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HIV-1 Reduces Aβ-Degrading Enzymatic Activities in Primary Human Mononuclear Phagocytes

Xiqian Lan,* Jiqing Xu,* Tomomi Kiyota,* Hui Peng,* Jialin C. Zheng,* and Tsuneya Ikezu*†‡

The advent and wide introduction of antiretroviral therapy has greatly improved the survival and longevity of HIV-infected patients. Unfortunately, despite antiretroviral therapy treatment, these patients are still afflicted with many complications including cognitive dysfunction. There is a growing body of reports indicating accelerated deposition of amyloid plaques, which are composed of amyloid-β peptide (Aβ), in HIV-infected brains, though how HIV viral infection precipitates Aβ accumulation is poorly understood. It is suggested that viral infection leads to increased production and impaired degradation of Aβ. Mononuclear phagocytes (macrophages and microglia) that are productively infected by HIV in brains play a pivotal role in Aβ degradation through the expression and execution of two endopeptidases, neprilysin (NEP) and insulin-degrading enzyme. In this study, we report that NEP has the dominant endopeptidase activity toward Aβ in macrophages. Further, we demonstrate that monomeric Aβ degradation by primary cultured macrophages and microglia was significantly impaired by HIV infection. This was accompanied with great reduction of NEP endopeptidase activity, which might be due to the diminished transport of NEP to the cell surface and intracellular accumulation at the endoplasmic reticulum and lysosomes. Therefore, these data suggest that malfunction of NEP in infected macrophages may contribute to acceleration of β amyloidosis in HIV-infected brains, and modulation of macrophages may be a potential preventative target of Aβ-related cognitive disorders in HIV-affected patients. The Journal of Immunology, 2011, 186: 6925–6932.
Macrophages and microglia express two major Aβ-degrading endopeptidases, namely neprilysin (NEP; also called neutral endopeptidase and enkephalinase, CD10, EC 3.4.24.15) (24, 25) and insulin (insulin-degrading enzyme [IDE], EC 3.4.24.56) (26). NEP appears to be the predominant protease that degrades Aβ in the brain (27–30). NEP is a type II membrane-bound zinc metalloendopeptidase localized primarily on the plasma membrane with its catalytic site exposed extracellularly (hence an ectopeptidase), making this peptidase a prime candidate for peptide degradation at extracellular sites of amyloid accumulation. Numerous studies have implicated NEP as a rate-limiting Aβ-degrading enzyme in the brain (25, 31, 32). Expression levels of the Aβ-binding scavenger receptors scavenger receptor A, CD36, and receptor for advanced-glycosylation end products, namely neprilysin, and matrix metalloprotease-9 are decreased 2–5-fold in microglia in the aged brain of an AD mouse compared with their littermate controls (33). NEP and IDE become inactivated and downregulated during the early stages of both AD and aging (34–36).

It was reported that HIV viral protein Tat-derived peptide inhibited NEP activity in vitro, and recombinant Tat added directly to brain cultures resulted in a 125% increase in soluble Aβ (37, 38). Nevertheless, HIV encodes at least nine proteins, so it will be more pathophysiologically relevant to examine the effect of the complete HIV viral infection on macrophage- and microglia-mediated Aβ catabolism. In this report, we examined whether Aβ clearance via degradation and the enzymatic activity of NEP in mononuclear phagocytes, both brain perivascular macrophages and microglia, is altered as a result of HIV infection. Our in vitro study demonstrates that HIV infection significantly impaired Aβ degradation in mononuclear phagocytes. Further, we confirmed that NEP exhibits the dominant endopeptidase activity toward Aβ degradation in mononuclear phagocytes. However, we found that HIV viral infection dramatically compromised NEP activity, but not NEP expression level, which was concomitant with significant reduction in the cell-surface level of NEP protein and intracellular NEP accumulation in the endoplasmic reticulum (ER) and lysosomes.

