Journal of NeuroVirology, 7: 454–465, 2001
 ISSN 1355–0284/01 $12.00+.00

Protective effect of glutathione in HIV-1 lytic peptide 1-induced cell death in human neuronal cells

Ji Hye Sung,[1] Soon Ah Shin,[1] Hae Kyung Park,[1] Ronald C Montelaro,[2] and Young Hae Chong[1]

[1]Department of Microbiology, College of Medicine, Division of Molecular Biology and Neuroscience, Medical Research Center, Ewha Womans University, Yangcheonku, Seoul, Korea; and [2]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

To elucidate the pathogenic mechanisms involved in neurodegeneration in AIDS patients with cognitive deficits, we have examined the toxic effect of the lentivirus lytic peptide 1 (LLP-1) corresponding to the carboxyl terminus of HIV-1 transmembrane glycoprotein gp41 on human neuronal and glial cell lines. LLP-1 induced a significant lactate dehydrogenase (LDH, a marker of cell death) release from these cells in a concentration- and time-dependent manner, while the noncytolytic LLP-1 analog 2 had little effect. Application of LLP-1 to SH-SY5Y, a well-characterized human neuronal cell line, caused the decline of intracellular glutathione (GSH) content that appeared to occur before a significant LDH release. Furthermore, LLP-1 elicited a significant loss of mitochondrial function as measured by mitochondrial transmembrane potential (MTP). Among the reducing agents and antioxidants tested, GSH and a GSH prodrug *N*-acetylcysteine (NAC) provided protection against LLP-1-induced neuronal cell death, evidently by restoring the intracellular GSH levels and blocking the disruption of mitochondrial integrity. Thus, gp41-derived LLP-1 may be a potential neurotoxic agent capable of causing the intracellular GSH depletion and disturbing the mitochondrial function, possibly contributing to the neurodegenerative cascade as seen in HIV-1-associated dementia. Our data indicate that restoring both GSH concentration and mitochondrial function may hold promise as possible therapeutic strategies for slowing disease progression of dementia in AIDS patients. *Journal of NeuroVirology* (2001) **7,** 454–465.

Keywords: HIV-1-associated dementia; gp41; glutathione depletion; mitochondrial transmembrane potential; N-acetyl cysteine

Introduction

The mechanisms responsible for human immunodeficiency virus 1 (HIV-1) invasion of the central nervous system (CNS) and its association with the generation of AIDS dementia remain undefined, despite extensive efforts in this area of research. Productive viral infection of CNS cells is limited almost exclusively to immune cells of monocytic lineage, including brain macrophages and microglia, whereas neurons and astrocytes may undergo nonproductive or restricted infection (Glass and Johnson, 1996). Nevertheless, neurons, astrocytes, oligodendrocytes, and microglia all undergo pathological changes in AIDS brains. Thus, indirect mechanisms involving viral gene products, cytokine dysregulation, and other cellular factors which are released by HIV-1 infected brain macrophages and microglia as well as reactive astrocytes are implicated in the severity of neurological impairment in HIV-1-associated dementia (Glass and Johnson, 1996). Earlier reports demonstrated that the extent of dendritic and neuronal damage in HIV encephalitis may be more closely correlated with the amount of HIV-1 transmembrane envelope protein gp41 in the brain (Masliah *et al*, 1992; Glass *et al*, 1995). Furthermore, recent studies indicate a strong association of highly increased HIV-1 gp41 levels in HIV-1-infected brains with the severe and rapidly

Address correspondence to Young Hae Chong, Department of Microbiology, College of Medicine, Ewha Womans University, 911–1, Mok-6-dong, Yangcheonku, Seoul, Korea, 158-056. E-mail: younghae@mm.ewha.ac.kr

Received 3 November 2000; revised 10 April 2001; accepted 7 May 2001.

progressive cognitive dysfunction in HIV-1-associated dementia (Adamson *et al*, 1996, 1999; Rostasy *et al*, 1999). These observations together appear to implicate gp41 as a key viral determinant involved in neurodegenerative cascade seen in AIDS patients with dementia.

HIV-1 gp41 participates in several important aspects of viral replication and appears to have an important role in cytopathogenesis. The amino terminus of HIV-1 gp41 contains a fusion peptide domain capable of mediating virion-cell fusion and capsid entry, inducing formation of multinucleated syncytial cells and directly damaging the cell membrane (Fermin and Garry, 1992). This region also includes multiple immune-modifying domains that are associated with inhibition of lymphoproliferation, complement activation, and cytokine dysregulation leading to suppression of cellular immunity (Ruegg and Strand, 1991; Haraguchi *et al*, 1995; Marschang *et al*, 1997; Chong and Lee, 2000). On the other hand, a strongly amphipathic and arginine-rich helical lytic peptide, designated lentivirus lytic peptide (LLP-1), present in the carboxy terminal cytoplasmic domain of HIV-1 gp41 (amino acids 828-855) has been demonstrated to be a potent eukaryotic and/or prokaryotic cytolytic agent (Miller *et al*, 1991, 1993b). LLP-1, both as synthetic peptides and full-length gp41, is also known to interact with calmodulin, an important calcium-binding protein and affect the intracellular distribution of this important regulatory protein in HIV-1-infected cells (Miller *et al*, 1993b; Srinivas *et al*, 1993; Tencza *et al*, 1995). A second amphipathic α-helical motif with lytic and calmodulin-binding properties, designated LLP-2 (amino acids 757-779), present near the carboxyl terminus of gp41 may also be involved in HIV-1-induced cytopathology (Miller *et al*, 1993a; Tencza *et al*, 1995; Beary *et al*, 1998). The cytolytic properties of these lytic peptides have been attributed in part to their ability to alter the electrophysiological properties of biological membranes in much the same manner as natural cytolytic peptides such as magainins, cecropins, and melitttin which are produced by certain amphibians and insects (Zasloff *et al*, 1988; Miller *et al*, 1991; Chernomordik *et al*, 1994; Comardelle *et al*, 1997). Although there have been extensive studies for multiple cytopathic and immune-modifying properties by the HIV-1 LLP in lymphoid cells, the potential cytopathic roles and mechanisms mediated by these gp41-derived peptides within HIV-infected brains have not been extensively explored.

Glutathione (GSH) present in the brain (1–2 mM) strongly modulates the redox state of the cell, a role that is critical for cell survival (Cooper, 1997). Thus, GSH depletion may be a common mechanism underlying various forms of cell death including necrosis, apoptosis, and excitotoxicity in distinct neural subsets in both normal aging and in various neurodegenerative disorders. Evidence for a role of diminished GSH status and oxidative stress-mediated neuronal loss is presented for not only HIV infection but also Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, each of which has been suggested to have increased oxidative stress (Bains and Shaw, 1997; Herzenberg *et al*, 1997). Earlier studies showed that low GSH levels both promote HIV-1 expression and impair T-cell function, suggesting GSH deficiency as a key pathogenic determinant in HIV-1 disease (Staal *et al*, 1992). Furthermore, HIV-1-infected people tend to have a systemic GSH deficiency with subnormal GSH levels in plasma and CSF (Castagna *et al*, 1995; Helbling *et al*, 1996). Clinical studies thus demonstrated that low GSH levels predict poor survival in otherwise indistinguishable HIV-infected subjects and that oral administration of the GSH prodrug *N*-acetylcysteine (NAC) replenishes GSH in these subjects, improving their survival (Herzenberg *et al*, 1997). These observations together implicate that a disturbed cellular GSH homeostasis plays an important role in the pathogenesis of HIV-1 infection. However, the exact mechanism(s) and viral factors causing systemic GSH deficiency during HIV-1 infection remain unclear.

