

To determine how the decrease of EAAT-2 may occur, Wang et al (27) demonstrated that HIV-1 or surface glycoprotein 120 induces transcriptional downmodulation of the EAAT-2 transporter gene in human astrocytes and attenuates glutamate transport by the cells in vitro. Although we did not find a correlation between the decrease of EAAT-2 and HIV, apoptosis seemed to be more frequent in cases with HIV. Many studies have reported that viral factors such as HIV-1/glycoprotein 120 and HIV transactivator (Tat) protein may induce neuronal damage and apoptosis through indirect and/or direct pathways in vitro as well as in vivo (28–34). Because high viral loads and increase of the soluble form of viral proteins in the blood as well as in the cerebrospinal fluid are common features in the late stage of

AIDS, it might be possible that an increase in soluble viral antigens in the frontal cortex through the blood-brain barrier results in the astrocytic abnormality/injury, thus permitting subsequent neuronal damage. On the other hand, Boycott et al (35) recently reported that the decrease of EAAT-2 in cultured astrocytes is mediated by TNF in a hypoxic condition. Although TNF was not detected in astrocytes and diffusely activated microglia in the area showing decrease of EAAT-2 in our study, an increase of TNF in the blood has been reported in the late stage of AIDS, and this might explain the decrease of EAAT-2 expression in astrocytes in the present study.

We also observed diffuse activation of microglia in the cortex; the numbers of activated microglia correlated with a

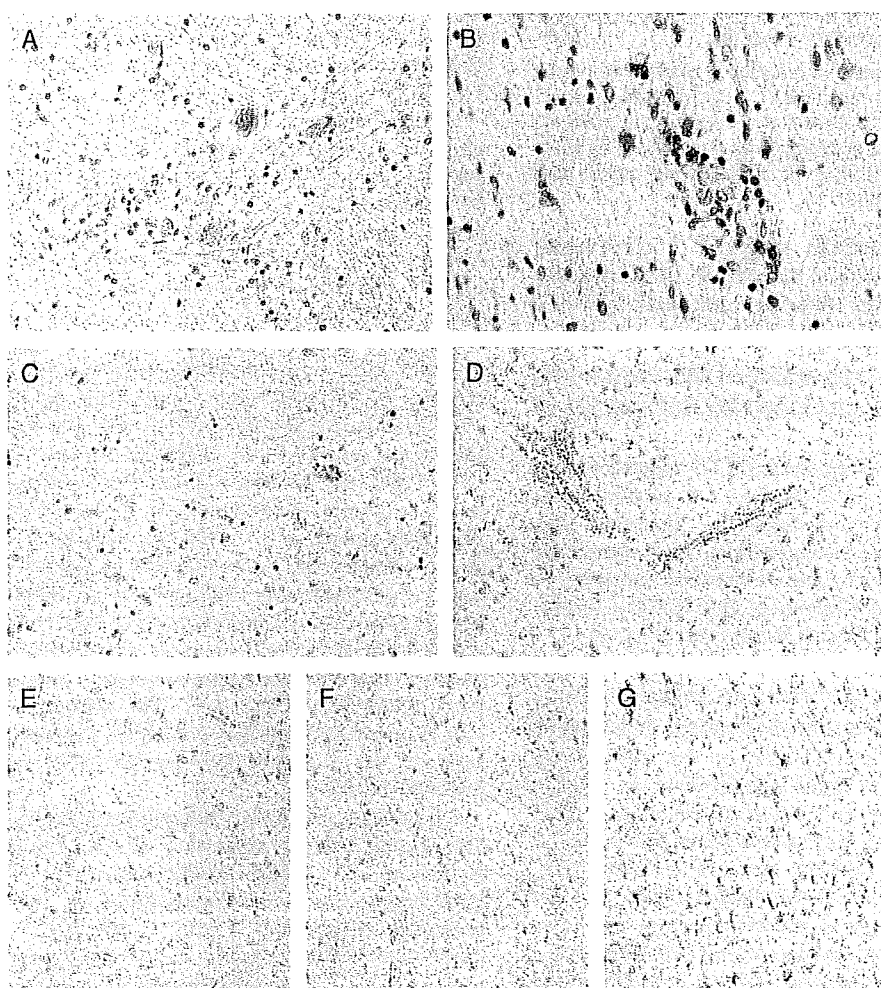


FIGURE 7. Interleukin-1 β (IL-1 β) and tumor necrosis factor (TNF) immunopositivity are restricted to the inflammatory lesions. **(A, B)** Frontal cortex white matter of Patient 6. Some IL-1 β -positive cells are detected in the perivascular infiltration and multinucleated giant cells (MNGCs) [**A**] original magnification: 100 $\times$). The TNF-positive cells are detected in some perivascular infiltrates and surrounding microglia [**B**] original magnification: 200 $\times$). **(C, D)** Frontal cortex of Patient 7. The IL-1 β -positive cells are detected in some MNGCs [**C**] original magnification: 200 $\times$). The TNF-positive cells are detected in the perivascular infiltrate [**D**] original magnification: 100 $\times$). **(E–G)** Frontal cortex of Patient 13. The IL-1 β [**E**] original magnification: 100 $\times$) and TNF [**F**] original magnification: 100 $\times$) are not detected in an area with numerous diffuse Iba1-positive microglia [**G**] anti-Iba1, original magnification: 40 $\times$).

decrease of EAAT-2. Some of the activated microglia were immunopositive for EAAT-2, but their expressions of TNF and IL-1 β were not detected by immunohistochemistry. In general, microglial cells are distributed ubiquitously throughout the CNS and become activated in response to harmful stimuli (36). Activated microglia release proinflammatory cytokines such as IL-1 β and TNF and thus have potential neurotoxic functions. In AIDS encephalopathy, HIV-infected macrophages and microglia may produce viral neurotoxins and neurotoxic cytokines that lead to neuronal dysfunction and death (37–40). Thus, activated microglia have been considered to be effectors of neuronal degeneration. On the other hand, activated microglia can also secrete some neurotrophic factors and serve neuroprotective functions (41). The expression of EAAT-2 by microglia has been reported in AIDS brains by others (41–42), and we confirm EAAT-2 expression by activated microglia in this study. These data suggest that similar to astrocytes, microglia might clear extracellular glutamate, thereby playing a neuroprotective role in AIDS brains.

Neuronal apoptosis is considered to be one of the major pathological changes in HIV infection (14, 43, 44). We observed apoptosis of some large pyramidal and small neurons in the frontal cortex of HIV-infected brains, whereas neuronal apoptosis was rarely noted in our previous study of SIV-infected animals. The difference might be explained by the difference in the stage of DPD. In human ADC, an autopsy is usually performed at the advanced stages of the disease, whereas the animals in our previous study were at a subclinical stage, and the findings observed might have been the early changes of DPD. We hypothesize that abnormalities of astrocytes and microglial activation might precede neuronal damage in early DPD and that when microglia fail to supply sufficient neuroprotection, neuronal damage becomes apparent later in DPD.

In summary, this is the first report of primary injuries to astrocytes (i.e. apoptosis and decreased EAAT-2 expression in the neuropil) in HIV-infected human autopsy brains. We also demonstrated an increase of activated microglia and apparently compensatory expression of EAAT-2 by microglia in the frontal cortex in these cases. These findings in the frontal cortex did not correlate with the severity of HIVE, which suggests that astrocytic degeneration and microglial activation may also play a role independent of HIVE in ADC.

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Original Article

In vivo expression of proinflammatory cytokines in HIV encephalitis: an analysis of 11 autopsy cases

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As the pathogenesis of AIDS dementia complex (ADC), cytokines such as TNF- α and IL-1 β have been thought to have toxic effects on CNS cells and induce neuronal cell death. However, many of the discussions have been based on the studies done by in vitro experiments. There are only a few reports which demonstrate proinflammatory cytokines directly in vivo in HIV encephalitis (HIVE) brains, and roles of these cytokines with relation to HIV-1 infection are not yet clarified. In the present study, we examined 11 autopsy cases of HIVE using immunohistochemistry, and explored which cell types expressed these cytokines and whether expression of cytokines was related to viral infection. IL-1 β was detected in the frontal white matter of all 11 cases where microglial nodules were observed to varying degrees, whereas TNF- α was detected in seven cases. IL-1 β - or TNF- α -positive cells were almost restricted to CD68-positive macrophages/microglia and mild expression of these cytokines by astrocytes was observed in two cases with severe HIVE. IL-1 β was detected in some HIVp24-positive multinucleated giant cells. However, we could not detect TNF- α expression in the HIVp24-positive cells, which indicates that IL-1 β is induced by HIV-1 infection. In conclusion, a macrophage/microglia lineage is the main cell type to release cytokines in HIVE, and IL-1 β expression by HIV-1-infected cells may be one of the important factors for induction of HIVE.

In addition, many non-infected macrophages/microglia as well as some astrocytes express IL-1 β and TNF- α , which might contribute to pathogenesis of ADC.

Key words: HIV encephalitis, HIVp24, IL-1 β , multinucleated giant cells, TNF- α .

