Journal of NeuroVirology, 10: 98–108, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490279816

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Brain aging in acquired immunodeficiency syndrome: Increased ubiquitin-protein conjugate is correlated with decreased synaptic protein but not amyloid plaque accumulation

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Two neuropathological changes that are linked with biological and pathological aging were examined in subjects with end-stage acquired immunodeficiency syndrome (AIDS). Autopsy brain specimens were examined from 25 people who died from complications of AIDS and 25 comparison subjects who were human immunodeficiency virus (HIV)-negative, matched for age, gender, ethnicity, and postmortem time interval. These adults were stratified into three age groups: elderly (62 to 75 years), intermediate (55 to 60 years), and young (21 to 42 years). Ubiquitin-stained dotlike deposits (Ub-dots) and diffuse extracellular plaques containing the beta-amyloid (Aβ) fragment of the amyloid precursor protein (Aβ plaque) were both increased significantly in the hippocampal formation of older subjects. In subjects with AIDS, Ub-dots were increased whereas Aβ plaque counts were not significantly different. Western blotting confirmed that high-molecular-weight ubiquitin-protein conjugates (HMW-Ub-conj) were increased in AIDS. The band intensity of one HMW-Ub-conj species with an approximate molecular mass of 145 kDa was correlated significantly with increased acute phase inflammatory protein (α-1-antichymotrypsin) and decreased synaptophysin and growth-associated protein-43 band intensities. These results raise the possibility that HIV-related brain inflammation disturbs neuronal protein turnover through the ubiquitin-proteasome apparatus, and might increase the prevalence of age-associated neurodegenerative diseases by decreasing synaptic protein turnover through the proteasome. *Journal of NeuroVirology* (2004) **10,** 98–108.

Keywords: amyloid precursor protein; autopsy; diffuse plaque; growth associated protein-43, HIV encephalitis; proteasome; synaptophysin; ubiquitin; Western blotting

Introduction

The acquired immunodeficiency syndrome (AIDS) epidemic began as a fatal disease of mostly young adults that often caused human immunodeficiency virus (HIV)-associated dementia (HAD). Most neurological and neuropathological data concerning HAD were collected from young adults or children at the start of the AIDS pandemic (Gelman *et al*, 1996; Navia *et al*, 1986; Petito, 1993). Those studies established that AIDS is associated with a high risk of neurocognitive dysfunction and dementia in relatively young adults and children. In many, but not all demented subjects, HIV encephalitis was present at autopsy (Wiley and Achim, 1994). When highly active antiretroviral therapy (HAART) was introduced about 13 years after the beginning of the epidemic (Sacktor *et al*, 2001), it suppressed virus replication in most infected people and prolonged life span (Hogg *et al*, 1997). As the mortality rate of HIV infection has declined, there is a steady increase in the number of

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The technical assistance of Monica Rodriguez-Wolf is acknowledged.

This work was supported by the National Institutes of Health R24-MH59656, R24-NS45491, R01-DC04749, and R01-MH69200.

Received 17 September 2003; revised 22 October 2003; accepted 4 November 2003.

older subjects who harbor persistent HIV infection. Thus, the average age of HIV-infected decedents at autopsy in the HAART era is increasing slowly (Gray *et al*, 2003; Jellinger *et al*, 2000; Masliah *et al*, 2000; Morgello *et al*, 2002). In the short term, there is evidence that HAART reverses neurocognitive deficits and dementia in many nonelderly adults with HIV infection (Chang *et al*, 1999; Dore *et al*, 1999; Dougherty *et al*, 2002; Sacktor *et al*, 1999, 2000). However, the lasting effect of HIV on brain function in elderly people remains unknown (Dore *et al*, 1999; Tozzi *et al*, 2001). Aging increases the risks of neurodegenerative disease and dementia independently (Band *et al*, 2002), and combined with concomitant HIV infection, those risks could increase still more. To meet the potential challenge of increased neurocognitive disability in elderly people with HIV infection, HIV-associated neurodegeneration needs to be evaluated for potential synergy with pathological and biological brain aging. Neuropathological analysis of brain tissue from elderly subjects is needed in order to establish a relevant basic research agenda for the future. The resources needed to study brain aging in AIDS remain very scarce because the average age at death remains relatively young (about 42 years old). The present study examined age-related neuropathological changes in people who died of AIDS at relatively older ages in the pre-HAART era of the AIDS epidemic. The goal was to determine whether neuropathological changes associated with brain aging were more pronounced in the most elderly people who died of AIDS.

Results

Ubiquitin-stained structures were observed in temporal lobe white matter that were identical to the uniformly distributed dotlike structures that were described previously in aging human brain (Figure 1A–C) (Dickson *et al*, 1990; Pappolla *et al*, 1989). In the oldest group, 90% had more than

