Accumulation of nuclear and mitochondrial DNA damage in the frontal cortex cells of patients with HIV-associated neurocognitive disorders

Yulin Zhang\textsuperscript{a,c,1}, Meixia Wang\textsuperscript{b,1}, Hongjun Li\textsuperscript{a}, Honghai Zhang\textsuperscript{a}, Ying Shi\textsuperscript{a}, Feili Wei\textsuperscript{a}, Daojie Liu\textsuperscript{c}, Kai Liu\textsuperscript{c}, Dexi Chen\textsuperscript{a,c,*}

\textsuperscript{a}Department of Medicine, Beijing You An Hospital, Capital Medical University, Beijing 100069, China
\textsuperscript{b}Clinical Drug Research Center, Beijing You An Hospital, Capital Medical University, Beijing 100069, China
\textsuperscript{c}Beijing Institute of Liver Disease, Beijing 100069, China

\textbf{ARTICLE INFO}

\textbf{Article history:}
Accepted 2 April 2012
Available online 11 April 2012

\textbf{Keywords:}
HAND
AIDS
ROS
HIV-1
Mitochondria
mtDNA
8-oxoG

\textbf{ABSTRACT}

Oxidative stress has been suggested to play a key role in the neuropathogenesis of HIV infection. HIV proteins (gp120, Tat) and proinflammatory cytokines can trigger the production of reactive oxygen species (ROS), resulting in DNA and RNA lesions. Among all the lesions induced by ROS, one of the most abundant lesions in DNA and RNA is 8-hydroxydeoxyguanosine (8-oxoG). Here, we studied accumulated DNA oxidative damage induced by ROS in the central nervous system (CNS) in tissue from neuro-AIDS patients. The frontal cortex of autopsy tissue from HIV-1 infected patients was adopted for analysis for HIV-1 subtype, nuclear and mitochondrial DNA lesions by immunofluorescence staining, qPCR and sequencing of PCR cloning. This study provides evidence that HIV infection in the CNS leads to nuclear and mitochondrial genomic DNA damage in the brain. High level of nuclear and mtDNA 8-oxoG damage were identified in the cortex autopsy tissue of HAND patients. Increased accumulation of mtDNA mutations and depletion occurs in brain tissue in a subset of HAND cases, and is significantly different from that observed in control cases. These findings suggest that higher level of ROS in the CNS of HAND patients would contribute to the HIV induced neuro-inflammation and apoptosis of neuronal and glial cells.

© 2012 Elsevier B.V. All rights reserved.

\textbf{1. Introduction}

In the central nervous system (CNS), apoptosis of neuronal and non-neuronal cells has been demonstrated in brain autopsy tissue from HIV-1 infected patients, in particular HIV-associated dementia (HAD) which is defined as severe cognitive, behavioral and motor difficulties (Everall et al., 1993; Garden and Morrison, 2005; Petito and Roberts, 1995). Early studies showed that patients with AIDS often developed cognitive and motor dysfunction resulting from HIV-1 associated damage to synapses and the death of neurons in brain regions such as the hippocampus and basal ganglia (Bassiri et al., 1995; Chiodi, 1995), suggesting that their neurological dysfunction, termed minor cognitive motor disorder (MCMD),

\textsuperscript{*} Corresponding author at: STD/AIDS Research Center and Department of Medicine, Beijing You An Hospital, Capital Medical University, Beijing, China, 100069. Fax: +86 10 63293371.
E-mail address: dxxi0963@yahoo.com (D. Chen).

\textsuperscript{1} These authors contributed equally to this work.

0006-8993/ – see front matter © 2012 Elsevier B.V. All rights reserved.
was due to chronic neuronal apoptosis (Kaul, 2008; Kaul and Lipton, 2006). The mechanisms that lead to neuronal apoptosis in the brain of AIDS patients are very complex and still unclear. It is believed that the process of MCMD begins with the infection of one or more populations of mitotic brain cells with HIV-1, which then release neurotoxic viral proteins (Ozdener, 2005). Prior to widespread use of highly active antiretroviral therapy (HAART), the prevalence of HAD was 20–30% among patients with advanced HIV and low CD4 lymphocyte counts. The widespread availability of HAART led to a marked decline in the reported incidence of HAD to 10/1000 person-years in 1996–1998 (Sacktor et al., 2002), but the total prevalence of HIV-associated neurocognitive disorders (HANDs) including HAD and MCMD may be as high as 37% (Robertson et al., 2007).