Recent studies have implicated a damaged mitochondrial function critical in neuronal death and a decline in the mitochondrial transmembrane potential (MTP) as a universal event that accompanies apoptotic and necrotic cell death (Ankarcrona *et al*, 1995; Zamzami *et al*, 1997; Kroemer and Reed, 2000). Furthermore, a possible link between the diminished GSH level and a mitochondria-mediated neurodegenerative cascade has been recently reported (Ju *et al*, 2000; Schuchmann and Heinemann, 2000). In this regard, it is important to determine the viral or cellular factors that could disturb GSH homeostasis and mitochondrial function in human brain cells, especially as it relates to potential therapeutic strategies to help compensate for such decreased redox status possibly linked to a mitochondrial dysfunction.

The aim of this study, therefore, was to examine the toxic effect of LLP-1 on human neuronal cells and to further elucidate the molecular mechanisms underlying LLP-1-mediated neurotoxicity. We specifically determined whether LLP-1 interacts with SH-SY5Y, a well-characterized human neuronal cell line, to affect the intracellular GSH level by measuring GSH concentration with the fluorescent dye, monochlorobimane (MBCl). Furthermore, modulation of mitochondrial function associated with LLP-1-induced neurotoxicity was also analyzed by monitoring changes in MTP using the membrane potential-sensitive probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). In addition, the various antioxidants and reducing agents including GSH and NAC were employed to test protection against LLP-1-induced neuronal cell death for evaluating possible therapeutic strategies of host defense functions in the control of HIV-1-associated neurological disease.

Results

The mechanism by which HIV-1 induces neurodegeneration in AIDS patients with cognitive deficit remains controversial, but may involve cytotoxic domains of viral proteins such as gp41-derived LLP-1. To address neuronal cell death induced by LLP-1, LDH release was measured in SH-SY5Y cells treated with increasing concentrations of LLP-1 for 20 h. Exposure to LLP-1 resulted in the LDH release from human neuronal and glial cell lines in dose-dependent manner (Figure 1A). In agreement with previous reports (Miller *et al*, 1991, 1993a), as assessed morphologically using a phase-contrast microscope, nearly 50% of total cells died after LLP-1 treatment at 10 μM concentration, correlating with an observed about 45% increase in LDH release from LLP-1-treated human neuronal SH-SY5Y cells compared to untreated controls. Further increases in LLP-1 concentrations proportionally increased the observed cell cytotoxicity and LDH release levels from treated cells and further increases of LDH release were observed at higher concentrations. The dose-dependent effect of LLP-1 on human glial T98G cells mimicked that observed with human neuronal SH-SY5Y cells, although a higher LLP-1 concentration (>25 uM) was required to reach similar level of cytotoxicity. The toxic effect of LLP-1 on both SH-SY5Y and T98G cells was time-dependent and a significant LDH release ($P < 0.001$) was observed as soon as 3 h followed by increasing and statistically significant LDH release by LLP-1 treatment (Figure 1B). Similar dose- and time-dependent patterns of cytotoxicity were also observed in other human neuronal SK-N-SH and human glial U251 cell lines (data not shown). However, LLP-1 analog 2, which has been substituted with

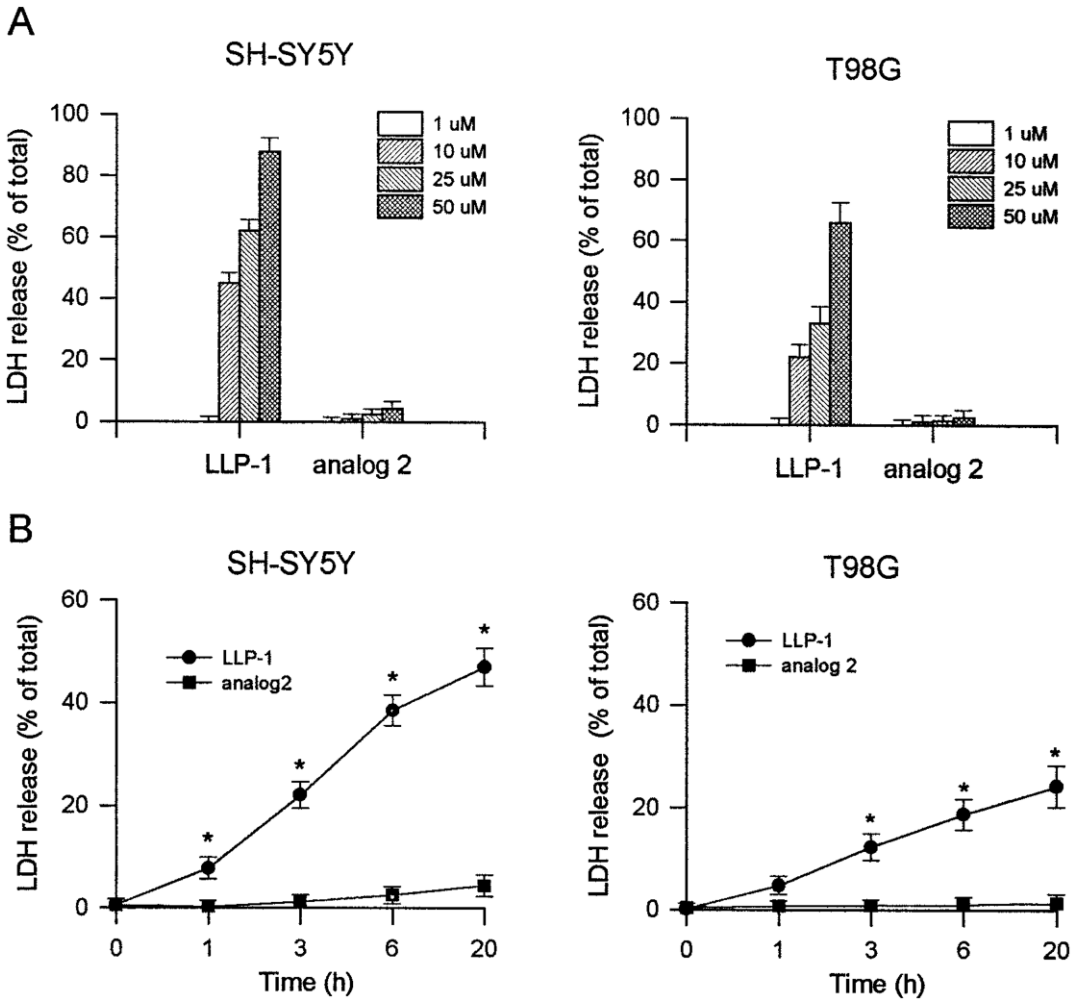

Figure 1 Effect of LLP-1 on LDH release from SH-SY5Y and T98G cells. LDH activity in the conditioned medium from human neuronal and glial cell lines, SH-SY5Y and T98G, was assayed at 20 h after treatment with indicated concentrations of LLP-1 or analog 2 (A) and after incubating cells with 10 μM LLP-1 or analog 2 for the indicated time (B). Data are mean ± SEM (bars) values obtained from four different wells in the same 96-well culture plate per experiment, determined in five to seven independent experiments ($n = 5$–7) and expressed as a percentage of total LDH released from detergent treated cells (Materials and methods). $^*P < 0.001$ by ANOVA compared with the appropriate untreated groups.

two glutamic acids for two arginine residues to abrogate peptide lytic activity, did not show any significant cytotoxic effects on these neuronal and glial cells. SH-SY5Y cell line was chosen for more detailed mechanistic studies because LLP-1 displayed a more prominent cytotoxic effect on neuronal cells as compared to glial cells.

