Neuronal loss is frequently found in brains of patients with human immunodeficiency virus (HIV)-encephalopathy. Extensive apoptosis of neurons is probably involved in the development of HIV-encephalopathy. The present study was designed to investigate the mechanism of neuronal apoptosis. For this purpose, we examined autopsy brains of two patients with HIV-encephalopathy. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells and active forms of caspase-3- and -8-positive cells, including neurons, were found in the perivascular regions of the brains. In these regions, TNF-related apoptosis-inducing ligand (TRAIL) + macrophages were also observed. We also examined brains of HIV-1-infected mouse model inoculated with human cells. In these brains, TUNEL + neurons were also found in the perivascular region, the site where infiltrated HIV-1-infected and TRAIL-expressing macrophages were observed. Using an in vitro-culture system, we also demonstrated that the HIV-1-infected monocyte-derived macrophages preferentially expressed TRAIL and that the addition of HIV-1-infected macrophages or human TRAIL-overexpressing mouse cells to cultured mouse primary neurons/glia resulted in neuronal apoptosis. Our results suggest the involvement of TRAIL expressed on HIV-1-infected macrophages in the induction of neuronal apoptosis in infected brain.

Key words: HIV-encephalopathy, TRAIL, neuronal apoptosis, caspase-8, HIV

Introduction

Human immunodeficiency virus (HIV)-encephalopathy is a cognitive/motor deficit complex in HIV-infected patients after long latent infection. The histopathological features of this encephalopathy include perivascular cuffing, microglial nodules, astrogliosis, neuronal degeneration, reduced synaptic density, myelin pallor, and occasional multinucleated giant cells. In the infected brain, HIV infection has been confirmed only in monocyte/macrophage-lineage such as infiltrating macrophages and microglia, but not in neurons. Although many factors induced by HIV-1 infection, such as viral proteins and host proteins, are thought to contribute to neuronal degeneration, the exact mechanism of this pathological process has not yet been identified. Based on a series of histopathological analyses in autopsy brains, apoptosis of neurons was reported to play a major role in HIV-encephalopathy. However, the pathways involved in this apoptotic process remain unknown at present.

TNF-related apoptosis-inducing ligand (TRAIL) is a new member of death-inducing ligands in the TNF family. This death ligand activates caspase-8 and subse-
quently caspase-3 through its receptor and efficiently induces apoptotic cell death. The human TRAIL molecule is capable of providing apoptosis signal to its murine receptors. This property allows examination of the therapeutic application of human TRAIL in vivo using mouse tissues. While it has been reported that TRAIL induces apoptosis of various tumor cells, the pathological roles of TRAIL are largely unknown. In addition, recent studies reported that certain viral infections can augment TRAIL expression and induce TRAIL-mediated apoptosis. Thus, it is possible that TRAIL is involved in HIV pathogenesis.

The present study was designed to examine the role of TRAIL expressed on HIV-infected macrophages in induction of neuronal apoptosis. Our results showed that apoptosis of neurons correlated with the presence of TRAIL-expressing macrophages in the brains of patients with HIV-encephalopathy and HIV-1-infected in vivo-murine model. Furthermore, the addition of HIV-1-infected macrophages or TRAIL-overexpressing mouse cells to cultured neurons/glia directly induced neuronal apoptosis. Our results suggest that TRAIL expressed on HIV-infected macrophages induces neuronal apoptosis in HIV-1-infected brain.

Materials and Methods

1. Brain specimens
Autopsy brains of two demented HIV-1-infected patients aged 48 and 25 years, who were free of opportunistic infection or tumor in the central nervous system (CNS), were kindly provided by Dr. K. Inoue (Faculty of Medicine, University of Tokyo). Postmortem tissues were fixed in 10% buffered formalin and embedded in paraffin. For negative control, non-demented HIV-1-uninfected brain was prepared. The study protocol was approved by the Human Ethics Review Committees of participating institutions.

2. Generation of human cell-transplanted mice and HIV-1 infection
Non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice were used at 6-8 weeks of age. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the participating institutions. The methods used for isolating human peripheral blood mononuclear cells (PBMC) and their infection with a green fluorescent protein (GFP)-expressing HIV-1NL-CSF3-EGFP virus were described previously. The GFP-expressing HIV-1NL-CSF3-EGFP virus was constructed by replacing the V3 sequence in the HIV-1NL-EGFP with the V3 sequence from HIV-1JRCSF. Seven days later, the infected NOD-SCID mice were injected intraperitoneally with 100 μg of lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA). The mice were sacrificed 3 days later and the brains were dissected out, fixed in 4% periodate-lysine-paraformaldehyde (PLP) fixative, and then subjected to histopathological analyses. For negative control, brains of mice both inoculated with human PBMC and uninfected with HIV-1 were prepared.

