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ORIGINAL ARTICLE

Increased Accumulation of Intraneuronal Amyloid β in HIV-Infected Patients

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Abstract In recent years, human immunodeficiency virus (HIV)-infected patients under highly active anti-retroviral therapy (HAART) regimens have shown a markedly improved general clinical status; however, the prevalence of mild cognitive disorders has increased. We propose that increased longevity with HIV-mediated chronic inflammation combined with the secondary effects of HAART may increase the risk of early brain aging as shown by intraneuronal accumulation of abnormal protein aggregates like amyloid β (Aβ), which might participate in worsening the neurodegenerative process and cognitive impairment in older patients with HIV. For this purpose, levels and distribution of Aβ immunoreactivity were analyzed in the frontal cortex of 43 patients with HIV (ages 38–60) and HIV− age-matched controls. Subcellular localization of the Aβ-immunoreactive material was analyzed by double labeling and confocal microscopy and by immunono-electron microscopy (EM). Compared to HIV− cases, in HIV+ cases, there was abundant intracellular Aβ immunostaining in pyramidal neurons and along axonal tracts. Cases with HIV encephalitis (HIVE) had higher levels of intraneuronal Aβ immunoreactivity compared to HIV+ cases with no HIVE. Moreover, levels of intracellular Aβ correlated with age in the group with HIVE. Double-labeling analysis showed that the Aβ-immunoreactive granules in the neurons co-localized with lysosomal markers such as cathepsin-D and LC3. Ultrastructural analysis by immuno-EM has confirmed that in these cases, intracellular Aβ was often found in structures displaying morphology similar to autophagosomes. These findings suggest that long-term survival with HIV might interfere with clearance of proteins such as Aβ and worsen neuronal damage and cognitive impairment in this population.

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Introduction

In the age of highly active antiretroviral therapy (HAART), the survival of patients with AIDS has increased, and the proportion of HIV patients over the age of 50 has become significant. A recent study (Valcour et al. 2004a) found that individuals over 50 years of age represent 11% of the HIV cases registered by the Centers for Disease Control and Prevention (CDC). Data from the Hawaii cohort suggest an association between increased neurocognitive impairment in older HIV patients compared with younger controls (Valcour et al. 2004a, b, c, 2005; Valcour and Shiramizu 2004). Moreover, in a study in Western Pennsylvania (Becker et al. 2004), the prevalence of cognitive disorder among HIV-positive patients over 50 years of age was significantly higher than in younger individuals. Factors leading to this association may include vascular pathology, age-related immunological changes, and limited compensatory brain capacity. This suggests that chronic HIV infection and aging have similar effects on global cognitive functioning. Progressive brain dysfunction associated with aging has been linked to synapto-dendritic damage, reduced neurotransmitter production, and increased neuro-inflammatory reactivity associated with formation of radical oxygen species (Agrawal et al. 2006; Platenik et al. 2001; Price et al. 2006; Reynolds et al. 2007; Visalli et al. 2007).

Also contributing to the neuronal injury in older HIV patients might be the chronic permanence of HIV in the CNS. The HIV reservoir in the brain is known to be more difficult to reach because of the limited penetrance of HAART, emergence of resistant species, and viral escape (Dunfee et al. 2006; Langford et al. 2004).

Thus, despite the beneficial effects of HAART at suppressing viral load peripherally, the continued prevalence of moderate neurocognitive alterations in patients with HIV (Ances et al. 2008; Tozzi et al. 2007) might be related to the persistence of HIV in the CNS. Therefore, HIV encephalitis (HIVE) has shifted from a subacute neuro-inflammatory condition with abundant multinucleated giant cells to a more chronic and protracted condition with moderate astrogliosis, microgliosis, and viral load but more extensive neurodegeneration.

