

Figure 5. Increased expression of inflammation-related molecules in coculture supernatant, as confirmed by immunofluorescence analysis and enzyme-linked immunosorbent assay (ELISA). **A** and **B**, Expression of intercellular adhesion molecule 1 (ICAM-1), CXCL-1, RANTES, interleukin-8 (IL-8), and interferon- γ -inducible 10-kd protein (IP-10) in salivary gland epithelial cells (SGECs) cocultured with HCT-5 cells (**A**) or Jurkat cells (**B**), as determined by immunofluorescence analysis. The third rows in each panel show merged views. Yellow signal indicates coexpression of signal from top and middle rows. Results are representative of 2 independent experiments with similar findings. GAG = human T lymphotropic virus type 1-related proteins p19, p28, and Gag. **C** and **D**, Concentrations of soluble ICAM-1 (sICAM-1), CXCL1/GRO α , CCR5/RANTES, CXCL8/interleukin-8 (IL-8) and CXCL10/IP-10, in supernatant of SGECs cocultured with HCT-5 cells (**C**) or Jurkat cells (**D**), as determined by ELISA. Values are the mean \pm SD ($n = 3$ samples). * = $P < 0.05$; ** = $P < 0.01$ versus 0 hour, by Student's t -test.

96-hour coculture with HCT-5 cells. However, the expression of ICAM-1, CXCL1, RANTES, IL-8, and IP-10 was not increased when SGECs were cocultured with Jurkat cells (Figure 5B). Accordingly, significant increases in the expression of sICAM-1, RANTES, and IP-10 in the cocultured supernatant were confirmed by ELISA (Figure 5C). In SGEC/Jurkat cell coculture supernatant, however, no significant increase in sICAM-1, RANTES, and IP-10 expression was observed (Figure 5D). Furthermore, these molecules as well as IL-8 were scarcely detectable in SGEC/Jurkat cell coculture supernatant. The results of immunofluorescence

analysis of apoptosis-related molecules also showed that the membranous expression of Fas on SGECs as well as the cytoplasmic expression of Bcl-2, cytochrome c, HO-2, and Hsp27 were up-regulated after 96-hour coculture with HCT-5 (Figure 6A). Compared with the results for HCT-5/SGEC coculture, no increase in apoptosis-related molecules was observed on SGECs cocultured with Jurkat cells (Figure 6B).

No detection of apoptosis of cocultured SGECs.

We previously reported that cultured SGECs are committed to apoptosis by several stimuli (15,16). Because in the present study the expression of proapoptotic mole-