Because recent studies indicated that the increased neuronal death might result from a diminished concentration of reduced GSH (Ju *et al*, 2000; Schuchmann and Heinemann, 2000), we next examined whether the decrease in intracellular GSH level was associated with LDH release from LLP-1-treated SH-SY5Y using the fluorescence dye MBCl. The MBCl dye rapidly and specifically reacts with GSH to form a highly fluorescent adduct even at low concentrations of 50–100 μM, whereas unbound MBCl has no fluorescence. Thus, it has been efficiently used to determine intracellular GSH in human living cells and in intact, cultured neurons and glial cells (Millis *et al*, 1997; Ju *et al*, 2000; Schuchmann and Heinemann, 2000). The GSH level in control SH-SY5Y cultures was about 4.20 $\pm$ 0.43 nmol/mg of protein (see the legend of Figure 2). A rapid decrease of 30–45% in intracellular GSH was observed in LLP-1-treated SH-SY5Y cells (Figure 2). In marked contrast, intracellular GSH levels were barely decreased by less than 5% in SH-SY5Y cells exposed to LLP-1 analog 2. The decrease of intracellular GSH levels appeared to precede the release of LDH because intracellular GSH levels in intact cells were decreased to nearly 45% of the control level within 3 h (Figure 2),

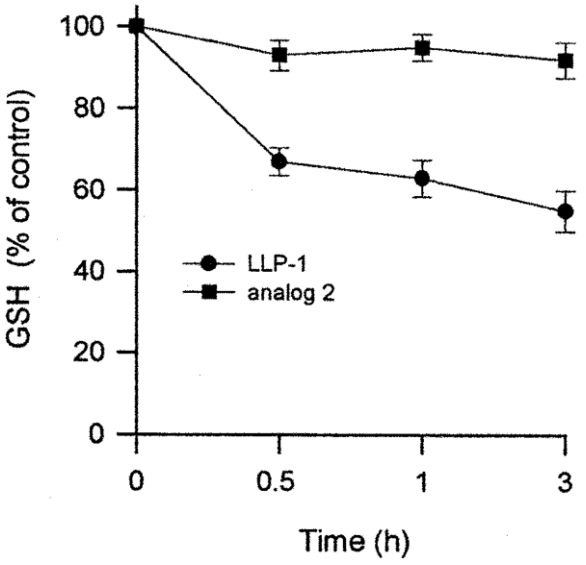

Figure 2 Effect of LLP-1 on GSH content in SH-SY5Y cells. Intracellular GSH levels were measured at the indicated intervals in SH-SY5Y cells after exposure to 10 μM LLP-1. The GSH level in the control (untreated) SH-SY5Y cells was 4.20 $\pm$ 0.43 nmol/mg of protein. Data are mean $\pm$ SD (bars) values ($n = 5$), expressed as a percentage of control, which represents the GSH level at each indicated time in untreated SH-SY5Y cells.

whereas LDH release was increased by 26% at 3 h (Figure 1B).

Both necrotic and apoptotic cell death are characterized by changes in mitochondrial function involving mitochondrial transmembrane potential (MTP) and mitochondrial pore formation (Ankarcrona *et al*, 1995; Zamzami *et al*, 1997; Kroemer and Reed, 2000). To determine whether mitochondrial damage correlated with the loss of viability of LLP-1-treated SH-SY5Y cells, we monitored a decrease of MTP using the cell-permeant, fluorescent dye JC-1. The reversible formation of J-aggregates from monomers in the matrix was previously shown to depend on the MTP (Reers *et al*, 1991); thus, the fluorescence intensity at 590 nm (J-aggregates) qualitatively characterizes MTP and decreasing red fluorescence (J-aggregates) indicates dissipation of MTP. The results of this experiment (Figure 3) showed that a depolarization of the MTP, as indicated by the loss of the red fluorescence, was only observed in 10 μM LLP-1-treated SH-SY5Y cell. The LLP-1 peptide caused an apparent decrease in J-aggregates (red fluorescence) and concomitant increase in J-monomers (green fluorescence) during the first hour, at which time levels of aggregates and monomers appeared to reach a steady state. In contrast to the effects observed with LLP-1 treatment, the cells treated with analog 2 displayed no significant change in J-aggregate or monomer levels over the 3-h observation period (Figure 3). However, the LLP-1 effect on MTP was substantially lower than that induced by 5 mM H_2O_2 that is known to strongly depolarize mitochondria, as evidenced by increasing monomer fluorescence and decreasing aggregate fluorescence (Figure 3). These results indicate that the mitochondrial damage is likely linked to LLP-1-induced neuronal cell death.

Imbalance of redox status or free radical scavengers could be a critical event for neuronal cell death signaling in the genesis and progression of neurodegenerative diseases (Coyle and Puttfarcken, 1993). Thus, we investigated the protective effects of the major brain redox regulator glutathione (GSH), the GSH prodrug *N*-acetylcysteine (NAC), the other thiol containing antioxidant, pyrrolidinedithiocarbamate (PDTC), and iron-chelating antioxidant deferoxamine (DFO) on LLP-1-induced cell death. Specific doses of these agents for the LLP-1 studies were chosen on the basis of *in vitro* experiments for their biological activities as described previously (Kalebic *et al*, 1994; Chong and Lee, 2000). Figure 4 demonstrates that cell viability was not significantly affected by treatment with the indicated concentration of the drug alone, except for PDTC that was itself slightly cytotoxic at 100 μM concentration. However, coincubation with either GSH or NAC did block LDH release from LLP-1-treated SH-SY5Y cells, showing a potent protective effect against LLP-1.

A marked contrast to this pattern could be observed with addition of PDTC that enhanced LLP-1-mediated LDH release, while DFO did not exert

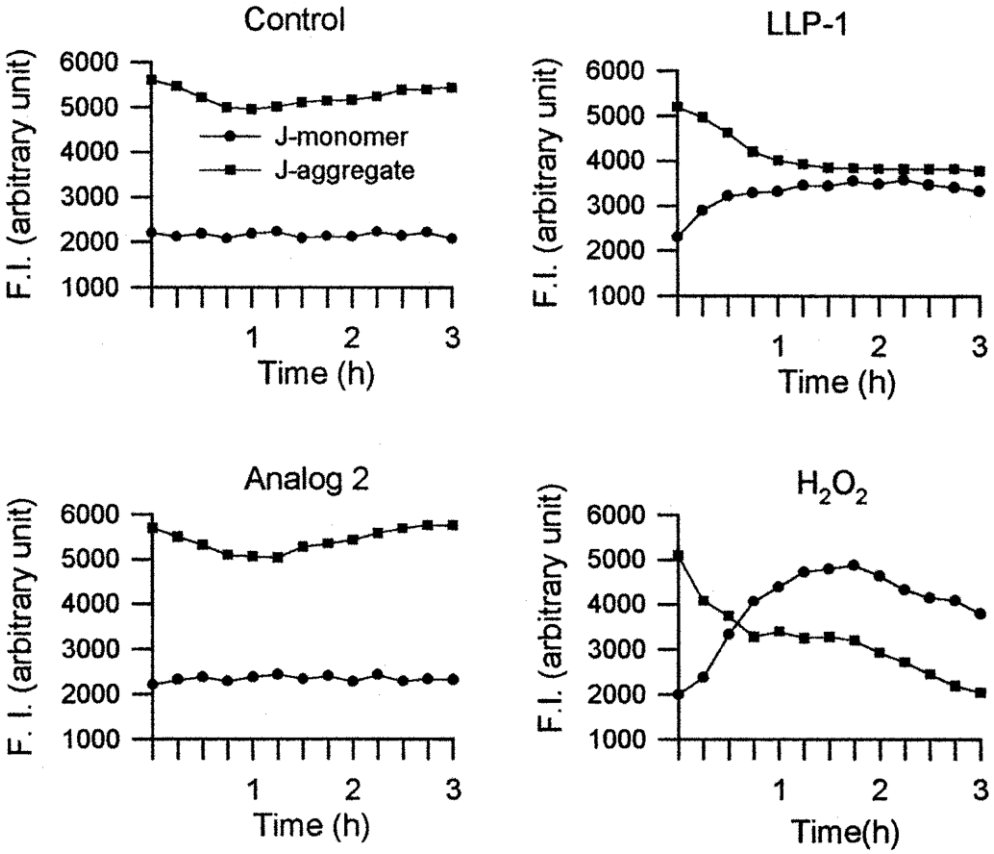

Figure 3 Effect of LLP-1 on the fluorescence of JC-1. SH-SY5Y cells were loaded with JC-1 (1.0 μg/ml) for 20 min and then incubated in the absence and presence of 10 μM LLP-1 or analog 2, as indicated. The fluorescence intensity (F.I.) values were measured at 530 (J-monomer) and 590 (J-aggregate) nm using a fluorescence microplate reader. Fluorescence values were corrected for autofluorescence. Data are mean values ($n = 4$).