3. Cell culture and staining
Cultures of mouse brain primary neurons/glia mixture were prepared from E 18 Balb/c mouse embryo as described previously. Cells were maintained in high glucose-DMEM (Sigma) containing B27 components (Gibco, Tokyo). Seven days after initiation of culture, HIV-1-infected monocyte-derived macrophages (MDM) or human TRAIL- or Fas ligand (FasL)-expressing mouse B cells (hTRAIL/2PK-3 and hFasL/2PK-3, respectively) were added and co-cultured for 4 days. The MDM had been enriched from PBMC of a HIV-1-seronegative healthy donor by plastic adherence and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 5% giant cell tumor supernatant (igen, Rockville, MD, USA). MDM were infected with HIV-1JRFL or HIV-1NL-CSF3-EGFP at 7 days after plating. Co-cultured cells were fixed with 4% paraformaldehyde and stained with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (Cy5, green), and anti-neuron-specific enolase (NSE) (red). NFP+ TUNEL+ apoptotic neurons were counted. Then, fixed MDM were incubated with anti-TRAIL antibody (Ab) (goat polyclonal IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Biotinylated donkey IgG against goat IgG (Polyscience Inc., Warrington, PA, USA) was used as secondary Ab, and followed by Alexa 568-conjugated streptavidin (Molecular Probes; FMP, Eugene, OR, USA).

4. Histopathological analyses
The fixed brain tissues were embedded in paraffin and cut into 6-μm thick sections. Sections were stained by hematoxylin and eosin (H&E), and immunohistochemically with the following Abs: anti-
HIVgag p24 mAb (clone Kal-1, Dako), anti-active form caspase-3 Ab (rabbit polyclonal IgG, R&D Systems, Minneapolis, MN, USA), anti-cleaved caspase-8 mAb (clone D384, mouse monoclonal IgG, Cell Signaling Technology, Beverly, MA, USA), or anti-TRAIL Ab (goat polyclonal IgG, Santa Cruz Biotechnology). For detection, the HISTOFINE indirect method (NICHIREI Co., Tokyo) or the TSA™-Indirect method (NEN) was performed as described previously. Non-immunized immunoglobulin was used as a negative control. TUNEL staining was carried out as described previously. Absence of TdT served as a negative control. DNase-treated sections served as a positive control. Dual-color staining, including TUNEL, was performed with Alexa 568-conjugated streptavidin and FluoroNissl Green (FNG, FMP). Triple-color analysis for GFP, CD68, and TRAIL was performed with horseradish peroxidase-conjugated anti-human CD68 mAb (clone PGM1, Dako) and anti-TRAIL Ab (goat polyclonal IgG, Santa Cruz Biotechnology), followed by biotinylated donkey IgG against goat IgG (Polyscience Inc.), Cy5-conjugated streptavidin (Amersham Pharmacia Biotech, Tokyo), and Cyanine 3-conjugated tyramide (NEN). Negative controls were prepared without the use of the primary Ab. Sections were covered with a Vectorshield-mounting-medium and examined under a confocal laser-scanning microscope (model TCS NT, Leica, Mannheim, Germany). A single 488-nm beam for GFP and FNG, 542-nm for Cyanine 3 and Alexa 568, 600-nm for Cy5 from an argon-krypton laser were used for excitation. Emission from GFP or FNG was detected through a long-pass filter (<540 nm) and was displayed as green color. Emission from Cyanine 3 or Alexa 568 was detected through a band-pass filter (600–70 nm) and was displayed as red. Emission from Cy5 was detected through a band-pass filter (740–70 nm) and was displayed as blue or green.

5. Statistical analysis
All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student’s t-test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results
1. Histopathological analyses of the brains of HIV encephalopathy patients
We initially performed histopathological analyses of brains of two HIV-dementia patients. H&E staining indicated astrocystosis, macrophage infiltration, microglial nodules and multinucleated giant cells in the infected brains as described previously (data not shown). HIV-1 p24+ cells, which might produce HIV-1 virions, were macrophage and microglia but not neurons (Fig. 1e). These findings corresponded well with those of previous reports. Importantly, we were able to
find many TUNEL$^*$ apoptotic cells including neurons (more than 20 cells per slide) in the brains of these patients (Fig. 1a and b).

To identify the apoptosis-cascade, we immunostained brain sections using Abs against the active form of caspase-3 and cleaved caspase-8. A significant number of active form caspase-3- and cleaved caspase-8-positive cells including neurons were found in the perivascular regions of the frontal lobe (Fig. 1c and d). These data suggested that cells including neurons underwent apoptosis through caspase-3 and -8-pathways. Since upregulation of TRAIL expression has been reported in HIV-1 infected macrophages$^{16}$, and in both HIV-infected patients and hu-PBMC-SCID mice$^{11,17}$, TRAIL is a possible death ligand molecule in the HIV-1-infected brain. In fact, many macrophages, especially those infiltrating the perivascular region of the frontal lobe, preferentially expressed TRAIL in these patients (Fig. 1f).