In the 1980s and early 1990s, illegal commercial plasma collection spread rapidly in China which allowed HIV to be transmitted among plasma donors and their spouses (Wu et al., 2006). Sequence analysis indicated that most of these plasma donors harbored B and C, however, other recombinant strains were detected (Liu et al., 2012; Su et al., 2003). Among them, CRF 01_AE/B was a mosaic HIV-1 genome composed of CRF01_AE and B subtypes due to recombination and the breakpoints of recombination could distribute in both polymerase and envelope genomes. By October 2010, over 370,000 people have been found to be infected with HIV in China. Sexual contact is becoming a major mode of HIV transmission, in particular among men who have sex with men (MSM), and ethnic minorities comprise the fastest growing segment of the HIV-infected population (National Center for AIDS/STD Control and Prevention, China CDC), but, up to now, very few reports have documented the prevalence of HAND. Our multi-center investigation disclosed that approximately 37% of HIV positive patients and 26.79% of former plasma donors (FPD) had HAND in China (data will be published). Heaton and his colleagues investigated 203 HIV positive FPD in rural China and found that 34.2% of HIV mono-infected patients and 39.7% of HIV and HCV co-infected patients had HAND (Heaton et al., 2008).

HIV-1 infected non-neuronal cells, such as macrophages and microglia, are required for Tat, Vpr and gp120-induced neurotoxicity via the production of immune-stimulated neurotoxins (Buzy et al., 1992; Jones et al., 2007; Yeung et al., 1995). These neurotoxins are excitotoxic, interacting with glutamate receptors, primarily of the N-methyl-D-aspartate subtype (NMDAR). Prior to widespread use of highly active antiretroviral therapy (HAART), the prevalence of HAD was 20–30% among patients with advanced HIV and low CD4 lymphocyte counts. The widespread availability of HAART led to a marked decline in the reported incidence of HAD to 10/1000 person-years in 1996–1998 (Sacktor et al., 2002), but the total prevalence of HIV-associated neurocognitive disorders (HANDs) including HAD and MCMD may be as high as 37% (Robertson et al., 2007).

With the progression of HAND, there are increased ROS production and oxidative mtDNA damage (mutation and deletion), disrupting mitochondrial oxidative phosphorylation, which further induces neuronal apoptosis and accelerates the progression of HAND (Eugenin et al., 2007; Kaul and Lipton, 1999; Lipton, 1996; O’Donnell et al., 2006; Saitoh et al., 2007). Among all ROS induced lesions discovered so far, one of the most abundant lesions in DNA and RNA is 8-hydroxyguanine (8-Oxoguanine, 8-oxoG), which can result in a mismatched pairing with G to T and C to A substitutions in the genome (Cattley and Glover, 1993; Cheng et al., 1992; Kanvah et al., 2010). In humans, it is primarily repaired by 8-Oxoguanine glycosylase (OGG1). In order to examine whether DNA oxidative damage in the frontal cortex as a result of HIV-1 infection in the CNS plays a role in neuronal injury, we began by investigating oxidative nuclear DNA damage and mtDNA damage in the HIV infected CNS. Furthermore, we sought to identify 8-oxoG level in the cortex autopsy tissue from AIDS patients as a biomarker for ROS level contributing to neuro-AIDS including HAND.

2. Results

2.1. Patients and tissues

Frontal cortex tissues from ten patients infected with HIV-1 and five normal controls were examined. Patients’ information such as age, gender, viral load, CD4 cells counts and dementia score are shown in Table 1. All frontal cortex autopsies were selected from patients who were infected with HIV-1 via transfusions with commercial plasma donors between 1994 and 1995. These patients died between 2006 and 2007. There were seven males and three females and with average age of 32.9 (8–56) years old. All final CD4 cell counts were very low. The mean of the CD4 cell count was 31.4 (13–56) ×10^6 cells/L. The HIV-1 subtype from these patients was HIV-1B CRF 01_AE/B. The mean of the last recorded viral loads in plasma was 102,400 (28,000–219,000) copies/mL; the viral load in CSF was unavailable. There were no records indicating that any of these patients had opportunistic infections (OIs) in the CNS, but three had pulmonary tuberculosis (TB), one had pneumocystic pneumonia (PCP), one had lymphoma and one had cytomegalovirus infection in the eye. Four patients did not have any OI records. HAND screen testing showed that five patients had a neurocognitive disorder (score ≤10). All five control samples were from normal tissues surrounding brain tumors that were removed by surgery and none of these patients had any records of OIs, HIV-1 infection, chemotherapy or radiotherapy.