Materials and Methods

Cell culture

Isolation and cultivation of monocytes were performed as previously reported (39). Briefly, human monocytes were recovered from PBMCs of HIV-1 and hepatitis B seronegative donors after leukopheresis and purified by countercurrent centrifugal elutriation (40). Monocytes were cultured in Medium A containing DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated human serum, 2 mM l-glutamine (2 mM), gentamicin (50 μg/ml), ciprofloxacin (10 μg/ml), and M-CSF (1000 U/ml; R&D Systems, Minneapolis, MN). Monocytes were cultivated in Medium A for 7 d, allowing their differentiation into macrophages, which were then referred to as monocyte-derived macrophages (MDM). MDM were maintained in Medium B, which is Medium A without M-CSF. Human microglia were isolated following the described protocols (39, 41, 42). Fetal brain tissue (gestational age 14–16 wk) was obtained from the Birth Defects Laboratory, University of Washington (Seattle, WA), in full compliance with the ethical guidelines of the National Institutes of Health (NIH) and the Universities of Washington and Nebraska Medical Center. At least three different donors were tested for the experiments using MDM and microglia.

Viral infection

MDM cultured in plates or on coverslips (intended for cell staining) were rinsed twice with 1× PBS, then incubated for 24 h with either Medium B alone in the case of the control group or with Medium B containing HIV-1pYU-2 (titrated as 1 pg HIV p24 protein/cell; NIH AIDS Research and Reference Reagent Program, Germantown, MD) in the infected group (43). Then cells were washed three times with 1× PBS and replaced with Medium B, monitored for cytopathic effects by nuclear staining of apoptotic cells, and maintained for 3, 7, or 10 d.

Aβ degradation in MDM and microglia

Primary cultured MDM or microglia were plated onto 96-well plates. Viral infection was carried out as above. Briefly, solid Aβ peptide (Aβ 1-42; Invitrogen) was dissolved in cold hexafluoro-2-propanol (Sigma-Aldrich,
MDM at 7 dpi (Sigma–Aldrich) at 250 μM and then diluted at 10 μM in Medium B. MDM were infected with HIV-1pYu2, and at 3 dpi, the cells were incubated with 10 μM Aβ for 1 h, then fixed with freshly depolymerized 4% paraformaldehyde (PFA) for 15 min. Standard immunofluorescence was performed using anti-Aβ–specific Abs NU-2 (1:100 dilution; mouse; Invitrogen) kindly provided by Dr. William Klein, Northwestern University, Evanston, IL) or OE10 (1:1000 dilution; mouse; Covance Research Product, Princeton, NJ) and Alexa Fluor 488-conjugated anti-mouse IgG (H+L–) (Molecular Probes/Invitrogen) secondary Abs. Cells were counterstained with Hoechst 33342 (1:2000 dilution; Invitrogen) and subjected for immunofluorescence microscopy and fluorescent intensity measurements.

Cellular NEP and IDE endopeptidase activity assay
MDM maintained in Medium B for 3–7 d were rinsed twice with ice-cold PBS and then lysed in lysis buffer (20 mM Tris-Cl [pH 7.4], 0.5% Triton X-100, 10% sucrose, 1 μg/ml aprotinin, and 10 μM phenylmethylsulfonyl fluoride; all from Sigma–Aldrich). The lysates were centrifuged at 15,000 × g for 15 min at 4°C, and protein concentrations of the supernatants were measured by standard BCA assay using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Endopeptidase activities were assayed in a 96-well plate. In each well, 10 μg lystate and 10 μM fluorogenic peptide Mca-RPPGFSAFK(Dnp)-OH (R&D Systems) were mixed in reaction buffer (100 mM Tris-Cl [pH 7.5], 50 mM NaCl, and 10 μM ZnCl2) and incubated in the dark for 1 h at 37°C. Insulin (10 μM; Invitrogen), a potent competitive inhibitor of IDE toward Aβ, or thiorphan (10 μM; Sigma–Aldrich), a specific NEP inhibitor, was added to distinguish specific enzyme activities in the cells lysates. Fluorescent intensity of the cleaved fragments was measured by fluorometry with excitation at 320 nm and emission at 405 nm. IDE activity was defined as the activity sensitive to insulin inhibition, whereas NEP activity was defined as the activity sensitive to thiorphan inhibition.