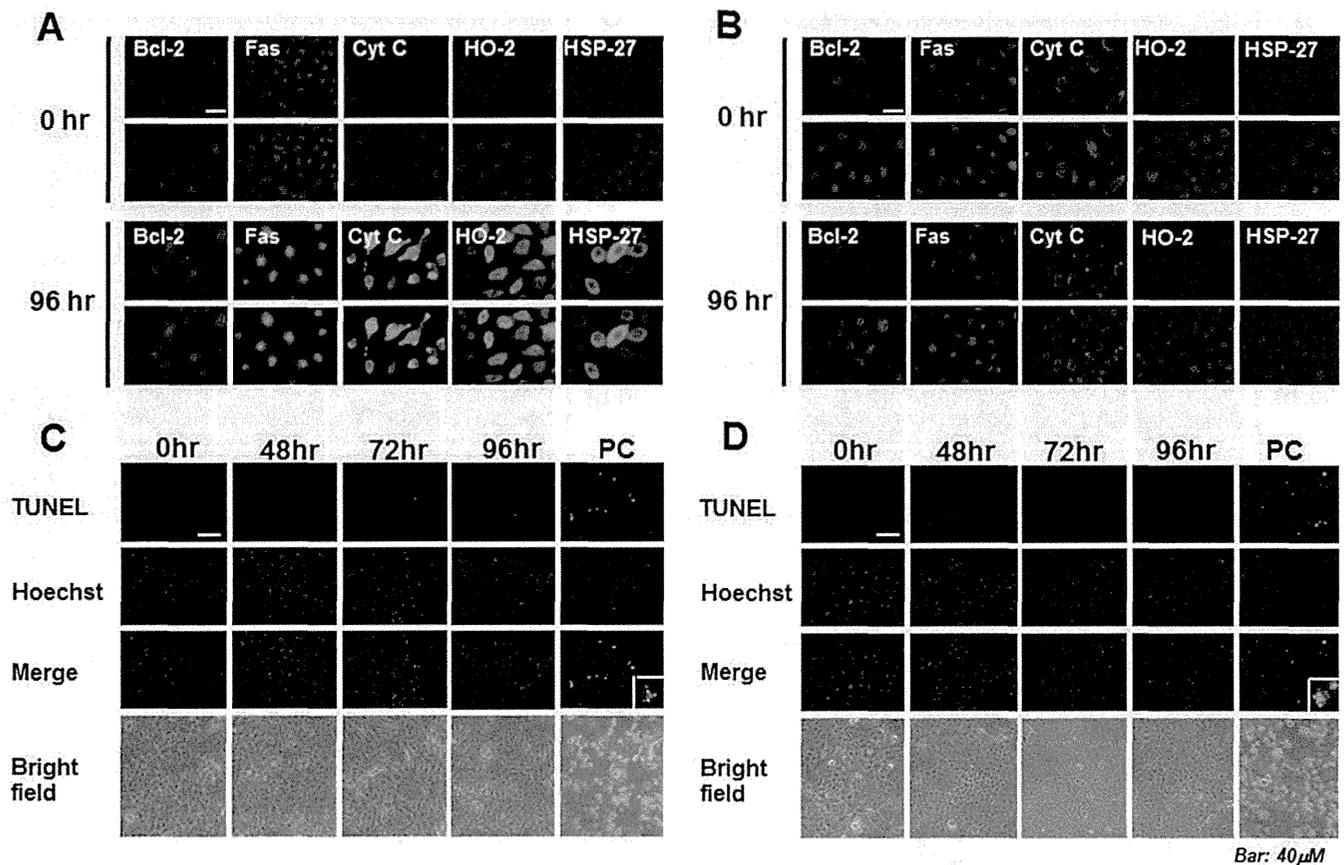


Figure 6. Expression of apoptosis-related molecules in cocultured salivary gland epithelial cells (SGECs). **A** and **B**, Expression of Bcl-2, Fas, cytochrome c (Cyt C), heme oxygenase 2 (HO-2), and Hsp27 in supernatant of SGECs cocultured with HCT-5 cells (**A**) or Jurkat cells (**B**), as determined by immunofluorescence analysis. **C** and **D**, Apoptosis of SGECs as evaluated by TUNEL staining. SGECs were cocultured with HCT-5 cells (**C**) or Jurkat cells (**D**) for 0–96 hours, followed by fixation in phosphate buffered saline containing 4% paraformaldehyde at 4°C and immersion in methanol at –20°C for 10 minutes, and were analyzed for TUNEL staining; Hoechst 33258 was used for nuclear staining. The fluorescein isothiocyanate–conjugated green signal indicates the presence of TUNEL-positive cells. As a positive control (PC), the SGECs were treated with TRAIL for 3 hours. Results are representative of 2 independent experiments with similar findings.

cles was increased by coculture with HCT-5 cells, it could be speculated that coculture with HCT-5 cells might induce apoptosis of SGECs. As we showed previously, the number of TUNEL-positive cells was clearly increased in SGECs stimulated with TRAIL (Figure 6C). In contrast, during 0–96-hour coculture, no TUNEL-positive staining was observed in SGECs cocultured with HCT-5 cells (Figure 6C). In addition, no obvious morphologic change was observed on brightfield views during coculture. Similarly, no TUNEL-positive staining was observed in SGECs during 0–96-hour coculture with Jurkat cells (Figure 6D).

DISCUSSION

With regard to the relationship between primary SS and retrovirus, Talal et al first reported that serum

antibodies against human immunodeficiency virus 1 were detected in 30% of sera from patients with primary SS (19), and the presence of retroviral particles was observed in salivary gland tissue from patients with SS (20). Retroviral particles were also observed in the LSGs of patients with SS (21).