2. Histopathological analyses of in vivo HIV-encephalopathy murine model

We also examined the brains of HIV-1-infected human PBMC-transplanted NOD-SCID mice after LPS inoculation. LPS is a potent activator of macrophage and can induce transmigration of these cells from the peripheral blood into CNS$^{18,19}$. In these experiments, we used GFP-carrying HIV-1 which efficiently infect CD68$^+$ macrophages, and then human cells including GFP$^+$ HIV-1-infected macrophages to invade the brain. TUNEL$^*$ apoptotic neurons, which show Nissl body in the cytoplasm (a neuron-specific marker), and TRAIL$^*$ GFP$^+$ CD68$^+$ macrophages were frequently found in the perivascular region (Fig. 2). These results indicate that neuronal apoptosis is functionally associated with TRAIL expressing cells (i.e., virus-infected macrophages) in HIV-1-infected brain.

Fig. 2. Histopathological examination of brains of HIV-encephalopathy in vivo-murine model. Hu-PBMC-NOD-SCID mouse was infected with HIV and LPS was administered 7 days after infection. Three days later, the brain was dissected out and stained for histopathological examination. (a) Neuronal apoptosis was identified as nuclear TUNEL$^*$ and cytoplasmic FNG$^*$ cells, indicated by arrows, in the cerebral cortex of brain of HIV$^{p24*}$-infected mouse. (b) TRAIL$^*$ GFP$^*$ human CD68$^*$ macrophages were found in the perivascular lesion of the cerebral cortex of GFP-carrying HIV$^{IL-10*CD8*}$-infected mouse.
Fig. 3. *In vitro* co-culture system with murine brain cells and HIV-1-infected human MDM. Neurons/glia culture was prepared from fetal mouse brains. Seven days later, HIV-1*JRFL*-infected, HIV-1*NL-CSFV3-EGFP*-infected or uninfected MDM were added to the culture. Four days after co-cultivation, cells were stained. (a) Neuronal apoptosis was evident in neurons/glia cultured with HIV-infected or uninfected human MDM. Apoptotic neurons are identified by the presence of yellow-colored nuclei. Arrows indicate neurofilament protein (NFP)*+* TUNEL*+* apoptotic neurons. Scale bars = 10 µm. (b) Proportion of apoptotic neurons calculated by counting NFP*+* TUNEL*+* cells and total NFP*+* cells. Data are mean ± SD of triplicate cultures. *, P < 0.05 by Student's t-test. (c) TRAIL expression in GFP-carrying HIV-infected MDM. Human MDM were isolated from a healthy HIV-1-seronegative donor, cultured and infected with HIV*NL-CSFV3-EGFP* virus. Many GFP*+* HIV-1-infected MDM expressed TRAIL. Scale bars = 20 µm.
3. Induction of neuronal apoptosis co-cultured with HIV-1-infected MDM in vitro

To test whether HIV-1-infected macrophages can induce neuronal apoptosis, cultures of primary neurons/glia mixture isolated from fetal mice were established and then co-cultured with HIV-1-infected human MDM. In these experiments, apoptotic neurons were identified by dual-immunostaining for NFP and TUNEL. The numbers of NFP+ TUNEL+ apoptotic neurons were significantly higher in co-cultures of HIVJRFL- and HIVNL-CSFV3-EGFP-infected human MDM than in uninfected MDM and cultures of primary neurons/glia cell mixtures (Fig. 3a, b). Furthermore, cultured GFP+ HIVNL-CSFV3-EGFP-infected MDM expressed high levels of TRAIL molecule (Fig. 3c). These results suggested that HIV-1-infected human MDM could efficiently induce apoptosis of mouse neurons through a TRAIL-dependent signal pathway in vitro.

4. TRAIL induces neuronal apoptosis in vitro

To test whether the TRAIL molecule contributes to the induction of apoptosis of neurons, cultures of mouse primary neurons/glia were incubated with human TRAIL-transfected mouse B cell lymphoma cells, 2PK-3. For comparison, cultures of mouse primary neurons/glia were also co-cultured with human FasL-expressing 2PK-3 cells. Overexpression of TRAIL or FasL on the cells was confirmed by FACS analysis (data not shown). Apoptotic neurons were identified by dual staining as described above. The number of NFP+ TUNEL+ apoptotic neurons was significantly higher in cultures containing human TRAIL-overexpressing cells than human FasL-overexpressing cells or mock-transfected cells (Fig. 4). These results indicated that human TRAIL is important to induce neuronal apoptosis in vitro.