HAART has modified the patterns of HIV-related neuropathology and clinical manifestations in the past 10 years with the emergence of new variants of HIV encephalopathy (Everall et al. 2006). A paramount co-pathogenic factor in the long-term survival of patients with HIV on HAART is age. During aging, there is a failure in the handling and clearance of misfolded proteins such as Aβ and α-synuclein. Misfolded proteins can be cleared via lysosomal (e.g., autophagy) and non-lysosomal pathways (e.g., proteasome; Williams et al. 2006). In Alzheimer's disease (AD) and other neurodegenerative disorders, this is manifested by increased accumulation of Aβ into toxic oligomers (Barghorn et al. 2005; Deshpande et al. 2006; Glabe 2008; Shin et al. 2008; Standridge 2006; Tsigelny et al. 2008; Watson et al. 2005; White et al. 2005; Wisniewski and Konietzko 2008; Yoshiike et al. 2003; Selkoe 2008). Recent studies have suggested that in addition to the extracellular deposition of Aβ, in AD, a smaller but significant fraction might accumulate intraneuronally (Arvanitis et al. 2007; Cuello 2005; Cuello and Canneva 2008; Echeverria and Cuello 2002; Grant et al. 2000; Green et al. 2005). Previous studies have shown that in aged HIV patients, there is increased amyloid deposition and plaque formation (Daily et al. 2006; Green et al. 2005; Rempel and Pulliam 2005). However, it was unclear what is the earliest site for Aβ accumulation in patients with HIV and how this might be related to the alterations in protein handling during aging. For this purpose, patterns of intracellular Aβ accumulation and its relationship with lysosomal clearance were analyzed in HIV cases ranging in age from 38–60 years old. Our results indicate that in this population, there is increased intraneuronal Aβ accumulation in lysosomal–autophagic structures. These findings suggest that longer-term survival with HIV might interfere with clearance of proteins such as Aβ that might worsen neuronal damage and cognitive impairment in this population.

Materials and methods

Subjects and neuropathological assessment For the present study, we included a total of 43 HIV+ cases from the HIV Neurobehavioral Research Center and California NeuroAIDS Tissue Network at the University of California San Diego. Cases had neuromedical and neuropsychological examinations within a median of 12 months before death. Most cases died as a result of acute bronchopneumonia or septicemia, and autopsy was performed within 24 h of death. Autopsy findings were consistent with AIDS, and the associated pathology was most frequently due to systemic CMV, Kaposi sarcoma, and liver disease. Subjects were excluded if they had a history of CNS opportunistic infections or non-HIV-related developmental, neurologic, psychiatric, or metabolic conditions that might affect CNS functioning (e.g., loss of consciousness exceeding 30 min, psychosis, substance dependence). In all cases, neuropathological assessment was performed in paraffin sections from the frontal, parietal, temporal cortices, hippocampus, basal ganglia, and brainstem stained with H&E or immunolabeled with antibodies against p24 and GFAP (Achim

et al. 1993; Masliah et al. 1992). The diagnosis of HIVE was bases on the presence of microglial nodules, astrogliosis, HIV p24-positive cells, and myelin pallor. Additional analysis was performed with a subset of five age-matched (non-HIV) cases from the UCSD—Medical center Autopsy Service.

Immunocytochemical analysis and image analysis To further evaluate the patterns of Aβ immunoreactivity in the brains of patients with HIV, briefly as previously described (Rockenstein et al. 2005) vibratome sections from the mid-frontal cortex (40 μm thick) were incubated overnight at 4°C with the mouse monoclonal antibody against Aβ (clone 4G8; 1:600, Senetek, Napa, CA, USA) or with the antibody against the N terminus of Aβ (aa 1–16; clone 82E1; Immuno-Biological Laboratories, Gunma, Japan) followed by incubation with secondary biotinylated anti-mouse IgG, followed by ABC and DAB. Sections were transferred to SuperFrost slides (Fisher Scientific, Tustin, CA, USA) and mounted under glass coverslips with anti-fading media (Vector Laboratories, Burlingame, CA, USA). All sections were processed under the same standardized conditions. From each case, an average of 50 neurons were imaged, and digital files were analyzed with the Image Quant system. Three immunolabeled sections were analyzed per case, and the average of individual measurements was used to calculate group means. Additional confirmation of the intracellular amyloid labeling was performed utilizing the Thioflavine-S staining in the vibratome sections (Rockenstein et al. 2001).