Immunocapture-based NEP endopeptidase activity assay
Goat anti-human NEP Ab (2 μg/ml; AF1182; R&D Systems) diluted in sodium bicarbonate (100 mM [pH 9]) was coated on Nunc MaxiSorp 96-well plates (Thermo Scientific) overnight at 4°C. The plates were washed six times with PBS containing 0.5% Tween-20 and nonspecific binding of Ab was blocked by incubation with 1% PBS-BSA (Sigma–Aldrich) for 3 h at room temperature. Plates were then washed six times with PBS containing 0.5% Tween-20. MDM lysates (200 μg) or microglia lysates (100 μg) were added to the coated plates and incubated at 4°C overnight. After washing six times, the fluorogenic peptide (10 μM) diluted in reaction buffer (100 mM Tris-Cl [pH 7.5], 50 mM NaCl, and 10 μM ZnCl2) was added and incubated at 37°C in the dark for 1 h, then fluorescence was measured with excitation at 320 nm and emission at 405 nm.

Effect of HIV viral proteins on endopeptidase activities of recombinant IDE and NEP
HIV-1 Tat protein (catalog number 2222) and HIV-1 gp120 (catalog number 7363) were obtained from the NIH AIDS Research and Reference Reagent Program. Tat protein (0.1, 1, and 10 μg/ml) or gp120 (1, 10, and 100 μM) was mixed with 20 μg recombinant human IDE (R&D Systems) or 10 ng recombinant human NEP (R&D Systems) in reaction buffer with shaking at room temperature for 10 min. This was followed by the addition of 10 μM fluorogenic peptide as described and incubated at 37°C in the dark for 1 h. Fluorescence was measured with excitation at 320 nm and emission at 405 nm.

Immunoblotting
Macrophage lysates were prepared as mentioned above. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific). Protein (25 μg) was electrophoresed on 10% SDS-PAGE and transferred to Immuno-Blot PVDF membranes (Bio-Rad, Hercules, CA), followed by incubation in blocking buffer (5% skim milk in TBST) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary Abs against IDE (1:500 dilution; rabbit polyclonal, PC730; EMD Chemicals, Gibbstown, NJ), NEP (1:100 dilution; mouse monoclonal, NCL-CD10-270; Novoceastra/Leica Microsystems, Bannockburn, IL), and β-actin (1:40,000 dilution; mouse monoclonal, AC-15; Sigma–Aldrich). Subsequently, membranes were incubated with HRP-conjugated secondary anti-rabbit or anti-mouse secondary Abs (1:10,000 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (GE Healthcare Biosciences, Piscataway, NJ) and captured with CL-X Posure Film (Pierce). For data quantification, the films were scanned with a CanonScan 9950F scanner (Canon USA, Lake Success, NY); the acquired images were then analyzed on a Macintosh computer using the public domain NIH image program (http://rsb.info.nih.gov/nih-image/).

Immunofluorescence
MDM were seeded onto 15-mm coverslips housed in 24-well plates. Seven dpi, cells were fixed with 4% PFA (Sigma–Aldrich), and immunocytochemistry was conducted as reported (44). Briefly, to visualize plasma membrane-localized NEP, fixed cells were not permeabilized prior to incubation with blocking buffer containing 5% BSA and 5% normal donkey serum in PBS for 1 h at room temperature. Afterwards, samples were incubated overnight with p24 Ab (1:50 dilution in PBS; mouse monoclonal, M8057; Dako North America, Carpinteria, CA) and NEP Ab (1:50 dilution in PBS; goat polyclonal; R&D Systems) at 4°C, followed by washing with PBS three times. Then samples were incubated for 30 min at room temperature with appropriate fluorescent-labeled secondary Abs (for p24, Alexa Fluor 647 donkey anti-mouse IgG; for NEP, Alexa Fluor 488.
donkey anti-goat IgG) diluted 1:500 in PBS. After washing with PBS three times, cells were also incubated for 20 min at room temperature with Alexa Fluor 594-conjugated phalloidin (1:40; Invitrogen) to visualize F-actin cytoskeleton and with Hoechst 33342 (1:5000; Invitrogen) to stain nuclear DNA. Images were acquired using a laser-scanning confocal microscope (510 Meta; Carl Zeiss MicroImaging, Thornwood, NY) at an objective of ×40 and a ×2 zoom view for the interested area. To stain NEP within all cellular compartments, cell staining followed the above procedures except that cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature prior to blocking for nonspecific binding. The average fluorescence intensity for NEP, phalloidin, and Hoechst 33342 was measured per region of interest (ROI) for each group (15 fields per group), and the intensity value was normalized by the number of cells as determined by the Hoechst 33342 staining as per 100 cells. Normalized NEP and phalloidin intensity value of the same ROI was used for the calculation of NEP/phalloidin ratio for the group comparison.