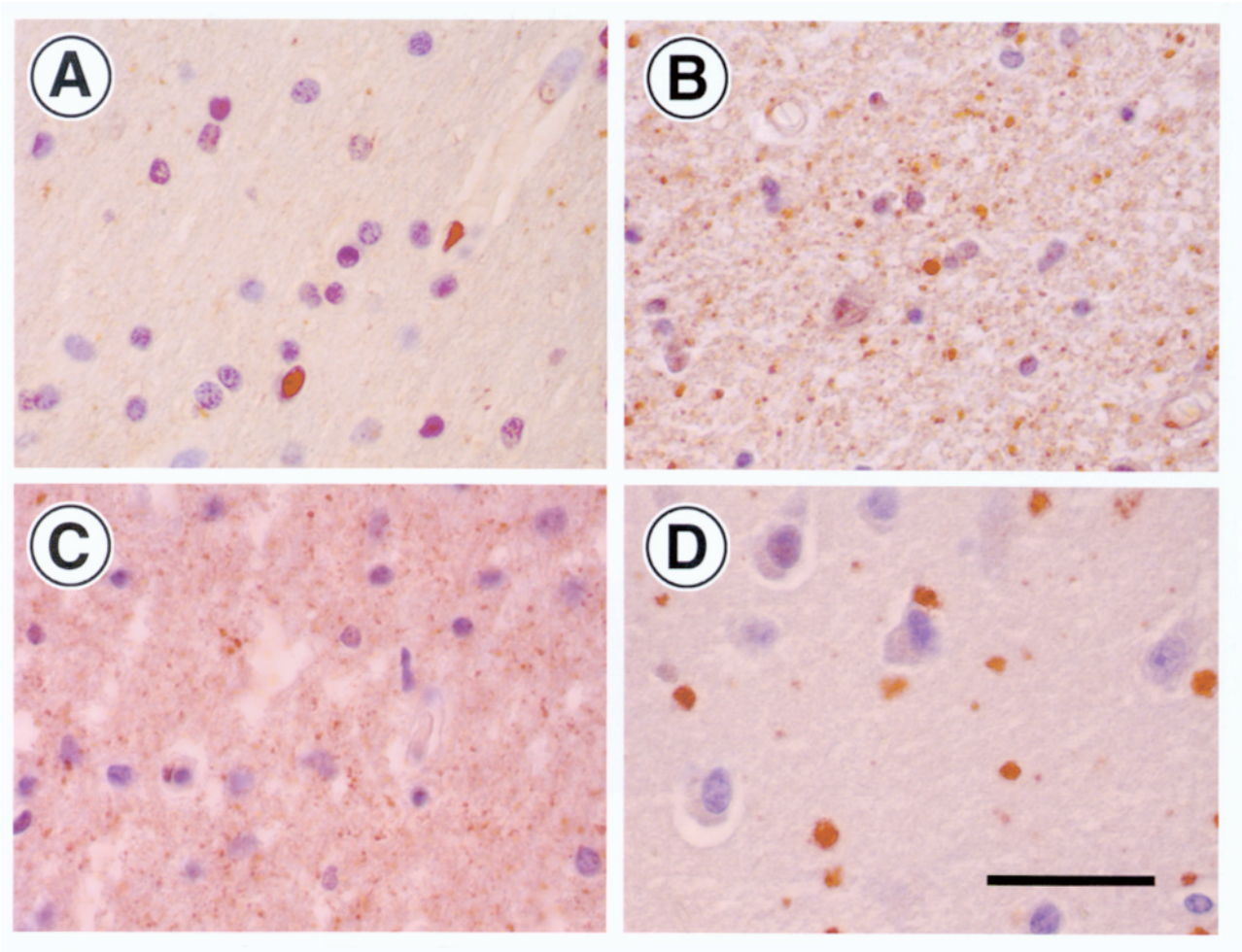

Figure 1 Ubiquitin immunostaining in the brain. White matter in the parahippocampal gyrus in a 42-year-old man without AIDS (**A**), a 42-year-old man with AIDS (**B**), and a 72-year-old man who did not have AIDS (**C**). Relative to the young adult in **A**, there are more ubiquitin-stained dots in the young adult with AIDS and in the elderly decedent. **D** shows ubiquitin-stained structures in entorhinal cortex of a young subject with HIV/AIDS. These structures are larger than those in white matter and were more numerous in AIDS. Scale bar = 50 μm.

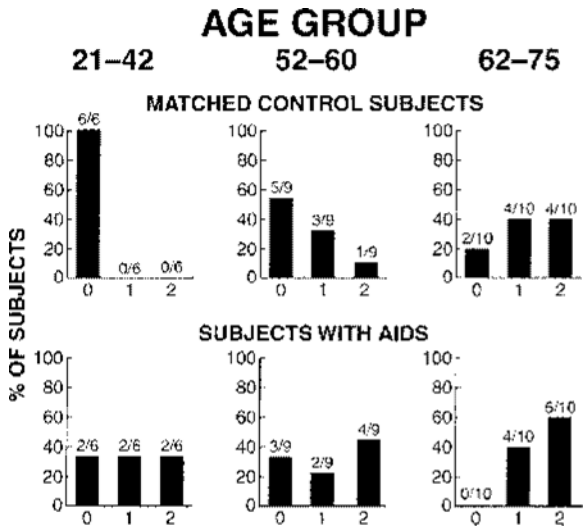

Figure 2 Ubiquitin-stained objects in temporal lobe white matter. Three age groups in the HIV-negative comparison group (top three bar graphs) are compared to decedents of comparable ages who did not have AIDS (bottom three bar graphs). At left are the young adults, middle graphs are of intermediate ages, and right graphs are the most elderly. Tallies were graded as described in Materials and Methods. The increase in the number of stained objects in AIDS cases and the older decedents both were statistically significant (see Results). See Figures 3 and 4 for Western blotting of ubiquitinylated protein.