Double immunolabeling and confocal laser microscopy To evaluate the co-localization between Aβ immunoreactivity and lysosomal markers, double immunocytochemical analysis was performed (Belinson et al. 2008). For this purpose, vibratome sections were immunolabeled with a monoclonal antibody against Aβ (clone 4G8; 1:600, Senetek, Napa, CA, USA) detected with FITC-conjugated secondary antibodies (1:75, Vector Laboratories) and the antibodies against cathepsin-D, Lamp2, and Rab5 (1:500, Dako, Carpinteria, CA, USA) detected with the Tyramide Signal Amplification™-Direct (Red) system (1:100, NEN Life Sciences, Boston, MA, USA; Pickford et al. 2008). All sections were processed simultaneously under the same conditions, and experiments were performed twice to assess reproducibility. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss, Germany) with an attached MRC1024 LSCM system (BioRad). To confirm the specificity of primary antibodies, control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted) or preimmune serum and primary antibody alone.

Immunoelectron microscopy analysis Briefly, vibratome sections immunostained as described above with the 4G8 antibody or a nonimmune IgG were fixed in 0.25% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and then pre-embedded with 50% Durcupan epoxy resin, and 50% ethanol (dry) for 30 min. Samples were then embedded in Durcupan mix epoxy resin and polymerized under a vacuum at 60°C for 48 h. After the resin was polymerized, tissues were mounted into plastic cylinders, sectioned with an ultra microtome (Reichert Ultracut E) at 60 nm thickness and collected in copper grids for ultrastructural analysis. The immunostained grids were post-stained using saturated uranyl acetate solution in 50% ethanol for 20 min at room temperature, washed in distilled water, and placed in bismuth nitrate solution for 10 min followed by a final wash in deionized water. The immunolabeled grids were analyzed with a Zeiss EM10 electron microscope and electron micrographs obtained at a magnification of 35,000.

Statistical analysis All the analyses were conducted on blind-coded samples. After the results were obtained, the code was broken, and data were analyzed with the StatView program (SAS Institute, Cary, NC, USA). Comparisons among groups were performed with unpaired Student's *T* test, Chi square analysis and simple linear regression analysis. All results were expressed as mean±SEM.

Results

Intraneuronal accumulation of Aβ in HIV patients A total of 48 cases were included, of which 43 were HIV seropositive, and five were HIV seronegative (Table 1). The age

Table 1 Summary of demographic and pathological findings

Group	Number (*N*)	Gender M/F	Risk factor MM/DU/other	Age mean (years)	Age range (years)	Postmortem interval (h)	Cases with intracellular Aβ	Cases with amyloid plaques
HIV+, no HIVE	18	15/3	12/5/1	49±1.5	38–60	18±2	7/18 (38%)	1/18 (5%)
HIV+ Yes HIVE	25	21/4	16/9/0	47±1	39–57	19±2	18/25 (72%)	2/25 (8%)
HIV-	5	4/1	0/1/4	47±2	38–55	15±2	0/0	0/0