Regarding HTLV-I infection in patients with primary SS, Mariette et al reported the presence of the HTLV-I *tax* gene in the LSGs of patients with primary SS; however, the LSGs of patients with other inflammatory diseases also contained this gene, suggesting that the HTLV-I *tax* gene contributes to the development of chronic inflammatory diseases including primary SS (22,23). In addition, Green et al reported that HTLV-I Tax–transgenic mice exhibited exocrinopathy involving the salivary glands, and Tax

protein was detected in their salivary glands and muscle specimens (24).

A recent study showed that HTLV-I p19 or Tax protein was expressed in 42.4% of LSG samples from patients with SS, and the clinical characteristics of these SS patients (including low levels of complement and high lymphocyte counts) were identified (25). Considering the accumulating evidence of a relationship between HTLV-I and SS, we speculate that HTLV-I may directly infect SGECs, a major cellular constituent of the salivary glands, and change their characteristics to an inflammatory phenotype, triggering the development of SS.

In the present study, we observed for the first time that HTLV-I appears to infect SGECs, although the expression of HTLV-I-related protein was <10% among cocultured SGECs. The migration of HTLV-I into SGECs was suggested to induce functional alterations of SGECs, because some of the SGECs became positive for nuclear NF- κ B p65, which is a transcription factor known to be activated by HTLV-I (26). Accordingly, the production of several inflammatory cytokines and chemokines increased during coculture of SGECs with HCT-5 cells in the current study. However, one or more pathways other than direct infection of SGECs by HTLV-I may be used, because a substantial population of SGECs showed no staining for HTLV-I-related proteins, HTLV-I proviral DNA, or nuclear NF- κ B p65 after coculture. Autocrine or paracrine interactions of cytokines and chemokines might be involved in these processes, in which cytokines and chemokines induce the production reciprocally (27).

Alternatively, transcription factors or activators other than NF- κ B p65, such as CREB/activating transcription factor and CREB-binding protein, which serves as a transcription activator, might be essential (28,29). Whether unique changes induced by HCT-5 are consequences attributable to direct infection of SGECs by HTLV-I or are an indirect effect of the molecules produced by neighboring activated cells (including HCT-5) is a crucial issue. In coculture, SGECs are spindle-shaped, and the intensity of Gag staining is not as strong as that in HCT-5 cells, suggesting that SGECs are distinguishable from HCT-5 cells. Some SGECs became double positive with Gag and inflammatory molecules in the coculture (Figure 5A). Because coculture of SGECs with the non-HTLV-I-infected T cell line Jurkat did not induce changes in the expression of functional molecules, cell-free HTLV-I virions might contribute to the changes in SGECs. Although no evidence of cell-free transmission of HTLV-I to any epithelial cells has been reported, HTLV-I virions have

the potential to infect myeloid and plasmacytoid dendritic cells (DCs) (30). A previous study also showed that DC-SIGN plays an important role in cell-free HTLV-I infection of DCs (31). Further studies investigating cell-free infection of SGECs by HTLV-I virions are needed in the future.

In the current study, in addition to inflammatory cytokines and chemokines, both proapoptotic and antiapoptotic molecules were augmented in SGECs after coculture with HCT-5 cells compared with coculture with Jurkat cells. However, we should also note that the results of the apoptosis analysis might be influenced by the remaining HCT-5 cells during coculture. As shown in Figure 2C, HCT-5 cells attached to SGECs during coculture, and ~5% of HCT-5 cells still remained at 96 hours. It is possible that HCT-5 cells remained in coculture because these cells had migratory and adhesive capacity; we previously reported that CD4+ T cells derived from patients with HAM showed strong trans-migrating activity (32).

The increased expression of these molecules may be induced via activation of transcription factors including NF- κ B p65 or by the cytokines and chemokines produced by SGECs themselves. It has been demonstrated that the expression of both proapoptotic molecules and antiapoptotic molecules is regulated by the mechanisms described above (33,34). Increases in the expression of antiapoptotic molecules such as Bcl-2, HO-2, and Hsp27 might antagonize the apoptosis-inducing capacities of Fas and cytochrome c in SGECs, indicating that apoptosis does not occur in SGECs.