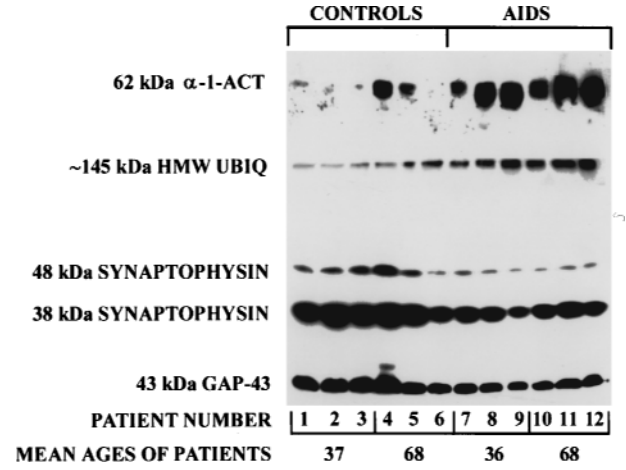

Figure 3 Immunoblotting of fresh-frozen frontal lobe samples. α-1-ACT is increased in AIDS (6% SDS-PAGE gel). The HMW-Ub-145 bands were increased in AIDS cases (14% SDS-PAGE gel). The synaptic proteins 38-kDa synaptophysin, 48-kDa synaptophysin, and GAP-43 all were decreased in AIDS (6% SDS-PAGE gels). Densitometry of all 60 bands is given in Figure 4. Synaptic protein and HMW-Ub-145 was measured in gray matter; ACT-62 is from white matter extracts, which demonstrated the phenomenon most intensely.

20 ubiquitin-stained dotlike deposits (Ub-dots) per high power field, as compared to 55% in the intermediate aged group, and 33% in the youngest group (Figure 2) ($\chi^2 = 11.32$; $df = 2$; $P = .0035$). In gray matter, ubiquitin-stained structures were not as numerous, and were larger (Figure 1D). The number of ubiquitin-stained structures in both gray and white matter was increased significantly in the subjects with AIDS. Twenty out of 25 of the subjects with AIDS (80%) had at least 20 Ub-dots per high power field, as compared to 12 out of 25 (48%) in the matched comparison group ($\chi^2 = 5.56$; $df = 1$; $P = .0184$) (Figure 2). Results for gray matter were equivalent and are not illustrated. It is likely that increased ubiquitin-stained objects are aggresomes (Taylor *et al*, 2003) that contain accumulated high-molecular-weight protein conjugated covalently to ubiquitin (versus ubiquitin monomer), as demonstrated using Western blotting in the brains of aged rodents (Marzban *et al*, 2002; Ohtsuka *et al*, 1995). To compliment and confirm the immunohistochemical findings, Western blotting on a subset of human brain specimens was performed. Ubiquitin-stained protein band intensities were clearly increased in AIDS. One such band had an approximate molecular mass of 145 kDa (HMW-Ub-145), and is illustrated in Figures 3 and 4C, D. Because HAD often is associated with brain inflammation (Glass *et al*, 1995; Williams and Hickey, 2002), we performed additional immunoblots and immunohistochemistry for the acute phase inflammatory response protein, α-1-antichymotrypsin (ACT-62) (Figure 5). ACT-62 is synthesized and accumulates in numerous brain cells and processes in response to inflammatory signals (Kanemaru *et al*, 1996; Licastro *et al*, 1999; McGeer and McGeer, 2001; Schreiber and Aldred, 1993), including a monkey model of HIV encephalitis (Roberts *et al*, 2003). Although ACT-62 tissue staining was more intense generally in the subjects with AIDS, the pattern of tissue staining was too complex to perform a quantitative analysis. In contrast, ACT-62 band intensities on immunoblots were readily quantified, and were sharply increased (Figures 3 and 4B). The increased ACT-62 band intensity in AIDS was correlated strongly with the increased HMW-Ub-145 ($r = .79$; $P < .0025$) (Figure 6A). Since the concentration of synaptic protein may be decreased in demented subjects (Masliah *et al*, 1997; Terry *et al*, 1991), we performed immunoblots for the synaptic proteins synaptophysin and growth-associated protein 43 (GAP-43). These protein band intensities were decreased in AIDS (Figures 3 and 4F, H, J) and the changes were correlated with increased HMW-Ub-145 and the acute inflammatory response protein ACT-62 (Figure 6B). It is possible that the increased ubiquitinylated protein in AIDS is a response to increased opportunistic infection or other stress, as suggested in Table 1, which shows a variety of neuropathological changes in AIDS. To explore that further, we compared subjects who had an opportunistic lesion in the brain to those that had minimal or no anomaly. In the decedents that were evaluated neurochemically, none of the six people with AIDS had an opportunistic brain lesion in the tissue sample; nearly all of them had the