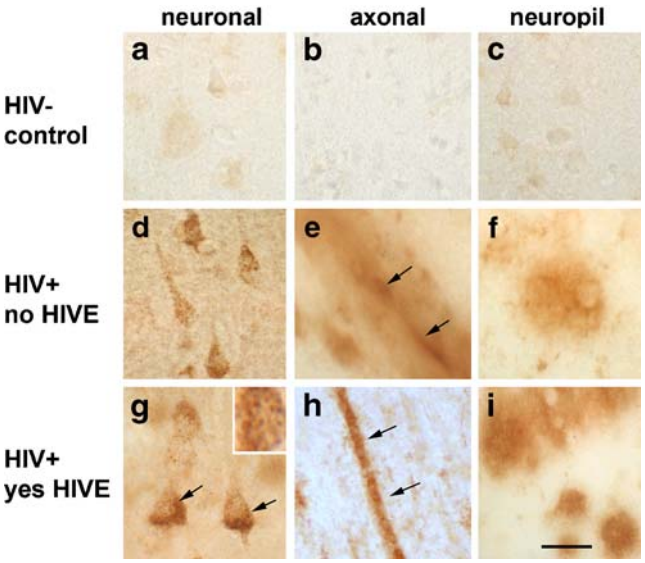

Fig. 1 Patterns of Aβ immunoreactivity in control and HIV+ cases. Panels are from the frontal cortex immunostained with the monoclonal antibody 4G8. **a**–**c** In an age-matched control HIV− case (42 year old) the neuronal cell bodies (**a**), axons in the white matter (**b**), and neuropil (**c**) are devoid of amyloid deposits. **d**–**f** Examples of intraneuronal (**d**) granular Aβ immunoreactivity in an older HIV+ case (47 year old). Aβ deposits can be found in axons (**e**) and in the neuropil (**f**) as diffuse plaques. **g**–**i** Examples of intraneuronal (**g**) and axonal (**h**) Aβ immunoreactivity (*arrows*) in an older HIV+ case (50 year old) with HIVE. The *inset* shows in greater detail the punctate appearance of the intraneuronal Aβ immunostaining. Diffuse amyloid plaques (**i**) were also detected in a few cases. Bar=10 μm

range varied between 38 and 60 years with a mean of 48± 2 years. Of the 43 HIV cases, 18 had no significant opportunistic infections or HIVE, and the other 25 had HIVE. Immunocytochemical analysis with the antibody against Aβ (4G8 clone) showed that compared to HIV− controls (Fig. 1A–C), in seven out of 18 HIV+ cases (38%) with no HIVE, there was intraneuronal immunolabeling (Fig. 1D). In contrast, in cases with HIVE, intraneuronal Aβ immunoreactivity was observed in 18 out of the 25 cases (72%; Fig. 1G). This difference was significant by Chi square analysis (X^2=4.7, p=0.029). The Aβ immunostaining was observed in pyramidal neurons in layers 2–3 and 5, had a granular cytoplasmic appearance that was concentrated in the neuronal cell body (Figs. 1D, G; 2B–E), although in some cases, extended to the axons (Fig. 1E, H) and dendrites (Fig. 2D). In the five control cases, there was very low or no detectable intracellular Aβ immunostaining (Figs. 1A–C, 2E). Image analysis of the levels of intracellular Aβ immunostaining in the individual cases showed significantly higher levels in the HIVE compared to HIV+ cases without HIVE (*t* test, p=0.005; Fig. 2). In one of the HIV+ cases with no apparent HIVE (Fig. 1F) and in two of the cases with HIVE, there was evidence of extracellular Aβ deposition (Fig. 1I). The plaques had a diffuse appearance and in some cases surrounded neuronal cell bodies or were observed along axonal tracts (Fig. 3A–C). Although these diffuse plaques were similar to those observed in AD, no abundant neuritic plaques or tangles were detected, thus ruling out the possibility of AD in these cases. Similar results were observed with the antibody against the N terminus of Aβ (82E1 clone) and with thioflavine-S (Fig. 3D–F). Linear regression analysis showed that there was a significant correlation between the levels of intracellular Aβ immunoreactivity and age in the HIV+ group with HIVE (Fig. 4A), but no correlation was observed in the HIV+ group with no HIVE (Fig. 4 B).

Co-localization of lysosomal markers with the intraneuronal Aβ in the brains of HIV patients Given the punctate cytoplasmic characteristics of the intraneuronal Aβ immunoreactivity in the HIV cases and that previous studies have suggested in experimental models that this might be localized