INTRODUCTION

AIDS dementia complex (ADC)^{1,2} is a clinical triad of progressive cognitive decline, motor dysfunction, and behavioral abnormalities induced by human immunodeficiency virus 1 (HIV-1), and occurs in approximately 20% of AIDS cases during the late stages of the HIV-1 infection.³ One of the histopathologic correlates of ADC is diffuse and nodular microgliosis with formation of multinucleated giant cells (MNGCs) in the white matter of the brain and is termed HIV encephalitis (HIVE),⁴⁻⁶ Myelin pallor⁴ and axonal damage⁷⁻⁹ with abundant HIV-infected macrophages and microglial cells have been demonstrated in the white matter.^{4,10} However, poor correlations between these findings and the clinical manifestations of ADC have been repeatedly reported.^{11,12} On the other hand, diffuse poliodystrophy (DPD) has been reported as another histopathologic evidence for ADC,^{4,5} and a variety of neuronal damages such as neuronal loss, apoptosis of neurons, and synaptic and dendritic simplification have been demonstrated in the cerebral cortex.^{4,5,13-19}

Our previous study using a Simian immunodeficiency virus (SIV)-infected macaque model demonstrated that two types of pathologic changes, an inflammatory process with virus-infected multinucleated giant cells in the white matter and degenerative changes of the cerebral cortex along with development of immunodeficiency, could occur

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independently, and the cell tropism of inoculated viruses may be involved in selection of the types of pathology.²⁰ Further analyses of the frontal cortex of these macaques with advanced AIDS demonstrated astrocytic degeneration and diffuse activation of microglia, suggesting roles of these cells in pathogenesis of DPD.²¹

Regarding pathogenesis of ADC, it has been discussed that proinflammatory cytokines such as TNF- α and IL-1 β may have toxic effects on CNS cells, inducing neuronal cell death.^{22,23} Many studies suggest that IL-1 β and TNF- α are released by microglia and macrophages and play a role in CNS injury.^{24,25} On the other hand, infection of astrocytes may also occur with limited virus replication,²⁶ and astrocytes could cause CNS injury by secreting these cytokines.²⁷⁻³⁰ However, *in vivo* expression of these cytokines in the microenvironment of HIVE, microglial nodules with MNGCs, are not yet clarified. Our previous study suggested that virus-infected microglia/macrophages do not always express IL-1 β and TNF- α in the macaque model.³¹

In order to clarify roles of these cytokines in pathogenesis of ADC, we examined human autopsy brains from HIV-1-infected individuals focusing on types of cells expressing IL-1 β and TNF- α in the microenvironment of HIVE.

MATERIALS AND METHODS

Autopsy cases

Among all autopsy cases of the Neurological Institute, Vienna University since 1983, there were altogether 429 who died from AIDS. The records of these cases were screened and the cases with opportunistic infection, neoplasm, and massive cerebrovascular sickness in the brain were excluded. Finally, 11 cases with HIVE in the brain were selected.

Histopathological examination

Paraffin serial sections of the frontal lobe and pons were processed for further histopathological examination. An analysis for degrees of HIVE was done using HE and KB stainings. We used the EnVision system (Dako, Carpinteria, CA, US) for immunohistochemistry. The following antibodies were used as first antibodies: mouse anti-human (TNF- α ; 1:400; Abcam, Cambridge, MA, US), rabbit polyclonal antibody TNF- α (1:200; Abcam), and rabbit polyclonal antibody IL-1 β (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, US) were used to detect the respective cytokines. We also performed GFAP, ubiquitin carboxyl-terminal esterase L (UCHL)-1, CD45 RB, and CD20 immunohistochemical staining for routine cell character-

ization. To identify activated microglia, we used a mouse monoclonal antibody to human macrophage CD68 (KP1, 1:50; Dako, Glostrup, Denmark) and a rabbit anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1; 1:500; Wako Chemicals, Osaka, Japan). For HIV-infected cells, we used an antibody HIVp24 (1:10, DaKoCytomation, Glostrup, Denmark). Antigen retrieval was performed by pretreatment in 0.067% protease (Sigma-Aldrich, St Louis, MO, US) in PBS for 3–5 min for immunohistochemistry of CD68, CD3, HIVp24, TNF- α , and IL-1 β . Immunoreactivity was visualized using either diaminobenzidine/peroxidase or 3-amino-9-ethylcarbazole (Dako) substrate-chromogen system (Dako). Light counterstaining was done with hematoxylin. For the TNF- α and IL-1 β immunostaining, tonsil sections with chronic inflammation were used as a positive control.

Double-label immunohistochemistry

To determine the phenotype of virus-infected cells, we performed a double-label immunohistochemistry for HIVp24 and either Iba1 or GFAP. We first performed immunohistochemistry for Iba1 or GFAP using avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, US) and then for HIVp24 using the EnVision system (Dako). The double-labeling was done using Vector blue/alkaline phosphatase followed by the 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase.

We performed a double-label immunohistochemistry for TNF- α and either GFAP, CD68, UCHL-1, or CD20 to determine the phenotype of the TNF- α positive cells. We first performed immunohistochemistry for either GFAP, Iba1, UCHL-1, or CD20 using avidin-biotin-peroxidase complex (Vector) and then followed with TNF- α using the EnVision system (Dako). The double-labeling was done using Vector blue/alkaline phosphatase followed by the 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase. To determine the phenotype of the IL-1 β positive cells, we performed a double-label immunohistochemistry for CD68 or UCHL-1 and IL-1 β using the same method. A double-label immunohistochemistry was performed for GFAP and IL-1 β by first performing immunohistochemistry for GFAP using the EnVision system (Dako), followed by immunohistochemistry using IL-1 β avidin-biotin-peroxidase complex (Vector). The double-labeling was performed using diaminobenzidine/peroxidase followed by Vector blue/alkaline phosphatase.

We performed a double-label immunohistochemistry for IL-1 β and HIVp24 to examine the expressions of IL-1 β correlating with the HIVp24-positive cells. This entailed performing immunohistochemistry for IL-1 β using the avidin-biotin-peroxidase complex (Vector), followed by

immunohistochemistry for HIVp24 using the EnVision system (Dako). The double-labeling was performed using Vector blue/alkaline phosphatase followed by diaminobenzidine/peroxidase. To examine the expressions of TNF- α correlating with the HIVp24-positive cells, we performed double-label immunohistochemistry for TNF- α and HIVp24 by first performing immunohistochemistry for HIVp24 using the EnVision system (Dako) and then for TNF- α using avidin-biotin-peroxidase complex (Vector). The double-labeling was performed using 3-amino-9-ethylcarbazole substrate-chromogen system (Dako; red)/peroxidase followed by the Vector blue/alkaline phosphatase.

Semiquantitative analysis

We performed semiquantitative assessments for routine histopathological changes as follows: 3; severe, 2; moderate, 1; mild, 0; none. We also semiquantitatively estimated

numbers of cells positive for the antibodies to HIVp24, CD68, TNF- α , and IL-1 β as following: 3; many, 2; moderate, 1; a few, 0; none.

RESULTS

Routine histopathological changes

Among 11 cases of the frontal white matter and pons from HIV-1 infected patients, all cases showed the typical pathology of HIVE such as glial nodules with multinucleated giant cells and mononuclear cell infiltration. A varying degree of white matter changes such as loosening of myelin staining, vacuolar changes, or astrocytic gliosis were also observed in all cases. HIVp24-positive cells were detected in all cases (Fig. 1). Semiquantitative judgments of histopathological findings of the frontal lobe and pons are summarized in Tables 1 and 2.

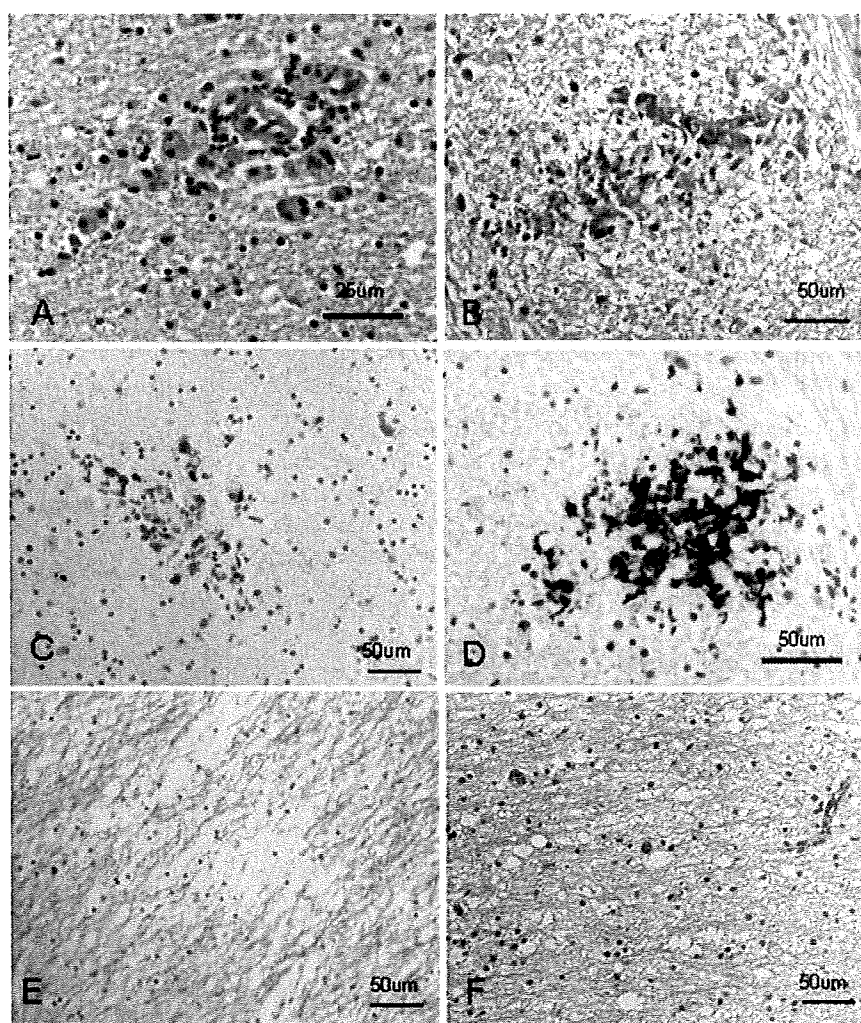


Fig. 1 Pathologic changes of HIV encephalitis. Glial nodules with multinucleated giant cells in the frontal white matter (A) as well as pons (B). HIVp24-positive cells were detected in the glial nodules seen in the frontal white matter (C), as well as pons (D). A varying degree of white matter changes such as loosening of myelin staining (E), vacuolar changes (F) seen in the frontal white matter. A–D, #32–92; E–F, #98–02; A, C, E–F, frontal white matter; B, D, pons; A, B, F, HE staining; C, D, anti-HIVp24; E, KB staining.

