cytoskeletal reorganization determines the intercellular transmission efficiency of HTLV-I. We previously reported that Rac and Cdc42 are more activated in HTLV-I-infected T-cell lines derived from HAM/TSP patients than in those derived from other origins,[7] suggesting that HTLV-I-infected T-cell lines derived from HAM/TSP patients have the potential of the efficient intercellular transmission of HTLV-I based on activated status of the cytoskeletal reorganization including actin polymerization.

The vasodilator-stimulated phosphoprotein (VASP) regulates signal transduction pathways involved in actin cytoskeleton dynamics.[8,9] VASP is a known substrate of serine/threonine kinases, such as cyclic adenosine monophosphate- (cAMP) or cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKA or PKG, respectively). Both kinases phosphorylate the Ser^{157} and Ser^{239} sites in VASP. It is known that phosphorylated VASP (p-VASP) acts as a negative regulator of actin dynamics. Phosphorylation of VASP catalyzed by either PKA or PKG inhibits actin polymerization, and conversely, dephosphorylation accelerates actin polymerization. That is, the degree of reorganization of the actin cytoskeleton is determined by the phosphorylated or dephosphorylated status of VASP. Therefore, the intracellular cAMP or cGMP concentration might regulate polarization of the actin cytoskeleton and affect the intercellular transmission efficiency of HTLV-I.

To test these hypotheses, we investigated the relationships between the intercellular transmission efficiency of HTLV-I, the intracellular cAMP or cGMP levels and the phosphorylation status of VASP in a comparative study of HTLV-I-infected T-cell lines derived from an HAM/TSP patient or an HTLV-I carrier.

Methods

Chemicals and antibodies

Latrunclin B was purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Forskolin was purchased from Applichem (Darmstadt, Germany). Both compounds were dissolved with dimethyl sulfoxide (DMSO) as the vehicle before experiments. Rabbit polyclonal anti-VASP, phosphorylated VASP (Ser^{157} or Ser^{239}; p-VASP), rabbit monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-HTLV-I tax or gp-46, α-tubulin and an HRP-conjugated goat anti-mouse immunoglobulin G antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines

Interleukin-2 (IL-2)-dependent HTLV-I-infected T-cell line derived from the cerebrospinal fluid of an HAM/TSP patient (HCT-5) and IL-2-independent HTLV-I-infected T-cell line derived from an HTLV-I carrier (TL-Su) were used in the present study.[7]

The H9/K30 *luc* reporter cell line was kindly provided by Professor Akio Adachi (University of Tokushima Graduate School, Tokushima, Japan). H9/K30 *luc* cells are lymphocytic H9 cells that have been stably transfected with a plasmid containing the gene encoding luciferase under the control of the HTLV-I long terminal repeat (LTR).[10] Therefore, as activation of LTR driven by HTLV-I tax induces luciferase expression, these reporter cells can detect the efficiency of HTLV-I transmission under co-cultivation with HTLV-I-infected cells by a luciferase assay system. Expression of the integrin αLβ2 and its ligand, ICAM-1, was confirmed in both cell lines by flow cytometric analysis.

The present study complied with the guidelines of the ethics committee of our institution.

Western blotting analysis

Each culture of HTLV-I-infected T cells was collected and lysed by the addition of M-PER mammalian protein extraction reagent (Thermo Scientific, Hanover Park, IL, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St, Louis, MO, USA) and a Halt phosphatase inhibitor cocktail (Thermo Scientific). Insoluble material was removed by centrifugation at 14 300 *g* for 30 min at 4°C, and the supernatant was analyzed by western blotting. An identical amount of protein for each lysate (20 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (ATTO, Tokyo, Japan). Subsequently, proteins were transferred onto a polyvinylidene difluoride membrane and immersed in 5% non-fat milk in Tris-buffered saline, 0.1% Tween 20 (TBST) at room temperature for 60 min to block non-specific binding sites. Anti-p-VASP, -VASP, -HTLV-I tax and -HTLV-I gp46 (all used at 1:1000 dilution) antibodies were used as primary detection reagents. Anti-GAPDH or α-tubulin (both used at 1:1000 dilution) antibodies for detection of an internal control protein were used for confirmation of equal protein loading. Membranes were

incubated overnight with specific primary antibodies at 4°C. After washing with TBST, membranes were incubated with appropriate secondary HRP-conjugated anti-species antibodies at room temperature for 1 h. After further washing with TBST, peroxidase activity was detected by using the ECL plus western blot detection system (Amersham, GE Healthcare, Little Chalfont, UK).

Measurement of intracellular cAMP or cGMP levels

Intracellular cAMP or cGMP levels in both cell lines were measured using enzyme-linked immunoassay kits [cyclic AMP complete assay (Stressgen, Ann Arbor, MI, USA) or Parameter cyclic GMP assay (R&D systems, Minneapolis, MN, USA), respectively], according to the instructions provided by the manufacturer. Briefly, equal numbers of HCT-5 or TL-Su cells and equal numbers of DMSO- or forskolin-treated HCT-5 were lysed with an equal volume of lysis buffer. After uniform lysis was confirmed by microscopy, the cellular debris was removed by centrifugation at 600 *g* for 10 min at 4°C, and the supernatant was used for assays. The intracellular cAMP or cGMP levels were determined in triplicate. Data were expressed as mean ± SD. The minimum detection limits of cAMP and cGMP in these assays were 0.039 pmol/mL or 1.14 pmol/mL, respectively.

Co-cultivation

HCT-5 or TL-Su (5×10^5 cells) were co-cultivated with H9/K30 *luc* reporter cells (3.5×10^5 cells; kindly provided by Professor Akio Adachi, University of Tokushima Graduate School) in a 24-well culture plate at 37°C under 5% CO_2. After co-cultivation for 6 h, luciferase activity was assessed by using a luciferase assay system (Promega, Madison, WI, USA) and Gene Light (Microtec, Tokyo, Japan). The relative luc activity was calculated according to the following formula: relative luminescent units (RLU) of co-cultivated sample/RLU of the H9 only cultivated sample. Data were expressed as mean ± SD of triplicate cultures.

Latrunclin B and forskolin treatment

Latrunclin B disrupts the actin cytoskeleton of cells by inhibiting actin polymerization.[11] Forskolin induces increased intracellular cAMP levels by activation of adenylyl cyclase.[12] HCT-5 cells were treated with 1.25 μmol/L latrunculin B or 15 μmol/L forskolin or DMSO. Treated HCT-5 cells were lysed at intervals up to 6 h for western blotting analysis and an assay of intracellular cAMP or cGMP levels. Concomitantly, after treatment for 90 min, HCT-5 cells were co-cultivated with H9/K30 *luc* cells. Analysis of cell viability using a modified MTT assay, MTS (Promega), showed that treatment with both compounds for up to 6 h was not associated with toxicity in either H9/K30 *luc* or HCT-5 cells.

Statistical analysis

Student's *t*-tests were used for statistical analysis. Differences were considered significant at $P < 0.05$.

Results

Effect of latrunculin B treatment on the intercellular transmission efficiency of HTLV-I

The relationship between the intercellular transmission efficiency of HTLV-I and actin polymerization was first analyzed. As shown in Fig. 1a, treatment of HCT-5 cells with latrunculin B significantly suppressed (approximately 70%) the relative luc activity (DMSO treated: 6.7 ± 0.3 *vs* latrunculin B treated: 2 ± 0.1, $P = 0.0014$) without downregulation of the expression of HTLV-I tax and gp46 (Fig. 1b). These observations suggest that actin reorganization plays

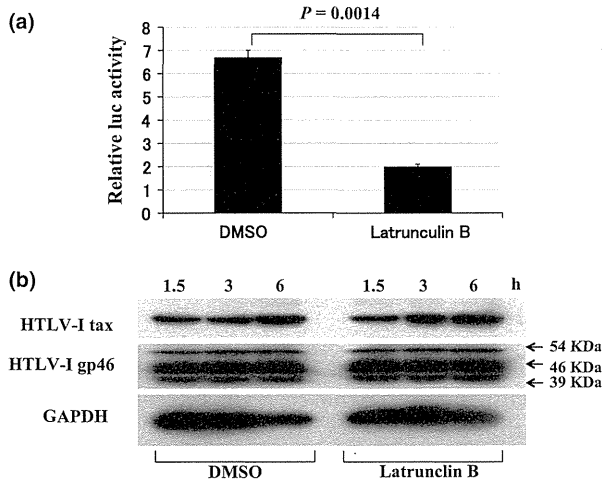

Figure 1 Effect of latrunculin B treatment on HCT-5 cells. HCT-5 cells treated with 1.25 μmol/L latrunculin B for 90 min (5×10^5 cells) were co-cultivated for 6 h with H9/K30 *luc* cells (3.5×10^5 cells) in a 24-well culture plate at 37°C in 5% CO_2. (a) Treatment with latrunculin B significantly suppressed (approximately 70%) the relative luc activity (dimethyl sulfoxide [DMSO] treated: 6.7 ± 0.3 *vs* latrunculin B treated: 2.0 ± 0.1, $P =$ (b) Latrunculin B treatment for up to 6 h did not affect the expression of human T-lymphotropic virus type I (HTLV-I) tax and gp46. Statistical significance was determined by Student's *t*-tests.

an important role in the intercellular transmission of HTLV-I.

Relationship between the intracellular cAMP concentration and the efficiency of the intercellular transmission of HTLV-I

Forskolin treatment of HCT-5 induced a significant increase in the intracellular cAMP concentration (DMSO treated, forskolin treated: 5.37 ± 0.47, 42.90 ± 0.95 pmol/mL, respectively, $P = 0.0047$; Fig. 2a). Concomitantly, upregulation of p-VASP (Ser^{157}) was observed by western blotting analysis (Fig. 2b). However, there were no differences in the expression of HTLV-I tax and gp46 between DMSO and forskolin treatment (Fig. 2b). As shown in Fig. 2c, analysis of the intercellular transmission efficiency of HTLV-I revealed a decrease in relative luc activity to 50% in forskolin-treated HCT-5 (3 ± 0.5), compared with DMSO-treated HCT-5 cells (6 ± 0.9; $P = 0.0299$).