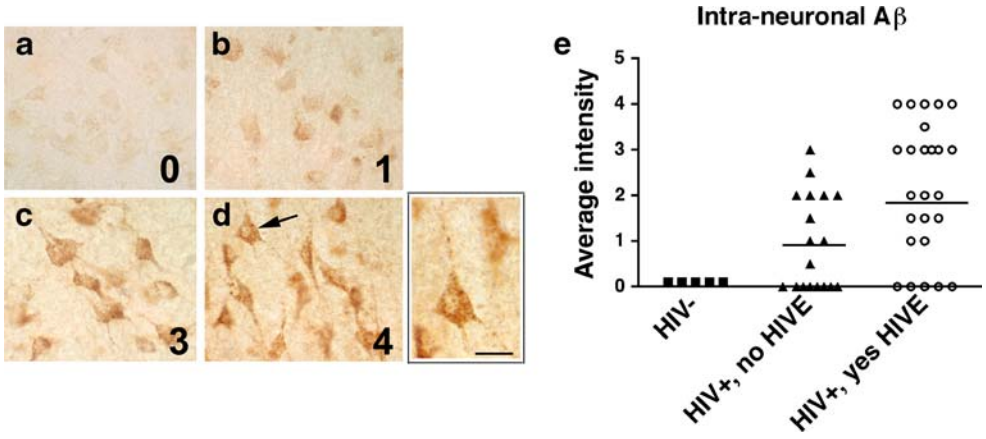

Fig. 2 Levels of intraneuronal Aβ immunoreactivity in older HIV+ cases. Images are from the frontal cortex immunostained with the monoclonal antibody 4G8. **a**–**d** Examples of the various levels (0–4) of intraneuronal Aβ immunoreactivity in HIV+ cases; the *arrow* indicates the enlarged image to the left displaying punctate appearance. **e** Compared to HIV+ with no HIVE, in cases with HIVE, there was an increase in the levels of intraneuronal Aβ immunoreactivity. Bar=5 μm

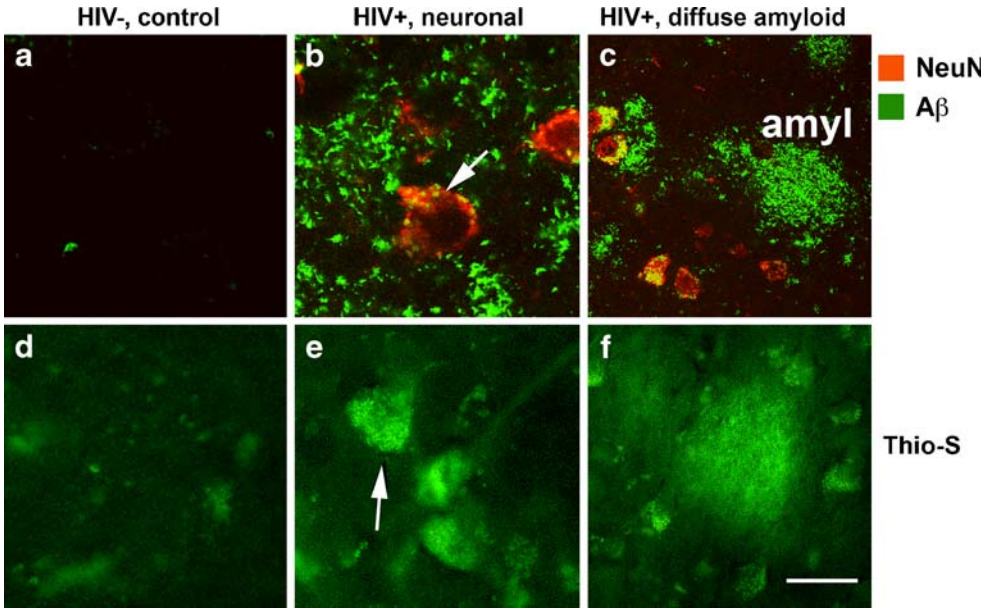

Fig. 3 Laser confocal microscopy imaging of the amyloid deposits in HIV+ cases. Examples are from the frontal cortex. **a** No evidence of amyloid deposits in HIV− age-matched control; **b**, **c** double-labeling with antibodies against the neuronal markers NeuN (*red*) and Aβ (*green*) showing neuronal amyloid deposits (**b**, *arrows*) and diffuse plaques (**c**, amyl) in HIV+ cases. **d**–**f** Comparative images in HIV− (**d**) and HIV+ (**e**, **f**) cases stained with thioflavine S. Bar=10 μm

in lysosomal structures, double labeling studies were performed. By confocal microscopy, the Aβ-immunoreactive structures co-localized with the lysosomal markers cathepsin-D (Fig. 5D–F) and LAMP2 (Fig. 5 G–I). Moreover, the Aβ-immunolabeled granular bodies co-localized with the autophagy marker LC3 (Fig. 5J–L). Overall, these granular structures were enlarged and clustered; in contrast, in control cases, discrete cathepsin-D lysosomal structures were identified that showed no Aβ immunoreactivity (Fig. 5A–C).

To further investigate the intracellular localization of Aβ, immunolabeled vibratome sections were analyzed by electron microscopy. Compared to control cases (Fig. 6A), in the HIV cases with intraneuronal Aβ immunoreactivity, aggregates were observed associated with structures reminiscent of lysosomes and autophagolysosomes in the cell body and in axonal structures (Fig. 6B, C).