Table 1 Routine histopathological changes

Patient no.	Frontal white mater				Pons			
	Microglial nodule	Myelin pallor	Vacuolation	Gliosis	Microglial nodule	Myelin pallor	Vacuolation	Gliosis
32-92	3	3	2	3	1	1	1	2
50-95	3	3	2	3	3	2	2	3
98-02	3	3	3	3	3	3	3	3
137-99	2	1	1	1	1	1	1	0
10-87	2	1	2	3	2	2	2	3
133-00	2	2	2	1	1	1	1	0
82-91	2	1	1	3	1	1	1	2
206-86	2	2	2	3	3	3	3	3
45-96	1	1	1	1	2	1	1	2
61-93	1	1	1	2	1	1	1	0
118-91	1	1	1	1	0	1	1	2

3, marked; 2, moderate; 1, slight; 0, none.

Table 2 Expression of proinflammatory cytokines TNF- α and IL-1 β in HIV encephalitis

Patient No.	HIVp24		IL-1 β of frontal white matter				TNF- α of frontal white matter			
	Frontal white mater	Pons	MNGC	Microglia	Macrophage	Astrocyte	MNGC	Microglia	Macrophage	Astrocyte
32-92	3	3	3	2	3	3	0	3	3	3
50-95	3	1	3	2	3	3	0	3	3	3
98-02	2	2	0	3	2	0	0	3	3	0
137-99	2	1	0	1	2	0	0	2	2	0
10-87	2	3	0	0	1	0	0	0	0	0
133-00	1	1	1	1	1	0	0	1	1	0
82-91	1	2	0	1	1	0	0	1	1	0
206-86	1	2	0	1	1	0	0	0	0	0
45-96	1	2	0	1	1	0	0	1	1	0
61-93	1	1	0	1	1	0	0	0	0	0
118-91	0	3	0	1	1	0	0	0	0	0

3, many; 2, moderate; 1, a few; 0: none; MNGC, multinucleated giant cells.

Detection of HIVp24 in microglial cells and macrophages as well as MNGCs, but not in astrocytes

To determine which cell types are infected with HIV-1, we performed double-label immunohistochemistry. The HIVp24 staining was detected in infiltrating macrophages, MNGCs, macrophages and microglia of glial nodules, as well as scattered activated microglia, but not in astrocytes in the frontal white matter (Fig. 2).

The expressions of IL-1 β by macrophages, microglial cells, and astrocytes from HIV

IL-1 β -positive cells were shown as intracytoplasmic labeling. Among 11 cases, IL-1 β was detected in the frontal white matter of all cases, and in the frontal cortex of one case. Most of the positive cells had morphological features of perivascular macrophages and multinucleated giant cells in the glial nodules (Fig. 3A,D). On the other hand, in two cases of 32-92 and 50-95, some IL-1 β positive cells showed morphological features of astrocytes in the white matter (Fig. 3B,C). In order to search expressions of IL-1 β , we compared IL-1 β and CD68, Iba1, UCHL-1 and

GFAP and HIVp24 in serial sections. IL-1 β -positive cells had the same shape and distribution as Iba1- and CD68-positive cells, although CD68-positive cells were more numerous (Fig. 3D-H). The double-label immunohistochemistry demonstrated that IL-1 β was detected in CD68-positive macrophages/microglia of most cases (Fig. 3I), and in GFAP-positive astrocytes of only two cases, 32-92 and 50-95 (Fig. 3J), and not in UCHL-1-positive cells (Fig. 3K).

The expressions of TNF- α by macrophages, microglial cells, and astrocytes from HIV

TNF- α -positive cells were shown as intracytoplasmic labeling. Among 11 cases, TNF- α was detected in seven cases in the frontal white matter. Positive cells were detected in some mononuclear cells of inflammatory lesions in the frontal white matter, as well as a few perivascular macrophages, and some TNF- α -positive cells showed morphological features of astrocytes in the white matter (Fig. 4A,B). The double-label immunohistochemistry demonstrated that the TNF- α -positive cells were restricted to CD68-positive macrophages/microglia in most cases (Fig. 4C),

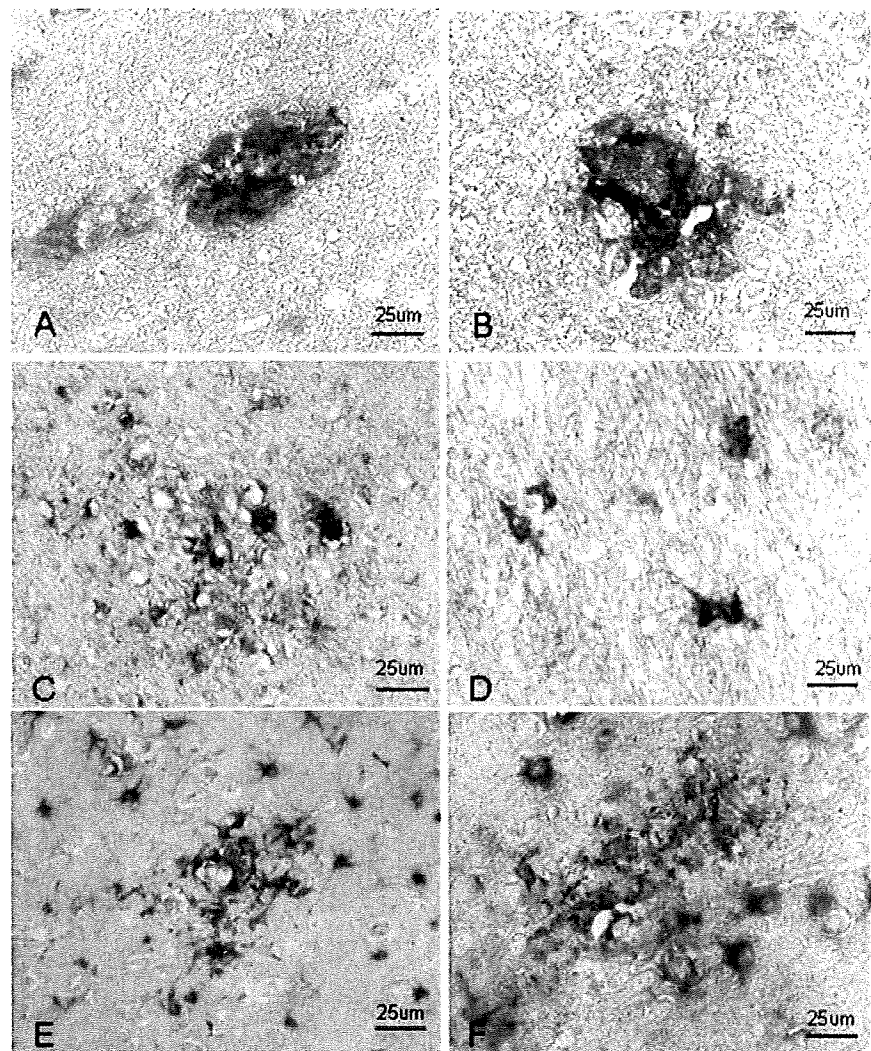


Fig. 2 Double-label immunohistochemistry for HIVp24 and anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1) or GFAP in HIV encephalitis. HIVp24 staining was detected in infiltrating macrophages (A), multinucleated giant cells (B), macrophages and microglia of glial nodules (C), as well as scattered activated microglia (D), but not in astrocytes (E, F) in the frontal white matter. A, B, F, #50–95; C–E, #32–92. A–D, double-label of HIVp24 (red) and Iba1 (blue); E–F, double-label of HIVp24 (red) and GFAP (blue).

and in some GFAP-positive astrocytes of only two cases, 32–92 and 50–95 (Fig. 4D), and not in UCHL-1-positive cells (Fig. 4E) and the CD20-positive cells (Fig. 4F).

Some HIVp24-positive cells express IL-1 β but not TNF- α

In order to investigate the relationship between expression of IL-1 β or TNF- α and virus infection, we performed double-label immunohistochemistry for IL-1 β or TNF- α and HIVp24. Interestingly, some IL-1 β -positive cells were HIVp24-positive multinucleated giant cells in the glial nodules. Several other IL-1 β -positive cells were also found around the HIVp24-positive cells, but they were negative for HIVp24 (Fig. 5A–C). Although the TNF- α -positive cells were found around the HIVp24-positive cells, they were negative for HIVp24 (Fig. 5D,E).