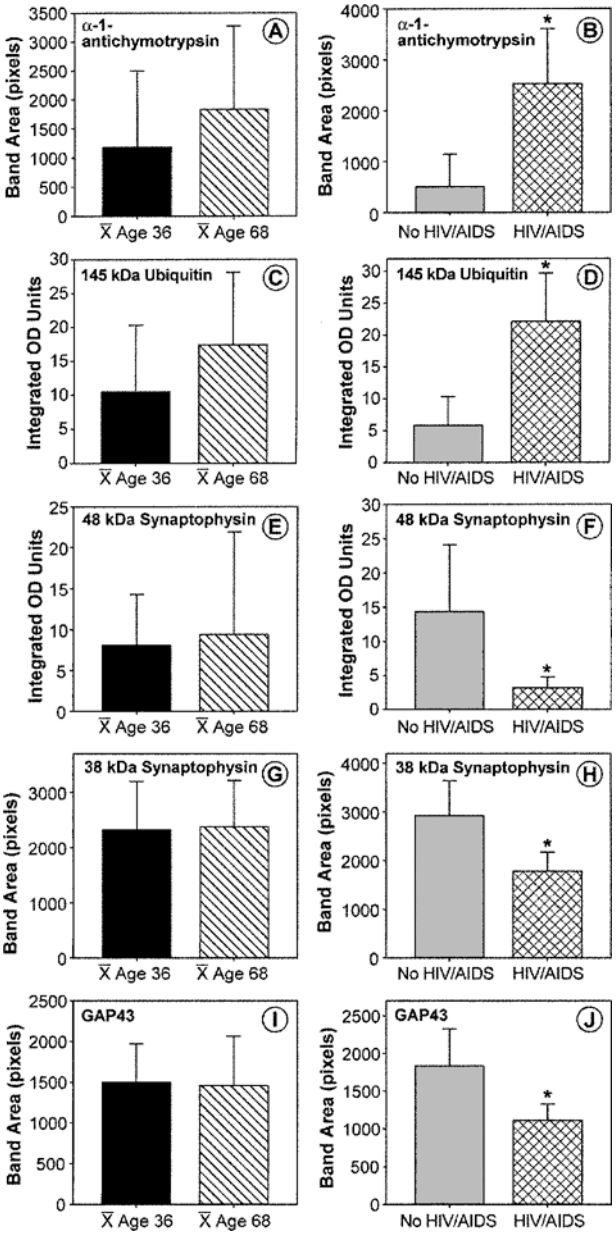

Figure 4 Densitometry of immunoblots depicted in Figure 3. Band intensities of ACT-62 and HMW-Ub-145 band both were strongly increased in AIDS. The band intensities of synaptic protein (38- and 48-kDa synaptophysin and GAP-43) were decreased in AIDS. The asterisk represents significance using the Student's *t* test assuming equal variances at a *P* value of .05 or lower.

biochemical anomaly. In the decedents that underwent immunostaining of tissue sections, the increase was evident in subjects with and without, opportunistic brain lesions (not illustrated). The cohort available to us for study was not homogeneous enough either to rule out or prove that opportunistic infection was contributing significantly to the increased ubiquitin concentration.

The accumulation of beta-amyloid (Aβ)-stained plaques in the coronal plane of midhippocampus, including entorhinal cortex and parahippocampal gyrus, was most prevalent in the more elderly subjects (Figure 7). Thus, 30% (6 out of 20) of the eldest group had substantial plaque accumulation (grade 2) versus 17% (3 out of 18) of the middle-aged group, and 8% (1 out of 12) of the young group (Figure 8). The ontogeny of diffuse Aβ plaque accumulation during natural brain aging has been extensively documented in the literature (Thal *et al*, 2000; Vogelgesang *et al*, 2002; Yamaguchi *et al*, 2001). Our results provided no evidence that the age of onset or the rate of accumulation of Aβ plaque was increased as a result of having AIDS. Twenty percent (5 out of 25) of the HIV-negative comparison group had substantial plaque accumulation (grade 2) as compared to 4% (1 out of 25) of the AIDS group (Figures 7 and 8).

Discussion

In the HAART era of the AIDS epidemic, a clinically relevant basic research agenda is needed to determine how HIV infection will influence cognitive disability in elderly people. This study suggests that protein turnover through the ubiquitin-proteasome apparatus could be a key focus for this field of investigation. Ubiquitin-stained structures were more prevalent in histological sections of brain specimens from people who died with AIDS, which augments previous observation (Adle-Biassette *et al*, 1999; An *et al*, 1997; Izycka-Swieszewska *et al*, 2000). Ubiquitin-stained structures in white matter increase progressively in brain tissue of older people who do not necessarily have a specific disease syndrome or a neuropathological anomaly associated with dementia (Dickson *et al*, 1990; Pappolla *et al*, 1989). Thus, they may reflect a natural aging process that occurs in the brain. It still is possible that the increased ubiquitinylated protein in AIDS is partly in response to increased opportunistic infection or other stress, as suggested by the variety of neuropathological changes (see Table 1). People with and without opportunistic infection both had evidence of increased staining, and the present cohort was not large enough to elucidate the influence of opportunistic infection further.

Ubiquitin-protein deposits in tissue often are referred to generically as "aggresomes," because they represent aggregations of ubiquitinated protein that fail to become degraded by the proteasome (Taylor *et al*, 2003). The ultrastructural localization of small white matter aggresomes in aging has eluded precise characterization. In the aging canine brain, they have been localized within axons (Dimakopoulos and Mayer, 2002). A human ultrastructural study suggested that localization within glial cells also was possible (Dickson *et al*, 1990). Three other reports have mentioned that ubiquitin-stained aggresomes are increased in young adults with AIDS (older subjects were not examined) (Adle-Biassette *et al*, 1999; An *et al*, 1997; Izycka-Swieszewska *et al*, 2000). One