Discussion

The present study showed that in HIV patients, there is a prominent intraneuronal accumulation of Aβ associated with lysosomal structures. The intraneuronal Aβ was more abundant in patients with HIVE compared to HIV cases with no significant pathology, and in the group with HIVE, the levels of intracellular Aβ increased with age. These findings suggest that long-term survival with HIV might interfere with clearance of proteins such as Aβ that might worsen neuronal damage and cognitive impairment in this population. Previous studies have shown in older HIV+ patients the presence of extracellular amyloid deposits similar to those detected in patients with AD (Green et al. 2005; Rempel and Pulliam 2005).

The first study to identify changes similar to mild AD was reported by Esiri et al. (1998) who found Aβ plaques in HIV patients by using argyrophilic and thioflavine stainings. Another study using a more comprehensive methodology that included staining with the 4G8 antibody, Congo red, and Thioflavine-S (Izycka-Swieszewska et al. 2000) showed that in 15 AIDS cases (five with HIVE), three had perivascular plaques positive for 4G8 but not Congo Red or Thioflavine-S. Based on these findings, the authors concluded that the neurodegeneration associated with HIV infection could be primarily of vascular origin. Our results suggest that while

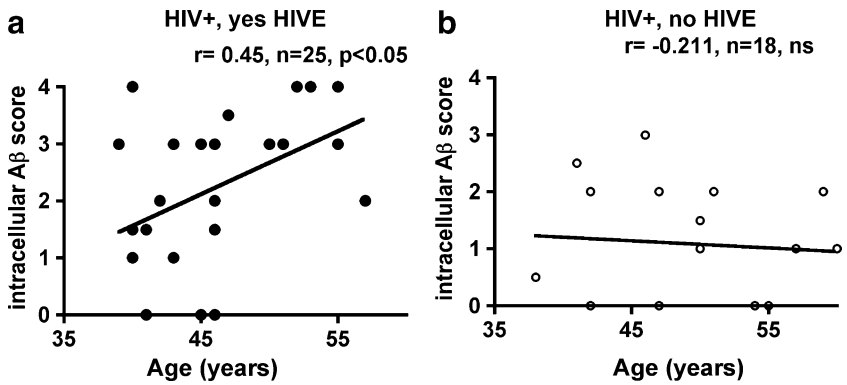

Fig. 4 Linear regression analysis between intracellular Aβ and age. **a** In cases with HIVE, there was a significant correlation. **b** In cases with no HIVE, there was no significant correlation