2.2. HIV-1 subtype and gp120 CCR5 tropism

Previous studies have shown that the V3 loop of gp120 plays a critical role in neuronal toxicity via its interaction with co-receptors of HIV-1 (Catani et al., 2000; Shino et al., 2000). Our lab and others have reported that HIV-1/gp120 co-receptor...
usage is associated with the disease status of the patients. The virus isolated from the plasma of long-term nonprogressor (LTNP) patients used the CCR5 co-receptor with serine at amino acid position 11 of the V3 loop (S11R). HIV-1 isolated from other types of AIDS patients used both CCR5 and CXCR4 co-receptors with arginine at amino acid position 11 of the V3 loop. This suggests that the S11R mutation is associated with syncytium formation. To identify the amino acid sequence of the V3 loop in these brain tissues, we isolated frontal cortex genomic DNA from the autopsy tissue and a single genome amplification (SGA) PCR method were used to amplify a 900 bp fragment using ED31/BH2 primers with serial dilution of genomic DNA. The C2–V5 region of gp120 was then amplified via nested PCR using primers DR7/DR8. The C2–V5 region of HIV-1 from these brain tissues was demonstrated to have a subtype B envelope (Fig. 1A). The neighbor phylogenetic assay

---

**Table 1 – Patient demographics and clinical characteristics.**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Gender</th>
<th>Age (year)</th>
<th>IHDs</th>
<th>CD4 (10⁶/L)</th>
<th>HIV subtype</th>
<th>Viral load (copies/mL)</th>
<th>OIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS-1</td>
<td>M</td>
<td>8</td>
<td>7</td>
<td>34</td>
<td>CRF 01_AE/B</td>
<td>47,000</td>
<td>PTB</td>
</tr>
<tr>
<td>AIDS-2</td>
<td>M</td>
<td>45</td>
<td>4</td>
<td>56</td>
<td>CRF 01_AE/B</td>
<td>139,000</td>
<td>None</td>
</tr>
<tr>
<td>AIDS-3</td>
<td>M</td>
<td>31</td>
<td>9</td>
<td>45</td>
<td>CRF 01_AE/B</td>
<td>30,000</td>
<td>None</td>
</tr>
<tr>
<td>AIDS-4</td>
<td>M</td>
<td>48</td>
<td>10</td>
<td>15</td>
<td>CRF 01_AE/B</td>
<td>219,000</td>
<td>PCP</td>
</tr>
<tr>
<td>AIDS-5</td>
<td>M</td>
<td>56</td>
<td>5</td>
<td>20</td>
<td>CRF 01_AE/B</td>
<td>110,000</td>
<td>None</td>
</tr>
<tr>
<td>AIDS-6</td>
<td>M</td>
<td>23</td>
<td>11</td>
<td>41</td>
<td>CRF 01_AE/B</td>
<td>121,000</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>AIDS-7</td>
<td>M</td>
<td>27</td>
<td>11</td>
<td>53</td>
<td>CRF 01_AE/B</td>
<td>32,000</td>
<td>None</td>
</tr>
<tr>
<td>AIDS-8</td>
<td>F</td>
<td>35</td>
<td>11</td>
<td>13</td>
<td>CRF 01_AE/B</td>
<td>200,000</td>
<td>PTB</td>
</tr>
<tr>
<td>AIDS-9</td>
<td>M</td>
<td>33</td>
<td>12</td>
<td>16</td>
<td>CRF 01_AE/B</td>
<td>98,000</td>
<td>PTB</td>
</tr>
<tr>
<td>AIDS-10</td>
<td>F</td>
<td>23</td>
<td>11</td>
<td>21</td>
<td>CRF 01_AE/B</td>
<td>28,000</td>
<td>Eye-CMV</td>
</tr>
<tr>
<td>Control-1</td>
<td>M</td>
<td>42</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Control-2</td>
<td>M</td>
<td>36</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Control-3</td>
<td>F</td>
<td>39</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Control-4</td>
<td>F</td>
<td>44</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
<tr>
<td>Control-5</td>
<td>M</td>
<td>55</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
</tbody>
</table>

Legend: IHDs: International HIV Dementia Score; OIs: Opportunistic infections; PTB: Pulmonary tuberculosis; PCP: Pneumocystic pneumonia; Eye-CMV: Cytomegalovirus infection in the eye; N/A: Not applicable.