It is interesting to note that HTLV-I infection of SGECs induces the niche of SS, because the expression pattern of cytokines, chemokines, proapoptotic molecules, and antiapoptotic molecules of SGECs cocultured with HCT-5 cells *in vitro* resembles the pattern observed *in vivo* in the salivary glands of SS patients (35). However, it is debatable whether the present *in vitro* results truly reflect *in vivo* observations in patients with anti-HTLV-I antibody-positive SS. In this regard, Ohyama et al reported that HTLV-I proviral DNA was not present in either acinar cells or ductal epithelial cells in the LSGs of patients with HTLV-I antibody-positive SS but was observed in the infiltrating T lymphocytes, as demonstrated by *in situ* PCR (36).

It has become evident that CD4+ T cells infected by HTLV-I resemble FoxP3+ Treg cells (37). Regulatory T cells produce regulatory cytokines such as IL-10 and transforming growth factor β (38), which might affect the migration of HTLV-I into ductal epithelial cells *in vivo*. Further studies are necessary to clarify the

differences and similarities of the in vitro role of HTLV-I infection in patients with SS.

In summary, we have shown that direct infection of human primary SGEs by HTLV-I induces the niche of the salivary glands in patients with SS. Our clinical and histologic examinations also revealed the characteristics of anti-HTLV-I antibody-positive SS patients, including the low rate of ectopic GC formation in LSGs and parotid gland destruction (7,8). Although the exact pathways used by HTLV-I in SS remain unclear, this study is the first investigation in humans showing that HTLV-I infects SGEs and thus has an impact on the induction of SS.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. H. Nakamura had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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ORIGINAL ARTICLE

Intracellular cyclic adenosine monophosphate regulates the efficiency of intercellular transmission of human T-lymphotropic virus type I

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Keywords

actin polymerization; cyclic adenosine monophosphate; human T-lymphotropic virus type I; human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis; vasodilator-stimulated phosphoprotein

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Abstract

Objective To investigate the relationship between the intercellular transmission efficiency of human T-lymphotropic virus type I (HTLV-I) and the signaling involved in actin polymerization during cytoskeletal reorganization in a comparative study of HTLV-I-infected T-cell lines derived from an HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patient or an HTLV-I carrier.

Methods HCT-5 and TL-Su cells derived from an HAM/TSP patient and an HTLV-I carrier, respectively, were used as HTLV-I-infected T-cell lines. After co-cultivation of each HTLV-I-infected T-cell line with H9/K30 *luc* reporter cells, the relative luc activities were calculated to analyze the efficiency of intercellular transmission of HTLV-I. The intracellular levels of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) were measured in enzyme-linked immunoassays. The expression of phosphorylated vasodilator-stimulated phosphoprotein (p-VASP) was analyzed by western blotting.

Results Treatment of HCT-5 cells with latrunculin B, an inhibitor of actin polymerization, significantly suppressed the relative luc activity. Western blotting analysis of HCT-5 cells treated with the adenylyl cyclase activator forskolin showed upregulation of p-VASP, with a concomitant and significant increase in the intracellular cAMP concentration. Furthermore, the relative luc activity was significantly decreased. The intracellular cAMP, but not cGMP levels, were significantly lower in HCT-5 than in TL-Su. Vasodilator-stimulated phosphoprotein appeared less phosphorylated in HCT-5 than in TL-Su. The relative luc activity was significantly higher in HCT-5 than in TL-Su.

Conclusions The intracellular cAMP concentration regulates the efficiency of intercellular HTLV-I transmission under the control of p-VASP expression, suggesting the intercellular transmission potential of HTLV-I-infected T cells of HAM/TSP patients is enhanced by downregulated intracellular cAMP levels. (Clin. Exp. Neuroimmunol. doi: 10.1111/cen3.12097, February 2014)

Introduction

Human T-lymphotropic virus type I (HTLV-I) infects cells by intercellular transmission through the virological synapse, which is composed of integrin/ligand complexes, such as lymphocyte function antigen-1 (α L β 2)/intercellular adhesion molecule-1 (ICAM-1),

and mediates cytoskeletal polarization.^{1–3} Integrin/ligand signaling induces the activation of small GTPases, such as Rho, Rac and Cdc42, and subsequently regulates cytoskeletal reorganization including actin polymerization, cell polarity and reorientation of the microtubule-organizing center.^{4–6} Therefore, it can be speculated that the status of