For immunofluorescence of subcellular NEP, control and HIV-infected MDM on coverslips were prepared as above and fixed with 4% PFA, followed by incubation with 0.5% Triton X-100 for 15 min at room temperature for membrane permeabilization. Then cells were incubated with blocking buffer (0.1% Triton X-100, 5% BSA, and 5% normal donkey serum in PBS) for 1 h at room temperature. To locate NEP at a subcellular level, double immunofluorescence was conducted by coincubating cells with NEP Ab (1:50; goat polyclonal; R&D Systems) and an Ab for established organelle markers, heat shock protein 90 kDa (Grp94), ER marker (1:100, rabbit polyclonal, ab3674; Abcam, Cambridge, MA), lysosomal-associated membrane protein 1 (LAMP1), lysosomal marker (1:100; rabbit polyclonal, ab24170; Abcam), GM130, cis-Golgi matrix protein, and Golgi apparatus marker (1:100; rabbit monoclonal, ab52649;}

**FIGURE 5.** HIV infection did not alter the expression of IDE/NEP in MDM. Cell lysates (25 μg/lane) from MDM with and without HIV-1pYu2 infection were subjected to SDS-PAGE and immunoblotting with anti-IDE (A), anti-NEP (C), or anti-β-actin control (A, C). B and D show the quantitative comparisons of the IDE or NEP intensities between the control group and HIV group, respectively.

**FIGURE 6.** Confocal imaging of plasma membrane-localized and cellular NEPP in MDM. A, Human MDM (2 × 10⁵ cells/cover slip in 24-well plates) were infected with HIV-1pYu2 (HIV group) or with culture medium only (Control group) for 24 h. Cells were fixed with 4% PFA at 7 dpi. Immunocytochemistry was carried without permeabilization to visualize surface-localized NEP (green) or permeabilization with 0.5% Triton X-100 to stain intracellular NEP, followed by permeabilization and immunostaining of HIV-1 p24 (white), phalloidin (red), and Hoechst 33342 (blue). Scale bar, 50 μm. Average immunofluorescent intensity per ROI was quantified for phalloidin (B), NEP (C), and NEP/phalloidin (D) fluorescent intensity percent ratio (n = 15 per group). *p < 0.05, **p < 0.01 versus control group as determined by ANOVA and Tukey post hoc.
Abcam). Subsequently, cells were incubated with appropriate fluorescent-labeled secondary Abs diluted 1:500 in PBS (for NEP, Alexa Fluor 488 donkey anti-goat IgG; for all three markers, Alexa Fluor 594 donkey antirabbit IgG) for 20 min at room temperature. Nuclei were stained with Hoechst 33342 (1:1000). Images were acquired using a Zeiss 510 Meta Confocal Laser Scanning Microscope (Carl Zeiss) at an objective of ×40 and a ×2 zoom view for the interested area.

Statistical tests

Data were presented as means ± SD unless otherwise noted. All experiments were repeated at least three times with different donors, and all data were evaluated statistically by the ANOVA, followed by Newman-Keuls multiple comparison tests using software (Prism 4.0; GraphPad, La Jolla, CA). In the case of single mean comparison, data were analyzed by t test. p values <0.05 were regarded as statistically significant.

Results

HIV reduced Aβ degradation in MDM

We have previously shown that Aβ degradation was reduced in HIV-infected MDM as determined by the clearance of aggregated [125I]-Aβ (5). To understand the changes in the clearance of monomeric Aβ, we have developed an experimental model for the clearance of monomeric Aβ after phagocytosis in MDM as described previously (45). Briefly, MDM were infected with HIV-1pYu-2 for 24 h, and at 3 dpi, the cells were incubated with 10 μM monomeric Aβ42 for 1 h; 24 h later, cells were then subjected to immunofluorescence of NU-2 for Aβ and Hoechst 33342 for nuclear staining (Fig. 1A–H). The intensity of NU-2 staining was enhanced by viral infection (Fig. 1C,1G), consistent with the significant difference as determined by fluorescent intensity measurement (Fig. 1I). These data suggest that monomeric Aβ clearance is reduced in HIV-1–infected MDM.