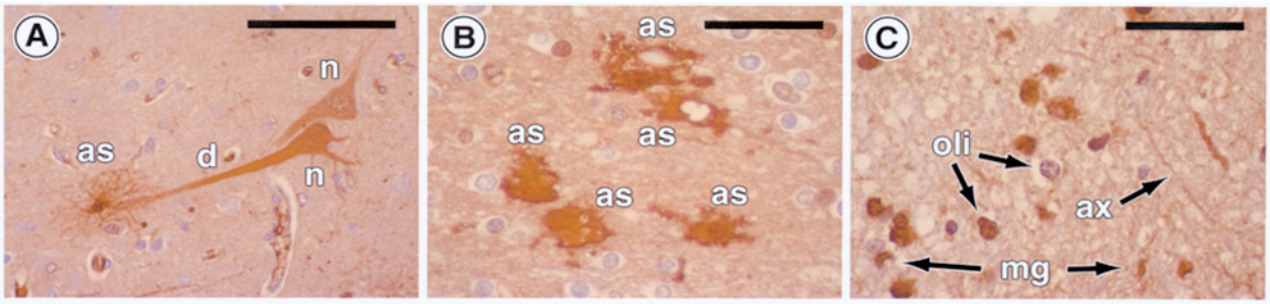

Figure 5 Immunostaining for the acute phase inflammatory protein ACT-62 in human brain. Gray matter (**A**) and white (**B**, **C**) matter of a person with AIDS and increased Ub-stained structures. Markings denote staining of neuronal perikarya (n), dendrites (d), astrocytes (as), axons (ax), microglial cells (mg), and oligodendrocytes (oli). Scale bar = 50 μm for **A**. Scale bar = 100 μm for **B** and **C**.

report refers to Ub-dots (Izycka-Swieszewska *et al*, 2000); the others described ubiquitin-stained swollen dystrophic axons ("spheroids"). Axon spheroids and Ub-dots both are more prevalent in the aging human brain, but are not identical structures (Dickson *et al*, 1990).

Immunoblotting confirmed that HMW-Ub-conj concentration was increased in AIDS. In aged mouse brain, HMW-Ub-conj concentration is increased sharply relative to young brains (Ohtsuka *et al*, 1995), and is analogous to what we have measured in human brain specimens. The chemical composition of these ubiquitin-stained structures in the human has not been determined beyond what we demonstrate. Immunoblots also showed that the increased HMW-Ub-conj in AIDS was linked significantly to decreased concentrations of synaptic protein. Genetic, molecular, and functional studies all indicate that the ubiquitin-proteasome apparatus plays a key role in the turnover of synaptic protein, and is linked with dementia (Ageta *et al*, 2001; Burbea *et al*, 2002; Buttner *et al*, 2001; Chapman *et al*, 1992, 1994; DiAntonio *et al*, 2001; Ehlers, 2003; Hegde and DiAntonio, 2002; Mahler, 1969; Wilson *et al*, 2002). Further, there is abundant evidence that the synaptic apparatus is very vulnerable to disturbance of the ubiquitin-proteasome system (Baker *et al*, 1992; DiAntonio *et al*, 2001; Ehlers, 2003; Jiang *et al*, 1998; Lopez-Salon *et al*, 2001). Autopsy studies also have established that ubiquitin-protein conjugation and aggresomes are involved in the pathogenesis of many neurodegenerative diseases (Bennett *et al*, 1999; Chung *et al*, 2001; Keller *et al*, 2000b; Lennox *et al*, 1988; Lowe *et al*, 1988, 2001; Ma *et al*, 2002; Mezey *et al*, 1998; Mizuno *et al*, 1998; Shimura *et al*, 2000; Tsuji and Shimohama, 2002). In many instances, abnormally conformed proteins in diseased human brain tissue directly inhibit the neuronal proteasome (Bence *et al*, 2001; Ma *et al*, 2002). Thus, the decreased ubiquitin-protein conjugation that occurs with brain age might "tip the balance" towards still more accumulation of misfolded toxic proteins that can inhibit the proteasome (Ma *et al*, 2002). The general scenario of an age-driven decrease in the disassembly of misfolded proteins via ubiquitin-protein conjugation has broad application to neurodegenerative diseases (Bence *et al*, 2001; Carrard *et al*, 2002; Keck *et al*, 2003; Keller *et al*, 2000a, 2000b, 2000c, 2000d, 2002; Keller and Markesbery, 2000; Klimaschewski, 2003; Sherman and Goldberg, 2001; Tsuji and Shimohama, 2002). Our data in HIV-infected people extend that concept still further by showing that neurodegeneration driven by chronic

