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FIGURE LEGENDS

Fig. 1. Characterization of HTLV-I-infected HCT-5 T cell line.

A: After fixation in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, HCT-5 cells were reacted with primary antibodies (anti-CD4, CD8, CD20, and mouse IgG1) followed by incubation with FITC-conjugated secondary antibody with Hoechst 33258 for counterstaining. **B:** HCT-5 for 0–96 h culture in keratinocyte-SFM were fixed and incubated with mouse anti-HTLV-I (p19, p28, and GAG) antibody and rabbit anti-NF-κB p65 antibody and then reacted with FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

Fig. 2. Detection of HTLV-I-related molecules in co-cultured SGECs.

A: After the SGECs co-cultured for 0–96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, immunofluorescence was evaluated to detect the presence of HTLV-I proteins (p19, p28, and GAG). The SGECs were initially incubated with anti-HTLV-I

antibody and NF-κB p65 followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining, respectively. In contrast to increased expression of HTLV-I proteins without NF-κB translocation **(96h-a)**, the translocation of NF-κB is shown in a different view **(96h-b)**. **B:** Low-magnification view at 96 h co-culture of SGECs with HCT-5 cells. **C:** After the SGECs co-cultured for 0–96 h were fixed and immersed, immunofluorescence was evaluated to show the presence of HTLV-I proteins (p19, p28, and GAG) and SGEC marker, cytokeratin 8/18. The SGECs were initially incubated with anti-HTLV-I antibody and anti-cytokeratin 8/18 antibody followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining, respectively. HTLV-I-infected SGECs were shown as yellow staining; meanwhile HCT-5 cells were indicated as green signal in merged view. Representative results of three independent experiments are shown.

Fig. 3. Detection of HTLV-I proviral DNA by *in situ* PCR.

A: For the positive control, HCT-5 cells were used after treatment with 1 μg/mL of PK, and five cycles of *in situ* PCR were performed. **B:** The fixed SGECs were treated with 1 μg/mL of PK, and five cycles of *in situ* PCR were then performed

in the presence and absence of primers for HTLV-I pX region as reported by Matsuoka et al. **(17)**. Representative results of two independent experiments with similar findings are shown.

Fig. 4. Semiquantitative analyses of inflammation-related molecules in the supernatant and apoptosis-related molecules in lysate during co-culture.

A and C: Co-cultured supernatant was assayed with a human cytokine dot-blot array kit. Data at 0–96 h are shown as the semiquantitative concentration of each molecule in culture medium (i.e., keratinocyte SFM) for SGECs after co-culture with HCT-5 cells **(A)** or Jurkat **(C)**. "HCT-5 only" and "Jurkat only" indicate the culture supernatant for HCT-5 and Jurkat, respectively. The expressions are noted as the ratio compared with control dot-blots. Representative results of two independent experiments with similar findings are shown.

B and D: Co-cultured SGECs, HCT-5 lysate and Jurkat lysate was analyzed using a human apoptosis dot-blot array kit. Data at 0–96 h co-cultured with HCT-5 **(B)** and Jurkat **(D)** are shown as semiquantitative concentrations of each molecule in recovered SGECs lysate. "HCT-5 only" and "Jurkat only" indicate data from HCT-5 cell lysate and Jurkat lysate, respectively. The expressions are

presented as the ratio compared with control dot-blots. Representative results of two independent experiments with similar findings are shown.

Fig. 5. Confirmation of the increase in the expression of inflammation-related molecules in co-culture by immunofluorescence and ELISA.

A and B: The SGECs were co-cultured with HCT-5 **(A)** and Jurkat **(B)** for 96 h. The SGECs at 0 and 96 h were fixed in PBS containing 4% PFA at 4°C, followed by immersion in methanol at −20°C for 10 min, and then an immunofluorescence analysis was performed. The SGECs were incubated with anti-ICAM-1, CXCL-1, RANTES, IL-8 and IP-10 antibodies followed by FITC- and TRITC-conjugated secondary antibodies with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

C and D: The SGECs were co-cultured with HCT-5 **(C)** and Jurkat **(D)** for 0-96 h. Then, ELISAs were performed using the co-cultured supernatant. The concentrations of sICAM-1, CXCL10/IP-10, CCR5/RANTES, CXCL1/GROα and CXCL8/IL-8 were detected by ELISA. Samples were collected from three independent patients, and the data shown are mean ± SD. $^{*}p<0.05$ and $^{**}p<0.01$

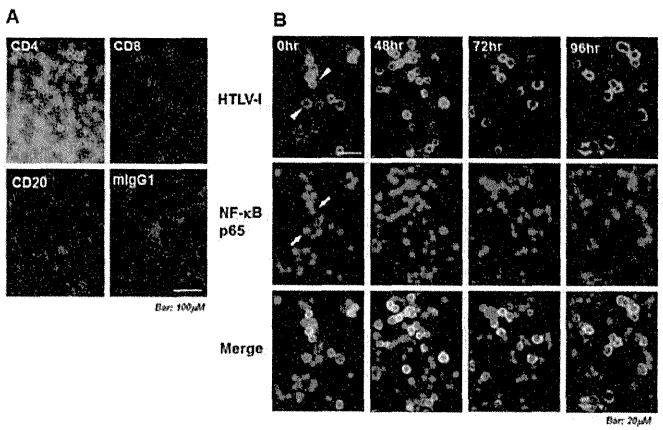
vs. 0 h (Student's *t*-test).