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 Ser¹⁵⁷ and Ser²³⁹ 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 (Ser¹⁵⁷ or Ser²³⁹; 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 α L β 2 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 μ g) 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 Paramete 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% CO₂. 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 latrunclin 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 latrunclin 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 latrunclin B significantly suppressed (approximately 70%) the relative luc activity (DMSO treated: 6.7 ± 0.3 vs latrunclin 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

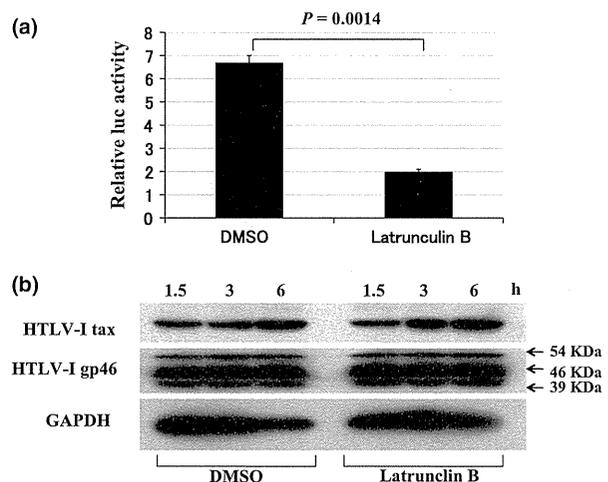


Figure 1 Effect of latrunclin B treatment on HCT-5 cells. HCT-5 cells treated with 1.25 μmol/L latrunclin B for 90 min (5×10^5 cells) were co-cultivated for 6 h with H9/K30 *luc* cells (3.5×10^5 cells) in a 24-well culture plate at 37°C in 5% CO₂. (a) Treatment with latrunclin B significantly suppressed (approximately 70%) the relative luc activity (dimethyl sulfoxide [DMSO] treated: 6.7 ± 0.3 vs latrunclin B treated: 2.0 ± 0.1 , $P =$) (b) Latrunclin 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 (Ser¹⁵⁷) 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-I of HCT-5 compared with that of TL-Su (Fig. 3c).

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

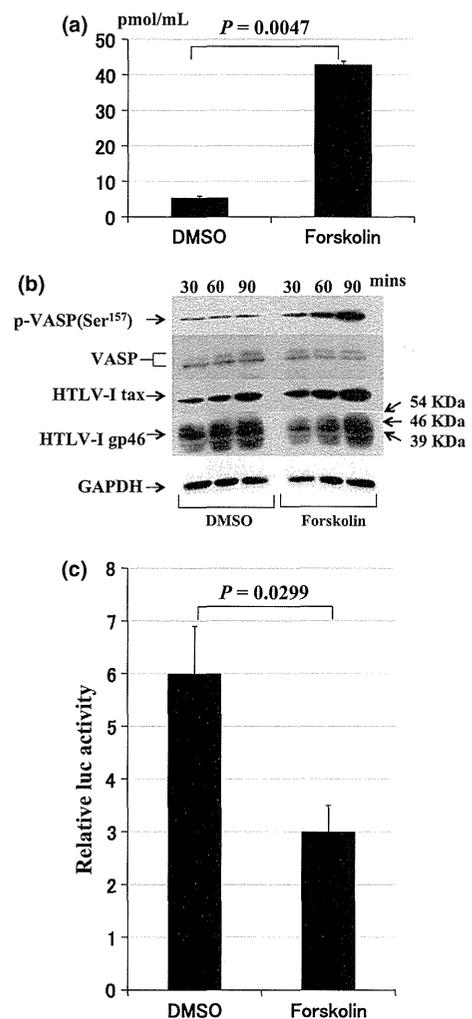


Figure 2 Effect of forskolin treatment on HCT-5 cells. (a) Treatment with 15 μ mol/L forskolin for 90 min induced a significant increase in the intracellular cyclic adenosine monophosphate concentration (dimethyl sulfoxide [DMSO] treated, forskolin treated: 5.37 ± 0.47 , 42.90 ± 0.95 pmol/mL, respectively). (b) Forskolin treatment gradually induced Ser¹⁵⁷ phosphorylation of vasodilator-stimulated phosphoprotein (VASP). However, there were no differences in the expression of human T-lymphotropic virus type 1 (HTLV-I) tax and gp46 between DMSO and forskolin treatment. (c) HCT-5 cells treated with 15 μ mol/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% CO₂. 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.