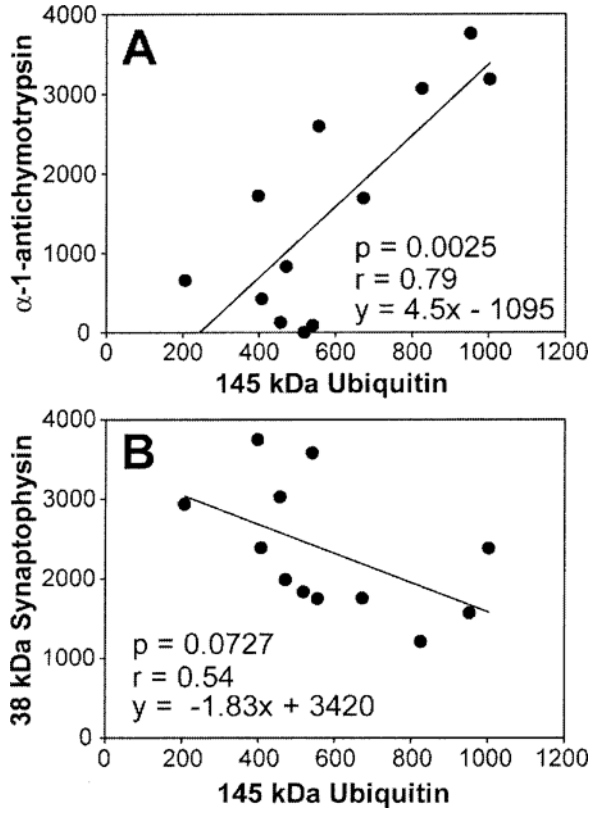

Figure 6 Linear regression analysis between band intensities of human brain protein, as shown in Figures 3 and 4. There was strong positive correlation between 145-kDa ubiquitinated protein versus the acute phase inflammatory protein ACT-62. In contrast, 145-kDa ubiquitin was negatively correlated with synaptic protein concentration.

Table 1 Characteristics of AIDS cases used in this study*

Case	*Age (years)*	*Gender*	*PMI (hours)*	*Race*	*Brain pathology*
1	75	Male	14.75	Afro-American	Cryptococcal meningitis
2	70	Male	10.50	Caucasian	Cerebral atrophy
3	69	Male	3.25	Afro-American	Treated toxoplasmosis, focal
4	69	Male	18.83	Caucasian	Microglial nodules, focal
5	65	Male	20.17	Caucasian	Myelin pallor and rare microglial nodules
6	65	Male	27.25	Caucasian	Cerebral atrophy
7	63	Male	18.17	Hispanic	HIV encephalitis
8	63	Female	16.83	Caucasian	Cytomegalovirus encephalitis
9	63	Male	9.25	Caucasian	Cerebral atrophy
10	62	Male	16.67	Caucasian	White matter gliosis; focal microglial nodules
11	60	Male	7.08	Caucasian	Old subarachnoid hemorrhage, focal
12	60	Male	11.25	Afro-American	HIV encephalitis
13	59	Male	6.80	Afro-American	Acute hypoxic/ischemic change
14	59	Male	20	Caucasian	Autolysis
15	55	Male	3.50	Caucasian	HIV encephalitis
16	55	Male	20.33	Hispanic	Cryptococcal meningitis
17	55	Male	6	Caucasian	Treated toxoplasmosis
18	55	Male	37.75	Caucasian	Cerebral atrophy
19	55	Male	16.13	Hispanic	Treated toxoplasmosis
20	42	Male	9.67	Afro-American	Progressive multifocal leukoencephalopathy
21	41	Male	11.5	Caucasian	Lymphocytic meningitis
22	33	Male	27.42	Caucasian	Single focus of CNS lymphoma
23	30	Male	11.5	Afro-American	HIV encephalitis
24	26	Male	7.83	Hispanic	Focal resolved lesion, probable treated toxoplasmosis
25	22	Male	6.6	Afro-American	Cerebral atrophy and myelin pallor

*Case numbers 1 to 10 are elderly, case numbers 11 to 19 are middle-aged, and case numbers 20 to 25 are young.

inflammation could exacerbate protein misfolding in senile dementias. In support of that concept, it is known that inflammatory stress and noninflammatory stress both decrease protein turnover systemically, and they specifically decrease activity of neuronal ubiquitin-protein conjugates (Goto *et al*, 2001, 2002; Grune, 2000; Keller *et al*, 2000c, 2000d, 2002; Merker and Grune, 2000; Merker *et al*, 2001; Ryazanov and Nefsky, 2002; Stolzing and Grune, 2001; Ward, 2000). Tumor necrosis factor-α and interferon-γ, two key inflammatory mediators that are increased in AIDS (Merrill *et al*, 1992; Walker *et al*, 1995), both decrease transcription of 20S proteasome α-subunits (Raasi *et al*, 1999). Protein turnover via ubiquitin-protein conjugation is decreased in senile dementias that contain activated microglia, such as Alzheimer's disease (AD) (Agarwal and Sohal, 1994; Bulteau *et al*, 2002; Davies, 2001; Keller *et al*, 2002). Thus, microglial cell activation could operate synergistically with brain aging to decrease turnover of synaptic protein, prevent removal of misfolded protein, or promote HMW-Ub-conj aggregation in the