DISCUSSION

The cytokines such as TNF- α and IL-1 β stimulate macrophages and endothelial cells to express chemokines such as monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) that attract leucocytes into the peripheral site of injury or infection.^{32–37} In HIV-1 infection, it has been considered that HIV-infected macrophages and microglia cells directly produce such cytokines and may have toxic effects on CNS cells and induce neuronal death, which has been discussed as the pathogenesis of ADC.^{22,24,29,38,39} Several in vitro studies have demonstrated that exposure of macrophages and microglia to either gp120 or Tat resulted in up-regulation of TNF- α expression^{40,41} and exposure of microglia to gp120 resulted in the production of IL-1 β .^{42,43} A number of hypotheses concerning the roles

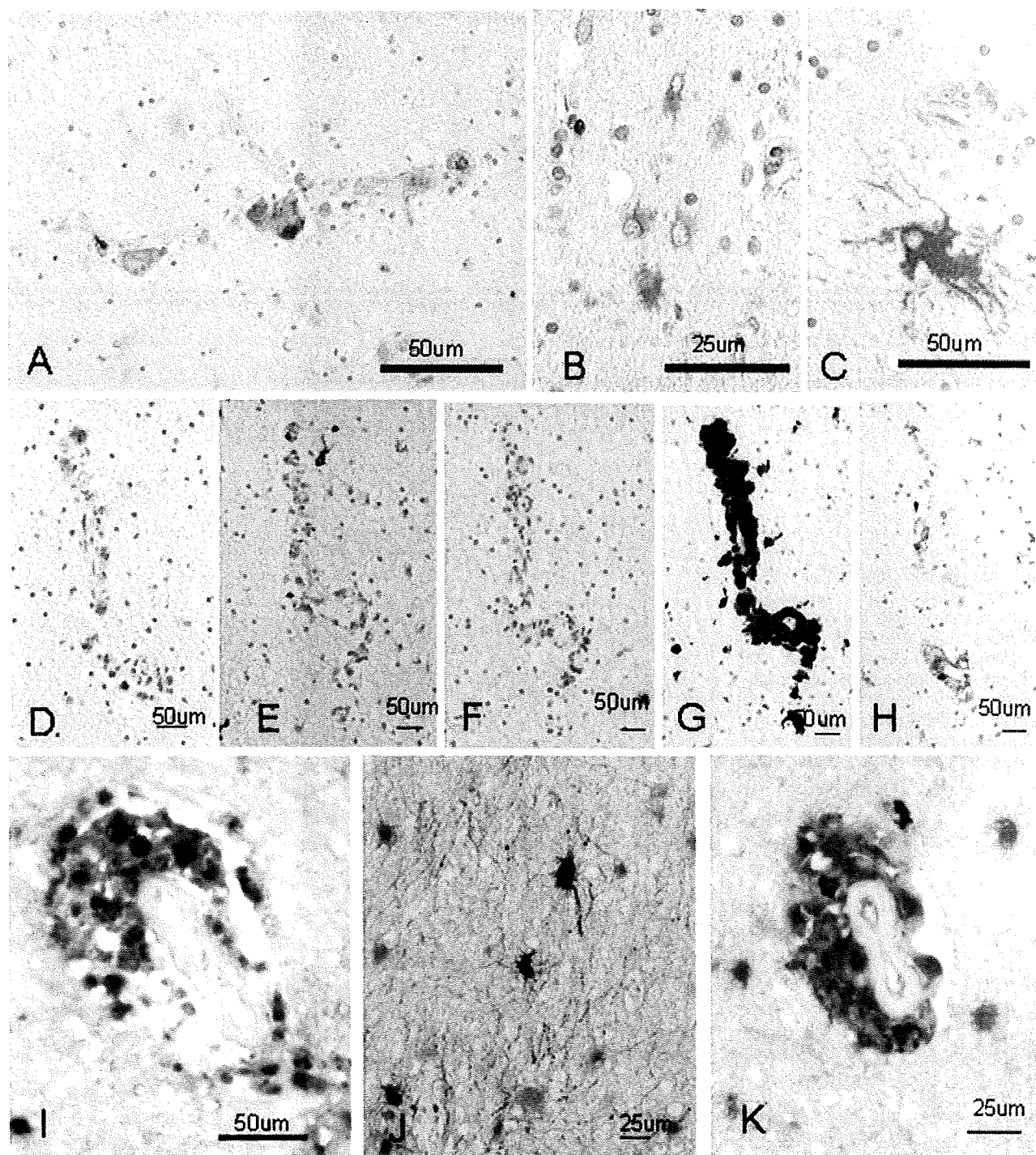


Fig. 3 Expression of IL-1 β in the frontal white matter of HIV encephalitis. IL-1 β immunoreactivity was detected in perivascular macrophages and multinucleated giant cells in the glial nodules (A), and mild expression of IL-1 β was also detected in some reactive astrocytes (B) and rarely in bizarre astrocytes (C). In serial sections (D–H), IL-1 β -positive cells had the same shape and distribution as anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1) and HIVp24-positive cells. Double-labelled immunohistochemistry (I–K), IL-1 β -positive cells were co-localized in CD68-positive macrophages/microglia (I) and GFAP-positive astrocytes (J), but not in UCHL-1-positive cells (K). A, D–K, #32–92; B, C, #50–95. A–D, anti-IL-1 β ; E, anti-Iba1; F, anti-HIVp24; G, anti-CD68; H, anti-UCHL-1; I, double-label of IL-1 β (red) and CD68 (blue); J, double-label of IL-1 β (dark blue) and GFAP (brown); K, double-label of IL-1 β (red) and UCHL-1 (blue).

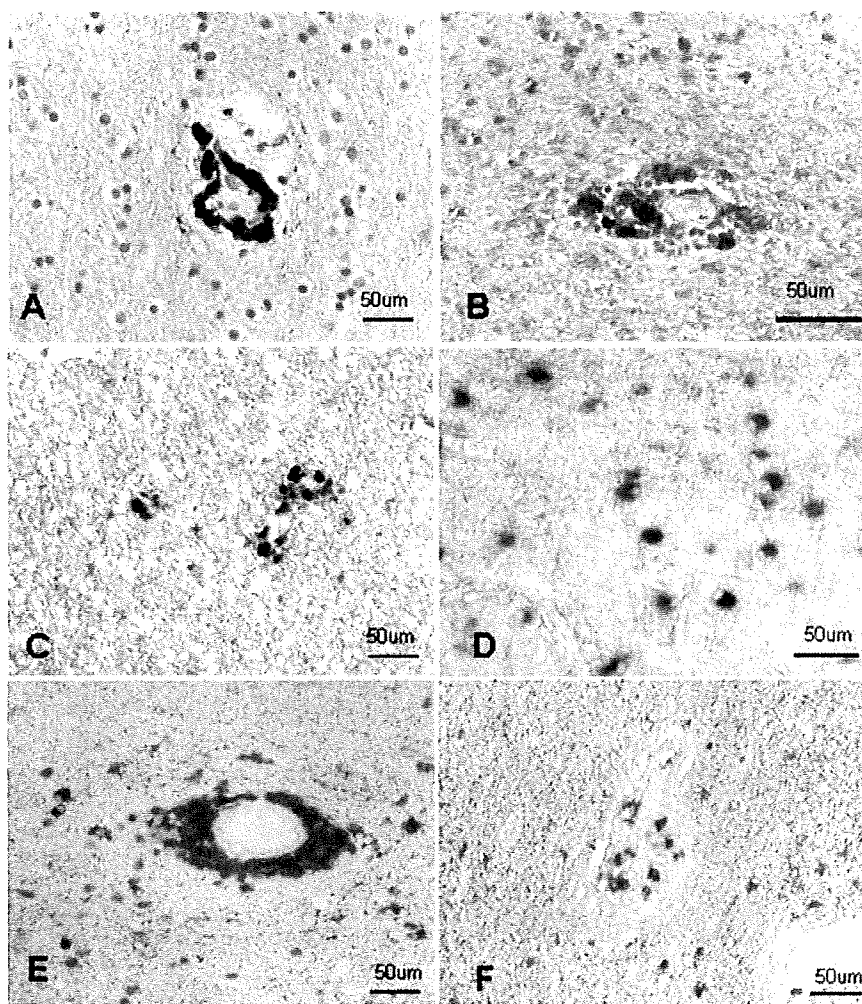


Fig. 4 Expression of TNF- α in the frontal white matter of HIV encephalitis. TNF- α immunoreactivity was detected in perivascular macrophages (A, B). Mild expression of TNF- α by astrocytes was detected in surrounding areas of inflammatory lesions (B). Double-labelled immunohistochemistry, TNF- α was detected in some CD68-positive perivascular macrophages/microglia (C) and some GFAP-positive reactive astrocytes (D), but TNF- α was not detected in UCHL-1-positive cells (E) as well as CD20-positive cells (F). A, C, #137-99; B, D-F, #50-95. A, B, anti-TNF- α ; C, double-label TNF- α (red) and CD68 (blue); D, double-label TNF- α (red) and GFAP (blue); E, double-label TNF- α (red) and UCHL-1 (blue); F, double-label TNF- α (red) and CD20 (blue).

of these cytokines are based on these in vitro experiments. However, little is known about in vivo expression of these cytokines in the microenvironment of HIVE, microglial nodules with MNGCs, and roles of these cytokines with relation to HIV infection are not yet clarified.

In the present study, we examined human autopsy brains from HIV-1 infected individuals and explored whether expression of cytokines such as IL-1 β and TNF- α can be detected in macrophages/microglia or astrocytes in HIVE, and whether expression of these cytokines is related to viral infection. Interestingly, IL-1 β was detected in the frontal white matter of all 11 cases where microglial nodules were observed to varying degrees. TNF- α was detected in seven cases, and we could not detect expression of TNF- α in four cases. IL-1 β -positive cells were mostly restricted to CD68-positive macrophages/microglia and some of them were HIVp24-positive multinucleated giant cells in the glial nodules. On the other hand, TNF- α positive cells were also restricted to mainly CD68-positive

macrophages/microglia; however, we could not detect TNF- α expression in the HIVp24-positive cells. These findings suggest that IL-1 β is induced by HIV-1 infection and may be one of the important factors for induction of HIVE.