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.

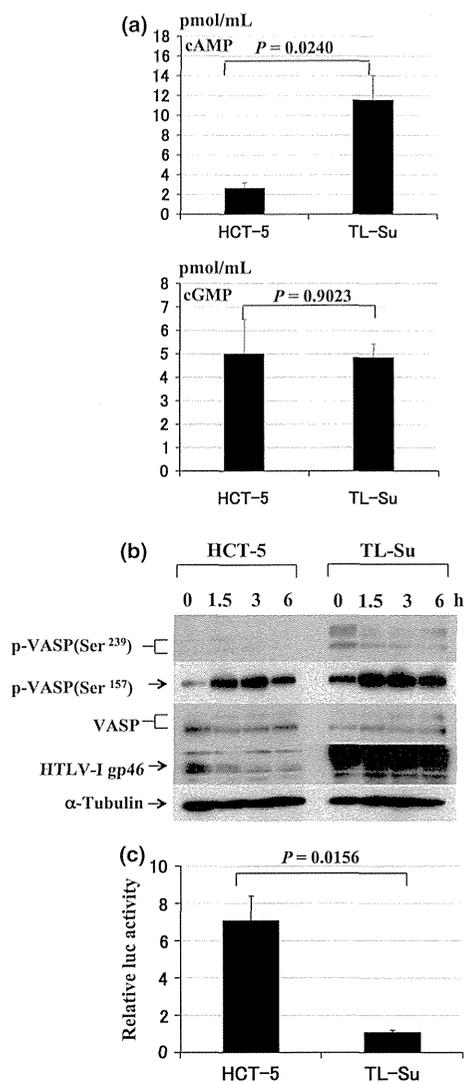


Figure 3 Comparative analysis of HCT-5 and TL-Su. (a) The intracellular cyclic adenosine monophosphate (cAMP) level was significantly lower in HCT-5 (2.67 ± 0.50 pmol/mL) than in TL-Su (11.58 ± 2.47 pmol/mL; $P = 0.0240$), although there was no significant difference in the intracellular cyclic guanosine 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 cells¹⁴ 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 interactions⁷, 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 transmemigrating 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.

Acknowledgements

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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-1 and 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 \pm SD; 24.2 \pm 15.1 years). With respect to the 10 ambulatory patients, the duration of illness ranged from 3 to 51 years (mean \pm SD; 21.0 \pm 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) UMIN00004492.

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 | | o | | | | | | | o | | | |
| One grade | | | o | o | o | o | o | | | | | |
| Two grades | o | | | | | | | | | | | o |

PSL prednisolone, IFN- α interferon-alpha

o indicates the status of the improvement of spasticity

^aOMDS score was 0–13 according to disability grade (Osame et al. 1989)

^bEvaluated at 1 week after final injection

^cImprovement 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'-ACTGTAGAGCTGAGCCGATAACG-3'), and β -actin-specific primers, forward primer (5'-GCCCTCATTTCCTCTCA-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-1 at 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 % CO₂. 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