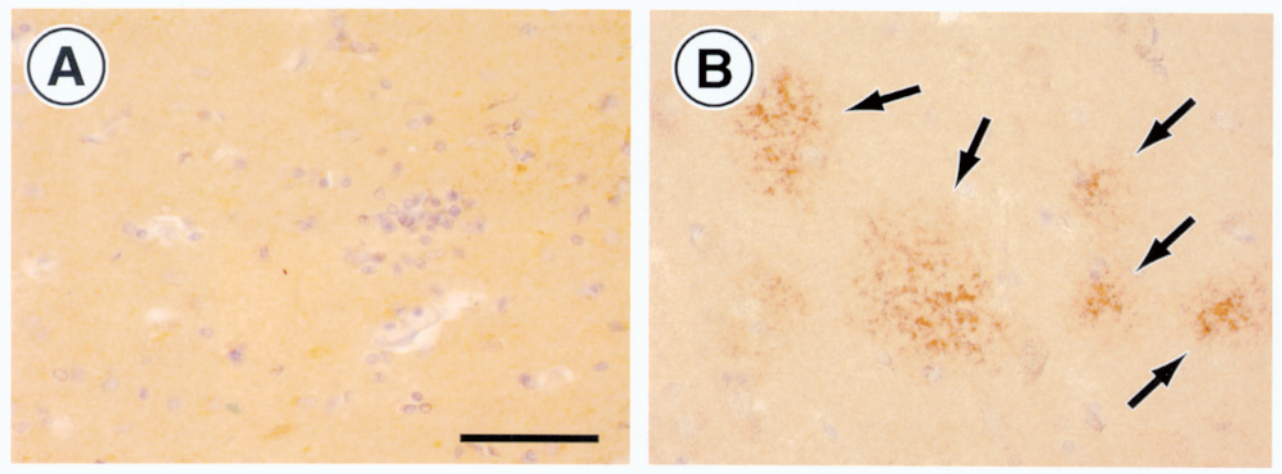

Figure 7 Immunostaining for Aβ plaque. Aβ stained diffuse plaque in the entorhinal cortex of a 65-year-old man with AIDS (**A**) is compared to his paired comparison subject, a 65-year-old man without AIDS (**B**). The image from the subject with AIDS contains a microglial nodule, but does not have any stained plaques (**A**). The age-matched comparison subject contains numerous stained plaques (**B**). Scale bar = 100 μm.

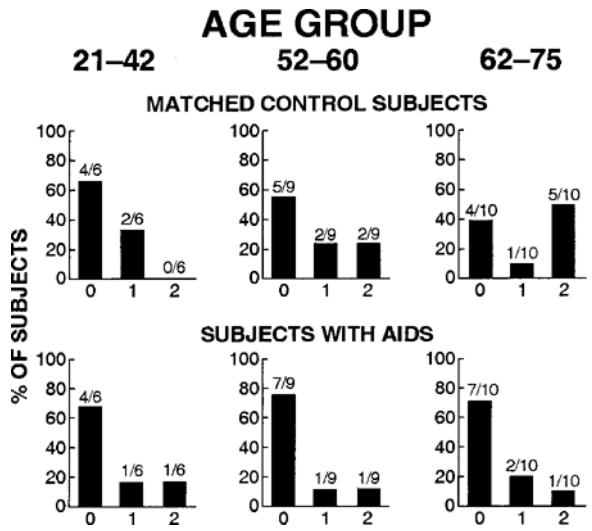

Figure 8 Extracellular Aβ-stained plaques in medial temporal lobe. Three age groups in the HIV-negative comparison group (top three bar graphs) are compared to decedents of comparable ages who had AIDS (bottom three bar graphs). At left are the young adults, middle graphs are intermediate ages, and right graphs are the most elderly. Grading scale is as described in Methods. Seven out of 25 subjects with AIDS have a score of "1" or "2" and had some diffuse plaque (28%), as compared to 12 out of 25 age-, gender-, and race-matched comparison subjects (48%). The AIDS subjects did not differ from the comparison group significantly ($\chi^2 = 2.12$; $P = .1452$).

brain (Figueiredo-Pereira *et al*, 2002; Li *et al*, 2003; Wyss-Coray and Mucke, 2002).

Aβ plaques are the probable precursor of senile plaques, which in turn, are a neuropathological hallmark of AD (Dickson, 1997; Mirra *et al*, 1993). In contrast to another report (Esiri *et al*, 1998), we did not detect a difference in the age of onset or rate of accumulation of diffuse Aβ-stained plaques in AIDS. Seven out of 25 subjects with AIDS had at least one stained Aβ plaque in the hippocampal formation (28%) as compared to 12 out of 25 of the comparison subjects (48%). We almost never observed Aβ plaques under the age of 50, an ontogenic pattern that mirrors closely other reports that employed similar Aβ staining technique (Thal *et al*, 2000; Vogelgesang *et al*, 2002; Yamaguchi *et al*, 2001). Just two control subjects had "neuritic" plaques in this cohort, which generally correlates with argyrophilic staining and neurocognitive dysfunction both. Thus, the influence of AIDS on neuritic, neurofibrillary, and argyrophilic changes that likely appear after Aβ plaques was not evaluated in our cohort. A more comprehensive survey is needed on elderly subjects from the HAART era in order to elucidate precisely the dynamics of Aβ plaque appearance in AIDS. Of perhaps more relevance to the pathogenesis of senile dementia, our results point to synaptic dysfunction as a potentially promising point of interaction between AIDS and the senile dementias. The loss of synapses is a critical facet of demented people with AD (Terry *et al*, 1991) and, possibly, in demented people with AIDS (Masliah *et al*, 1997). An interaction between AIDS and aging at the neuronal proteasome is suggested because the process of synapse maintenance is very sensitive to manipulation of the neuronal proteasome (Ageta *et al*, 2001; Baker *et al*, 1992; Burbea *et al*, 2002; Buttner *et al*, 2001; Chapman *et al*, 1992, 1994; DiAntonio *et al*, 2001; Ehlers, 2003; Hegde and DiAntonio, 2002; Jiang *et al*, 1998; Lopez-Salon *et al*, 2001; Mahler, 1969; Wilson *et al*, 2002). The decreased synaptic protein concentration in AIDS as measured neurochemically that we report here is a fairly novel observation. The loss of synaptic protein in AIDS, and its correlation with increased acute phase inflammatory protein, suggest that chronic inflammation, in the setting of long survival and persistent HIV infection, could exacerbate senile dementia by disturbing synaptic protein turnover through the proteasome.