Comparative analysis of intracellular cAMP concentrations, p-VASP expression, and the intercellular transmission efficiency of HTLV-I between HCT-5 and TL-Su

As shown in Fig. 3a, the intracellular cAMP level was significantly lower in HCT-5 (2.67 ± 0.50 pmol/mL) than in TL-Su (11.58 ± 2.47 pmol/mL; $P = 0.0240$), although there was no significant difference in the intracellular cGMP levels between the cell lines (HCT-5, TL-Su; 5.02 ± 1.46, 4.86 ± 0.57 pmol/mL, respectively, $P = 0.9023$). Consistent with this result, VASP appeared to be less phosphorylated in HCT-5 than in TL-Su (Fig. 3b). Comparison of the intercellular transmission efficiency of HTLV-I showed that the relative luc activity was significantly higher in HCT-5 (7.1 ± 1.3) than that in TL-Su (1.1 ± 0.1; $P = 0.0156$). These data show the higher intercellular transmission efficiency of HTLV-1 of HCT-5 compared with that of TL-Su (Fig. 3c).

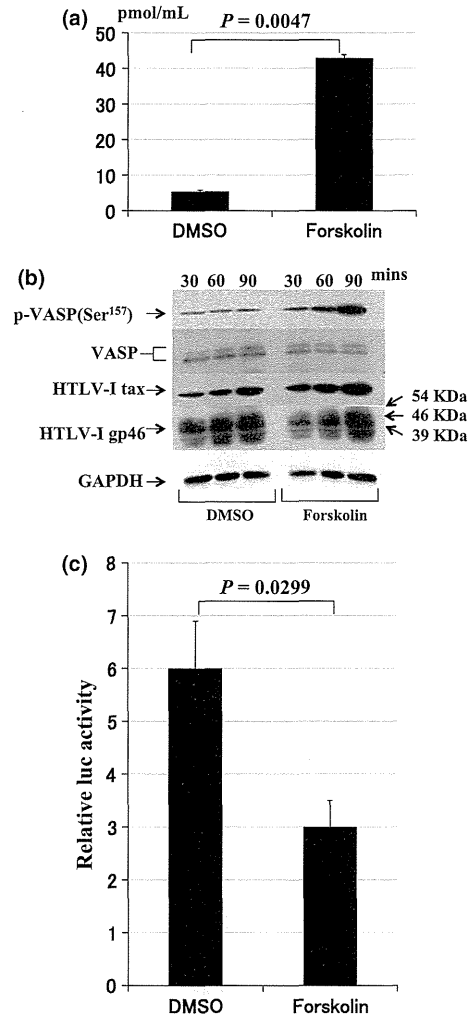

Figure 2 Effect of forskolin treatment on HCT-5 cells. (a) Treatment with 15 μmol/L forskolin for 90 min induced a significant increase in the intracellular cyclic adenosine monophosphate concentration (dimethyl sulfoxide [DMSO] treated, forskolin treated: 5.37 ± 0.47, 42.90 ± 0.95 pmol/mL, respectively). (b) Forskolin treatment gradually induced Ser^{157} phosphorylation of vasodilator-stimulated phosphoprotein (VASP). However, there were no differences in the expression of human T-lymphotropic virus type I (HTLV-I) tax and gp46 between DMSO and forskolin treatment. (c) HCT-5 cells treated with 15 μmol/L forskolin for 90 min (5×10^5 cells) were co-cultivated for 6 h with H9/K30 *luc* cells (3.5×10^5 cells) in a 24-well culture plate at 37°C under 5% CO_2. The relative luc activity decreased to 50% in forskolin-treated HCT-5 cells (3.0 ± 0.5), compared with DMSO-treated HCT-5 cells (6.0 ± 0.9). Statistical significance was determined by Student's *t*-tests.

Discussion

In the present study, we showed that intracellular cAMP regulates the efficiency of intercellular transmission of HTLV-I through control of VASP phosphorylation in HTLV-I-infected cells. The present results suggested that the reorganization of actin, which is a major component of the cytoskeleton, plays an important role in HTLV-I transmission. However, the absence of any significant difference in the intracellular cGMP concentrations between HCT-5 and TL-Su cells indicates that intracellular cGMP, which is also involved in VASP phosphorylation through PKG, does not influence the efficiency of HTLV-I transmission. To our knowledge, this is the first report of signaling molecule involvement in the efficacy of intercellular transmission of HTLV-I.

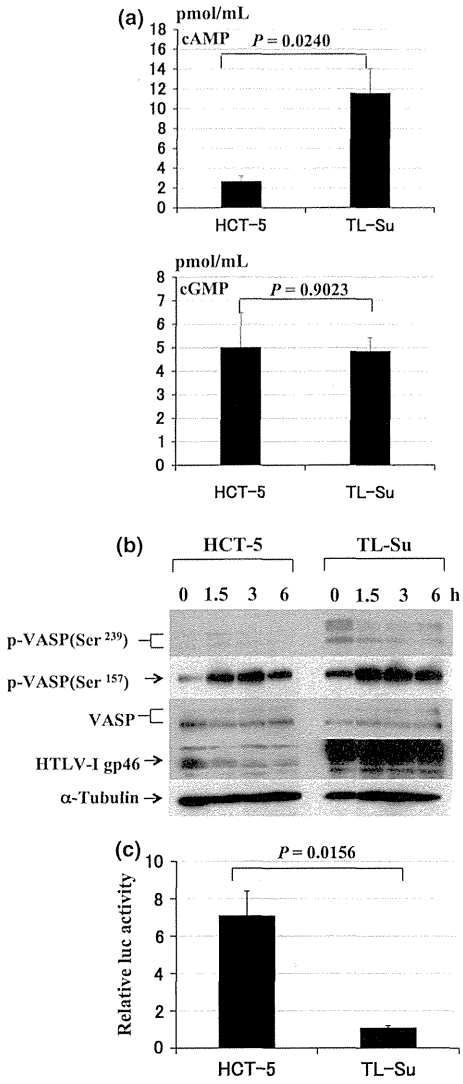

Figure 3 Comparative analysis of HCT-5 and TL-Su. (a) The intracellular cyclic adenosine monophosphate (cAMP) level was significantly lower in HCT-5 (2.67 ± 0.50 pmol/mL) than in TL-Su (11.58 ± 2.47 pmol/mL; $P = 0.0240$), although there was no significant difference in the intracellular cyclic guanosine monophosphate (cGMP) levels between both cell lines (HCT-5, TL-Su: 5.02 ± 1.46, 4.86 ± 0.57 pmol/mL, respectively, $P = 0.9023$). (b) Vasodilator-stimulated phosphoprotein (VASP) appeared to be less phosphorylated in HCT-5 than in TL-Su. (c) Comparison of the relative luc activity of HCT-5 and TL-Su. Either HCT-5 or TL-Su (5×10^5 cells) were co-cultivated for 6 h with H9/K30 *luc* cells (3.5×10^5 cells) in a 24-well culture plate at 37°C under 5% CO_2. The relative luc activity was significantly higher in HCT-5 cells (7.1 ± 1.3) than that in TL-Su cells (1.1 ± 0.1; $P = 0.0156$). Statistical significance was determined by Student's *t*-tests.

It is well known that a high HTLV-I proviral load in the peripheral blood is the most important prerequisite for the development of HAM/TSP.[13,14] Factors such as the relatively lower activity of HTLV-I-specific CD8+ cytotoxic T cells against HTLV-I-infected CD4+ T cells[14] and the active replication of HTLV-I[15,16] have been proposed as reasons for the induction of high HTLV-I proviral load in HAM/TSP patients. However, the increased proliferation of HTLV-I-infected cells[3,17,18] seems to play a highly important role in this effect, and it can be speculated that efficient intercellular transmission of HTLV-I is also partially responsible. Indeed, we previously reported that HTLV-I production by HAM/TSP patient-derived HTLV-I-infected T-cell lines, in which Rac and Cdc42 are activated, is downregulated by blockade of integrin/ligand interactions[7], suggesting that the extracellular release of HTLV-I from these HTLV-I-infected T-cell lines depends on the reorganization status of the cytoskeleton after activation of integrin/ligand signaling. Therefore, HAM/TSP-derived HTLV-I-infected T-cell lines might have the activity of efficient intercellular transmission of HTLV-I. Indeed, in the present study, we showed that HCT-5 cells have the activity of efficient intercellular transmission of HTLV-I with downregulated p-VASP expression following downregulation of intracellular cAMP level compared with TL-Su cells. Although this observation requires confirmation in other HTLV-I-infected T-cell lines, it suggests that HTLV-I-infected cells in HAM/TSP patients have the potential for efficient transmission of HTLV-I to non-infected cells, and its potential is partially responsible for the induction of a high HTLV-I proviral load in the peripheral blood observed in HAM/TSP patients.

Interestingly, the intracellular cAMP concentration was found to be lower in an HTLV-I-infected T-cell line derived from an HAM/TSP patient than that in an HTLV-I carrier derived T-cell line. Recently, Kress et al. reported that cAMP levels are elevated by decreased expression of phosphodiesterase, which hydrolyzes the phosphodiester bond in cAMP, in HTLV-I-infected transformed T-cell lines derived from patients with adult T-cell leukemia.[19] Thus, the regulation of intracellular cAMP level might be different among HTLV-I-infected T-cell lines from HAM/TSP patients, HTLV-I carriers and adult T-cell leukemia patients.