NEP activity was inhibited by HIV infection of MDM

NEP and IDE are two extensively characterized Aβ-degrading enzymes, although their enzyme activities in human MDM have not been well characterized. We have compared the activities of these enzymes in MDM using the fluorogenic peptide Mca-RPPGFSAFK(Dnp)-OH and inhibitors for IDE and NEP (insulin and thiorphan, respectively). NEP showed ~4-fold higher endopeptidase activity than IDE in MDM at both days 3 and 7 after M-CSF treatment (Fig. 2), suggesting that NEP is a dominant Aβ-degrading enzyme during the monocytic differentiation into MDM. We next examined the effect of viral infection on NEP activity in MDM at 3, 7, and 10 dpi. NEP endopeptidase activity was significantly decreased by HIV infection by 28.6, 58.4, and 67.6% at 3, 7, and 10 dpi, respectively (Fig. 3). IDE activity was relatively very low and unchanged by viral infection (data not shown). The infection efficiency was determined by HIV-1 p24 staining of MDM postinfection (Fig. 4, green), which was 68–100% (average 89%) at 3 dpi and 100% at 7 and 10 dpi, regardless of donors.

To understand the mechanism responsible for reduced NEP activity in HIV-infected MDM, we have examined the protein expression levels of IDE and NEP by Western blotting. Neither IDE nor NEP protein levels were reduced after HIV infection (Fig. 5).
This suggests that the alteration in NEP activity is not due to the changes in its expression level, but is potentially due to its post-translational modifications or alterations in its cellular distribution. Because HIV-Tat protein has been shown to inhibit NEP activity (37), we have examined the direct effect of different doses of intact Tat protein (Tat1-72, 0.1–10 pg/ml) and gp120 (1–100 μM) on the activities of purified recombinant human NEP and IDE proteins in vitro. However, neither Tat nor gp120 protein inhibited NEP or IDE activity, ruling out the potential that these proteins directly inhibit the Aβ-degrading enzymes (data not shown).

Reduction of cell-surface NEP in HIV-infected MDM

NEP activity is sensitive to the changes of pH and has maximum activity at neutral pH, suggesting that its distribution on the cell surface is critical for its maximum activity as compared with its intracellular distribution in acidic environments, such as endosomes, lysosomes, or ER, where trafficking of extracellular peptides are negligible (46). Thus, we have examined if viral infection can alter cellular distribution of NEP and hence its activity. MDM were infected with HIV-1pYu2 and fixed for immunofluorescence of NEP under both permeabilized and nonpermeabilized conditions at 7 dpi (Fig. 6). Infection efficiency of the MDM was ~100% as determined by HIV-1 p24 staining. Using an Ab that recognizes the extracellular domain of NEP, we revealed that NEP was prominently expressed on the cell surface in the control group (Fig. 6A). However, almost no NEP was detected on the surface of MDM after viral infection (90% inhibition versus control, Fig. 6A, 6B). In contrast, cytoplasmic NEP intensity was stronger in HIV-infected MDM when compared with that of the uninfected group (247% increase versus control, Fig. 6A–D). In addition, phalloidin staining (red) representing the F-actin signal was consistent among MDM with or without infection (Fig. 5B).

To further understand the redistribution of NEP after viral infection, we performed laser-scanning confocal microscopy to detect the colocalization of NEP with organelle markers. In control uninfected MDM, intracellular NEP was mainly colocalized with the ER (GRP94), lysosomes (LAMP1), and, to a lesser extent, the Golgi apparatus (GM130) (Fig. 6). Viral infection of MDM enhanced the cytoplasmic NEP signal intensity relative to the uninfected control group, part of which shows colocalization in the ER and lysosomes (arrows in GRP94 and LAMP1 panels, Fig. 7). However, NEP was also found to accumulate in the cytoplasmic organelles other than either the ER or lysosome, presumably endosomes. These results show that HIV infection dramatically restrained the cell surface expression of NEP, resulting in accumulation of cytoplasmic NEP in the ER and lysosomes. This could be due to impaired posttranslational modification of NEP at the ER or enhanced endocytosis of cell-surface NEP to lysosome due to viral infection, leading to NEP inactivation.