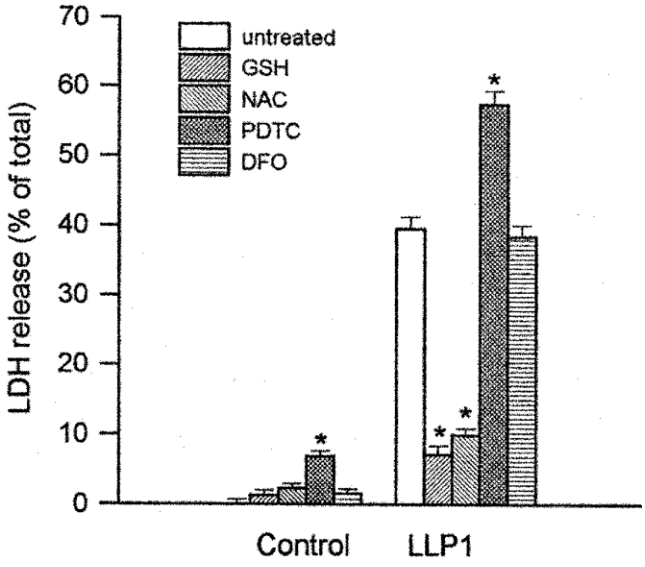

Figure 4 Effects of the antioxidants and reducing agents on LLP-1 induced neuronal cell death. SH-SY5Y cells were exposed to LLP-1 in the absence or presence of GSH (10 mM), NAC (20 mM), PDTC (100 μM), or DFO (10 μM) and then LDH release was measured 6 h after exposure as described in Figure 1. Data are mean ± SEM (bars) values ($n = 4$–7). $^*P < 0.001$ by ANOVA compared with the appropriate untreated groups.

any significant effect on LDH release by LLP-1 treatment. The observed LLP-1-induced cytotoxicity and the effects of the various antioxidants were corroborated with alterations in cell morphology and viability as measured by phase-contrast microscopy (Figure 5). Compared to the untreated control cells (Figure 5A), SH-SY5Y cells treated with 10 μM LLP-1 for 6 h exhibited a high degree of necrotic morphological signs with increasing cellular debris (Figure 5C), whereas cells treated with analog 2 rarely showed substantial degenerative changes (Figure 5B). In contrast to the effect seen with LLP-1 treatment alone, cells incubated with GSH or NAC displayed minimal morphological changes, indicating an effective protection from the deleterious effects of LLP-1 (Figure 5E, 5F). GSH (Figure 5D) or NAC (data not shown) alone did not significantly affect cell morphology as compared to the control, which validated results obtained by LDH assay (Figure 4). Compared to cells treated with LLP-1 or PDTC (Figure 5H) alone, the addition of PDTC increased the level of morphological alterations induced by LLP-1 treatment (Figure 5G), whereas DFO had no apparent effect on LLP-1 alteration of cell morphology (data not shown).

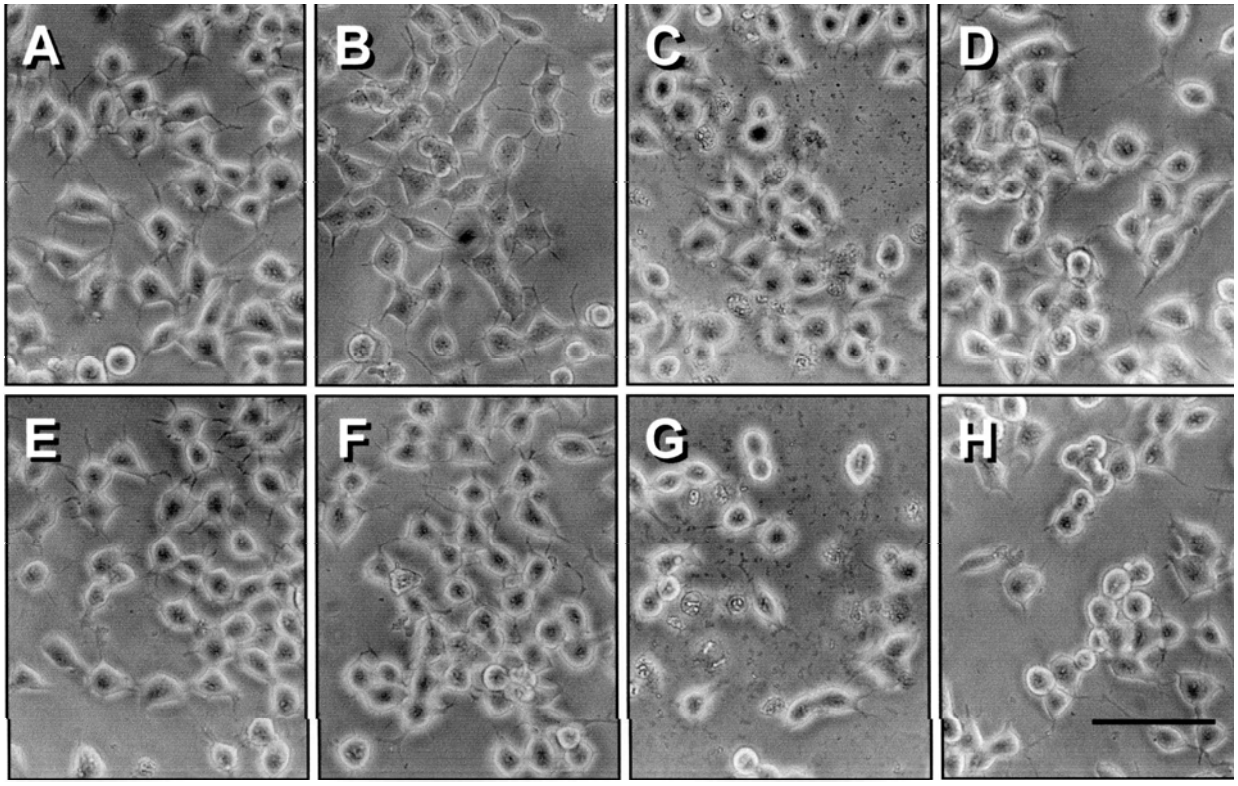

Figure 5 Effects of GSH and NAC on morphology of LLP-1-treated SH-SY5Y cells. Morphological changes of SH-SY5Y cells induced by LLP-1 in the presence or absence of various agents for 6 h were analyzed by phase-contrast microscopy: SH-SY5Y cells treated with PBS (A), 10 μM analog 2 (B), 10 μM LLP-1 (C), 10 mM GSH (D), 10 μM LLP-1 plus 10 mM GSH (E), 10 μM LLP-1 plus 20 mM NAC (F), plus 10 μM LLP-1 plus 100 μM PDTC (G), and 100 μM PDTC (H). The photographs shown are representative of five similar experiments. Bar = 50 μm.

To elucidate the protective mechanism of GSH or NAC on LLP-1-induced neuronal cell death, intracellular total GSH levels were measured in SH-SY5Y cells in the course of LLP-1 treatment. As shown in Figure 6A, exogenous addition of either GSH or NAC to the culture medium could prevent the LLP-1 mediated decrease of GSH level. In contrast, neither PDTC nor DFO treatments prevented LLP-1-induced reduction in intracellular GSH levels. Furthermore, changes in MTP could be also determined as either an increase in the monomer signal or as a decrease in the aggregate signal using the JC-1 ratio (the aggregates/monomer), an indication of the MTP (Reers *et al*, 1991). Addition of either GSH or NAC significantly restored the JC-1 ratio in response to LLP-1 when assayed at 1.5 h (Figure 6B), thus reducing depolarization and resulting in subsequent neuronal survival. Incubation with either GSH or NAC alone did not significantly affect the intracellular GSH level but resulted in a slight decrease of the JC-1 ratio, whereas DFO alone did not elicit any significant effects. In contrast, PDTC itself elicited neurotoxicity, as shown in Figure 4, and resulted in decreases in both intracellular GSH contents and JC-I ratio (Figure 6A, 6B). Thus, the protective mechanism by GSH or NAC against LLP-1-induced neurotoxicity appears to involve blocking both GSH depletion and MTP depolarization.