The reorganization of actin is also involved in cell adhesion and migration.[20,21] We previously reported the increased adherent activity of peripheral blood CD4+ T cells of HAM/TSP patients to human endothelial cells.[22] Subsequently, we showed the heightened transmigrating activity of peripheral blood HTLV-I-infected T cells through a reconstituted basement membrane in HAM/TSP patients.[23] The low levels of intracellular cAMP in HTLV-I-infected

T cells in HAM/TSP patients might induce a tendency toward the reorganization of actin, thus accounting for the increased adhesion and transmigrating activities of HTLV-I-infected T cells in HAM/TSP patients.

In conclusion, we showed that the intracellular cAMP concentration regulates the efficiency of intercellular HTLV-I transmission through the regulation of VASP phosphorylation. Further investigations of the signaling molecules and pathways involved in the regulation of the intracellular cAMP concentrations in HTLV-I-infected T cells are required to elucidate the mechanisms underlying this effect. In addition, based on the results of a comparative study between HTLV-I-infected T-cell lines derived from an HAM/TSP patient and an HTLV-I carrier in the present study, comparative studies of peripheral blood HTLV-I-infected T cells in HAM patients and HTLV-I carriers are required to confirm our observations.

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Competing interests

The authors declare that they have no competing interests.

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Pentosan polysulfate treatment ameliorates motor function with increased serum soluble vascular cell adhesion molecule-1 in HTLV-1-associated neurologic disease

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Abstract The main therapeutic strategy against human T lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) characterized by lower extremity motor dysfunction is immunomodulatory treatment, with drugs such as corticosteroid hormone and interferon-α, at present. However, there are many issues in long-term treatment with these drugs, such as insufficient effects and various side effects. We now urgently need to develop other therapeutic strategies. The heparinoid, pentosan polysulfate sodium (PPS), has been safely used in Europe for the past 50 years as a thrombosis prophylaxis and for the treatment of phlebitis. We conducted a clinical trial to test the effect of subcutaneous administration of PPS in 12 patients with HAM/TSP in an open-labeled design. There was a marked improvement in lower extremity motor function, based on reduced spasticity, such as a reduced time required for walking 10 m and descending a flight of stairs. There were no significant changes in HTLV-I proviral copy numbers in peripheral blood contrary to the inhibitory effect of PPS in vitro for intercellular spread of HTLV-I. However, serum soluble vascular cell adhesion molecule (sVCAM)-1 was significantly increased without significant changes of serum level of chemokines (CXCL10 and CCL2). There was a positive correlation between increased sVCAM-1and reduced time required for walking 10 m. PPS might induce neurological improvement by inhibition of chronic inflammation in the spinal cord, through blocking the adhesion cascade by increasing serum sVCAM-1, in addition to rheological improvement of the microcirculation. PPS has the potential to be a new therapeutic tool for HAM/TSP.

Keywords HTLV-I · HAM/TSP · Pentosan polysulfate · Soluble adhesion molecule

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Introduction

Human T lymphotropic virus (HTLV)-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic progressive myelopathy characterized by bilateral pyramidal tract involvement with sphincter disturbances (Osame et al. 1987). HTLV-I infects 10–20 million people worldwide, mainly in large endemic areas such as southern Japan, the Caribbean, Central and South America, Middle East, Melanesia, and equatorial regions of Africa (de-Thé and Bomford 1993; Hollsberg and Hafler 1993). However, only a small proportion of HTLV-I-infected individuals develop HAM/TSP. The primary neuropathological feature of HAM/TSP is chronic inflammation caused by transmigration of HTLV-I-infected cells in the spinal cord (Nakamura et al. 2009). Immunomodulatory drugs, such as prednisolone and interferon (IFN)-α, have been prescribed for HAM/TSP (Nakamura et al. 2009). Unfortunately, such drugs often have insufficient effects and various side effects and are expensive for long-term treatment. Progressive neurological symptoms, such as lower extremity motor dysfunction with urinary disturbances, develop in patients with HAM/TSP and lead to deterioration in quality of life. Therefore, we now urgently need to develop strategies that allow treatment to commence as soon as possible after development of HAM/TSP and that are also tolerable even for long-term or lifelong treatment.

Pentosan polysulfate sodium (PPS) is a semisynthetic drug manufactured from European beech-wood hemicellulose by sulfate esterification (Ghosh 1999). PPS was developed as a heparin-like agent and has been used in Europe for about 50 years for thrombosis prophylaxis and treatment of phlebitis. Therefore, PPS is safe and has also been approved by the US Food and Drug Administration as the active ingredient in ELMIRON®, an oral medication for treating interstitial cystitis. HTLV-I-infected T cells are the first responders in the immunopathogenesis of HAM/TSP (Nakamura 2009). Therefore, therapeutic approaches aimed at targeting HTLV-I-infected cells are reasonable in HAM/TSP. HTLV-I infection is spread via cell-to-cell contact (Igakura et al. 2003). Polysulfate has the potential to inhibit intercellular spread of HTLV-I by blocking binding of the virus to heparan sulfate proteoglycans through its function as a polyanion (Ida et al. 1994; Jones et al. 2005; Araya et al. 2011). Thus, PPS also might have a similar potential and the treatment by PPS might induce the decrease of HTLV-I-infected cells in the peripheral blood of HAM/TSP patients.

The main regions in which pathological changes occur in HAM/TSP are in the lower thoracic spinal cord (Izumo et al. 1989). These regions are anatomical watershed zones, where the stagnant lymphocytes can easily transmigrate to the tissues and evoke immune reactions because of decreased blood flow. Bystander damage of the surrounding spinal cord tissues during the interaction between HTLV-I-specific cytotoxic T cells and HTLV-I-infected T cells might be involved in the spinal cord pathology in HAM/TSP (Ijichi et al. 1993). This pathological event is initiated by adhesion of HTLV-I-infected cells to vascular endothelial cells (ECs) (Nakamura 2009). Therefore, manipulation of the microcirculation and interaction between lymphocyte integrins and their receptors on vascular ECs might be therapeutic mechanisms in HAM/TSP. Indeed, strategies that inhibit lymphocyte trafficking to tissues are effective in treating other inflammatory diseases, such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (Weiner and Hafler 1988; Irony-Tur-Sinai et al. 2003).

We previously reported the efficacy of heparin treatment against HAM/TSP (Nagasato et al. 1993). However, whether heparin is safe for the long-term treatment of HAM/TSP is still unknown. As mentioned above, PPS is a safe drug even for long-term use. We therefore investigated the efficacy of PPS treatment of HAM/TSP.

Patients and methods

Patients

We enrolled 12 HAM/TSP patients (nine women and three men), whose ages ranged from 49 to 77 years and who fulfilled the criteria described previously (Osame 1990), as the outpatients. The duration of illness ranged from 3 to 52 years (mean±SD; 24.2±15.1 years). With respect to the 10 ambulatory patients, the duration of illness ranged from 3 to 51 years (mean±SD; 21.0±13.4 years). Motor function was rated from 0 to 13 according to the motor disability score of Osame et al. (1989). The medical history of the patients is summarized in Table 1. Concomitant immunomodulatory treatment that was received prior to and during this study (on the condition that the dosage was kept constant during the study period) included three doses of three million international unit of IFN-α per week with 7.5 and 5 mg oral prednisolone on alternate days in the case of patient 1 and 5 mg/day oral prednisolone for patients 5 and 10. No medication was changed during the trial. We excluded patients who had experienced prolonged activated partial thromboplastin time (APTT) or prothrombin time (PT), prior hemorrhagic diseases, bleeding tendencies with anticoagulant drugs, or active gastric/duodenal ulcers. Informed written consent was obtained from all patients who participated in the study. This trial was approved by the Institutional Clinical Review Board of Nagasaki University Hospital, Nagasaki, Japan, and was registered with the UMIN Clinical Trials Registry (UMIN-CTR) UMIN000004492.

Table 1 History of HAM/TSP patients and effect of PPS treatment on lower-extremity motor function and spasticity

	1	2	3	4	5	6	7	8	9	10	11	12
Age (years)	57	62	61	49	64	76	63	67	77	62	73	63
Sex	Male	Female	Female	Female	Male	Male	Female	Female	Female	Female	Female	Female
Duration of illness (years)	51	28	30	29	11	23	15	3	17	12	52	19
Concomitant therapy	PSL/IFN-α	No	No	No	PSL	No	No	No	No	PSL	No	No
OMDS[a]												
Before treatment	6	10	5	5	5	6	4	3	5	4	10	4
After treatment[b]	5	10	5	5	5	6	4	3	5	4	10	4
Spasticity of lower extremities												
Before treatment	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes
Improvement[c]												
None		○										
One grade			○	○	○	○	○		○			
Two grades	○											○

PSL prednisolone, *IFN-α* interferon-alpha

○ indicates the status of the improvement of spasticity

[a] OMDS score was 0–13 according to disability grade (Osame et al. 1989)

[b] Evaluated at 1 week after final injection

[c] Improvement in spasticity of more than one grade according to the Modified Ashworth Scale (Bohannon and Smith 1987) was evaluated at 1 week after final injection

PPS treatment

PPS (pentosan polysulfate SP 54; bene-Arzneimittel GmbH, Munich, Germany) was administered subcutaneously once weekly. A dose of 25 mg was administered in treatment week 1 (commencement), 50 mg in week 2, and 100 mg in weeks 3–8. This PPS dosage schedule was the same as that used in a previous clinical trial for patients with knee osteoarthritis (Kumagai et al. 2010). We monitored activated clotting time (ACT), APTT, and PT international normalized ratio (PT-INR) 1 h after the administration of PPS.

Assessment of effects of PPS treatment

Neurological assessment

Each week, we monitored changes in motor function score using Osame's Motor Disability Scale (OMDS) (Osame et al. 1989). Spasticity of the lower extremities was also graded each week with the Modified Ashworth Scale (Bohannon and Smith 1987). We also calculated the percentage reduction in the time required to walk 10 m or down a flight of stairs, as follows: (time required at commencement of PPS treatment−time required/time required at commencement of PPS treatment)×100. The clinical investigators were blinded to the laboratory investigations and assessments such as HTLV-I proviral load, soluble adhesion molecules, and chemokines.