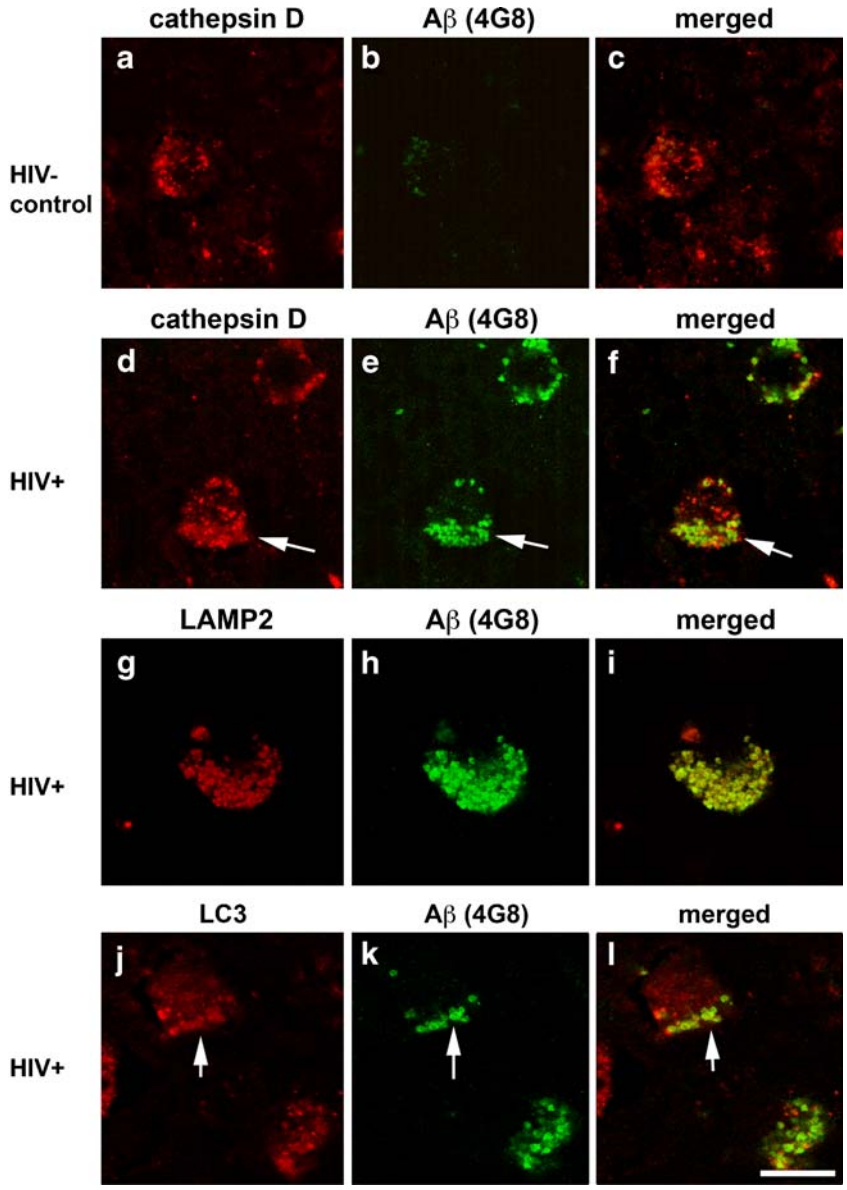

Fig. 5 Laser confocal microscopy imaging of the intraneuronal Aβ and lysosomal markers in older HIV+ cases. Sections are from the frontal cortex double labeled with the Aβ (4G8) antibody (*green*) and lysosomal proteins cathepsin-D, LAMP2, and LC3 (*red*). **a**–**c** HIV− control case. **d**–**l** Co-localization (*arrows*) of intraneuronal Aβ and cathepsin-D (**d**–**f**), LAMP2 (**g**–**i**), or LC3 (**j**–**l**) in an HIV+ case. Bar=5 μm

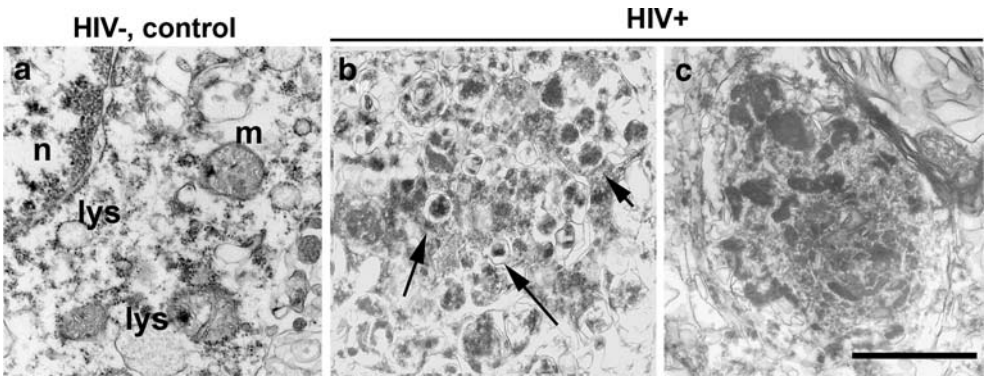

Fig. 6 Ultrastructural analysis of the Aβ deposits in HIV+ cases. Sections from the frontal cortex were immunostained pre-embedding with 4G8 and analyzed with the electron microscope. **a** In a control, HIV− case, evidence of preservation of neuronal organelles such as lysosomes (lys) and mitochondria (m). **b** In an HIV+ case, abnormal electrodense membrane-bounded organelles (*arrows*) displaying Aβ immunoreactivity accumulate in the neuronal cell body and axons. **c** Additional view of abnormal electrodense membrane-bounded organelles displaying intraneuronal Aβ immunoreactivity in an HIV+ case. Bar=2 μm

the vascular hypothesis may explain some of the plaques seen in the AIDS brains, given the intraneuronal accumulation and the neuronal origin of Aβ, this supports a possible cerebral source of the parenchymal amyloid.