Discussion

The biological functions of HIV-1 gp41 remain critical to unraveling the mechanism underlying cognitive deficits in AIDS patients. Previous studies demonstrated that gp41-derived LLP-1 has a capacity to induce cellular toxicity through the disturbance of cell membrane integrity in various cell lines (Miller *et al*, 1991, 1993a; Chernomordik *et al*, 1994; Tencza *et al*, 1995; Comardelle *et al*, 1997). The cytolytic properties of HIV-1 LLP-1 peptides require a conservation of the highly positively charged amphipathic helical structure, as even minor changes in charged residues completely abrogates cytolytic activity (Miller *et al*, 1993a). In addition to the channel-inducing and cytotoxic effects of LLP-1, a recent study reported its role specific to the central nervous system (CNS) in excitotoxic damage to neurons in HIV-1 infection through impairment of excitatory amino acids transport in glial cells (Kort, 1998). We are here reporting that LLP-1 of HIV-1 gp41 significantly elicited a highly specific

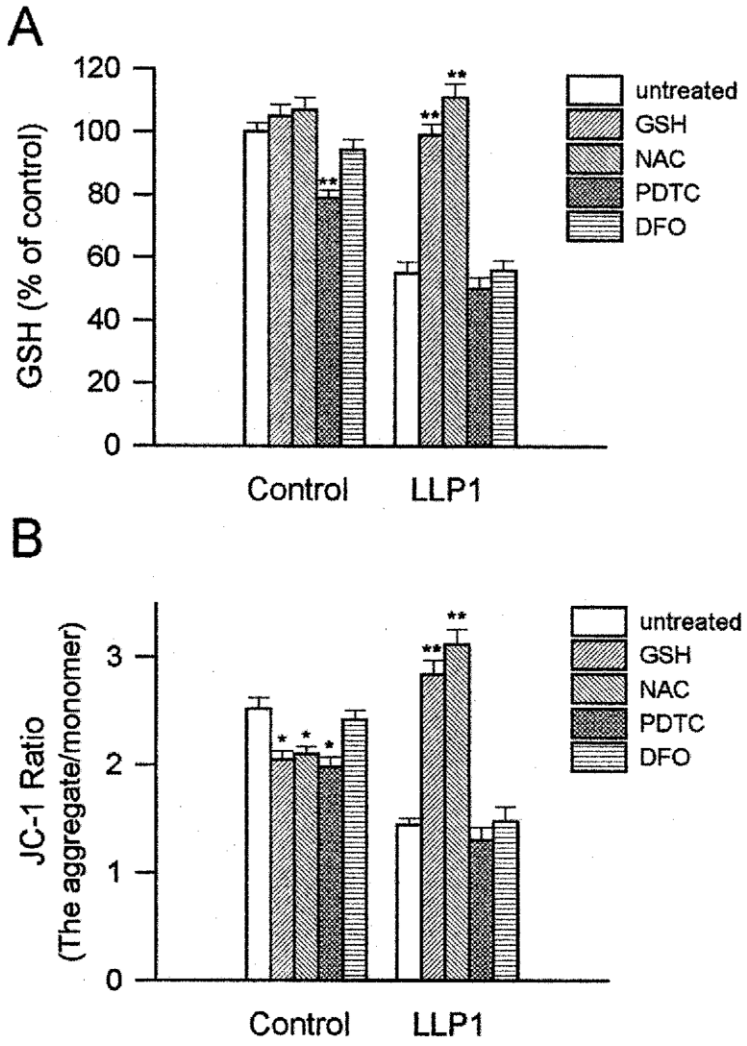

Figure 6 Effects of GSH and NAC on GSH contents and the JC-1 ratio in LLP-1-treated SH-SY5Y cells. Intracellular GSH levels (A) and fluorescence of JC-1 (B) were measured at 3 h and 1.5 h in SH-SY5Y cells treated with 10 μM LLP-1 as described in Figures 2 and 3, respectively, in the absence or presence of GSH (10 mM), NAC (20 mM), PDTC (100 μM), or DFO (10 μM). GSH levels were expressed as described in Figure 2 and F.I. values monitored at 530 (J-monomer) and 590 (J-aggregate) nm were expressed as the JC-1 ratio (the aggregate/monomer). Data are mean ± SEM (bars) values ($n = 4$–7). $^{*}P < 0.01$, $^{**}P < 0.001$ by ANOVA significantly different from the appropriate untreated controls.

cellular toxicity in both human neuronal cells and human glial cells that was not observed with a noncytolytic analog of this peptide that differed by only two amino acid residues. The molecular mechanism underlying LLP-1-mediated neurotoxicity appears to involve both the depletion of intracellular GSH and the loss of mitochondrial integrity. Furthermore, the exogenous addition of either GSH or NAC were shown to be able to attenuate both intracellular GSH depletion and MTP depolarization, thus blocking LLP-1-mediated neuronal cell death.

The *in vivo* relevance of the present finding using exogenous addition of peptide that evidently enters target cells (Montelaro, unpublished observations) can only be speculative at this time. The LLP domain of HIV-1 is located in the intracytoplasmic tail of the gp41 envelope protein and is believed to be predominantly associated with the inner surface of the plasma membrane of virus infected cells. There does not appear to be any direct evidence of an interaction between HIV-1 gp41 and mitochondria, although it is not clear that this possibility has been examined carefully. Thus it is possible that minor amounts of HIV gp41 may in fact associate with mitochondrial membranes, especially in cells that may be degenerating from viral infection. Direct killing of HIV-1-infected cells such as macrophages and microglia within CNS by necrosis could also release cellular components capable of stimulating an inflammatory response in brain tissues through induction of chemokines and other inflammatory mediators (Taub *et al*, 1996). Macrophages and microglia further recruited in this inflammatory milieu could then be infected to produce high levels of

HIV-1 replication and cell death that release extracellular gp41 or envelope proteolytic fragments containing the LLP-1 segment. Consequently, LLP-1 at high concentrations within brain tissues could induce bystander killing of neuronal and glial cells as it does in cultured cells. Cells dying by necrosis would release cellular components, further exacerbating inflammatory reaction ultimately triggering a neurodegenerative cascade and disease progression as seen in AIDS patients with dementia (Glass and Johnson, 1996). Several studies demonstrating that gp41 is readily detected in the nanomolar range in the brains of HIV-1-infected patients and that the levels of gp41 are closely correlated well with the severity and rapid progression of AIDS-associated dementia support the *in vivo* relevance of this present study (Masliah *et al*, 1992; Glass *et al*, 1995; Adamson *et al*, 1996, 1999; Rostasy *et al*, 1999). In addition, the present study demonstrated that neuronal cells were more vulnerable to LLP-1 treatment than glial cells, although the mechanism of this differential susceptibility is not clear at present. One possibility is that some differences in membrane lipid or protein composition or membrane physiology between neurons and glial cells might be responsible for the different sensitivity to LLP-1. The cellular determinants of LLP-1 susceptibility present an interesting area of future research.