HTLV-I proviral load in peripheral blood mononuclear cells

We monitored the changes in HTLV-I proviral load in peripheral blood mononuclear cells (PBMCs) at the commencement of PPS treatment (treatment week 1), treatment week 3, and at 1 and 5 weeks after the final injection. For quantitative analysis of 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, as described previously (Nishiura et al. 2009). Genomic DNA samples from PBMCs from HAM/TSP patients were prepared using a Genomic DNA Extraction kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). DNA samples were subjected to real-time PCR in a LightCycler PCR system using Tax-specific primers, forward primer (5′-AAACAGCCCTGCAGATACAAAGT-3′), and reverse primer (5′-ACTGTAGAGCTGAGCCGA TAACG-3′), and β-actin-specific primers, forward primer (5′-GCCCTCATTTCCCTCTCA-3′) and reverse primer (5′-GCTCAGGCAGGAAAGACAC-3′). The PCR conditions for the Tax-specific primers were 40 cycles of denaturation (95 °C, 15 s), annealing (55 °C, 5 s), and extension (72 °C, 10 s), and those for the β-actin primers were 32 cycles of denaturation (95 °C, 15 s), annealing (62 °C, 5 s), and extension (72 °C, 15 s). The HTLV-I proviral load per 10,000 cells was calculated according to the following formula: (copy number of Tax/copy number of β-actin/2)×10,000.

Measurement of soluble adhesion molecules and chemokines in serum

The concentration of soluble vascular cell adhesion molecule VCAM (sVCAM)-1, soluble intercellular adhesion molecule (sICAM)-1, chemokine CXC ligand (CXCL)10, and chemokine CC ligand (CCL)2 in serum was measured using an ELISA kit (MILLIPLEX® MAP kit, Millipore Corporation, Billerica, MA, USA) on MAGPIX with xPONENT software (Merck Millipore Co., USA) in accordance with the manufacturer's instructions. All samples were analyzed 20-fold diluted for sVCAM-1 and sICAM-1 and 4-fold diluted for CXCL10 and CCL2 in duplicate and on the same plate. The detection ranges for these assays were 61–250,000 pg/ml for sVCAM-1 and sICAM-1 and 3.2–10,000 pg/ml for CXCL10 and CCL2, respectively. We calculated the percentage increase for serum sVCAM-1 level as follows: (serum level of sVCAM-1at 1 week after final injection−level at commencement of PPS treatment/level at commencement of PPS treatment)×100.

Co-cultivation

We used HCT-5 which is an HTLV-I-infected T cell line derived from the cerebrospinal fluid of an HAM/TSP patient (Fukushima et al. 2008) and H9/K30 *luc* reporter cells which are lymphocytic H9 cells stably transfected with a plasmid containing the gene encoding luciferase under the control of the HTLV-I long terminal repeat (LTR) (Yoshida et al. 2005) (kindly provided by Prof. Akio Adachi, University of Tokushima Graduate School, Japan). HCT-5 (5×10^5 cells) were co-cultivated in the presence of various concentrations of PPS with H9/K30 *luc* reporter cells (3.5×10^5 cells) in 24-well culture plate at 37 °C under 5 % CO_2. After co-cultivation for 24 h, luciferase activity was assessed by using a luciferase assay system (Promega, Madison, USA) and Luminometer TD-20/20 (Turner Designs Instrument, USA). The relative luc activity was calculated according to the following formula: relative luminescent units (RLU) of co-cultivated sample/RLU of the H9-only-cultivated sample. Data were expressed as mean±SD of triplicate cultures.

Statistical analysis

Data were analyzed using Student's *t* tests and the Wilcoxon signed-rank test. Data for the reduction in time required to walk 10 m or down a flight of stairs were analyzed using the Wilcoxon signed-rank test or the Kruskal–Wallis test. If significance was detected, Steel's test for post hoc comparison was performed to compare the effect of treatment with the data at the commencement of treatment. Correlation analysis was performed by use of nonparametric Spearman's rank correlation test. Differences were considered statistically significant for $p<0.05$.

Results

Clinical effects

Improvement of spasticity

Improvement of motor disability score was observed only in patient 1 when evaluated at 1 week after the final injection (Table 1). However, according to evaluation with the Modified Ashworth Scale, among the nine patients in whom lower extremity spasticity was observed before treatment, six showed improvement of one grade following treatment, and two patients demonstrated an improvement of two grades, when evaluated at 1 week after the final injection (Table 1).

Improvement in time required to walk 10 m and down a flight of stairs

In the 10 ambulatory patients, except 2 and 11 who could not perform the test because of high-grade OMDS score, the time required to walk 10 m was significantly reduced from 14.7 (SE 2.6) s at commencement of PPS treatment to 12.7 s (SE 2.6) at 1 week after the final injection and 13.2 s (SE 2.6) at 5 weeks after the final injection ($p=0.017$ and $p=0.011$, respectively). The reduction rate in the time required to walk 10 m increased gradually from the commencement of treatment until 1 week after the final injection and was 14.2 % (SE 3.2) at that point. The reduction rate was 12.0 % (SE 2.3) even at 5 weeks after the final injection. These values were significant compared with the time required to walk 10 m at the commencement of treatment ($p=0.003$ and $p=0.015$, respectively) (Fig. 1a).

In the eight patients, except 2, 4, 6 and 11 who were not able to perform the test because of high-grade OMDS score, the time required to walk down a flight of stairs was also significantly reduced from 9.3 s (SE 2.0) at the commencement of treatment to 7.6 s (SE 1.6) at 1 week after the final injection, and 8.5 s (SE 2.1) at 5 weeks after the final injection ($p=0.018$ and $p=0.035$, respectively). The reduction rate in the time required to walk down a flight of stairs gradually increased from the commencement of treatment until 1 week after the final injection and was 15.4 % (SE 3.6) at that point. Although the reduction rate at 5 weeks after the final injection [10.8 % (SE 2.9)] only showed a tendency to be increased compared with the time required at the commencement of treatment ($p=0.054$), the value at 1 week after the final injection was significant compared with the time required at the commencement of treatment ($p=0.003$) (Fig. 1b).

Change in HTLV-I proviral copy number in PBMCs

PPS might have the potential to inhibit intercellular spread of HTLV-I. Therefore, before clinical trial with PPS, we

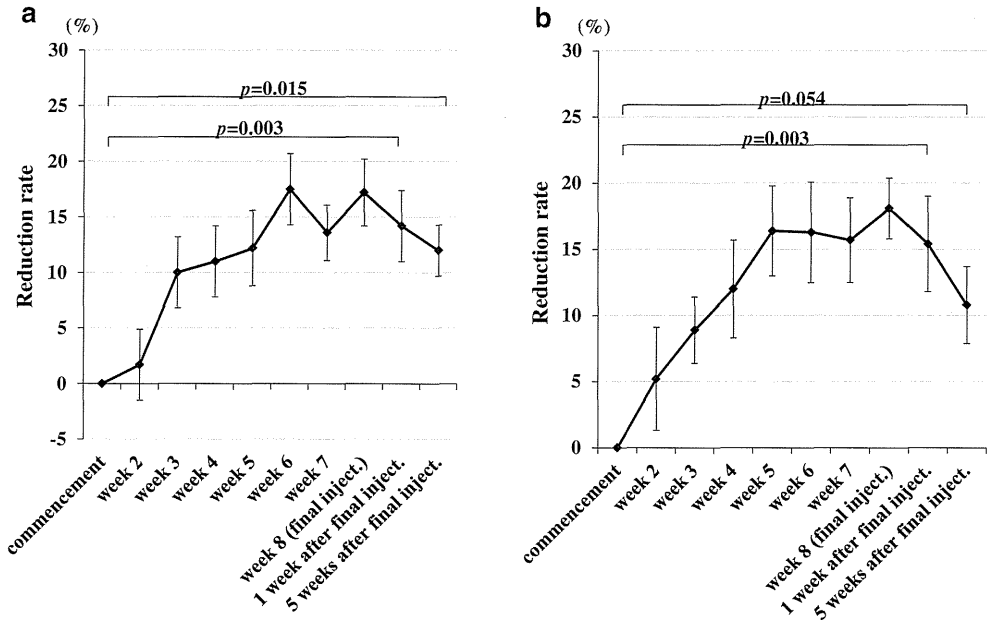

Fig. 1 Reduction rate in time required for HAM/TSP patients to walk 10 m and down a flight of stairs. **a** The reduction rate increased gradually from the commencement of treatment and was 14.2 % (SE 3.2) and 12.0 % (SE 2.3) at 1 and 5 weeks after the final injection, respectively. These values were significant compared with the time required at the commencement of treatment. **b** The reduction rate in time required to walk down a flight of stairs increased gradually from commencement of treatment and was 15.4 % (SE 3.6) and 10.8 % (SE 2.9) at 1 and 5 weeks after the final injection, respectively. Although the value at 5 weeks after the final injection only showed a tendency to increase compared with the commencement of treatment, the value at 1 week after the final injection was significant compared with the commencement of treatment. Data were analyzed using the Wilcoxon signed-rank or Kruskal–Wallis test. If significance was detected, Steel's test for post hoc comparison was performed to compare the effect of treatment with the data at the commencement of treatment. inject.=injection

investigated whether or not PPS has its activity using co-cultivation of HCT-5 with H9/K30 *luc* reporter cells in vitro. The relative luc activity significantly decreased in a dose-dependent manner, suggesting that PPS can inhibit intercellular spread of HTLV-I (Fig. 2). From this data, we expected that PPS treatment would induce the decrease of HTLV-I proviral copy number in PBMCs. Indeed, the decrease of HTLV-I proviral copy number ranged from 0.6 to 44.7 % was observed at 1 week after the final injection in cases 1–9 (Fig. 3a). However, the decrease of it was not observed at 1 week after the final injection in cases 10–12 (Fig. 3a). Overall, HTLV-I proviral copy number in PBMCs was 1,268 (SE 186), 1,156 (SE 148), 1,153 (SE 176), and 1,241 (SE 200) per 10^4 PBMCs at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively (Fig. 3b). Although the HTLV-I proviral copy number at 1 week after the final injection was decreased by about 9 % compared with that at the commencement of treatment, the decrease in HTLV-I proviral copy number at each point compared with at the commencement of treatment did not reach statistical significance.