---

**Fig. 1 – Phylogenetic analyses of envelope proteins (C2–V5) and V3 and CCR5 tropism in frontal cortex autopsy tissue from HIV-1 infected patients.** A. Phylogenetic trees were constructed by the neighbor-joining method using BioEdit. The branch lengths are proportional to the distance between the sequences. B. V3 loop domains of gp120 proteins in autopsy tissue from frontal cortex of 10 HIV-1 infected patients. C. Fluorescence immunocytochemistry of expression and sub-localization of CCR5 and gp120 proteins in autopsy tissue from frontal cortex of with and without HAND patients.
showed that the C2-V5 region of HIV from these ten samples displayed very low viral divergence by comparing the nucleotide distance of C2-V5 in each patient, with that of HIV-1B CRF 01_AE/B from 0.0994 (Case 2) to 0.1673 (Case 10). The average nucleotide distance of C2-V5 was not significant different between patients with HAND (0.1319) and non-HAND (0.1468). Previous reports from patients infected with this subtype of HIV have suggested that there was a relationship between the non-syncytium inducing (NSI) and the syncytium inducing (SI) phenotype and the V3 loop domain. In this study, we also analyzed the V3 loop domains of HIV-1/gp120 for all patients and the results are shown in Fig. 2B. We found that the amino acid in position 11 of the V3 loop was serine in all ten of frontal cortex autopsy tissues. Comparing these samples with the original “Thailand B (1501B.TH99.99TH),” we found that patients with HAND (Cases 1-5) had no significant mutations, and no patients showed any mutations associated with the syncytium-inducing phenotype (SI, X4). Based on the deduced amino acid sequences, all samples had nonsyncytium-inducing (NSI, R5) phenotypes. The lower levels of diversity from the CNS may be due to host immune response and environmental factors within the CNS that influence selection pressures. In order to confirm the association of gp120 and CCR5, we then examined gp120 and CCR5 expression by immunofluorescence. The results showed that gp120 positive cells in all ten frontal cortex autopsy tissues from patients colocalized with CCR5 (Fig. 1C). These observations indicate that gp120 is associated with CCR5 positive cells in these samples.

2.3. Cortex autopsy tissue from patients with HAND showed an increased loss of mtDNA

It is well known that mtDNA oxidative damage and mtDNA depletion are serious injuries, resulting in the decline of mitochondrial respiratory function and cell apoptosis. Furthermore, chronic neuron apoptosis is the main pathogenesis of HAND. In this study, the autopsy tissues came from patients who had been chronically infected with HIV-1. Thus, the analysis of mtDNA loss in these samples should help us to understand the link between mtDNA loss and the progression of HAND. The difference in mtDNA copy number from autopsy tissues was analyzed by real-time qPCR among five HAND patients, five non-HAND patients and five controls as our previous description (Wu et al., 2009). Firstly, we confirmed the linear correlation between actual DNA amount and relative fold change of DNA calculated from qPCR Ct value, which is necessary for using this method (Fig. 2A). Then the results showed that, compared with controls, the mtDNA copies in HAND and non-HAND samples were decreased to 0.69±0.18 and 0.74±0.15 respectively (Fig. 2B). This suggests that the mtDNA copy number in HIV-1 infected brain was significantly reduced during the chronic infection process. However, there is no significant difference between HAND and non-HAND patients.

2.4. DNA oxidative marker 8-oxoG is significantly increased in frontal cortex autopsy tissue from AIDS patients

HIV-1 infection leads to an increase in ROS production. Several antioxidants, including l-deprenyl and ebesselen, can block ROS toxicity in the CSF, suggesting a key role for oxidative stress in the neuronal death that occurs in HIV-1 neuropathy. ROS can interact with a spectrum of cellular macromolecules, resulting in lipid peroxidation, protein oxidation and oxidative DNA damage. Like the polyunsaturated fatty acids within cell membranes, DNA is also particularly vulnerable to be attacked by ROS. The most common type of DNA oxidative damage is the production of 8-hydroxyguanine (8-oxoguanine, 8-oxoG), which is highly mutagenic to genomic DNA. In this study, we detected 8-oxoG in the frontal cortex of autopsy tissues by anti-8-oxoG specific monoclonal antibodies and in situ immunofluorescence. The results are shown in Fig. 3A. We found that the 8-oxoG levels increased in tissues from both patients with and without HAND. However, 8-oxoG could not be detected in the control frontal cortex tissue. We then analyzed the density of 8-oxoG staining in the ten HIV-1 and five control samples. The results are shown in Fig. 3B (data from three independent experiments). There was an average of 45% 8-oxoG positive cells in tissues from patients with HAND and 30% from patients without HAND. However, there was only an average of 4% 8-oxoG positive cells in the controls. Due to 8-oxo-G being a confirmed marker for ROS, these results, at least in part suggest that nuclear DNA damage exists in the frontal cortex of AIDS patients due to high levels of ROS which