Because GSH plays multiple roles in the nervous system, including as an intracellular reductive and antioxidative agent, a free radical scavenger, a redox modulator of some ionotropic receptor currents, and a potential neurotransmitter, it may be crucial in protecting neurons from various stressors associated with various neurodegenerative diseases (Bains and Shaw, 1997). Thus, LLP-1-induced GSH depletion in neuronal cells may cause a substantial loss of redox poise, which could be a strong contributory secondary factor leading to neuronal death in situations requiring a higher demand for GSH, even if not directly causal. Multiple factors and mechanisms may contribute to systemic GSH deficiency in HIV disease, including the production and release of viral proteins, inflammatory cytokines and excessive use of GSH-depleting drugs (van der Ven *et al*, 1998). In particular, Tat, a transactivator encoded by HIV-1, has been recently shown to cause GSH depletion *in vitro* and *in vivo*, at least partially through mechanisms involving modulation of GSH biosynthetic enzymes (Westendorp *et al*, 1995; Choi *et al*, 2000). These observations and the present study suggest that high expression of viral factors such as gp41-derived LLP-1 or Tat may cause the systemic GSH deficiency observed in humans with HIV-1 infection (Castagna *et al*, 1995; Helbling *et al*, 1996). Further study is required to define the mechanisms by which LLP-1 alters the GSH status and to determine the possible role of GSH deficiency in redox regulation associated with AIDS dementia.

Recent studies have demonstrated that a reduction in mitochondrial transmembrane potential (MTP) and changes in mitochondria morphology in T-lymphocytes from HIV+ donors during both acute cytopathic and chronic HIV-1 infection (Macho *et al*, 1995; Cossarizza *et al*, 1996; Plymale *et al*, 1999b). In particular, gp41-derived LLP-1 has been shown to disrupt mitochondrial integrity of $CD4^+$ T-lymphoblastoid cells, inducing the cell death cascade that culminates in apoptosis or necrosis depending on the concentration of peptide employed (Plymale *et al*, 1999a). Modest changes in mitochondrial function as measured by MTP induced by sublytic concentration of LLP-1 may predispose cell to death by apoptosis, whereas more extensive mitochondrial dysfunction, which occurs at a higher LLP-1 concentration, may be required to reach the threshold for necrosis. In good agreement with this data, our current study demonstrated that a dose of 10 μM LLP-1 could specifically induce a reduction of MTP as measured by the membrane potential probe JC-1 in human neuronal cells that is accompanied by the cell death cascade mostly culminating in necrosis. Further experiments are required to elucidate the morphological or biochemical changes characteristic of apoptosis, which could occasionally occur in cells exposed to higher concentration of LLP-1 (Plymale *et al*, 1999a). Although of lower magnitude, the decline of MTP induced by LLP-1 is comparable to the strong depolarization of mitochondria by H_2O_2 that occurs only at very high concentrations, ultimately leading to cell death by necrosis as recently reported (Hoyt *et al*, 1997). These observations together suggest that mitochondria may be the target site of subcellular damage in LLP-1-induced cell death of target cells by either necrosis or apoptosis, depending on the tissue concentration of transmembrane concentration. Thus, the relative magnitude of reduction of MTP correlating with necrosis or apoptosis is consistent with other studies implicating that cellular signaling pathways could converge on mitochondria, leading to changes of mitochondrial function as an early universal event for either apoptosis or necrosis (Ankarcrona *et al*, 1995; Zamzami *et al*, 1997; Kroemer and Reed, 2000). Our study also supports the current hypothesis that severe ATP depletion resulting from extensive impairment of mitochondrial function favors the adoption of a more necrotic phenotype because the essential components of apoptosis require ATP (Kroemer *et al*, 1998; Kroemer and Reed, 2000). Interestingly, a recent study demonstrated that increased Bax protein induced by p53 activation in HIV-1-infected $CD4^+$ lymphocytes also resulted in a reduction in MTP and mitochondrial-mediated apoptosis, suggesting possible multiple effects of HIV-1-infection on MTP that can contribute to $CD4^+$ depletion (Genini *et al*, 2000).

Application of GSH or NAC, which is currently used therapeutically as the GSH prodrug to replenish GSH in treatment of HIV disease to improve survival of AIDS patients (Droge, 1993; Herzenberg *et al*, 1997), markedly attenuated LLP-1-induced

neurotoxicity, significantly blocking both a decline of intracellular GSH level and a loss of mitochondrial integrity. Microscopic examinations confirmed that the magnitude of LDH release well correlated with morphological degeneration of the neuronal cells and that substantial protective effects resulted from GSH or NAC treatment. However, exposure to PDTC, the other thiol-containing antioxidant, which itself downregulated the GSH level and caused the MTP depolarization, significantly enhanced LLP-1-mediated neuronal cell death. Thus, specific role of PDTC as an inhibitor of NF-κB signaling pathway could be responsible for amplification of LLP-1-mediated neuronal cell death, suggesting that maintenance of NF-κB activation is critical in neuronal survival (Mattson *et al*, 2000). Elucidating the possible relevance of NF-κB to the modulation of intracellular GSH level and mitochondrial integrity warrants further studies. In contrast, a cell-membrane-permeable iron chelator, DFO, neither significantly blocked GSH depletion nor restored MTP depolarization in LLP-1-treated neuronal cells. Thus, neuronal survival rate was insensitive to an iron-chelating antioxidant DFO, which could protect against the oxidative injury by inhibiting the formation of reactive oxygen species. Based on these observations and the fact that both LLP-1 and analog 2 are capable of producing reactive oxygen species in a similar level (Chong, unpublished observations), the protective mechanism by GSH or NAC against LLP-1-induced neuronal cell death is evidently at least in part mediated by their redox modulating activity. Further study will clarify their possible roles in blocking pore-forming and calmodulin-binding properties of LLP-1 leading to neuronal survival. The current finding is also consistent with recent data suggesting that diminished GSH levels may represent an intermediate stage in a mitochondria-mediated neurodegenerative cascade (Ju *et al*, 2000; Schuchmann and Heinemann, 2000). The potential role of GSH depletion linked to MTP depolarization in inducing neuronal cell death is further supported by earlier studies demonstrating that inhibition of GSH synthesis in rats caused a striking enlargement of mitochondria in the brain and severe brain damage accompanied inherited deficiencies of GSH synthesis in humans (Jain *et al*, 1991).

Taken together, these studies clearly demonstrate for the first time changes in mitochondrial function involving extensive reduction of mitochondrial transmembrane potential and decreased intracellular GSH levels are associated with LLP-1 cytotoxicity of human neuronal cells. These observations would be relevant to understanding the fundamental neuropathology and the progression of AIDS-associated dementia. Chronic exposure of neuronal cells to LLP-1 in concentrations correlating with the high level of HIV-1 envelope expression and virus replication in the CNS of AIDS patients could mediate glutathione redox imbalance and induce a loss of mitochondrial membrane integrity that culminate in the neuronal cell death. Consequently, cells dying by necrosis could release cellular constituents including damaged mitochondria, further exacerbating the inflammatory reaction and ultimately contributing to neurodegenerative cascade as seen in AIDS patients with dementia. Accordingly, the possible therapeutic value of strategies for restoring GSH level and mitochondrial function in HIV-1 infection may hold promise for slowing the disease progression in AIDS patients with dementia.

Materials and methods

Materials

Reduced glutathione (GSH), N-acetylcysteine (NAC), pyrrolidinedithiocarbamate (PDTC), and deferoxamine (DFO) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GibcoBRL (Grand Island, NY). Monochlorobimane (MBCl) and the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), were purchased from Molecular Probes (Eugene, OR).

Cell culture and virus

SH-SY5Y and SK-N-SH originating from human neuroblastoma were obtained from Dr YH Suh of Seoul National University, whereas T98G and U251 originating from human glioblastoma were obtained from ATCC (Rockville, MD) and Dr YH Suh of Seoul National University, respectively. Cells were seeded at 1×10^4/96-well culture plate and propagated in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 37°C incubator as previously described (Chong and Lee, 2000). The day after plating, the medium was changed to serum-free DMEM supplemented with 0.5% glucose, and cells were treated with synthetic peptides representing either the cytolytic LLP-1 (RVIEVVQGACRAIRHIPRRIRQGLERIL) or the noncytolytic LLP-1 analog 2: (EVIEVVQGACRAIRHIPREIRQGLERIL) at indicated concentrations for various intervals. Peptide production and characterization have been described in detail previously by Miller *et al* (1993a).