Change in serum soluble adhesion molecules and chemokines

An increase of serum sVCAM-1 level ranged from 1 to 87.9 % was observed at 1 week after the final injection in all 12 patients. As shown in Fig. 4a, serum level of sVCAM-1 was 587 (SE 81.4), 615.8 (SE 91.5), 762.2 (SE 118.6), and 757 ng/ml (SE 127.3) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. It was significantly increased at 1 week after the final injection and 5 weeks after the

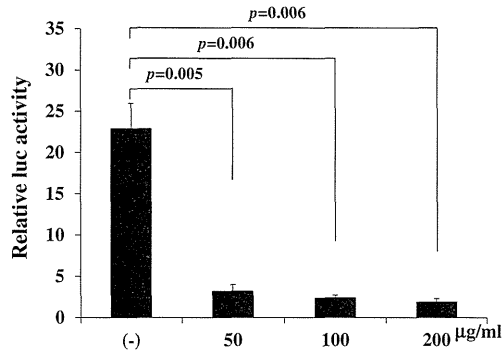

Fig. 2 The inhibitory effect for the intercellular spread of HTLV-I by PPS in vitro treatment. HCT-5 (5×10^5 cells) were co-cultivated in the presence of various concentrations of PPS with H9/K30 *luc* reporter cells (3.5×10^5 cells) in 24-well culture plate at 37 °C under 5 % CO_2. After co-cultivation for 24 h, luciferase activity was assessed by using a luciferase assay. The relative luc activity significantly decreased in a dose-dependent manner. The relative luc activity was calculated according to the following formula: relative luminescent units (RLU) of co-cultivated sample/RLU of the H9-only-cultivated sample. Data were expressed as mean±SD of triplicate cultures. Data were analyzed using Student's *t* tests

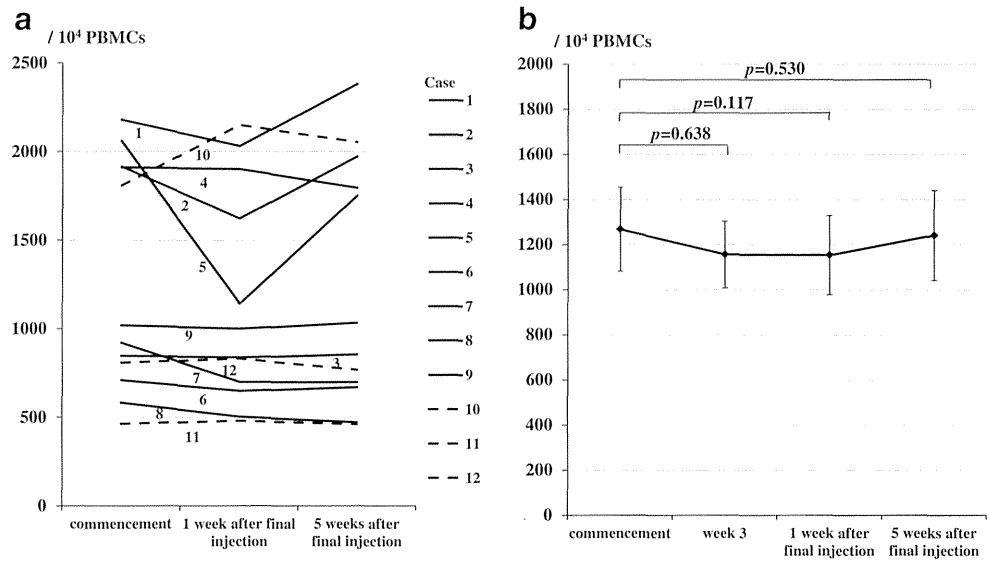

Fig. 3 Change in HTLV-I proviral copy numbers in PBMCs. **a** Changes in HTLV-I proviral copy numbers per 10^4 PBMCs at 1 week after the final injection in each case. The decrease of HTLV-I proviral copy number ranged from 0.6 to 44.7 % was observed at in cases 1–9 (indicated as *line*). However, the decrease of it was not observed in cases 10–12 (indicated as *dotted line*). **b** HTLV-I proviral copy numbers per 10^4 PBMCs were 1,268 (SE 186), 1,156 (SE 148), 1,153 (SE 176), and 1,241 (SE 200) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. The decrease in HTLV-I proviral copy number at each point was not significant compared with that at commencement of treatment. Data were analyzed using the Wilcoxon signed-rank test

final injection (p=0.002 and 0.015 vs the commencement of treatment, respectively). Serum level of sICAM-1 was 185.3 (SE 23.3), 184.4 (SE 23.8), 207.1 (SE 28), and 204.6 ng/ml (SE 24.9) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. Although the increase did not reach statistical significance, a slight trend towards an increase was observed at 1 and 5 weeks after the final injection (p=0.060 and 0.050 vs the commencement of treatment, respectively) (Fig. 4b). Serum level of CXCL10 was 209.8 (SE 56.9), 247.6 (SE 67.7), 186.2 (SE 47.3), and 242.7 pg/ml (SE 76.7) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. Serum level of CCL2 was 736.5 (SE 50.5), 751.1 (SE 56.0), 738.3 (SE 51.5), and 762.8 pg/ml (SE 55.4) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. Thus, there were no significant changes in serum levels of these two chemokines during PPS treatment (Fig. 4c, d).

Correlation between increase in sVCAM-1 and reduction in time required to walk 10 m

We evaluated the relationship between percentage increase in serum sVCAM-1 level and percentage reduction in time required to walk 10 m at 1 week after the final injection, compared with at commencement of treatment. As shown in Fig. 5, there was a moderately positive correlation between them (rs=0.648, p=0.043).

Adverse effects

All patients had a small amount of subcutaneous bleeding at the injection site. In coagulation studies 1 h after administration, the highest values were 177 s and 1.22 in ACT and PT-INR, respectively. For APTT, the highest value was 63.3 s. In blood and biochemical analyses, abnormal findings were not observed. Overall, no serious adverse effects were experienced by the HAM/TSP patients upon treatment with PPS.

Discussion

We demonstrated that PPS treatment safely improves motor disability by decreasing spasticity in the lower extremities of patients with HAM/TSP. Considering the relatively long (~21 years) mean duration of illness of the 10 ambulatory patients in our study, the efficacy of PPS treatment is a particularly interesting outcome. These results suggest that the pathological processes in the spinal cord of HAM/TSP patients is partially reversed and is treatable even if the tissues are damaged over a long period of time. We previously reported the therapeutic efficacy of heparin in HAM/TSP patients (Nagasato et al. 1993). However, we did not test whether heparin could provide safe and effective long-term treatment of HAM/TSP. Therefore, we conducted the present clinical trial with the heparinoid, PPS, the safety of which is established in Europe and the USA, even for long-term administration. The present study confirmed that PPS treatment induces effects similar to those of heparin.

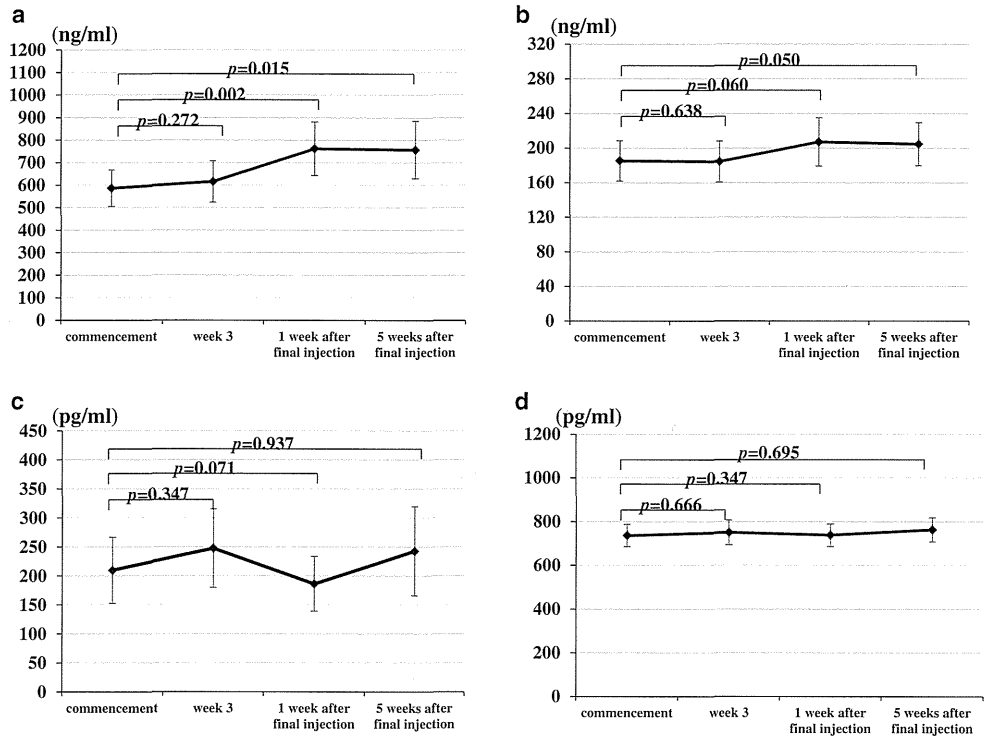

Fig. 4 Change in serum levels of sVCAM-1 and sICAM-1 and CXCL10 and CCL2. **a** Serum level of sVCAM-1 was 587 (SE 81.4), 615.8 (SE 91.5), 762.2 (SE 118.6), and 757 ng/ml (SE 127.3) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. It was significantly increased at 1 and 5 weeks after the final injection (p=0.002 and 0.015, vs the commencement of treatment, respectively). **b** Serum level of sICAM-1 was 185.3 (SE 23.3), 184.4 (SE 23.8), 207.1 (SE 28), and 204.6 ng/ml (SE 24.9) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, respectively. Although the increase did not reach significance, a slight trend towards an increase was observed at 1 and 5 weeks after the final injection (p=0.060 and 0.050, vs the commencement of treatment, respectively) (Fig. 3b). **c**, **d** There were no significant changes in serum levels of CXCL10 and CCL2 during PPS treatment. Statistical significance was determined by the Wilcoxon signed-rank test