Aβ is the result of the proteolytic processing of APP by β- and γ-secretases (Findeis 2007; Ghosh et al. 2005; Hoe and Rebeck 2008; Postina 2008; Russo et al. 2005; Thinakaran and Koo 2008; Vetrivel and Thinakaran 2006; Walsh et al. 2007; Wolfe 2006, 2008a, b). APP is ubiquitously distributed; in the CNS, APP is most abundantly produced by neurons and astroglial cells (Schmechel et al. 1988). Interestingly, the initial studies in the CNS of patients with HIV have focused on the levels of APP as a marker of neuronal injury. Several reports have described a significant increase in brain APP in AIDS cases, specifically in the axons of the subcortical white matter tracts. One leading theory is based on the inflammatory response in HIV infection of the brain parenchyma, where activated microglia are considered by many to be the likely source of mediators of disease that can promote overproduction and accumulation of APP (Adle-Biassette et al. 1999; Dickson et al. 1993). Among the brain macrophage-secreted factors mediating the overproduction of neuronal APP, a leading candidate is IL-1 (Stanley et al. 1994).

The association between the brain pathology characteristic of HIVE, including viral proteins and the presence of APP aggregates, often as intra-axonal globules, was reported in several studies (Giometto et al. 1997; Nebuloni et al. 2001; Raja et al. 1997). These results were further confirmed in the simian immunodeficiency virus (SIV) model of HIVE where Mankowski et al. have also found APP accumulation in degenerating axons (Mankowski et al. 2002). While the majority of these studies show a strong correlation between the topography of HIV-associated pathology and degenerating axons, suggesting a more local, intraparenchymal effect on APP accumulation, some investigators disagree. In one of the first studies of APP in the HIV brain, Scaravilli and colleagues (An et al. 1997) did not find a good correlation between APP and microgliosis and concluded that the axonal degeneration in HIV infection, may be due to systemic factors (e.g. cytokines).

The mechanisms through which HIV infection in the CNS might lead to increased accumulation of Aβ are not completely clear. Recent studies suggest that HAART resistance, chronic HIV infection, HAART toxicity and reduced clearance of misfolded proteins might play a role (Hult et al. 2008). Aβ is degraded by proteases such as neprilysin, insulin-degrading enzyme, and endothelin (Eckman and Eckman 2005; Miners et al. 2008; Nalivaeva et al. 2008; Wang et al. 2006). The HIV Tat protein has been shown to interfere with neprilysin activity, and HAART has been shown to alter the metabolic routes for Aβ and insulin processing (Daily et al. 2006; Nath and Hersh 2005; Rempel and Pulliam 2005). Neprilysin is not only capable of degrading extracellular but also intracellular Aβ (Spencer et al. 2008). While the clinical benefit in reducing viral burden is indisputable, HAART is also reported to induce metabolic dysregulation resulting in a syndrome of lipodystrophy (LD) in up to 83% of treated individuals (Carr et al. 1999). Features associated with LD include insulin resistance with hyperinsulinemia, centripetal lipohypertrophy with concurrent subcutaneous peripheral lipoatrophy, and hypertriglyceridemia. The metabolic complications underlying LD include insulin resistance.

Another important mechanism involved in the clearance of Aβ is the authophagy pathway (Jellinger 2006; Jellinger and Stadelmann 2001; Li et al. 2007; Lunemann et al. 2007; Nixon 2007; Shacka et al. 2008; Wilson et al. 2004; Yu et al. 2004, 2005; Zheng et al. 2006). This lysosomal pathway has been shown to be defective in patients with HIV (Delhaye et al. 2007; Fredericksen et al. 2002; Levine and Sodora 2006; Talloczy et al. 2008), and recent studies in AD patients have shown that alterations in autophagy leads to increased intracellular and extracellular Aβ (Pickford et al. 2008). Consistent with this possibility, in the present study, we showed the intracellular Aβ co-localized with lysosomal markers and accumulated in structures reminiscent of lysosomes and abnormal autophagosomes.

In summary, in HIV patients, there is extensive intraneuronal accumulation of Aβ suggesting that long-term survival with HIV might interfere with clearance of proteins such as Aβ that might worsen neuronal damage and cognitive impairment in this population.

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