Fig. 2 – Depletion of mtDNA was detected in frontal cortex autopsy tissue from HIV-1 infected patients. A. The mtDNA qPCR results (in fold change) are in the inner region of the curve with total DNA concentration. B. Compared with control, mtDNA copies were reduced in frontal cortex autopsy tissue from HAND and non-HAND patients. The data represent the average of three independent experiments.
probably contribute to neuronal injury. It is well known that the efficient removal of 8-oxoG damaged DNA via the short-patch base excision repair (BER) pathway is initiated by the enzyme DNA glycosylase 8-oxo-20-deoxyguanosine glycosylase 1 (OGG1) (Boiteux and Radicella, 1999). Studies on aging and neurodegenerative disease have found that there is an inverse correlation between OGG1 activity and 8-oxoG accumulation (Bolin et al., 2006). In this study, OGG1 was also detected in the autopsy tissue samples via immunofluorescence (Fig. 3C). We found that the level of OGG1 is significantly lower in the autopsy tissue from patients with HIV-1 than in controls, but no difference was found between patients with HAND and without HAND. These results suggest that both ROS and OGG1 effect the accumulation of 8-oxoG in the CNS of patients with HIV-1. Unfortunately, we did not identify the different cell types with 8-oxoG damage or OGG1 expression in our study. According to an experiment for identifying neurons (anti-MAP2 antibody) and astrocytes (anti-GFAP antibody), we found more than 90% DAPI positive cells matched with anti-MAP2 antibody staining (data not shown). This suggests that the results in this experiment mainly represent the 8-oxo-dG and OGG1 levels in the neurons.

2.5. Increase in mtDNA mutations in frontal cortex autopsy tissue from AIDS patients

High levels of ROS induced by both antiretroviral therapy and HIV-1 proteins are associated with mtDNA depletion and mutations (Gingelmaier et al., 2009). The chronic oxidative stress in neurons of HIV-1 infected patients can induce neuronal death and accelerate the progression of HAND. We identified high levels of 8-oxoG in autopsy tissues from HIV-1 patients, and the question of more mtDNA mutations occurs in the cortex of patients with HAND is raised. After preparing total genomic DNA from the frontal cortex autopsy tissue of patients with AIDS, PCR was performed on the D-loop regions (bp 1 to 583), followed by the
nucleotide distance assays of the mtDNA D-loop sequence, using BioEdit software (Fig. 4A), and aligned with the mtDNA sequence NC_012920 from GenBank. The results showed that the autopsy tissue from AIDS patients had an increased level of mutations. The distance to the standard mitochondrial DNA sequence (NC_012920) was 0.0471 (0.0122–0.1227). In the five control samples, however, results indicated that only two of them had a deletion (D310C), and none of them had mutations. Our data revealed that, compared with the control, HAND autopsy tissues had a significantly higher frequency of mtDNA mutations in the mtDNA D-loop sequences (Fig. 4B). The average number of single nucleotide mutations was 39 in HAND tissues and 13 in non-HAND tissues, and only two in control samples. The actual sequences/alignments results are shown in the supplementary materials (Fig. S1). This high level of single nucleotide mutations in the frontal cortex cells of patients with HAND could be associated with mitochondrial dysfunction which further increases the ROS level of cells.

2.6. Presence of superoxide anion level in cerebrospinal fluid from AIDS patients

Dihydroethidium (HE) was usually used to detect superoxide anion level in cells and tissues by fluorescence-based techniques. HE itself shows a blue fluorescence (absorption/emission: 355/420 nm) in cell cytoplasm until oxidation to form 2-hydroxyethidium (2-OH-E’) which becomes red fluorescent (absorption/emission: 518/605 nm) upon DNA intercalation (Kaufman and Jing, 2002). Only once it is internalized and dehydrogenated (oxidized) to 2-OH-E’, can it intercalate into DNA. In order to further confirm increased oxidative damage in the CNS of AIDS patients, as well as HAND patients, in this study, human leukemia K562-EGFP cell line which stably expresses EGFP green fluorescence was co-incubated with fresh CSF of AIDS patients including AIDS with or without HAND for 15 min respectively. Then, HE level in K562-EGFP cell was detected by flow cytometry. The results disclosed that HE detection rates which represented superoxide in CSF increased as HAND development in that 4% in negative control, 28% to 40% in 5 non-HAND patients, 37% to 65% in 5 HAND patients, and 99% in 0.3% H2O2 as positive control (Figs. 5A and B). The results suggested that the ROS levels in the CSF of HAND patients were significantly higher than that in the CSF of non-HAND patients.