Barak *et al.*⁴⁴ reported that intra-cerebroventricular administration of HIV-1, gp120 induced the expression of IL-1 β , but not TNF- α mRNA in the rat hypothalamus, 3 h after injection. Zhao *et al.*⁴⁵ reported that IL-1 β was expressed at high levels in areas of microglial nodules and some MNGCs were positive for IL-1 β in HIVE and they suggested that IL-1 β was induced by HIV-1 infection. We confirmed that the HIV-1 infection of macrophages/microglia induced the expression of IL-1 β , but not TNF- α for the first time in the brain lesions of HIV-1 encephalitis.

There are some in vitro studies suggesting that infection of astrocytes may also occur with limited virus replication,²⁶ and astrocytes could cause CNS injury by secreting cytokines.²⁷⁻³⁰ In the present study, we detected mild

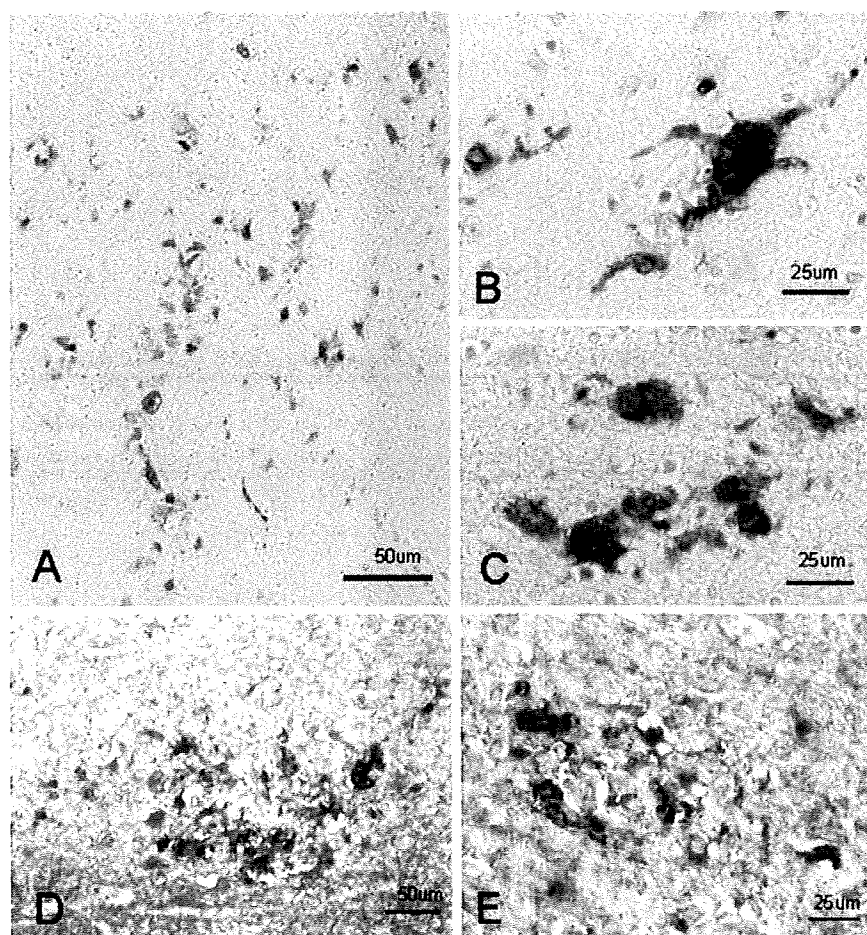


Fig. 5 Double-label immunohistochemistry for HIVp24 and IL-1 β or TNF- α in HIV encephalitis. IL-1 β positive cells were found around the HIVp24-positive cells and some of them were also positive for HIVp24 (A). Some HIVp24-positive multinucleated giant cells were positive for IL-1 β in the glial nodules (B, C). TNF- α -positive cells were found around the HIVp24-positive cells, but they were negative for HIVp24 (D, E). A–C, #32–92; D–E, #5095. A–C, double-label HIVp24 (brown) and IL-1 β (blue); D–E, double-label HIVp24 (red) and TNF- α (blue).

expression of IL-1 β or TNF- α by astrocytes in only two cases with severe HIVE. Our results are similar to the report of Wesselingh *et al.*²⁵ in which IL-1 β -positive cells were restricted to CD68-positive macrophages/microglia and rare GFAP-positive astrocytes. These findings suggest that the cells of macrophages/microglia lineage are main cells to release cytokines such as IL-1 β and TNF- α in the lesions of HIV-1 encephalitis. Recent studies reported that latent or low-level infection of astrocytes may occur in HIV-1 infected brains.⁴⁶ Cytokine-expressing astrocytes seen in the present study might be infected with HIV-1 in a latent manner, although our double immunohistochemistry could not detect HIVp24 in these astrocytes.

On the other hand, in our present study, IL-1 β was detected also in many HIVp24-negative cells, and most TNF- α -expressing cells were negative for HIVp24. Expression of these cytokines on HIVp24-negative cells is consistent with our previous study on the macaque model,³¹ and suggests that non-infected microglia/macrophages as well as astrocytes might have a role in the pathogenesis of ADC. Interestingly it has been proposed that uninfected mac-

rophages and microglia also release these neurotoxic cytokines, thus explaining why apoptotic neurons do not co-localize with infected macrophages and microglia.^{22,47,48} Our previous studies on the frontal cortex pathology also suggested that cortical degeneration and presence of virus-infected cells are independent of each other in the SIV model²¹ as well as in human autopsy brains.⁴⁹

In conclusion, our present study demonstrated that HIV-1-infected macrophages/microglia express IL-1 β but not TNF- α in HIVE inflammatory lesions. In addition, many non-infected macrophages/microglia as well as some astrocytes express IL-1 β and TNF- α , which may contribute to the pathogenesis of ADC.

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Original article

Disulfide-mediated apoptosis of human T-lymphotrophic virus type-I (HTLV-I)-infected cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis

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Background: This study was conducted to construct a basis for a therapeutic strategy against human T-lymphotropic virus type-I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) using a compound that contained a disulfide moiety, prosultiamine, which is a homologue of allithiamine originally synthesized by allicin and thiamine-thiol, for the targeting of HTLV-I-infected cells.

Methods: First, we analysed the apoptotic pathway in allicin or prosultiamine treatment against an HTLV-I-infected T-cell line (HCT-1), derived from an HAM/TSP patient, by flow cytometry and western blot. Second, we evaluated the effect of targeting HTLV-I-infected cells in a prosultiamine *in vitro* treatment and in a clinical trial in HAM/TSP patients by quantitative PCR analysis of HTLV-I proviral load.

Results: Prosultiamine, like allicin, induced caspase-dependent apoptosis against HCT-1 cells. The fact that

the loss of mitochondrial membrane potential was recovered in z-VAD-fmk-pretreated HCT-1 cells with prosultiamine treatment suggested that prosultiamine can induce caspase-dependent apoptosis through the mitochondrial pathway. On the basis of data showing that prosultiamine *in vitro* treatment against peripheral blood CD4⁺ T-cells of HAM/TSP patients induced a significant decrease of HTLV-I proviral copy numbers by apoptosis of HTLV-I-infected cells, we treated six HAM/TSP patients with intravenous administration of prosultiamine for 14 days. As a result of this treatment, the copy numbers of HTLV-I provirus in peripheral blood decreased to approximately 30–50% of their pretreatment levels with some clinical benefits in all patients. **Conclusions:** Our results suggest that prosultiamine has the potential to be a new therapeutic tool that targets HTLV-I-infected cells in HAM/TSP.

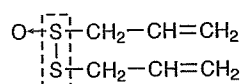
Introduction

Human T-lymphotropic virus type-I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive myelopathy characterized by the involvement of bilateral pyramidal tracts with sphincteric disturbances [1]. The primary pathological feature of HAM/TSP is chronic inflammation of the spinal cord characterized by perivascular cuffing and parenchymal infiltration of lymphocytes [2]. Although the exact mechanism of the pathogenesis of HAM/TSP is still obscure, immunological abnormalities (for example, the increased number of activated T-cells and T-helper 1 activation arising from a high HTLV-I proviral load) in peripheral blood play an important role in the pathological process of spinal

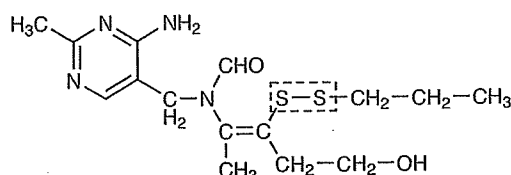
cord lesions in HAM/TSP patients [3,4]. Therefore, on the basis of this evidence, immunomodulatory therapy (such as prednisolone [1,5] and interferon- α [IFN- α] [6,7] treatment) has been the main treatment administered to HAM/TSP patients. Indeed, as it is conceivable that the immune-activated status of the peripheral blood involved in the process of chronic inflammation of the spinal cord is one of the targets of the treatments, these treatments produced good results in their own ways; however, their efficacy is still controversial. In addition, whether these treatments are tolerable as a long-term or lifelong treatment is uncertain. Most importantly, in a therapeutic strategy against HAM/TSP, the ideal treatment is the elimination of HTLV-I-

Figure 1. The structures of allicin and prosultiamine

A



B



(A) Allicin and (B) prosultiamine. Prosultiamine has a disulfide moiety in its structure, like allicin.

infected cells from the peripheral blood because HTLV-I-infected CD4⁺ T-cells are the first responders in the immunopathogenesis of HAM/TSP [4,8].