Cell viability assays

The toxicity of LLP-1 and LLP-1 analog 2 was evaluated by the morphological examination of cells using phase-contrast microscopy and quantified by measuring the amount of lactate dehydrogenase (LDH). Following the appropriate incubation time with peptides, LDH released into the culture medium was measured using a Cytotox96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's protocol. Basal LDH release (generally <10% of total LDH) from untreated control in

medium was subtracted from the LDH release levels before calculating the LDH release as % of the detergent-treated control to yield the LDH signal specific to experimental injury. Total LDH, which corresponds to complete cell death by 1% Triton X-100, was measured and cell death was expressed as percentage of total LDH. To examine whether various antioxidant agents could attenuate the cytotoxicity of LLP-1, cultures were treated with 10 μM of LLP-1 in the presence of indicated concentrations of various drugs for indicated times. LDH activity in the culture medium was determined as described above. The concentration of each agent was chosen on the basis of preliminary experiments that effects were observed. For all findings, each condition represents four separate wells per experiment and is repeated in four to seven independent experiments.

GSH measurements

GSH was measured using MBCl under the experimental conditions where concentration and loading periods were optimized in such a way as to maximize the amounts of MBCl-GSH formed with minimal nonspecific binding as previously described by Fernández-Checa and Kaplowitz (1990) with some modifications (Ju *et al*, 2000). Cells were washed with phosphate-buffered saline solution (PBS), and then incubated at 37°C for 10 min with 100 μM MBCl (Eugene, OR, U.S.A.). Cells were washed with PBS and then lysed with PBS containing 0.2% Triton X-100. The fluorescence of the MBCl-GSH complex was measured with a fluorescence microplate reader (FL600, Bio-tek Instruments, Inc, Winooski, VT) (excitation wavelength of 400 nm and emission wavelength of 480 nm). The GSH content of the samples was calculated from the standard curve prepared from GSH standards incubated in the presence of MBCl and glutathione transferase (Sigma Chemical Co, St. Louis, MO). The concentration of GSH was expressed as nmol/mg protein; protein content was determined using the bicinchoninic acid.

Measurements of mitochondrial transmembrane potential (MTP)

JC-1 has been recently proved as a more reliable fluorescent probe to assess changes of MTP in intact cells (Salvioli *et al*, 1997) and its efficiency has been recently studied in human neuroblastoma cell line SH-SY5Y (Nuydens *et al*, 1999). Thus, MTP is determined by JC-1 fluorescence according to the procedure of Reers *et al* (1991) with minor modifications (Ju *et al*, 2000). In brief, SH-SY5Y cells on 96-well culture plates were loaded for 20 min at 37°C with JC-1 (1.0 μg/ml), mitochondrial membrane-specific fluorescence dye, in culture medium. At depolarized (−100 mV) membrane potentials, JC-1 exists as green monomers with emission peak around 530 nm. As the membrane is hyperpolarized (−140 mV), JC-1 forms J-aggregates and the emission shifts towards 590 nm. Thus, depolarization of MTP was assessed by measuring the fluorescence intensities at 530 and 590 nm emission wavelength (with excitation at 485 nm), corresponding to the fluorescence peak of the monomer and that of the aggregate, respectively, using a fluorescence microplate reader (FL600; Bio-tek Instruments, Winooski, VT, USA). During the measurements, cells were maintained at 37°C and protected from light. Fluorescence intensity was measured every 5 min for <2 s to minimize photobleaching. All fluorescent measurements were corrected for autofluorescence; autofluorescence of cells not loaded with JC-1 was constant throughout the experiment. In control experiments, no photobleaching was observed during fluorescence monitoring.

Statistical analysis

Data are expressed as the mean ± SEM values and analyzed by two-tailed Student's *t*-tests for unpaired observations and ANOVA to study the relationship between the different variables. Values of $P < 0.05$ were considered to be significant.

Acknowledgements

This research was supported by the MOST through National Research Program (00-N6-01-01-A-05) for Ewha Womans University (1999–2001) and by the Brain Korea 21 project (1999–2001) to Young Hae Chong. We thank Jeong Jin Choi and Dr Won Ki Kim for valuable technical assistance.

References

Adamson DC, McArthur JC, Dawson TM, Dawson VL (1999). Rate and severity of HIV-associated dementia (HAD): Correlations with Gp41 and iNOS. *Mol Med* **5:** 98–109.

Adamson DC, Wildemann B, Sasak MI, Glass JD, Mcarthur JC, Christov VI, Dawson TM, Dawson VL (1996). Immunologic NO synthase: Elevation in severe AIDS dementia and induction by HIV-1 gp41. *Science* **274:** 1917–1921.

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995). Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15:** 961–973.

Bains JS, Shaw CA (1997). Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Rev* **25:** 335–358.

Beary TP, Tencza SB, Mietzner TA, Montelaro RC (1998). Interruption of T-cell signal transduction by lentivirus lytic peptides from HIV-1 transmembrane protein. *J Peptide Res* **51:** 75–79.

Castagna A, Le Grazie C, Accordini A, Giulidori P, Cavalli G, Bottiglieri T, Lazzarin A (1995). Cerebrospinal fluid S-adenosylmethionine (SAMe) and glutathione concentrations in HIV infection: Effect of parenteral treatment with SAMe. *Neurology* **45:** 1678–1683.

Chernomordik L, Chanturiya A, Suss-Toby E, Nora E, Zimmerburg J (1994). An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. *J Virol* **68:** 7115–7123.

Choi J, Liu RM, Kundu RK, Sangiorgi F, Wu W, Maxson R, Forman HJ (2000). Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J Biol Chem* **275:** 3693–3698.

Chong YH, Lee MJ (2000). Expression of complement inhibitor protein CD59 in human neuronal and glial cell lines treated with HIV-1 gp41 peptides. *J NeuroVirol* **6:** 51–60.

Comardelle AM, Norris CH, Plymale DR, Gatti PJ, Choi B, Fermin CD, Haislip AM, Tencza SB, Mietzner TA, Montelaro RC, Garry RF (1997). A synthetic peptide corresponding to the carboxy terminus of human immunodeficiency virus type 1 transmembrane glycoprotein induces alterations in the ionic permeability of Xenopus laevis oocytes. *AIDS Res Hum Retro* **13:** 1525–1532.

Cooper JJL (1997). Glutathione in the brain: Disorders of glutathione metabolism. In: *The molecular and genetic basis of neurological disease*. Barchi RL, Kunk LM (eds). Butterworth-Heinemann: Boston, pp 1195–1230.

Cossarizza A, Mussini C, Mongiardo N, Borghi V, Sabbatini A, De Rienzo B, Franceschi C (1996). Mitochondrial alterations and a dramatic tendency to undergo apoptosis in peripheral blood lymphocytes during acute HIV syndrome. *AIDS* **11:** 19–26.

Coyle JT, Puttfarcken P (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262:** 689–695.

Droge W (1993). Cysteine and glutathione deficiency in AIDS patients: A rationale for the treatment with N-acetylcysteine. *Pharmacology* **46:** 61–65.

Fermin CD, Garry RF (1992). Membrane alterations linked to early interactions of HIV with the cell surface. *Virology* **191:** 941–946.

Fernández-Checa JC, Kaplowitz N (1990). The use of monochlorobimane to determine hepatic GSH levels and synthesis. *Anal Biochem* **190:** 212–219.

Genini D, Sheeter D, Rought S, Zaunders JJ, Susin SA, Kroemer G, Richman DD, Carson DA, Corbeil J, Leoni LM (2001). HIV induces lymphocyte apoptosis by a p53-initiated, mitochondrial-mediated mechanism. *FASEB J* **15:** 5–6.