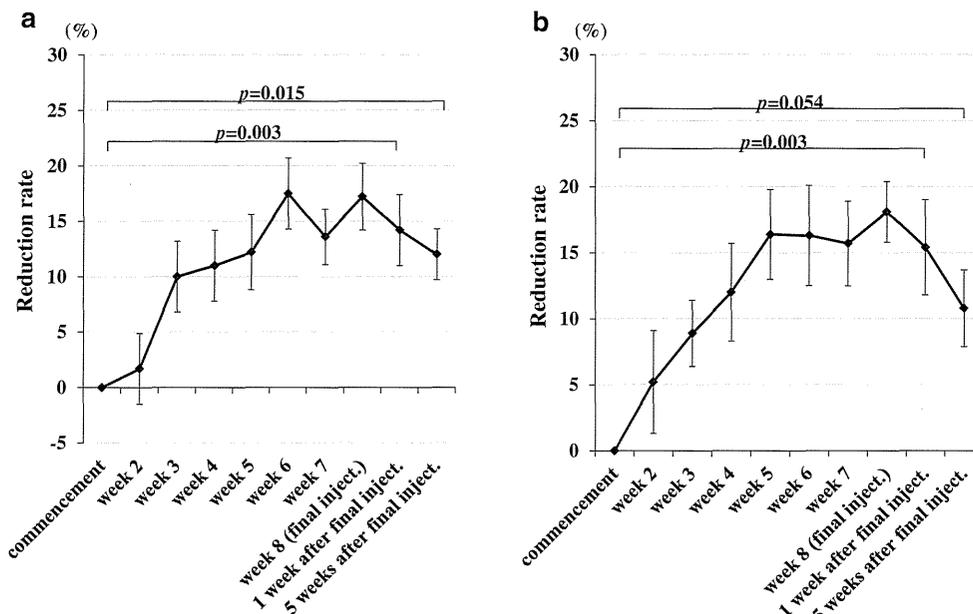


Fig. 1 Reduction rate in time required for HAM/TSP patients to walk 10 m and down a flight of stairs. **a** The reduction rate increased gradually from the commencement of treatment and was 14.2 % (SE 3.2) and 12.0 % (SE 2.3) at 1 and 5 weeks after the final injection, respectively. These values were 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 cocultivation 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 10⁴ 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.

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

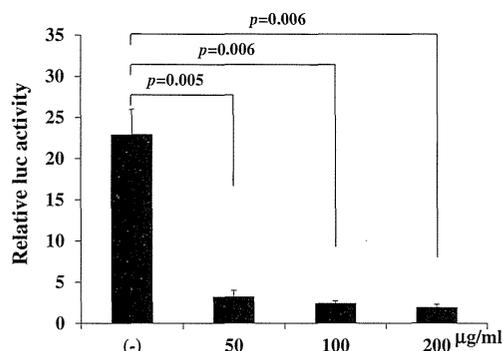


Fig. 2 The inhibitory effect for the intercellular spread of HTLV-I by PPS in vitro treatment. HCT-5 (5×10^5 cells) were co-cultivated in the presence of various concentrations of PPS with H9/K30 luc reporter cells (3.5×10^5 cells) in 24-well culture plate at 37 °C under 5 % CO₂. After co-cultivation 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

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

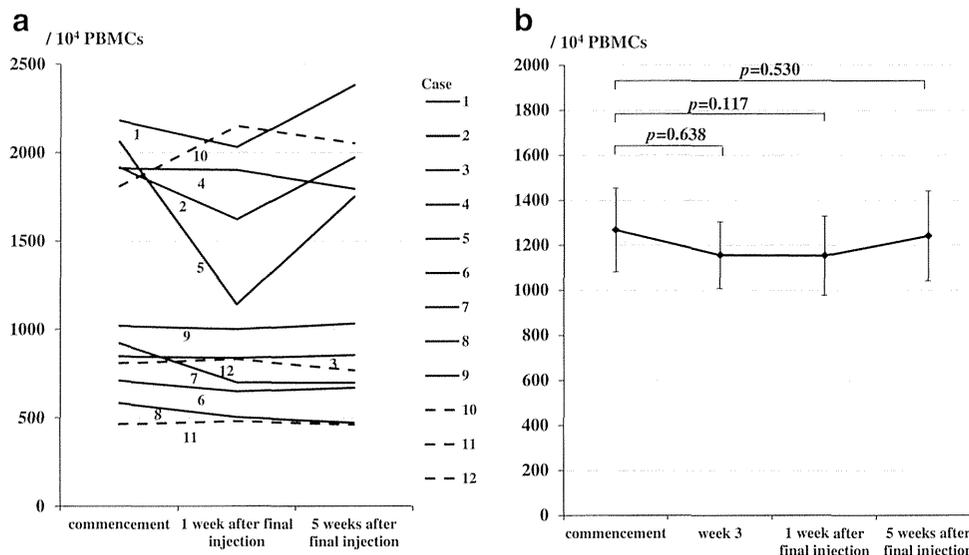


Fig. 3 Change in HTLV-I proviral copy numbers in PBMCs. **a** Changes in HTLV-I proviral copy numbers per 10⁴ PBMCs at 1 week after the final injection in each case. The decrease of HTLV-I proviral copy number ranged from 0.6 to 44.7 % was observed at in cases 1–9 (indicated as *line*). However, the decrease of it was not observed in cases 10–12 (indicated as *dotted line*). **b** HTLV-I proviral copy numbers per 10⁴

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 ($r_s=0.648$, $p=0.043$).

