40 μ g/ml) was added to selected cultures for 2 h before termination of HIV-1 exposure. Green fluorescence corresponding to Aβ(1–40) HiLite was assessed by fluorescence microscopy. DAPI staining was performed to visualize the nuclei (blue staining). HIV-1-induced Aβ HiLite accumulation was attenuated by pretreatment with the RAGE neutralizing antibody.

Figure 7. Schematic diagram illustrating the effects of HIV-1 on A β accumulation in brain endothelial cells

Exposure to HIV-1 results in RAGE overexpression, which stimulates the transfer of A β from the blood into the brain. Simvastatin (Sim) protects against these effects. Exposure to Sim also enhances expression of LRP1, which transports A β from the brain into the bloodstream. Treatment with HIV-1 alone does not affect LRP1 expression.