Materials and methods

Selection of elderly AIDS patients

We screened the autopsy archive of The University of Texas Medical Branch, which contained over 600 autopsy reports that indicated that the cause of death was AIDS. We identified the case numbers of the most elderly decedents for study. The search was restricted to decedents who did not have a history of successful suppression of HIV by HAART prior to death. Attention was focused on the pre-HAART era so that the influence of HAART itself on virus replication rate would not be a significant confounding factor in the baseline analysis. The time period covered was 1987 to 1997. We found 10 decedents who were at least 62 years old when they died with end-stage AIDS (Table 1). We found another 9 who were between 55 and 60 years of age. We selected another 6 cases who had low postmortem times and were between 21 and 42 in order to broaden the spectrum of age groups represented, for a total of 25 cases that died of AIDS.

Selection and pairing of comparison group

To obtain a comparison group, we paired each one of the 25 cases selected above *a priori* with 25 decedents who were not HIV positive and did not have AIDS. Over 3500 autopsy reports were screened from the same time period as above (1987 to 1997) to find suitable pairings for all 25 cases in the AIDS group. Pairing selection was based on identifying subjects with the same ages, genders, races, year of death, and postmortem times of the AIDS patients. The causes of death in the comparison group varied substantially relative to the AIDS group because it was not feasible to match them for the exact causes of death. For example, we could not obtain HIV-negative controls who died with *Pneumocystis carinii* pneumonia or Kaposi's sarcoma. We excluded comparison subjects who had either a neuropathological lesion or clinical history of a neurodegenerative disease. When

combined with the AIDS patients, the cohort totaled 50 cases and was stratified into three age groups: The oldest group available for study ($n = 20$) had a mean age of 67 years (range 62 to 75); the intermediate group ($n = 18$) had a mean age of 57 years (range 55 to 60 years); the youngest group ($n = 12$) had a mean age of 31 years (range 21 to 42). To detect neuritic changes in these plaques, sections were stained using the Bielschowsky method. Because the cohort was relatively young, just two HIV-negative controls contained any neuritic change, too few to draw a conclusion.

Immunohistochemistry of age-associated phenotypic changes

A paraffin block containing midhippocampus plus inferior temporal gyrus was retrieved and serial 7-micron sections were prepared. Immunostaining for the Aβ fragment of the amyloid precursor protein was performed after pretreatment by heating in 0.01 M citrate buffer (pH 6), followed by 90% formic acid. The primary antibody was mouse monoclonal 10D5 (gift from Dale Schenk, Athena Neurosciences, San Francisco, CA) used at a dilution of 1:500, followed by biotinylated anti-mouse immunoglobulin G (IgG) and avidin-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA). Monoclonal 10D5 is an IgG_1 that was raised against the 1–28 peptide fragment of Aβ (Anderson *et al*, 1991); it recognizes both Aβ1–40 and Aβ1–42. Slides were developed using 3,3′-diaminobenzidine (DAB) as substrate and Mayer's hematoxylin as a counterstain. The number of Aβ-stained plaques was screened in the entire hippocampus, entorhinal cortex, and parahippocampal gyrus and scored as: "0" = no plaques present in the entire section; "1" = 1 to 20 plaques present; "2" = more than 20 plaques. Because no difference in plaque number was evident after screening all the slides, assessing plaque area was deemed to be noncontributory. Ubiquitin staining was performed using rabbit anti-cow ubiquitin as the primary antibody (Dako, Carpinteria, CA) and ABC followed by detection using DAB as above. Ubiquitin-stained structures were counted in temporal lobe white matter in 10 high power fields in all 5 layers of entorhinal cortex, and in another 10 fields in white matter of the parahippocampal gyrus. Scoring was done analogous to the above, as follows: "0" = less than 20 stained objects per high power field; "1" = 21 to 100 objects; "2" = more than 100 stained objects.

Immunoblotting

Fresh frozen brain tissue from only six subjects with AIDS and six age-matched controls could be obtained for Western blotting. Subjects were divided into two groups: young (mean age = 36, $n = 6$) and elderly (mean age 68, $n = 6$). Gray matter and white matter from middle frontal gyrus was available for study. Total protein for each sample was determined using a micro-BCA kit (Pierce Biotechnology, Rockford, IL). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (Wolf *et al*, 1997) using either 6% or 14% SDS-polyacrylamide gels, depending on the molecular mass of the protein. Immunoblotting was performed using antibodies directed against: ACT-62 (rabbit anti–α-1-antichymotrypsin, 1:100, Dako); ubiquitinylated protein (rabbit anti-ubiquitin, 1:50, Dako); synaptophysin (anti-synaptophysin, clone SY 38, 1:100, Boehringer Mannheim, Indianapolis, IN); and GAP-43 (anti-growth associated protein-43, clone 91E12, 1:100, Boehringer Mannheim). Relative band intensities from scanned immunoblots were measured using Scanalytics software (CSPI, Billerica, MA) and Adobe Photoshop 6 (Adobe Systems, San Jose, CA) and represented as integrated densitometry units, or pixels per area, both calibrated to a secondary standard. The relative intensities of stained protein in young and elderly groups were compared using the Student's *t* test; comparison was made between the AIDS and HIV-negative control groups in the same way.

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