We expected that PPS treatment would decrease the number of HTLV-I-infected cells in PBMCs through its function as a polyanion (Ida et al. 1994; Jones et al. 2005; Araya et al. 2011). Indeed, the decrease of HTLV-I proviral copy number was observed at 1 week after the final injection in 9 cases, suggesting that the intercellular spread of HTLV-I was partially blocked by PPS treatment even in vivo in these cases. However, the decrease of it was not observed at 1 week after the final injection in another 3 cases. Totally, the decrease in HTLV-I proviral copy number did not reach statistical significance in this study. This finding suggests a limitation of the protocol used in the present study. Alternatively, HTLV-I proviral load in PBMCs in HTLV-I-infected individuals including HAM/TSP patients might be mainly maintained by the proliferation of HTLV-I-infected cells (Wattel et al. 1995).

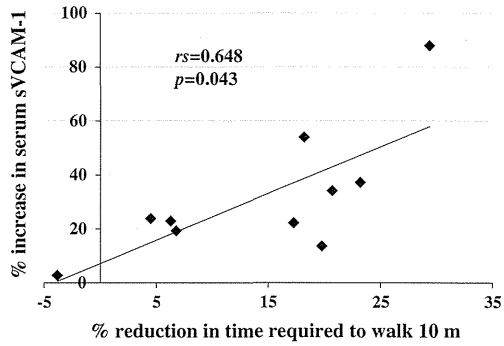

Fig. 5 Positive correlation between percentage increase in sVCAM-1 and percentage reduction in time required to walk 10 m at 1 week after the final injection compared with at commencement of treatment. There was a moderately positive correlation between them (rs=0.648, p=0.043). Correlation analysis was performed by use of nonparametric Spearman's rank correlation test

However, we demonstrated that serum levels of sVCAM-1 during treatment were significantly increased compared with pretreatment values, with a slight trend for sICAM-1 to increase. Calabresi et al. (1997) previously reported an increase in serum sVCAM-1 in MS patients treated with IFNβ-1b, which was correlated with a decrease in the number of contrast-enhancing lesions on magnetic resonance imaging. Moreover, Kallmann et al. (2000) also showed that preincubation of PBMCs with sVCAM-1 blocked their adhesion to human cerebral ECs in vitro. These results strongly suggest that sVCAM-1 can operate on very late antigen-4

(VLA-4), which is the ligand for VCAM-1, on the surface of lymphocytes as a bioactive antagonist. However, the biological functions of soluble adhesion molecules have not been entirely elucidated. It was previously reported that serum level of sVCAM-1, sICAM-1, and CXCL10 was increased, but CCL2 level was decreased, based on the immune-activated status of HAM/TSP patients (Matsuda et al. 1995; Tsukada et al. 1993; Best et al. 2006; Guerreiro et al. 2006). The precise mechanism of how serum sVCAM-1 level is increased by PPS treatment was not clear in the present study. However, there were no significant changes in serum levels of chemokines such as CXCL10 and CCL2, which is Th1-associated and Th2-associated chemokine, respectively (Best et al. 2006; Guerreiro et al. 2006), during PPS treatment. Therefore, PPS treatment does not appear to exacerbate the immune-activated status in HAM/TSP patients. We showed a correlation between the increase in sVCAM-1 and the reduction in the time required to walk 10 m at 1 week after the final injection compared with at commencement of treatment. Thus, the improvement in lower extremity motor function in HAM/TSP patients by PPS might depend on inhibition of transmigration of HTLV-I-infected cells or activated T cells inducing an inflammatory status in the lower thoracic spinal cord. Previously, an immuno-histopathological analysis in the spinal cord of HAM/TSP patients revealed that VCAM-1/VLA-4 interaction may play an important role for lymphocyte migration into the tissues (Umehara et al. 1996). In addition, as mentioned above, the main pathological regions in the central nervous tissues of HAM/TSP patients are anatomical watershed zones which lead to a slow blood flow (Izumo et al. 1989; Aye et al. 2000). Therefore, it is strongly suggested that the improvement in lower extremity motor function in HAM/TSP patients by PPS is mediated through blocking the adhesion cascade, which leads to trafficking into the tissues, by increased serum sVCAM-1 and rheological improvement of the microcirculation in the spinal cord. However, in order to confirm it, we need further analyses in the cerebrospinal fluid samples such as the changes of HTLV-I proviral load, inflammatory cytokines/chemokines, neopterin, etc. Very recently, Ando et al. (2013) clearly demonstrated that chronic inflammation in the spinal cord of HAM/TSP patients is induced by transmigration of HTLV-I-infected cells or activated T cells to the nervous tissues through CXCL10–CXCR3 inflammatory positive feedback loop. In the case of PPS treatment, this positive feedback loop might be attenuated by the inhibition of transmigration process of these cells by an increase of sVCAM-1 in the sera.

In conclusion, our results suggest that PPS, which is well tolerated in long-term administration, has the potential to be a new therapeutic tool for the treatment of HAM/TSP. Therefore, further studies are warranted to evaluate the efficacy of PPS treatment of HAM/TSP in a large randomized controlled study.

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Conflict of interest The authors declare that they have no conflict of interest.

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Familial Clusters of HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

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Abstract

Objective: HTLV-1 proviral loads (PVLs) and some genetic factors are reported to be associated with the development of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). However, there are very few reports on HAM/TSP having family history. We aimed to define the clinical features and laboratory indications associated with HAM/TSP having family history.

Methods: Records of 784 HAM/TSP patients who were hospitalized in Kagoshima University Hospital and related hospitals from 1987 to 2012 were reviewed. Using an unmatched case-control design, 40 patients of HAM/TSP having family history (f-HAM/TSP) were compared with 124 patients suffering from sporadic HAM/TSP, who were admitted in series over the last 10 years for associated clinical features.

Results: Of the 784 patients, 40 (5.1%) were f-HAM/TSP cases. Compared with sporadic cases, the age of onset was earlier (41.3 vs. 51.6 years, $p<0.001$), motor disability grades were lower (4.0 vs. 4.9, $p=0.043$) despite longer duration of illness (14.3 vs. 10.2 years, $p=0.026$), time elapsed between onset and wheelchair use in daily life was longer (18.3 vs. 10.0 years, $p=0.025$), cases with rapid disease progression were fewer (10.0% vs. 28.2%, $p=0.019$), and protein levels in cerebrospinal fluid (CSF) were significantly lower in f-HAM/TSP cases (29.9 vs. 42.5 mg, $p<0.001$). There was no difference in HTLV-1 PVLs, anti-HTLV-1 antibody titers in serum and CSF, or cell number and neopterin levels in CSF. Furthermore, HTLV-1 PVLs were lower in cases with rapid disease progression than in those with slow progression in both f-HAM/TSP and sporadic cases.

Conclusions: We demonstrated that HAM/TSP aggregates in the family, with a younger age of onset and a slow rate of progression in f-HAM/TSP cases compared with sporadic cases. These data also suggested that factors other than HTLV-1 PVLs contribute to the disease course of HAM/TSP.

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Introduction

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is characterized by slow progressive spastic paraparesis and positivity for anti-HTLV-1 antibodies in both serum and cerebrospinal fluid (CSF) [1,2]. Worldwide, at least 10–20 million people are infected with HTLV-1 [3]. However, although the majority of infected individuals remain lifelong asymptomatic carriers, approximately 2%–5% develop adult T-cell lymphomas [4,5] and another 0.25%–3.8% develop HAM/TSP [1,2]. Although the mechanisms underlying the development of HAM/TSP are not fully understood, several risk factors are closely associated with HAM/TSP. In particular, HTLV-1 proviral loads (PVLs) are significantly higher in HAM/TSP patients than in asymptomatic carriers and are also higher in genetic relatives of HAM/TSP patients than in non-HAM-related asymptomatic carriers [6]. Host genetic factors, including human leukocyte antigen (HLA) and non-HLA gene polymorphisms affect the occurrence of HAM/TSP [7], indicating that HTLV-1 PVLs and genetic backgrounds may influence individual susceptibility to HAM/TSP. Although several reports of familial adult T-cell lymphoma have been published [8,9], to our knowledge, there is only one case report of patient with HAM/TSP having family history (f-HAM/TSP) [10]. Hence, little is known about the prevalence and character of f-HAM/TSP cases. In this study, the characteristic clinical and laboratory features of f-HAM/TSP cases are defined and compared with those of sporadic cases.

Methods

Ethics Statement

This study was approved by the Institutional Review Boards of Kagoshima University. All participants provided written informed consent.

Design

We used an unmatched case-control design to identify the phenotypic features of f-HAM/TSP. f-HAM/TSP cases were identified as patients with multiple family members suffering from HAM/TSP. Controls were defined as HAM/TSP patients who were not genetically related to other HAM/TSP patients.