Allicin (diallyl thiosulfinate; Figure 1), a natural organosulfur compound derived from garlic (*Allium sativum*), has diverse biological activities, including anticarcinogenic, antibacterial and antifungal activity [9,10]. Although the mechanisms by which cytotoxic effects are induced by organosulfur compounds are poorly understood, a disulfide moiety in their structures seems to play an important role in triggering cell death [11]. The disruption of the intracellular redox system induced by the chemical reaction of a disulfide moiety with thiol-containing intracellular molecules, such as thioredoxin (Trx), Trx reductase and glutathione, might be involved in the cytotoxicity [9,11]. However, allicin is a very unstable compound and disappears rapidly after intravenous administration [12,13]. Therefore, its use in therapeutic trials for HAM/TSP is limited.

Prosultiamine (*N*-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-*N*-[4-hydroxy-1-methyl-2-(propyldithio)-1-butenyl]-formamide; Alinamin®), a product of Takeda Pharma Co., Inc. (Osaka, Japan), is a homologue of allithiamine, originally synthesized by thiol-type vitamin B1 and allicin [14]. To enhance stability in the blood and the efficient access of vitamin B1 to the tissues, prosultiamine was developed after allyl disulfide, which is derived from allicin, and was substituted with propyl disulfide in the structure of allithiamine [15]. Thus, prosultiamine has a disulfide moiety in its structure, like allicin (Figure 1). The fact that prosultiamine is reduced to a part of thiamine and propyl disulfide by the intracellular reducing system after penetration to the cells might indicate that this compound is expected

to have a pharmacological action similar to that of allicin in the cells. Importantly, prosultiamine is pharmacologically stable and is very frequently prescribed for vitamin B1 deficiency in Japan. As such, this drug is potentially available for immediate clinical trials in HAM/TSP patients.

Considering the above, we first investigated the cytotoxic effect of prosultiamine against various HTLV-I-infected T-cell lines derived from HAM/TSP patients compared with that of allicin through analyses of apoptotic signalling. Secondly, based on the data showing that prosultiamine *in vitro* treatment against peripheral blood CD4⁺ T-cells of HAM/TSP patients induced a significant decrease in HTLV-I proviral copy numbers via apoptosis of HTLV-I-infected cells, we treated six HAM/TSP patients with intravenous administration of prosultiamine. As a result of this treatment, the copy numbers of HTLV-I provirus in peripheral blood decreased to approximately 30–50% of their levels at pretreatment with some clinical benefits in all patients. We herein propose prosultiamine treatment as a new therapeutic strategy against HAM/TSP.

Methods

Cell lines and peripheral blood CD4⁺ T-cells

HTLV-I-infected T-cell lines (HCT-1, HCT-4 and HCT-5) derived from the cerebrospinal fluid of three HAM/TSP patients were used [16]. These cell lines were HTLV-I-producing cell lines. Jurkat cells were also used as an HTLV-I-non-infected T-cell line. HCT-1, HCT-4 and HCT-5 cells were interleukin (IL)-2-dependent and were maintained in RPMI 1640 containing 20% fetal bovine serum (FBS) supplemented with 100 units/ml of recombinant human IL-2 (kindly provided by Shionogi & Co., Ltd, Osaka, Japan). Jurkat cells were maintained in RPMI 1640 containing 10% FBS. The peripheral blood CD4⁺-enriched T-cells of HAM/TSP patients were separated from the negative selection by the depletion of CD8⁺ T-cells from macrophage- and B-cell-depleted mononuclear cells using magnetic beads coated with anti-CD8 monoclonal antibody (Dynabeads CD8; Dynal Biotech, Oslo, Norway) as previously described [17]. CD4⁺-enriched T-cells were used as CD4⁺ T-cells.

Cytotoxicity assays

Each cell line was cultured at the concentration of 2×10^5 cells/ml for 24 h in the presence of allicin (LKT Laboratories, Inc., Saint Paul, MN, USA) or prosultiamine (kindly provided by Takeda Pharma Co., Inc., Osaka, Japan) at various concentrations in 96-well culture plates. As a control, each cell line was cultured in the presence of vehicle

alone. Cultures were studied in quadruplicate. Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]2-2-[4-sulfophenyl]-2H tetrazolium (MTS) non-radioactive cell proliferation assays (Promega, Madison, WI, USA) [17].

Apoptosis assays

Flow cytometry

HCT-1 cells were cultured at the concentration of 2×10^5 cells/ml in the presence of 40 μ M prosultiamine or vehicle for 1, 3, 5, 8 and 24 h. HCT-1 cells were then collected at each time point and stained either by the potential sensitive fluorescent dye 3,3'-dihexyloxacarbocyanine iodide (Nacalai Tesque, Kyoto, Japan) as previously described [18] or by fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, San Diego, CA, USA) according to the instructions provided by the manufacturer; the loss of the mitochondrial membrane potential ($\Delta\Psi$ m) or the percentages of apoptotic cells, respectively, were analysed by flow cytometry (Epics XL System II; Beckman Coulter, Fullerton, CA, USA).

Effect of pretreatment with z-VAD-fmk against apoptosis

To examine whether apoptosis was dependent on caspase activation, HCT-1 cells were pretreated with 200 μ M z-VAD-fmk (a pan-caspase inhibitor; Calbiochem, La Jolla, CA, USA) for 1 h and then treated with 40 μ M of either allicin, prosultiamine or vehicle for 24 h; the cell viability and the loss of $\Delta\Psi$ m were determined by MTS non-radioactive cell proliferation assays and flow cytometry, respectively.

Western blot analyses

HCT-1 cells were collected at various time points after treatment with 40 μ M allicin, prosultiamine or vehicle and lysed by M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Sigma, Saint Louis, MO, USA). Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4°C and the supernatant was used for western blotting. An identical amount of protein for each lysate (10 μ g) was subjected to 10% SDS-PAGE (ATTO, Tokyo, Japan). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After overnight blocking with TBS with 0.1% Tween-20 containing 5% skimmed milk (Becton, Dickinson & Co., Hunt Valley, MD, USA) at 4°C, the PVDF membrane was incubated in the presence of mouse anti-caspase-3 antibodies (1:1000 dilution; Cell Signaling, Danvers, MA, USA) at 4°C overnight. After overnight incubation with donkey anti-mouse immunoglobulin G (IgG)-horseradish peroxidase-linked species-specific whole antibodies (Amersham Biosciences, Little Chalfont, UK) at 4°C overnight, proteins were

visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

Quantification of HTLV-I proviral load

For quantitative analysis of the HTLV-I proviral load, real-time quantitative PCR was performed in a LightCycler® FastStart DNA Master (Roche Diagnostics, Mannheim, Germany) based on general fluorescence detection with SYBR® Green. Briefly, genomic DNA samples from CD4⁺ T-cells or peripheral blood mononuclear cells (PBMCs) from HAM/TSP patients prepared using the Genomic DNA Purification kit (Promega) were subjected to real-time PCR in a LightCycler® PCR system using Tax-specific primers (forward primer 5'-AAACAGCCCTGCAGATACAAAGT-3' and reverse primer 5'-ACTGTAGAGCTGAGCCGATAACG-3') and β -actin-specific primers (forward primer 5'-GCCCTCATTTCCTCTCA-3' and reverse primer 5'-GCTCAGGCAGGAAAGACAC-3'). The PCR conditions for Tax were 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 5 s and extension at 72°C for 10 s; and the condition for β -actin were 32 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 5 s and extension at 72°C for 15 s. The HTLV-I proviral load per 10,000 cells was calculated according to the formula (copy number of Tax)/(copy number of β -actin/2) $\times$ 10,000.

In vitro treatment with prosultiamine against peripheral blood CD4⁺ T-cells of HAM/TSP patients

The peripheral blood CD4⁺ T-cells of HAM/TSP patients were cultured at a concentration of 1×10^6 cells/ml in 24-well culture plates in the presence of 5 μ M prosultiamine for 48 h. As a control, the cells were cultured in the presence of vehicle alone. After the cells were collected, the dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA, USA) according to the instructions provided by the manufacturer and viable cells were collected. Total cellular DNA samples prepared from viable cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analyses. To confirm apoptosis of HTLV-I-infected cells, cultured cells of an HAM/TSP patient were stained with anti-HTLV-I (p19, p28 and gag) monoclonal antibodies (Chemicon International, Inc., Temecula, CA, USA) followed by the incubation with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) supplemented with Hoechst33258 stain (Sigma) as previously described [19] and then analysed by confocal microscopy.

Clinical trials with prosultiamine

We enrolled six HAM/TSP patients (two men and four women) whose ages ranged from 51 to 73 years and

who fulfilled the criteria described previously [20]. The profiles of the patients are shown in Table 1. Informed written consent was obtained from all patients in the study. This research was approved by the review boards of the Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan.

Prosultiamine was diluted with 50 ml of physiological saline and administered intravenously at dosages of 40 mg daily for 14 days. The treatment that two patients were already receiving (1 g of vitamin C per day in patient 1 and 3 doses of 3 million IU of IFN- α per week with 7.5 and 5.0 mg of oral prednisolone per alternate days in patient 3) were continued on the condition that the dosage was kept constant concomitant with prosultiamine treatment during the study period. None of the patients except these two had received any immunomodulators. The clinical endpoints selected for the assessment of treatment were motor function score and time required to walk 10 or 30 m. The classifications of motor function score were made according to the disability grade [21]. Prior to the administration of prosultiamine, the patients were instructed to walk a fixed distance and the administration was begun only after the walking time became constant. We monitored the change of both motor function and HTLV-I proviral load in PBMCs.