Glass JD, Fedor H, Wesselingh SL, McArthur JC (1995). Immunocytochemical quantitation of human immunodeficiency virus in the brain: Correlations with dementia. *Ann Neurol* **38:** 755–762.

Glass JD, Johnson RT (1996). Human immunodeficiency virus and the brain. *Annu Rev Neurosci* **19:** 1–26.

Haraguchi S, Good RA, Day NK (1995). Immunosuppressive retroviral peptides: cAMP and cytokine patterns. *Immunol Today* **16:** 595–603.

Helbling B, von Overbeck J, Lauterburg BH (1996). Decreased release of glutathione into the systemic circulation of patients with HIV infection. *Eur J Clin Invest* **26:** 38–44.

Herzenberg LA, De Rosa SC, Dubs JG, Roederer M, Anderson MT, Ela SW, Deresinski SC, Herzenberg LA (1997). Glutathione deficiency is associated with impaired survival in HIV disease. *Proc Natl Acad Sci USA* **94:** 1967–1972.

Hoyt KR, Gallagher AJ, Hastings TG, Reynolds IJ (1997). Characterization of hydrogen peroxide toxicity in cultured rat forebrain neurons. *Neurochem Res* **22:** 333–340.

Jain A, Martensson J, Stole E, Auld PA, Meister A (1991). Glutathione deficiency leads to mitochondrial damage in brain. *Proc Natl Acad Sci USA* **88:** 1913–1917.

Ju C, Yoon KN, Oh YK, Kim HC, Shin CY, Ryu JR, Ko KH, Kim WK (2000). Synergistic depletion of astrocytic glutathione by glucose deprivation and peroxynitrite: Correlation with mitochondrial dysfunction and subsequent cell death. *J Neurochem* **74:** 1989–1998.

Kalebic T, Masiero L, Onisto M, Garbisa S (1994). HIV-1 modulates the expression of gelatinase A and B in monocyic cells. *Biochem Biophy Res Commun* **205:** 1243–1249.

Kort JJ (1998). Impairment of excitatory amino acid transport in astroglial cells infected with the human immunodeficiency virus type 1. *AIDS Res Hum Retro* **14:** 1329–1339.

Kroemer G, Dallaporta B, Resche-Rigion M (1998). The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* **60:** 619–642.

Kroemer G, Reed JC (2000). Mitochondrial control of cell death. *Nat Med* **6:** 513–519.

Macho A, Castedo M, Marchetti P, Aguilar JJ, Decaudin D, Zamzami N, Girard PM, Uriel J, Kroemer G (1995). Mitochondrial dysfunctions in circulating T lymphocytes from human immunodeficiency virus-1 carriers. *Blood* **86:** 2481–2487.

Marschang P, Kruger U, Ochsenbauer C, Gurtler L, Hittmair A, Bosch V, Patsch JR, Dierich MP (1997). Complement activation by HIV-1 infected cells: The role of transmembrane glycoprotein gp41. *J AIDS Hum Retrovirol* **14:** 102–109.

Masliah E, Achim CL, Ge N, Eeteresa RD, Terry RD, Wiley CA (1992). Spectrum of human immunodeficiency virus-associated neocortical damage. *Ann Neurol* **32:** 321–329.

Mattson MP, Culmsee C, Yu Z, Camandola S (2000). Roles of nuclear factor κB in neuronal survival and plasticity. *J Neurochem* **74:** 443–456.

Miller MA, Cloyd MW, Liebmann J, Rinaldo C, Islam KR Jr, Wang SZ, Mietzner TA, Montelaro RC (1993a). Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virology* **196:** 89–100.

Miller MA, Garry RF, Jaynes JM, Montelaro RC (1991). A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. *AIDS Res Hum Retro* **7:** 511–519.

Miller MA, Mietzner TA, Cloyd MW, Robey WG, Montelaro RC (1993b). Identification of a calmodulin-binding and inhibitory peptide domain in the HIV-1 transmembrane glycoprotein. *AIDS Res Hum Retro* **9:** 1057–1066.

Millis KK, Lesko SA, Gamcsik MP (1997). Formation, intracellular distribution and efflux of glutathione-bimane conjugates in drug-sensitive and -resistant MCF-7 cells. *Cancer Chemother Pharmacol* **40:** 101–111.

Nuydens R, Novalbos J, Dispersyn G, Weber C, Borgers M, Geerts H (1999). A rapid method for the evaluation of compounds with mitochondria-protective properties. *J Neurosci Methods* **92:** 153–159.

Plymale DR, Comardelle AM, Fermin CD, Martin DS, Costin JM, Norris CH, Tencza SB, Mietzner TA, Montelaro RC, Garry RF (1999a). Concentration-dependent differential induction of necrosis or apoptosis by HIV-1 lytic peptide 1. *Peptides* **20:** 1275–1283.

Plymale DR, Ng Tang DS, Comardelle AM, Fermin CD, Martin DS, Lewis DE, Garry RF (1999b). Both necrosis and apoptosis contribute to HIV-1-induced killing of CD4 cells. *AIDS* **13:** 1827–1839.

Reers M, Smith TW, Chen LB (1991). J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* **30:** 4480–4486.

Rostasy K, Monti L, Yiannoutsos C, Kneissl M, Bell J, Kemper TL, Hedreen JC, Navia BA (1999). Human immunodeficiency virus infection, inducible nitric oxide synthase expression, and microglial activation: Pathogenetic relationship to the acquired immunodeficiency syndrome dementia complex. *Ann Neurol* **46:** 207–216.

Ruegg CL, Strand MA (1991). Synthetic peptide with sequence identity to the transmembrane protein Gp41 of HIV-1 inhibits distinct lymphocytes activation pathways dependent on protein kinase C and intracellular calcium influx. *Cell Immunol* **137:** 1–13.

Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A (1997). JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett* **411:** 77–82.

Schuchmann S, Heinemann U (2000). Diminished glutathione levels cause spontaneous and mitochondria-mediated cell death in neurons from trisomy 16 mice: A model of Down's syndrome. *J Neurochem* **74:** 1205–1214.

Srinivas S, Srinivas R, Anantharamaiah G, Segrest J, Compans R, Segrest J (1993). Cytosolic domain of the HIV envelope glycoprotein binds to calmodulin and inhibits calmodulin-regulated proteins. *J Biol Chem* **268:** 22895–22899.

Staal FJ, Ela SW, Roeder M, Anderson MT, Herzenberg LA, Herzenberg LA (1992). Glutathione deficiency and human immunodeficiency virus infection. *Lancet* **339:** 909–912.

Taub DD, Anver M, Oppenheim JJ, Longo DL, Murphy WJ (1996). T lymphocyte recruitment by interleukin-8 (IL-8). IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both *in vitro* and *in vivo*. *J Clin Invest* **97:** 931–1941.

Tencza S, Miller M, Islam K, Mietzner T, Montelaro RC (1995). Effect of amino acid substitutions on calcodulin binding and cytolytic properties of the LLP-1 peptide segment of HIV-1 transmembrane protein. *J Virol* **69:** 5199–5202.

van der Ven AJ, Blom HJ, Peters W, Jacobs LE, Verver TJ, Koopmans PP, Demacker P, van der Meer JW (1998). Glutathione homeostasis is disturbed in CD4-positive lymphocytes of HIV-seropositive individuals. *Eur J Clinic Invest* **28:** 187–193.

Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V (1995). HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. *EMBO J* **14:** 546–554.

Zamzami N, Hirsch T, Dallaporta B, Petit PX, Kroemer G (1997). Mitochondrial implication in accidental and programmed cell death: Apoptosis and necrosis. *J Bioenerg Biomembr* **29:** 185–193.

Zasloff M, Martin B, Chen HC (1988). Antimicrobial activity of synthetic magainin peptides and several analogues. *Proc Natl Acad Sci USA* **85:** 910–913.