Subjects

f-HAM/TSP cases were extracted from our database of individuals diagnosed with HAM/TSP in Kagoshima University Hospital and related hospitals from 1987 to 2012. Controls included consecutive patients with sporadic HAM/TSP who were evaluated in our department between January 2002 and June 2012. HAM/TSP was diagnosed according to the World Health Organization diagnostic criteria, and the updated criteria of Castro-costa Belem [11]. Clinical information was obtained from the medical records of patient attendance at our hospital. In other cases, clinical data were obtained from the clinical records of patients or directly from the referring clinicians. Clinical variables included sex, age, age of onset, and initial symptoms. Neurological disabilities were assessed using Motor Disability Grading (MDG), modified from the Osame Motor Disability Scale of 0 to 10, as reported previously [12]. Motor disability grades were defined as follows: 5, needs one-hand support while walking; 6, needs two-hand support while walking; and 7, unable to walk but can crawl. We used a different assessment for the subgroup of more than grade 6 because their disease state significantly interfered with their lifestyle and necessitated the use of wheelchairs in daily life. The subgroup of patients with rapid progression was defined by deterioration of motor disability by more than three grades within two years. Anti-HTLV-1 antibody titers in serum and CSF were detected using enzyme-linked immunosorbent assays and particle agglutination methods (Fijirebio Inc, Tokyo, Japan). HTLV-1 PVLs in peripheral blood mononuclear cells (PBMCs) were assayed using quantitative PCR with the ABI PRISM 7700TM sequence detection system as reported previously [6].

Statistical Analysis

Data were analyzed using SPSS-20 (SPSS, Chicago, Illinois). Statistical analyses were performed using parametric (t-test) and non-parametric tests (Mann–Whitney test) for continuous variables and χ^2 (Pearsonχ^2 test/Fisher exact test) for categorical variables. Significant differences were then adjusted for potential confounders (age and sex) using multiple linear regression analysis. Survival was estimated according to the Kaplan–Meier method. The final endpoint was defined by a MDG score of 6. Patients with MDG scores of 6 almost wheelchair bound in daily life. The log rank test was used in Kaplan–Meier analyses. Differences were considered significant when $p<0.05$.

Results

Clinical characteristics of f-HAM/TSP

Of the 784 patients diagnosed with HAM/TSP between January 1987 and June 2012, 40 (5.1%) were f-HAM/TSP. The sex ratio was 33 males : 7 females. Of these 40 cases, 10 had parents or children (25.0%), 27 had siblings (67.5%), and three had other relatives (7.5%) diagnosed with HAM/TSP. Three individuals from one family were diagnosed with HAM/TSP, whereas only two individuals were diagnosed with HAM/TSP in all other families. In f-HAM/TSP cases, the age of onset was earlier (41.3 vs. 51.6 years, $p<0.001$), cases with rapid progression were fewer (10.0% vs. 28.2%, $p = 0.019$), motor disability grades were lower (4.0 vs. 4.9, $p = 0.043$) despite longer duration of illness (14.3 vs. 10.2 years, $p = 0.026$), and time elapsed between onset and wheelchair use in daily life was longer (18.3 vs. 10.0 years, $p = 0.025$) compared with sporadic cases. Sex and initial symptoms did not differ significantly between f-HAM/TSP and sporadic cases (Table 1). Twelve patients of f-HAM/TSP, and 38 of the 128 sporadic cases reached endpoint MDG scores of 6. Significant differences were then adjusted for potential confounders (age and sex) using multivariate analysis. Age of onset, duration of illness, MDG scores, and time elapsed between onset and wheelchair use in daily life remained significantly different after multivariate analysis (Table 1). The proportion of patients with rapid progression did not differ significantly between the groups, although there was a trend toward a higher proportion in sporadic cases. Kaplan–Meier analyses revealed that approximately 30% of both f-HAM/TSP and sporadic cases needed a wheelchair in daily life in 15 years after onset, and approximately 50% of patients from both groups needed it in 20 years after onset (Figure 1). Although sporadic patients needed wheelchairs earlier in most cases, the difference in the ratio of the patients with MDG score above six was not statistically significant between the groups. Finally, we compared differences in the age of onset between parent–child and sibling cases in f-HAM/TSP cases. Age of onset in parent–child f-HAM/TSP cases was significantly younger than that in sibling f-HAM/TSP cases (29.9 ± 10.0 vs. 45.1 ± 13.0 years, $p = 0.002$).

Laboratory parameters and PVLs in f-HAM/TSP cases

Protein levels in CSF were significantly lower in f-HAM/TSP cases than in sporadic cases (29.9 vs. 42.5 mg/dl, $p<0.001$). This difference in CSF protein level remained significant after multivariate analysis. Anti-HTLV-1 antibody titers in serum and CSF, and cell numbers and neopterin levels in CSF were not significantly different between two groups. Moreover, HTLV-1 PVLs did not differ significantly. (Table 2).

Clinical and laboratory findings in patients with rapid disease progression

Previous studies suggest that an older age of onset is associated with rapid disease progression. Similar findings are found in the present study. The percentage of rapid progression tended to increase with older age of onset in both f-HAM/TSP and sporadic groups (Figure 2). We compared the characteristics of 124 sporadic HAM/TSP patients with rapid and slow progression who were admitted to Kagoshima University Hospital in series during the last 10 years (Table 3). Patients with rapid progression were significantly older at onset than those with slow progression (62.3 vs. 47.4 years, $p<0.001$), although sex and initial symptoms did not differ significantly between rapid and slow progression groups. However, the time elapsed between onset and wheelchair use in daily life was markedly shorter among patients with rapid progression (1.5 vs. 14.4 years, $p<0.001$). Cell numbers, protein levels, and anti-HTLV-1 antibody titers in CSF were significantly higher in patients with rapid progression than in those with slow progression (11.6 vs. 3.2, $p<0.001$; 55.3 vs. 36.7 mg/dl, $p<0.001$; 1,251 vs. 416, $p<0.014$, respectively). Interestingly, HTLV-1 PVLs were significantly lower in patients with rapid progression than in those with slow progression (370 vs. 1,245 copies, $p<0.001$). Furthermore, we compared the differences between women and men in patients with rapid progression because the reason remains unknown why HAM/TSP is common in female

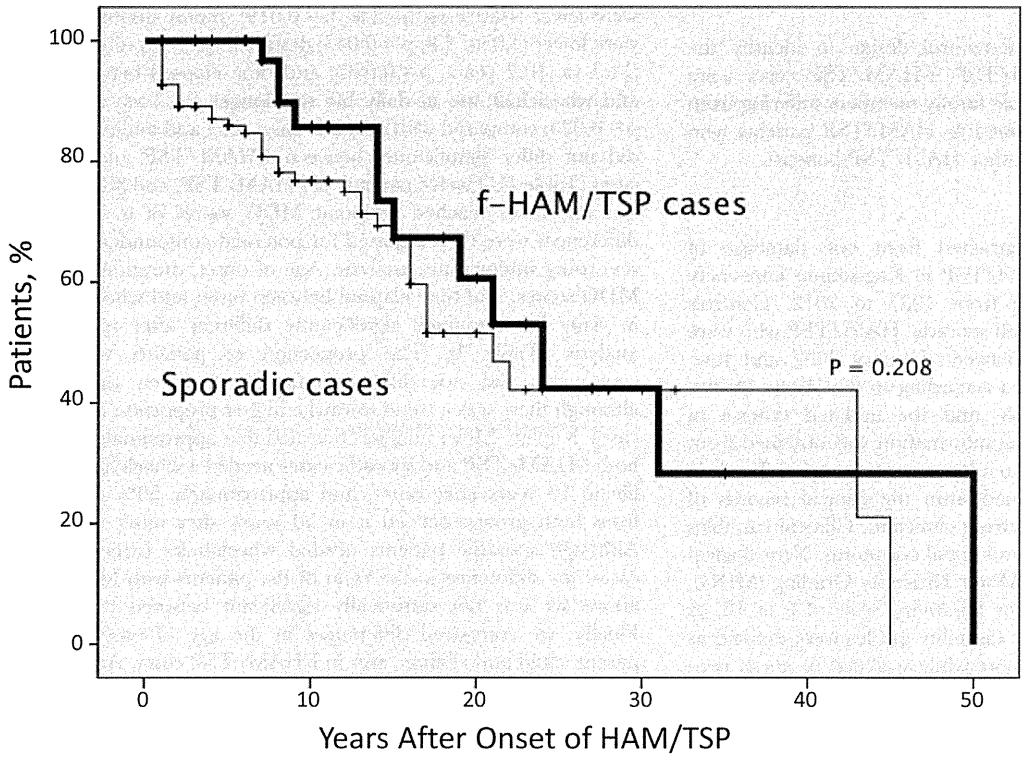

Figure 1. Kaplan–Meier estimates of the time from disease onset to assignment of motor disability scores of 6. In sporadic cases, more patients reached the score of six at an early stage; however, the difference was not significant. Approximately 30% of both f-HAM/TSP cases and sporadic cases needed a wheelchair in daily life in 15 years after onset and approximately 50% of patients from both groups needed a wheelchair in 20 years after onset.
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than in male. There was no significant difference between women and men in the age of onset (61.5 y.o.±12.6 vs. 62.7 y.o.±12.5), in the incidence of rapid progression (26.3% vs. 32.3%) and in MDG score (5.4 vs. 5.0; mean).