Statistical analyses

Student's *t*-test and Wilcoxon signed-rank test were used for statistical analyses. Differences were considered significant at $P < 0.05$.

Results

The cytotoxic effect of allicin and prosultiamine against HTLV-I-infected T-cell lines derived from HAM/TSP patients

The cell viability of the three HTLV-I-infected T-cell lines (HCT-1, HCT-4 and HCT-5) or the Jurkat cell line decreased as a result of allicin treatment for 24 h in a dose-dependent manner (Figure 2A). Prosultiamine treatment caused an effect similar to that of allicin

treatment (Figure 2A). These results indicate that prosultiamine has the potential to induce a cytotoxic effect against these cell lines, much like allicin. Of the cell lines treated, the HTLV-I-infected T-cell lines were more sensitive to the treatment with each compound than the Jurkat cell line. As shown in Figure 2B, western blot analysis revealed that the proteolytic cleavage of caspase 3 appeared between 3 and 5 h after the start of treatment in allicin- or prosultiamine-treated HCT-1 cells. Next, in order to confirm the involvement of caspases in the cytotoxicity against HCT-1 cells induced by these compounds, HCT-1 cells were treated with 40 μ M of either allicin or prosultiamine after this cell line was pretreated with 200 μ M z-VAD-fmk for 1 h. As shown in Figure 2C, the cell viability was recovered almost completely in z-VAD-fmk-pretreated HCT-1. These results strongly suggested that these compounds can induce caspase-dependent apoptosis in HTLV-I-infected cells derived from HAM/TSP patients.

Prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HTLV-I-infected cells

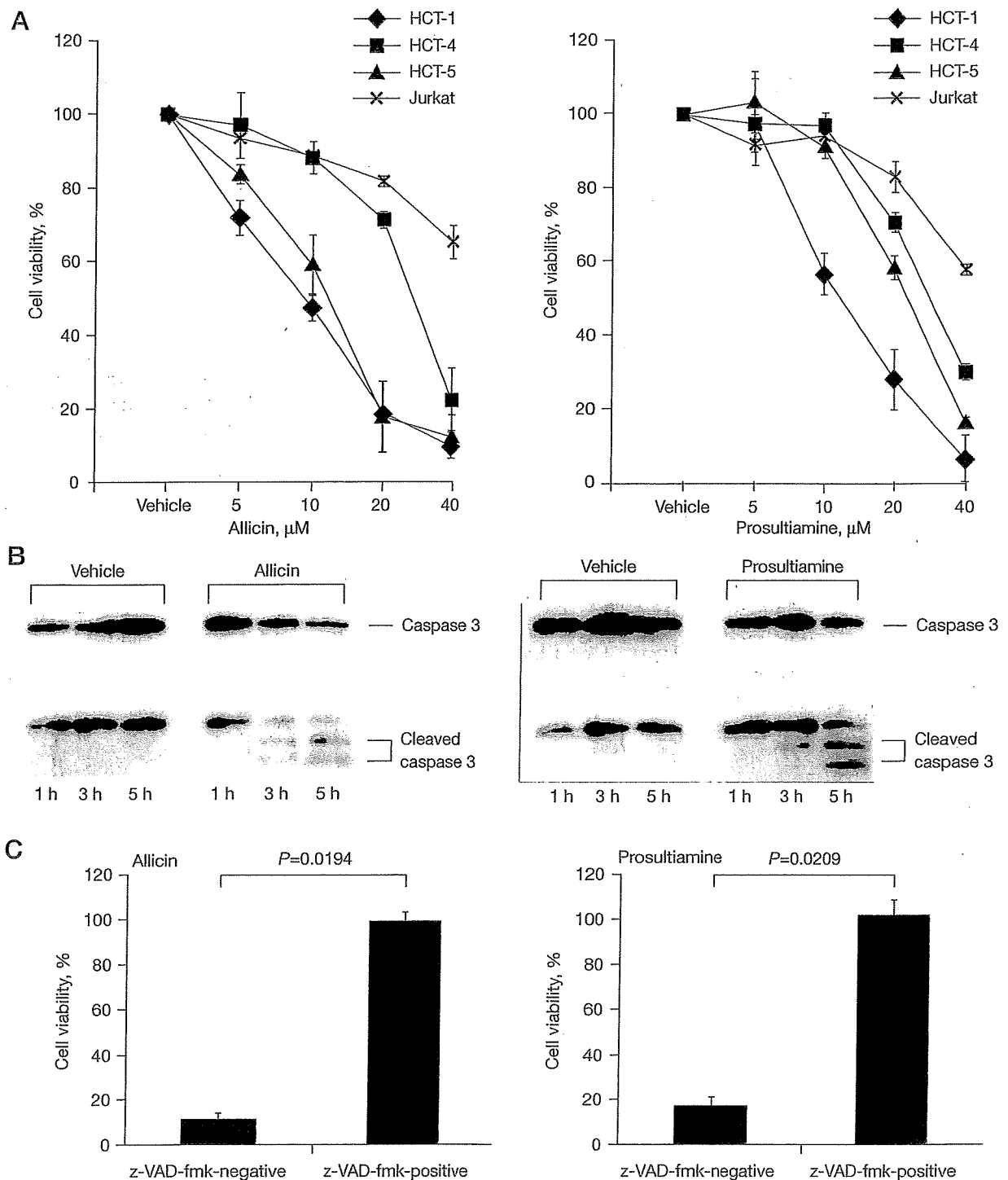
As mentioned earlier, because allicin is an unstable compound [12,13], it is very difficult to use as a therapeutic regimen in the treatment of HAM/TSP patients. However, prosultiamine is pharmacologically stable and is very frequently prescribed as a safe treatment regimen for thiamine deficiency. Therefore, we investigated the mechanisms of the induction of caspase-dependent apoptosis *in vitro* in prosultiamine-treated HCT-1 cells before performing a clinical trial with prosultiamine treatment. First, we performed a time course study of the loss of $\Delta\Psi_m$ concomitant with the appearance of annexin-V-positive cells in prosultiamine-treated HCT-1 cells. As shown in Figure 3A, the loss of $\Delta\Psi_m$ was detected in approximately 29.4%, 78.9% and 95.8% of prosultiamine-treated HCT-1 cells at 5, 8 and 24 h, respectively. In parallel with this data, annexin-V-positive cells were detected in 9.9% and 97.2% of prosultiamine-treated HCT-1 cells at 8 and 24 h, respectively (Figure 3B). These

Table 1. The profiles of patients and the clinical efficacy of prosultiamine treatment

Patient	Age, years	Gender	Duration of illness, years	Changes in motor function score*	
				Before	After
1	58	Female	24	4	4
2	51	Male	13	8	8
3	52	Male	46	4	4
4	73	Female	13	4	4
5	59	Female	12	4	4
6	53	Female	2	4	2

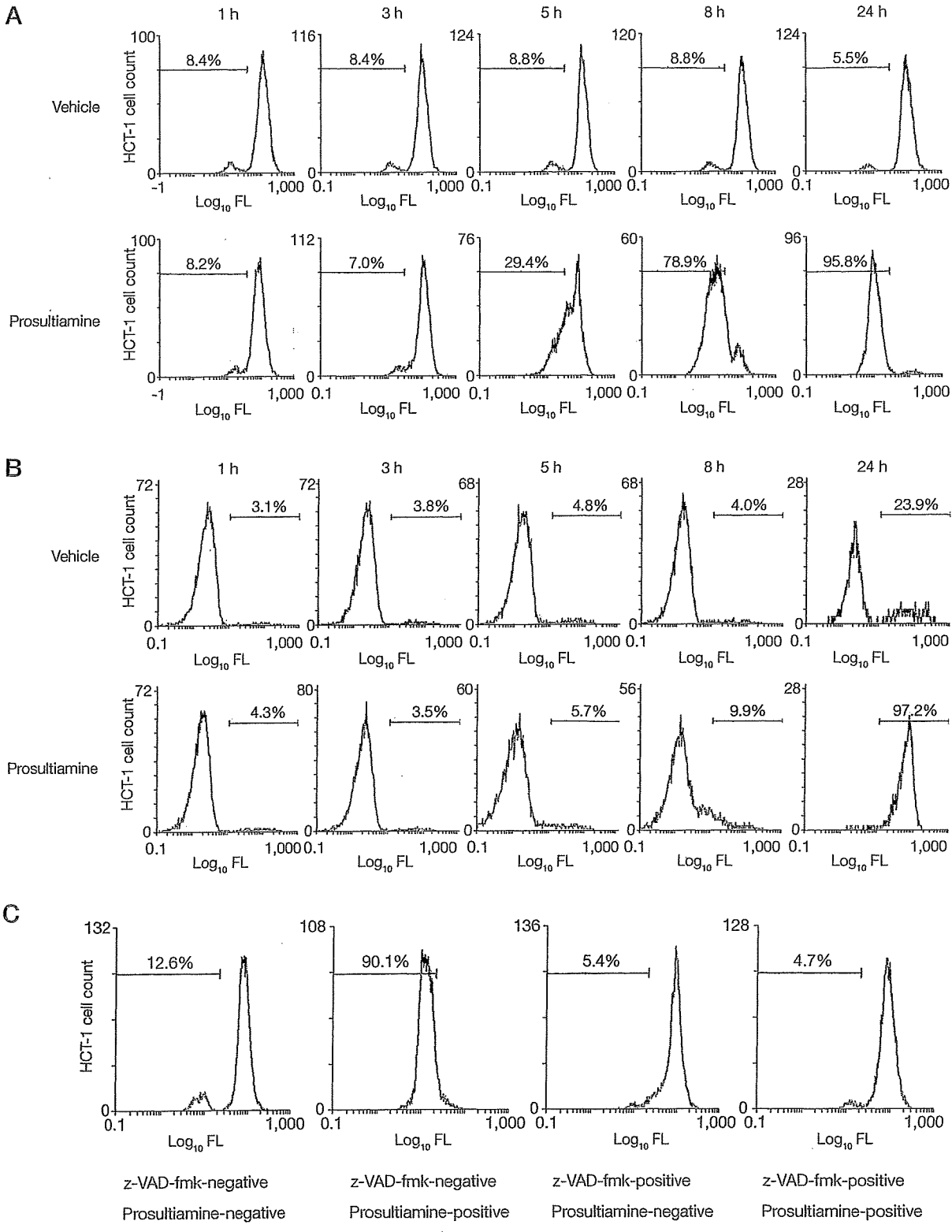
*Motor function score was rated from 0 to 10 according to the scale of reference [21].