3. Discussion

China appears to be on the brink of a tremendous upsurge in cases of human immunodeficiency virus type 1 (HIV-1) infection
Most cases of HIV-1 infection due to blood transfusions from PBD are in the province of Henan and its adjacent areas. The epidemic of HIV-1 in this particular population began in the middle of 1980s and peaked between 1992 and 1996. There are few reports of HAND in these patients. We selected 10 samples of HIV-1 infected cortex autopsy tissue from this population, including five with HAND, with the average length of HIV-1 infection being 12 years.

Previous studies have shown that HIV-1 co-receptor usage from plasma samples of this group obeyed the V3 loop 11/25 rule (SI phenotype and presence of basic amino acids at positions 11 and 25 on the V3 loop) (Chun et al., 2009). All of the HIV-1 isolated from patients with the NSI phenotype displayed R5 tropism. Reversely, most of the HIV-1 isolated from patients with the SI phenotype displayed R5X4 tropism and induced syncytium formation (Saitoh et al., 2007). Both Geno2pheno and PSSM prediction programs predicted that autopsy cortex tissue from the patients had the R5 tropism, based on V3 sequences of HIV-1 pro-viruses. This suggests that HIV-1 co-receptor usage could be different at AIDS stage between blood and CNS. Recently, we analyzed the characteristic of HIV 1 env gene in cerebrospinal fluid and plasma of infected Chinese paid blood donors. We found that the level of diversity from CNS was significantly lower than those from blood. According to 11/25 charge rule, all viral quasispecies from the CNS of all patients use CCR5 as co-receptor (Liu et al., 2012; Wei et al., 2011). Previous studies have demonstrated that HIV-1 sequences clustered together by organ in phylogenetic analyses, brain-specific evolution may be due to host immune response and environmental factors within the CNS that influence selection pressures. Some studies also showed that HIV-1 isolated from the CSF of HAD patients had both R5 and CXCR4 tropisms, and HAD pathogenesis was associated with the CXCR4 tropism. However, their study did not identify how many of their HIV-1 samples were from the CSF or brain tissue. In this study, we did not have access to plasma and CSF samples from these patients, and no data was collected regarding tropisms in the CSF when the patients were alive.

Previous studies suggest that oxidative stress probably plays a key role in the neuropathogenesis of HIV infection. Viral proteins, cytokines or the virus itself can stimulate neuronal and glial cells to produce ROS. One of the most frequent and mutagenic products of the oxidation of genomic DNA is 8-oxoG. It has been recognized as an indicator of the level of DNA oxidative damage and cellular oxidative stress. It is an identifying biomarker of toxicity of ROS. One study found that high levels of 8-oxoG and low levels of DNA glycosylase activity existed in CD4+ T cells in HIV-1 infected patients. However, in CD8+ T cells, the 8-oxoG levels were similar to those in healthy controls (Aukrust et al.,

Fig. 5 – Presence of superoxide anion level in cerebrospinal fluid from AIDS patients. The superoxide anion level in K562-EGFP cells was detected in different conditions via HE fluorescence-based flow cytometry. X-axis showed EGFP fluorescence and y-axis showed 2-hydroxyethidium (2-OH-E+) red fluorescence. The results disclosed that HE detection rates which represented superoxide in CSF were increased as HAND development in that 4% in negative control, 28% to 40% in non-HAND, 37% to 65% in HAND and 99% in 0.3% H2O2 as positive control.
Our study in the frontal cortex of HIV-1 autopsy tissue showed that 8-oxoG staining positive cells accounted for up to 45% of the total DAPI positive cells, but only 4% in healthy controls. This accumulation of oxidative DNA damage in HIV-1 autopsy brain tissue implies high level of ROS in the CNS of AIDS patients which could play a critical role in neurodegeneration.