HIV infection inhibited Aβ degradation in microglia

To understand if Aβ clearance is compromised by viral infection in the CNS, we have tested if HIV also inhibits degradation of monomeric Aβ in microglia. For that purpose, primary cultured human microglia were infected with HIV-1pYu2 for 3 d, followed by incubation with different doses of monomeric Aβ and immunofluorescence with anti-Aβ Ab 6E10 after 24 h incubation. Consistent with the result found in MDM, HIV infection of microglia significantly increased the fluorescence intensity of Aβ at both 1 and 5 μM doses (55 and 132% increase versus control, respectively), suggesting that reduced Aβ clearance occurs in primary microglia as well (Fig. 8A). In accord, NEP activity was also significantly reduced by viral infection at 3 dpi (12% reduction versus control, Fig. 8B), although the difference was much smaller than the one we saw in virus-infected MDM. This suggests that total NEP activity is relatively unchanged and does not directly reflect the reduced Aβ clearance in virus-infected microglia, consistent with our finding that the alteration in cellular distribution of NEP plays a role in Aβ clearance, as demonstrated in MDM. These data suggest that Aβ clearance and NEP activity are also compromised by HIV infection of human microglia.

Discussion

Understanding the regulation of amyloid clearance in the context of an HIV infection is critical for the prevention of the early onset of senile dementia in affected populations. Perivascular macrophages play an important role in the clearance of Aβ exported from the brain, which might be compromised by HIV viral infection. In this present study, the clearance of monomeric Aβ is significantly inhibited by viral infection of MDM, which express both IDE and NEP as Aβ-degrading enzymes. NEP activity is more potent than IDE activity in uninfected, differentiated MDM, and the viral infection significantly compromises the enzyme activity as determined by the virus-infected MDM lysates. Although the viral infection does not alter the cellular protein expression of NEP or IDE, it greatly reduces the cell-surface NEP level. We have shown that HIV infection induces an intracellular redistribution of NEP primarily to the ER and lysosomal compartments. An additional, intracellular compartment of NEP may also include endosomal compartments considering an internalization mechanism of the enzyme.
Because NEP is a neutral zinc-dependent metalloprotease and its activity is diminished at lower pH or in the absence of zinc (46), ER, lysosomal, or endosome-localized NEP might not have optimal endopeptidase activity. Therefore, HIV infection leading to a redistribution of NEP to intracellular compartments with lower than normal physiologically pH is likely to impair NEP enzyme activity. Indeed, redistribution of NEP to specific intracellular compartments by fusing recombinant NEP to different organelle-specific molecules results in diminished NEP activity; the wild-type NEP has the most enzyme activity, whereas the NEP fused to ER-targeting molecule demonstrated the least activity (47). Indeed, it has been reported that PBMCs from HIV-1–infected subjects have abnormal cell-surface enzyme kinetics (including NEP), and the subcellular distribution of these enzymes was also markedly changed (48). Thus, it is possible that impairment of Aβ clearance at early time points may be attributed to the reduction in cell-surface level of NEP (where NEP is situated in optimal pH).

The mechanism of virus-induced redistribution of NEP is unknown. One possibility is the endocytosis of NEP along with viral infection of MDM. However, so far, there is no specific viral capsid or envelope protein reported to interact with NEP for the induction of endocytosis. HIV gp120 had no effect on either purified NEP or IDE activity in vitro. Alternatively, NEP may be endocytosed along with viral endocytosis as a bystander membrane protein, effectively inducing the imbalance of cell-surface NEP and recycling/endocytosed NEP after viral infection. HIV-1 Tat can bind to NEP and inhibit its enzyme activity in primary cultured neurons and purified enzyme systems (37, 38). However, intact Tat protein failed to inhibit purified recombinant NEP in our system. Although there is a possibility that Tat protein is partially processed in the cells and has NEP inhibitory activity in MDM, these data suggest that the reduced NEP activity by HIV infection is not due to the direct effect of viral proteins, but could be due to their interaction with NEP for its endocytosis or impairment of posttranslational modification of NEP as part of viral-suppression mechanisms.