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.

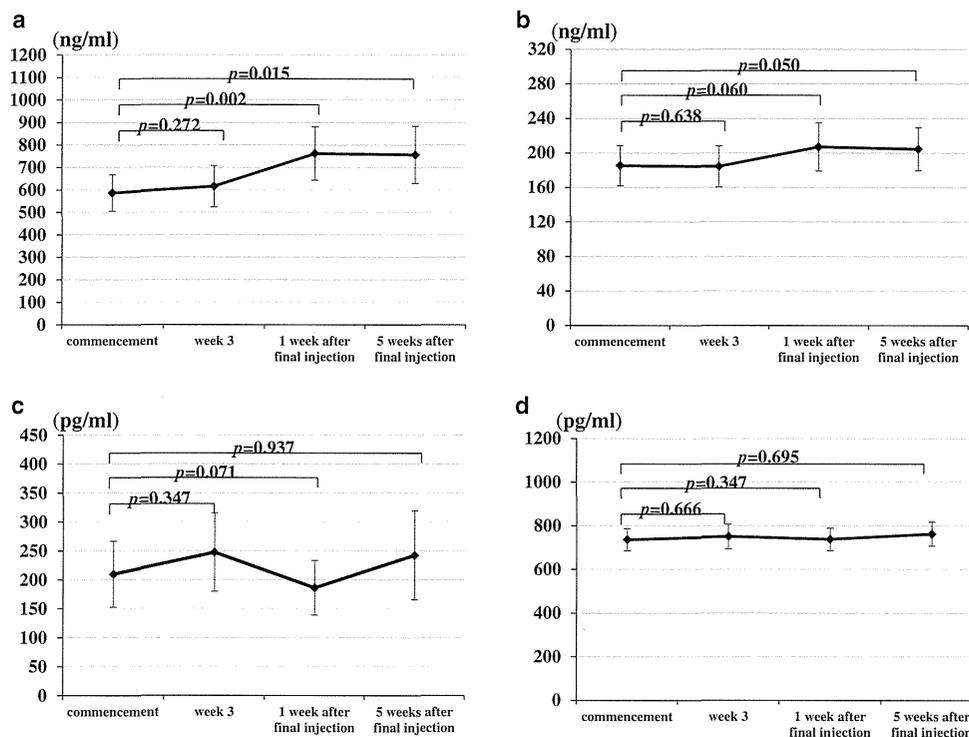


Fig. 4 Change in serum levels of sVCAM-1 and sICAM-1 and CXCL10 and CCL2. **a** Serum level of sVCAM-1 was 587 (SE 81.4), 615.8 (SE 91.5), 762.2 (SE 118.6), and 757 ng/ml (SE 127.3) at the commencement of treatment, treatment week 3, 1 week after the final injection, and 5 weeks after the final injection, 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).

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 IFN β -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

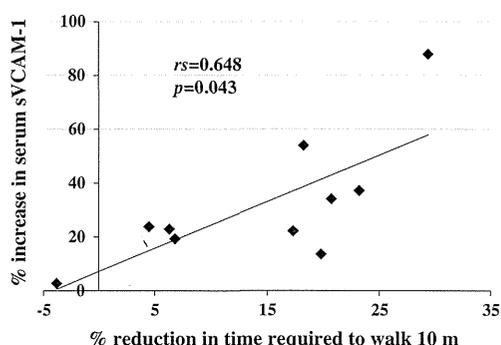


Fig. 5 Positive correlation between percentage increase in sVCAM-1 and percentage reduction in time required to walk 10 m at 1 week after the final injection compared with at commencement of treatment. There was a moderately positive correlation between them ($r_s=0.648$, $p=0.043$). Correlation analysis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

(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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