In clinical studies, mtDNA mutations and loss have been shown to increase with age and in common neurodegenerative diseases including Alzheimer disease (AD) (Reeve et al., 2008). HIV infection and treatment with nucleoside analogue reverse transcriptase inhibitors (NRTIs) are two important elements involved in mitochondrial DNA mutation and mitochondrial dysfunction (Casula et al., 2005; Kohler and Lewis, 2007). Targeted expression of HIV Tat to cardiomyocytes can damage the mitochondria. Additionally, over expression of HIV Tat represses mitochondrial superoxide dismutase expression (Raidel et al., 2002). The HIV-1 virus can enter the CNS in the early stages of infection. In the subsequent chronic pathological processes in the HIV-1 infected brain, the neural immune activation and the direct toxicity of viral proteins will increase the level of oxidative stress on neuronal and glial cells, resulting in a large number of ROS which includes hydroxyl radical, superoxide anion, and singlet oxygen (Pocernich et al., 2005). Our initial results suggest that superoxide in CSF increased as HAND development. Because of the high levels of ROS in the mitochondrial matrix, mtDNA mutations are frequent in cells. Furthermore, HIV infection alone can produce oxidative stress and stimulate the overexpression of mitochondrial glutaminase that induces glutamate-mediated neuronal apoptosis during HAND (Allard et al., 1998; Elbim et al., 2001; Tian et al., 2008). Thus, we were interested in determining whether mtDNA mutations and mtDNA loss were associated with HAND. Our results showed a higher level of mtDNA mutation and loss in HIV-1 infected autopsy tissue. Some studies have reported that HIV-1 infection and viral proteins such as Tat and Vpr play an important role in inducing high level of mitochondrial ROS which was relevant to the progression of HAND (Opii et al., 2007; Velsor et al., 2004). In our study, the mitochondrial ROS which was associated with HAND. Our results showed a higher level of mitochondrial ROS which was relevant to the progression of HAND (Opii et al., 2007; Velsor et al., 2004). In our study, the mitochondrial ROS which was relevant to the progression of HAND. Our results showed a higher level of mitochondrial ROS which was relevant to the progression of HAND (Opii et al., 2007; Velsor et al., 2004).

4. Experimental procedures

4.1. Antibodies and assays

Antibodies used in these studies were: mouse monoclonal anti-8-oxoG antibodies (Trevigen, USA); Anti-CCR5 and anti-gp120 antibodies (Santa Cruz Biotech, USA); and anti-ogg1 polyclonal antibodies (Novus Biologicals, Littleton, CO, USA). FITC-labeled anti-rabbit IgG and CY3-labeled anti-mouse IgG were obtained from Sigma in China (Sigma, Beijing, China). Dihydroethidium were obtained from Invitrogen in China (Invitrogen, Beijing, China). Viral load assays were performed with a NUCLISENS EasyQ Analyzer (Nuclisens EasyQ HIV-1.1, bioMérieux bv) and CD4 count assays were performed with the TriTEST CD4 FITC/CD8 PE/CD3 PerCP reagent (BD FACSCalibur, USA).

4.2. HAND scale test

The HAND was identified with International HIV Dementia Scale Score (IHDS) including memory-registration-recall; motor speed and psychomotor speed based on previous report (Sacktor et al., 2005). A total summed IHDS score is 12. IHDS score below or equal 10 suggests HAND, with lower scores indicating more severity.

4.2.1. Autopsy brain tissue

The frontal cortex from autopsies of AIDS patients was obtained from a group of patients who were infected with HIV-1 via commercial blood donation (PBD). This study was undertaken at the HIV/AIDS research center, Beijing You An Hospital, Capital Medical University, with institutional approval from the Beijing You An Hospital (IRB protocol [2009-1]). A total of 10 HIV positive frontal cortex autopsy tissue samples were obtained, five of which were from patients with confirmed HAND at various grades, and five of which were from patients without HAND. Five autopsy samples of normal frontal cortex brain tissue, used as controls, were obtained from the Department of Neuronal Surgery, Beijing Chao Yang Hospital, Capital Medical University. Tissues were fixed in 10% formalin. General patient information is provided in Table 1.

4.3. Immunofluorescence staining

Tissues on slides were frozen in –80 °C. Immunolabeling for 8-oxoG, OGG1, CCR5, and GP120 was performed by serial incubation of tissues with anti-8-oxoG, anti-OGG1, anti-CCR5, and anti-GP120 antibodies at 4 °C overnight followed by FITC or Cy3 labeled secondary IgG antibody (1:300) for 1 h at 37 °C. The nucleus was stained with DAPI before mounting on cover slips. Slides were viewed using a fluorescence microscope and images were collected using a Xillix digital camera. The 8-oxoG positive level was counted via the ratio of 8-oxoG positive nucleus/DAPI positive nucleus; and the relative OGG1 density was calculated with Image Pro Plus 6.0 software.
4.4. DNA isolation and quantitative real-time PCR