Some posttranslational modification of NEP can also be altered by viral infection. An alternative mechanism affecting NEP surface expression is glycosylation, which plays a critical role in regulating transport of NEP to the cell surface as well as the enzymatic activity of NEP (46). Decreased glycosylation results in reduced transport of NEP to the cell surface; thus, HIV infection may somehow inhibit the glycosylation level of NEP. Unfortunately, the large bands of the NEP on the immunoblotting data do not allow us to observe m.w. modifications, precluding from the conclusion. Another possibility lies in that HIV infection enhances endocytosis by viral infection and entry, and this may then lead to the enhanced relocation of NEP into endosomes (49). However, the detailed mechanisms responsible for disrupting NEP translocation from the plasma membrane by HIV infection remain to be elucidated in future studies.

One caveat of this NEP redistribution theory is that it does not explain the mechanism of reduced NEP activity in the virus-infected MDM lysate, which is assayed at an optimum pH condition (pH 7.5). HIV-infected cells are in a cellular redox state, which is closely linked to an increased level of ceramide, sphingomyelin, and 4-hydroxynonenal. These oxidative stress markers were increased in HIV encephalitis (48, 49). It was shown that NEP is modified by 4-hydroxynonenal adducts, resulting in decreased activity in the brain of AD patients and cultured cells (50–52). Thus, IDE and NEP may undergo oxidation-mediated modifications after viral infection and are therefore inactivated.

In addition, HIV viral infection may also compromise activity of NEP and IDE by virtue of deregulating apolipoprotein E (ApoE), which plays a role in facilitating the proteolytic clearance of soluble Aβ from the brain by NEP and IDE (53). It was reported that in HIV-infected brains, binding of viral Tat protein to low-density lipoprotein receptor-related protein resulted in substantial inhibition of neuronal binding, uptake, and degradation of physiological ligands for low-density lipoprotein receptor-related protein such as ApoE (54). Hence, it would be beneficial to examine if there is an alteration of ApoE levels in HIV-infected brains. Additionally, brain ApoE expression may be reduced by HIV infection because it has been shown that HIV downregulates renal ApoE expression (55).

Interestingly, antioxidant or anti-inflammatory supplements could potentially alleviate the oxidation-mediated impairment of NEP activity and enhance Aβ clearance in HIV-afflicted brains. Indeed, it has previously been shown that simvastatin, an anti-inflammatory compound, can attenuate virus-induced Aβ accumulation in brain endothelial cells (56). Taken together, restoration of NEP activity in the brain at the early stages of HIV-associated dementia may represent an effective strategy to prevent or attenuate disease progression.

Finally, it should be noted that viral infection of mononuclear phagocytes induces innate immunity responses, which are characteristic proinflammatory activation. This leads to enhanced production of proinflammatory cytokines, such as IFN-γ and TNF-α, which enhances production of Aβ through upregulation of APP and β-site APP-converting enzyme 1 from neurons and astrocytes (23). Proinflammatory cytokines also potently suppress Aβ clearance from human mononuclear phagocytes via downregulation of Aβ-degrading enzymes and chaperone molecules involved in protein refolding (39). Thus, bystander effect of brain parenchyma activation should be taken into account for understanding the overall mechanism of β-amyloidosis in brain.

In summary, we demonstrate that monomeric Aβ degradation is reduced by HIV infection of human MDM as early as 3 dpi. This is accompanied by redistribution of cell-surface NEP to intracellular compartments at 3 dpi and reduction in NEP activity in cell lysates at 7 dpi without alteration of cellular expression levels of NEP or IDE. Viral Tat or pg120 has no effect on purified IDE or NEP enzyme activity in vitro. Comparable results are also found in human primary cultured microglia, suggesting that viral infection also compromises microglial Aβ clearance in the brain. These data suggest that modulation of amyloid clearance by peripheral macrophages is a potential preventative target of Aβ-related cognitive disorders in affected populations.

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Disclosures
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