Discussion

We demonstrated that among 784 HAM/TSP patients, 40 (5.1%) had family members with the disease. The lifetime risk of developing HAM/TSP is 0.25% of HTLV-1 carriers in Japan

Table 1. Clinical features of f-HAM/TSP cases or sporadic cases of HAM/TSP.

	f-HAM/TSP cases (40 cases)	Sporadic cases (124 cases)	p value	p value†
Female ratio (%)	78.8% (7 males : 33 females)	66.4% (31 males : 93 females)	NS	
Age	55.6±13.0 (23–79)	61.8±12.5 (15–83)	**0.008**	
Age of onset	41.3±13.9 (14–65)	51.6±15.9 (13–78)	**<0.001**	**0.017**
Duration of illness (years)	14.3±11.4 (1–49)	10.2±9.6 (0–45)	**0.026**	**0.017**
Initial symptoms				
Gait disturbance	50.0%	52.4%	NS	
Urinary disturbance	32.5%	26.6%	NS	
Sensory disturbance	12.5%	14.5%	NS	
Others	5%	6.5%	NS	
Rapid disease progression	4 cases (10.0%)	35 cases (28.2%)	**0.019**	0.069
Motor disability score	4.0±2.0 (0–7)	4.9±1.5 (0–8)	**0.043**	**0.036**
Score more than 6	12 cases (30.0%)	38 cases (30.7%)	NS	
Time elapsed between onset and wheelchair use in daily life (years)	18.3±12.4 (7–50)	10.0±10.4 (1–45)	**0.025**	**0.020**

Data are presented as mean values ± s.d., (range),
†Adjusted for age and sex.
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Table 2. Laboratory findings of familial clusters or sporadic cases of HAM/TSP.

	f-HAM/TSP cases (40cases)	Sporadic cases (124 cases)	p value	p value†
Anti-HTLV-1 antibodies*				
Titer in Serum	20,787±31,004, N = 37	31,009±36,075, N = 109	NS	
Titer in CSF	2,310±11,741, N = 31	672±1,274, N = 111	NS	
Cerebrospinal fluid				
Cell number (/mm^3)	3.0±2.5, N = 25	5.7±10.0, N = 109	NS	
Protein (mg/dl)	29.9±9.4, N = 22	42.5±19.3, N = 109	**<0.001**	**0.007**
Neopterin (pmol/ml)	83.2±118.1, N = 18	38.3±56.8, N = 35	NS	
HTLV-1 proviral loads (Copies/10^4 PBMCs)	930±781, N = 32	968±1,746, N = 101	NS	

* Particle Aggregation Method.
Data are presented as mean values ± s.d., N = sample number,
†Adjusted for age and sex.
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[13]. Although clustering of familial adult T-cell lymphomas has been reported [8,9], to our knowledge the prevalence of familial clusters of HAM/TSP has not been described. A study in Peru showed that 30% of HAM/TSP patients have family members with paralytic neurological disorders, but the cause of paralysis was not evaluated [14]. In the present study, we included f-HAM/TSP diagnosed in medical institutions and excluded cases with a family history of neurological disorders. Thus, the actual incidence rates of f-HAM/TSP may be higher than those reported here. Interestingly, although HTLV-1 PVL has been associated with the development and clinical progression of HAM/TSP [15–17], there was no significant difference between f-HAM/TSP and sporadic cases in the present study. Because previous studies reported that HTLV-1 PVLs of asymptomatic carriers in relatives of HAM/TSP patients were higher than those in non-HAM-related asymptomatic carriers [6], relatives of HAM/TSP are believed to be at a higher risk of developing HAM/TSP. Interestingly, our data suggest that HAM/TSP patients aggregate in families and factors other than HTLV-1 PVLs may contribute to HAM/TSP.

Compared with sporadic HAM/TSP, the clinical characteristics of f-HAM/TSP have a younger age of onset and longer time elapsed between onset and wheelchair use in daily life. Although we were unable to identify the reason for earlier onset among f-HAM/TSP cases, one can speculate that mild symptoms, such as urinary and sensory disturbances, may be identified earlier by family members who are familiar with HAM/TSP symptoms. However, the present data show no difference in initial symptoms

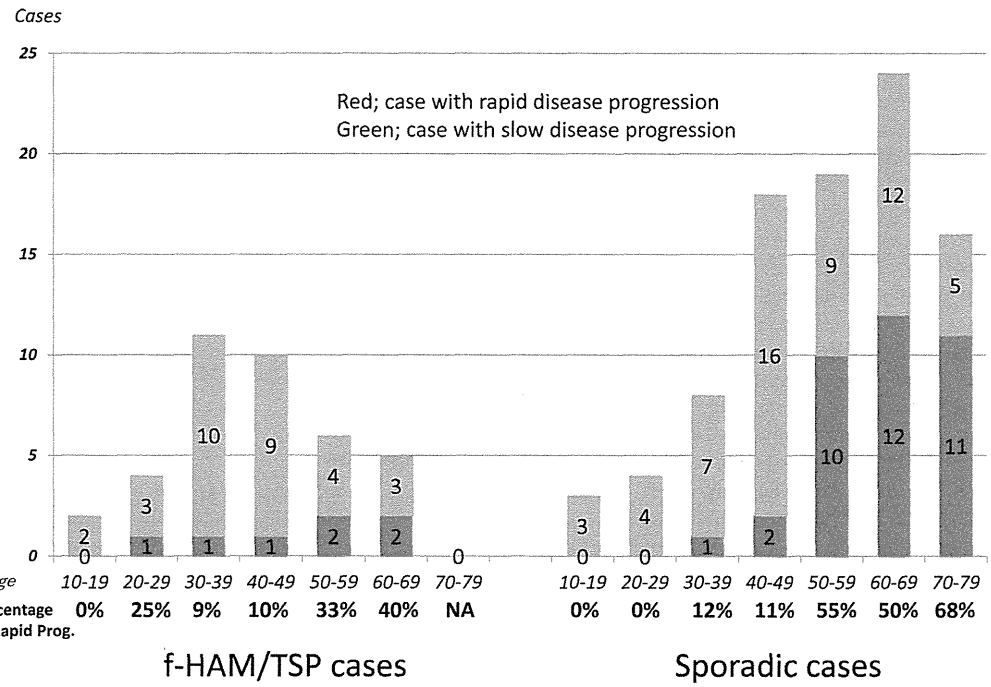

Figure 2. Age-specific proportions of rapid disease progression. The proportion of cases with rapid disease progression tended to increase with the older age of onset.
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Table 3. Clinical and laboratory findings of sporadic HAM/TSP with rapid/slow disease progression.

Type of disease progression	Rapid progression	Slow progression	p value
Female ratio (%)	71.4% (10 males : 25 females)	76.4% (21 males : 68 females)	NS
Age of onset	62.3±9.6, N = 35	47.4±15.9, N = 89	**<0.001**
Age of onset of f-HAM/TSP cases	60.5±3.7, N = 4	39.2±12.9, N = 36	**0.002**
Duration between onset and inability to walk alone (years)	1.5±0.9, N = 13	14.4±10.4, N = 25	**<0.001**
Anti-HTLV-1 antibodies*			
Titer in Serum	31,894±36,845, N = 34	30,608±35,965, N = 75	NS
Titer in CSF	1,251±1,800, N = 34	416±852, N = 77	**0.014**
Cerebrospinal fluid			
Cell number (/mm^3)	11.6±16.6, N = 34	3.2±3.5, N = 75	**<0.001**
Protein (mg/dl)	55.3±24.3, N = 34	36.7±13.0, N = 75	**<0.001**
Neopterin (pmol/ml)	74.9±107.9, N = 8	27.4±23.4, N = 27	0.255
HTLV-1 proviral loads (Copies/10^4 PBMCs)	370±327, N = 32	1,245±2,046, N = 69	**<0.001**

* Particle Aggregation Method.
Data are presented as mean values ± s.d., N = sample number.
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between f-HAM/TSP and sporadic cases. In all cases, the age of onset and initial symptoms of HAM/TSP were evaluated by the neurologists during hospitalization. Because inflammatory processes are less marked in f-HAM/TSP cases, as indicated by significantly lower protein levels in CSF, f-HAM/TSP cases may show slow progression of disease.

We need to discuss the possibility that the two groups compared represent different mode of HTLV transmission, i.e. vertical vs. sexual transmission. To clarify genetic backgrounds, sporadic HAM/TSP with seropositive carrier family members may be a more appropriate control, but are not available at present. The incidence of female cases showing no significant differences between f-HAM/TSP and sporadic cases, and between rapid and slow disease progression, might suggest less possibility of sporadic cases due to sexual transmission.

Although the subgroup of patients with rapid progression has not been clearly defined, previous studies suggest that rapid progression occurs in 10%–30% of all patients with HAM/TSP [12,14,16], and is associated with an older age of onset [14–16]. In the present study, the age of onset in patients with rapid progression was significantly older than that in patients with slow progression between f-HAM/TSP and sporadic cases, and the proportion of patients with rapid progression increased with the older age of onset (Figure 2). Among sporadic cases, cell numbers and protein levels in CSF were significantly higher in patients with rapid progression, suggesting that inflammation is more active in the spinal cords of patients with rapid progression and that cytotoxic T-lymphocyte (CTL) immune responses may be more intensive. Therefore, lower PVLs in PBMCs of patients with rapid disease progression may be attributed to the strong killing ability of the CTL. However, PVLs were higher in PBMCs of patients with HAM/TSP than in asymptomatic carriers [6]. In addition, the killing ability of CTLs in patients with HAM/TSP does not differ from that in asymptomatic carriers [18]. Hence, strong immune responses may be associated with the disease course. The onset of disease may require other factors that lead to strong immune responses. A late onset may also be associated with alterations of the immune function in HTLV-1-infected patients. Indeed, an increased age has been associated with autoimmune disorders, such as myasthenia gravis and rheumatoid arthritis, and may be partly explained by immune intolerance and accumulation of autoantibodies in older individuals [19,20].

In conclusion, we demonstrated that patients with HAM/TSP aggregate in some families. Compared with sporadic cases, the age of onset was younger and rates of disease progression were slower among familial cases, whereas HTLV-1 PVLs did not differ between f-HAM/TSP and sporadic groups. The present data suggest that factors other than HTLV-1 PVLs contribute to the disease course of HAM/TSP. Our data also suggested strong immune responses in the spinal cord of HAM/TSP patients with rapid progression. Further studies on HTLV-1, immune response to HTLV-1 and genetic factor in patients with rapid progression might provide new insights into HAM/TSP pathogenesis.

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Author Contributions

Conceived and designed the experiments: HT SI OW. Performed the experiments: SN EM. Analyzed the data: SN EM. Contributed reagents/materials/analysis tools: SN EM TM RK. Wrote the paper: SN EM.

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