Total DNA was isolated using a kit from QIAGEN China Co., Ltd. (QIAGEN, Shanghai, China) following the recommended protocol. Quantitative PCR (qPCR) assays were performed as previously described with minor modifications (Wu et al., 2009). The TaqMan 7900HT system was used to perform real-time PCR amplification of the mtDNA Coxl region using the following primers and probes: (Forward Primer: 5′-CCCAACATTGGCT-TAAAAACAGAT-3′, Reverse Primer: 5′-TATACCCCGTCGTTG-TAGCGGT-3′, Probe: 5′-FAM-CAATTTGCACTTAAACCC-AACACTTTC-TAMRA-3′). GAPDH was used as an internal control (Forward Primer: 5′-CGGGGCTTCAGAACATC-3′, Reverse Primer: 5′-ATGACCTTGCCCACAGCCT-3′, Probes: 5′-FAM-CCTGCTCTACTGGCGCTGCC-TAMRA-3′). All primers and probes were obtained from Invitrogen (Shanghai, China). The real-time PCR reactions were performed in triplicate for each sample. qPCR was performed in TaqMan 7900HT Fast Real-time PCR System with probe and primer concentrations of 250 and 300 nM, respectively, in the final PCR reaction mix. Cycling variables were: 3 min at 95 °C and then 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Controls were performed under the same conditions. Data analysis was performed using Microsoft EXCEL software.

4.5. Cloning and sequencing of mtDNA of D-loop region

PCR amplification was performed using 10 ng of total DNA, according to a standard protocol, using high fidelity Platinum Taq polymerase (Invitrogen, Shanghai, China). PCR products were cloned into pGEM-18 T vectors (Tankra, China) according to the manufacturer’s instructions. All sequences were obtained by cycle sequencing executed by Biotake Co. Ltd. (Beijing China) using an ABI 3130 sequencer (Applied Biosystems, USA). Sequence analysis was conducted using NCBI BLAST and BioEdit software. Multiple alignments were performed using Clustal W Multiple Alignment program in Bioedit, version 7.0.5.3.

4.6. Single genome amplification (SGA) for HIV-1 subtype assay

Genomic DNA was isolated and purified from formalin fixed brain tissue using a DNA purification kit from Qiagen (Shanghai China). Purified genomic DNA was serially diluted and distributed in replicates of 8 PCR reactions in MicroAmp 96-well plates, so as to identify a dilution where PCR positive wells constituted less than 30% of total number of the reactions to get the genomic DNA concentration for the PCR product from a single cDNA molecule. PCR amplification was carried out in the presence of High Fidelity Platinum Taq PCR system (Invitrogen). The sequences of ED31/BH2 primers and DR7/DR8.8 used in C2-V5 PCR were described in a previous report (Chen et al., 2009). Briefly, ED31/BH2 primers were used to amplify 900 bp of the envelope gene, followed by a nested PCR amplification of the C2–V5 region using primers DR7/DR8.8. Amplicons were directly sequenced by cycle-sequencing using BigDye Terminator chemistry and protocols recommended by the manufacturer (Applied Biosystems, Foster City, CA). The sequences were subjected to heteroduplex mobility assays (HMA) using a kit supplied by the NIH AIDS Repository program. The Accession Numbers from case 1 to case 10 respectively are: JQ218937–JQ21846.

4.7. Superoxide detection in K562 cells incubated with CSF from AIDS patients

Human leukemia K562-EGFP cells had been constantly preserved in our laboratory, and were cultured in 6 well plates in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO2 incubator. For the purpose of detection of superoxide anion level in CSF of AIDS patients, 1×10⁵ K562-EGFP cells were co-incubated with the 200 μL CSF of AIDS patients for 15 min. K562-EGFP cells co-incubated with 0.03% H₂O₂ for 5 min were selected as positive control as well as without treatment K562-EGFP cells as negative control. Then the medium was refreshed with PBS and HE which was in a stock solution of 10 mg/mL in dimethyl sulfoxide (Polysciences, Warrington, PA) was added directly to the culture medium at a final concentration of 50 μg/mL. The culture was incubated for 10 min at 37 °C, in the dark. Finally, the cells were analyzed using BD FACSDiva flow cytometry (BD FACS Canto™ II). HE was excited at 490 nm and detected at 620 nm. List mode data from 10,000 cells were stored and precessed with CellQuest software.

4.8. Data analysis

Each sample was analyzed for 8-oxoG, and cell counts were obtained from ten randomly selected 20× microscopic fields of frontal cortex tissue. All statistical analyses were performed with Sigma Plot 11.0 software using one-way ANOVA to compare individual means with significant differences at a confidence level of p<0.05. Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.brainres.2012.04.001.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (20910103915 and 30870853), Beijing Natural Science Foundation (7092045 and 7101005) and Twelfth Key Science and Technology Five Year Plan of China (2012ZX1001-002, 2012ZX1001-003 and 2012ZX10001-004).

References