Figure 2. The cytotoxic effect of allicin and prosultiamine against HTLV-I-infected T-cell lines derived from HAM/TSP patients based on caspase-dependent apoptosis



(A) Cell viability of three human T-lymphotropic virus type-I (HTLV-I)-infected T-cell lines (HCT-1, HCT-4 and HCT-5) or a Jurkat cell line decreased as a result of either allicin or prosultiamine treatment for 24 h in a dose-dependent manner. HTLV-I-infected T-cell lines were more sensitive to treatment with each compound than the Jurkat cell line. (B) Western blot analysis of caspase 3 activation. The proteolytic cleavage of caspase 3 appeared between 3 and 5 h after the start of the treatment in HCT-1 cells treated with 40 μM of either allicin or prosultiamine. (C) HCT-1 pretreated with 200 μM z-VAD-fmk (a pan-caspase inhibitor) for 1 h were treated with 40 μM of either allicin or prosultiamine for 24 h. Cell viabilities were recovered almost completely in z-VAD-fmk-pretreated HCT-1. As a cell viability assay, 3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]2-2-[4-sulfophenyl]-2H tetrazolium non-radioactive cell proliferation assays were performed. Cell viability was determined as follows: each optical density (OD) titre at a wavelength of 490 nm in quadruplicate cultures in the presence of allicin or prosultiamine was divided by the mean OD titre (at a wavelength of 490 nm) of the quadruplicate cultures under vehicle alone, and its mean $\pm$ SD was presented as the cell viability. HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis.

Figure 3. Prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HCT-1 cells



The time course study of (A) the loss of the mitochondrial membrane potential ($\Delta\Psi_m$) and (B) the appearance of annexin-V-positive cells in prosultiamine-treated human T-lymphotrophic virus type-I (HTLV-I)-infected T-cell line (HCT-1) cells by flow cytometric analyses. Prosultiamine treatment of HCT-1 cells induced the loss of $\Delta\Psi_m$ with the appearance of annexin-V-positive cells. (C) The effect of pretreatment with z-VAD-fmk (a pan-caspase inhibitor) on the recovery of the loss of $\Delta\Psi_m$ in prosultiamine-treated HCT-1 cells. The loss of $\Delta\Psi_m$ in HCT-1 cells resulting from treatment with 40 μ M prosultiamine was almost completely recovered by pretreatment with 200 μ M z-VAD-fmk for 1 h. FL, fluorescent intensity.

results suggest that prosultiamine induces caspase-dependent apoptosis through the mitochondrial pathway in HCT-1 cells. In order to confirm this finding, we analysed the effect of pretreatment with z-VAD-fmk on the recovery of the loss of $\Delta\Psi_m$ in prosultiamine-treated HCT-1 cells. As shown in Figure 3C, the loss of $\Delta\Psi_m$ in HCT-1 as a result of treatment with 40 μM prosultiamine was almost completely recovered by pretreatment with 200 μM z-VAD-fmk for 1 h. Overall, these results strongly suggest that the cytotoxicity of prosultiamine against HTLV-I-infected cells is mainly based on caspase-dependent apoptosis through the mitochondrial pathway.

Prosultiamine *in vitro* treatment against peripheral blood CD4⁺ T-cells of HAM/TSP patients

A confocal microscopic study showed that prosultiamine treatment against peripheral blood CD4⁺ T-cells from a HAM/TSP patient induced the condensation of chromatin in HTLV-I-infected cells only in prosultiamine-treated peripheral blood CD4⁺ T-cells, as shown in the double staining with anti-HTLV-I monoclonal antibodies and Hoechst33258 stain (Figure 4A). Next, we evaluated the effect of prosultiamine treatment on the reduction of HTLV-I-infected cells in peripheral blood CD4⁺ T-cells from HAM/TSP patients. As shown in Figure 4B, prosultiamine treatment induced a decrease in the numbers of HTLV-I proviral copies ranging from 11.2% to 70.1% (mean 42.9%) in comparison with the vehicle treatment. Allicin *in vitro* treatment induced the same effect as prosultiamine *in vitro* treatment (data not shown). We also evaluated the cell viability using MTS non-radioactive cell proliferation assays and found that prosultiamine treatment did not affect the viability of total peripheral blood CD4⁺ T-cells, indicating that the effect did not depend on a non-specific cytotoxic effect (data not shown). These data suggest that prosultiamine *in vitro* treatment can selectively induce apoptosis in HTLV-I-infected peripheral blood CD4⁺ T-cells from HAM/TSP patients.

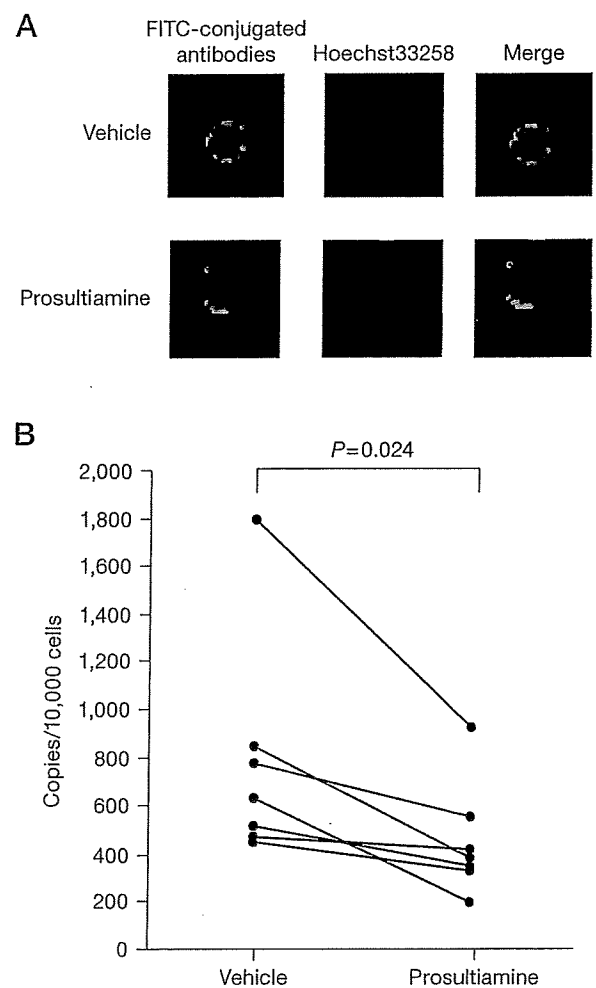
Clinical trials with prosultiamine

On the basis of the data mentioned above, we treated six HAM/TSP patients by intravenous administration of prosultiamine. As shown in Table 1, although the motor function score did not change among five of six HAM/TSP patients (patients 1–5), some clinical improvement was observed in individuals, including a decrease in the walking time in patient 1, the acquisition of an ability to maintain a standing position from a wheelchair-dependent state for a short period in patient 2 and the improvement of spasticity in patients 3–5. By contrast, patient 6 showed marked improvement of motor function, such as a change of motor

disability grade from 4 to 2 (Table 1). No adverse events were observed.

We monitored the change in the copy numbers of HTLV-I provirus in the PBMCs of HAM/TSP patients at pretreatment (day 0), during treatment (days 7 and 14) and 7 days after treatment (day 21). The decrease of

Figure 4. Effect of *in vitro* treatment with prosultiamine on HTLV-I-infected cells of HAM/TSP patients



Peripheral blood CD4⁺ T-cells of human T-lymphotropic virus type-I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients was cultured in the presence of 5 μM prosultiamine or vehicle for 48 h. (A) A confocal microscopic study in prosultiamine- or vehicle-treated peripheral blood CD4⁺ T-cells of an HAM/TSP patient. The condensation of chromatin in HTLV-I-infected cells was shown in only prosultiamine-treated peripheral blood CD4⁺ T-cells by double staining with anti-HTLV-I antibodies (followed by fluorescein isothiocyanate [FITC]-conjugated anti-mouse immunoglobulin G) and Hoechst33258 stain. (B) Decrease of HTLV-I proviral copy numbers in peripheral blood CD4⁺ T-cells of HAM/TSP patients as a result of the *in vitro* treatment with prosultiamine. After the dead cells induced by prosultiamine *in vitro* treatment were removed using the Dead Cell Removal kit (Miltenyi Biotec, Auburn, CA, USA), total cellular DNA samples prepared from viable cells were subjected to the measurement of HTLV-I proviral copies by quantitative PCR analyses. Prosultiamine treatment induced a significant decrease of HTLV-I proviral copies compared with vehicle treatment.