![Fig. 4. Neuronal apoptosis of murine neurons/glia co-cultured with TRAIL-transfectant in vitro. Primary neurons/glia were co-cultured with human TRAIL/2PK-3, human FasL/2PK-3, or 2PK-3 cells for 4 days. The cells were then immunostained simultaneously with anti-NFP mAb and TUNEL. (a) NFP+ neurons are shown in red. Nuclei of apoptotic cells are shown in green. Arrows indicate apoptotic neurons with a red cytoplasm and a yellow nucleus (merging of green with red). Scale bars = 10 μm. (b) The proportion of apoptotic neurons was calculated as described above. Data are mean ± SD of triplicate cultures. *P < 0.05 by Student’s t-test.](image-url)


Discussion

1. Role of TRAIL in the brain

In this study, we found significant numbers of TRAIL-expressing cells in the autopsy brains of patients with HIV-encephalopathy and in those of HIV-1-infected hu-PBMC-NOD-SCID mice. In vitro experiments of infected macrophages indicated that HIV-1 infection augmented the TRAIL expression. TRAIL expression in the brain tissue has not yet been examined because this molecule has only been recently identified. The TRAIL molecule is a death ligand, however, its pathological role has not been fully examined. Previous studies indicated that TRAIL expression is upregulated by ischemia in the rat brain. In another study, injection of a TRAIL antagonist induced exacerbation of experimental allergic encephalomyelitis although no TRAIL-expressing cells were demonstrated in these experiments. On the other hand, Nitsch et al. reported induction of apoptosis of cultured human brain cells following the addition of soluble TRAIL. More recently, Dorr and colleagues identified the expression of apoptosis-mediating TRAIL receptors on human neurons. Our results add further support to the importance of TRAIL as a key mediator of apoptosis in the brain, especially in HIV-encephalopathy.

2. Caspase-mediated neuronal apoptosis

There is sufficient evidence for the role of caspase-3 in neuronal apoptosis in the brains of patients with HIV-encephalopathy. In particular, significantly high levels of the active form of caspase-3 were found in children with HIV-encephalopathy, suggesting that either death ligand-mediated or mitochondria-mediated apoptosis occurs in infected brains. In the present study, we identified activation of caspase-8 in brains of our HIV-encephalopathy patients. Caspase-8 is an earlier mediator of the death pathway and is activated through the TNF receptor family molecules. Apoptosis of neurons cultured with HIV-1 Vpr protein is mediated through caspase-8 activation, while activation of caspase-8 has not yet been confirmed in the brain of patients with HIV-encephalopathy. In other CNS diseases, activation of caspase-8 in neurons was reported in vitro in reovirus-infections, Alzheimer disease, prion disease, Huntington disease, and in vivo in ischemic brain. Down syndrome, Parkinson disease, and amyotrophic lateral sclerosis.

3. HIV-encephalopathy in vivo and in vitro-murine system

Our in vivo mouse model with HIV-1-systemic infection developed neuronal apoptosis, which was similar to that described in patients with HIV-encephalopathy. Persidsky et al. described a HIV-encephalopathy murine model established by intracranial inoculation of HIV-1-infected MDM. In comparison with their model, our mouse model closely resembles HIV-encephalopathy in human because it involves the induction of HIV-1 systemic infection and no artificial trauma of the brain. Using our model, it is possible to examine the mechanism of transmigration of infected cells and systemic neuronal apoptosis in CNS. Our data suggest that transmigration of HIV-1-infected macrophages is induced by certain stimuli and that neuronal apoptosis is augmented by the TRAIL signal on HIV-infected macrophages without infection of murine cells.

We also used a unique in vitro human and mouse co-culture system. Since HIV infects only human cells but not murine cells, this is a suitable system to examine the indirect effect of HIV infection. However, it is necessary to consider that human molecules may influence murine cell function, which may limit the use of this system.

4. Infection of macrophages

Our histopathological studies of autopsy brain samples of patients with HIV-dementia indicated that HIV-1 producing cells were limited to monocyte/macrophage lineage. Several studies have suggested that virus-infected macrophages play critical roles in the pathogenesis of encephalopathy and dementia. Recent studies of simian immunodeficiency virus (SIV)-infected animals showed the presence of infiltrating blood-derived, virus-containing macrophages in the perivascular regions and indicated that these pathological changes were seen in all animals with encephalopathy. We extended these results in the present study by demonstrating that the infiltrated macrophages might provide TRAIL signal for neuronal apoptosis.

In conclusion, we have demonstrated in the present study that TRAIL expressed on human macrophages could induce neuronal apoptosis in vitro. Since both apoptotic neurons and TRAIL-expressing macrophages were observed in vivo in brains of patients with HIV-encephalopathy and in our murine model, a similar mechanism may be involved in the pathogenesis of HIV-encephalopathy. These results provide a strong evidence for the involvement of TRAIL expressed on
HIV-infected macrophages in induction of apoptosis of neurons of HIV-infected brain both in vitro and in vivo.

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