Fig. 6. Apoptosis of SGECs during co-culture with HCT-5 cells.

A and B: The SGECs were co-cultured with HCT-5 **(A)** and Jurkat **(B)** for 96 h. After the SGECs at 0 and 96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, an immunofluorescence analysis was performed to reveal apoptosis-related molecules. The SGECs were incubated with anti-Bcl-2, Fas, cytochrome C (Cyt C), HO-2 and HSP-27 antibodies followed by FITC-conjugated secondary antibody with Hoechst 33258 for counterstaining. Representative results of two independent experiments with similar findings are shown.

C and D: The SGECs were co-cultured with HCT-5 **(C)** and Jurkat **(D)** for 0-96 h. The SGECs at 0–96 h were fixed in PBS containing 4% PFA at 4°C followed by immersion in methanol at −20°C for 10 min, then analyzed for TUNEL staining with Hoechst 33258 for nuclear staining. The FITC-conjugated green signal suggested the presence of TUNEL-positive cells. Before the TUNEL assay, observations in the bright field were also made. For the positive control (PC), the SGECs were treated with TRAIL for 3 h as reported previously **(15)**.

Representative results of two independent experiments with similar findings are shown.



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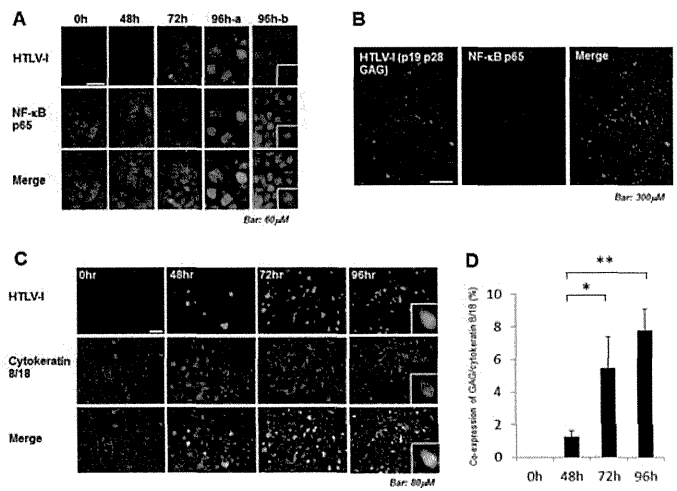

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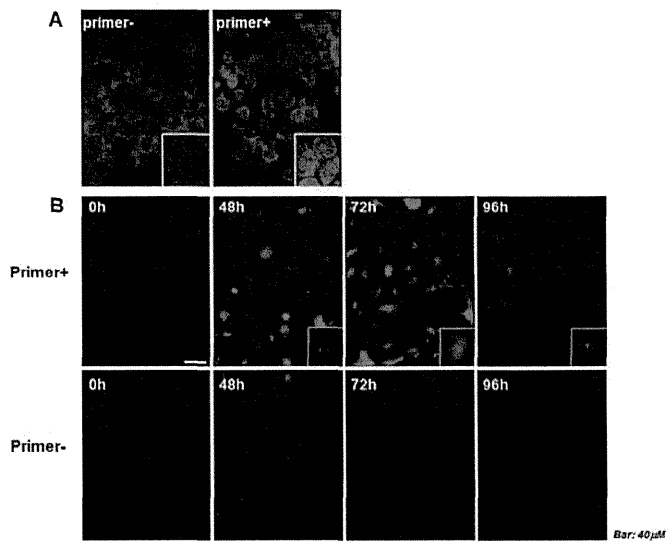

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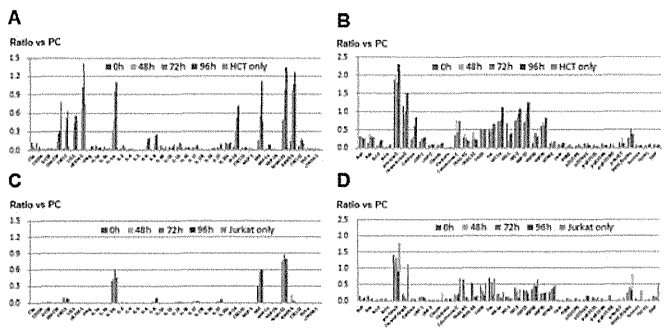

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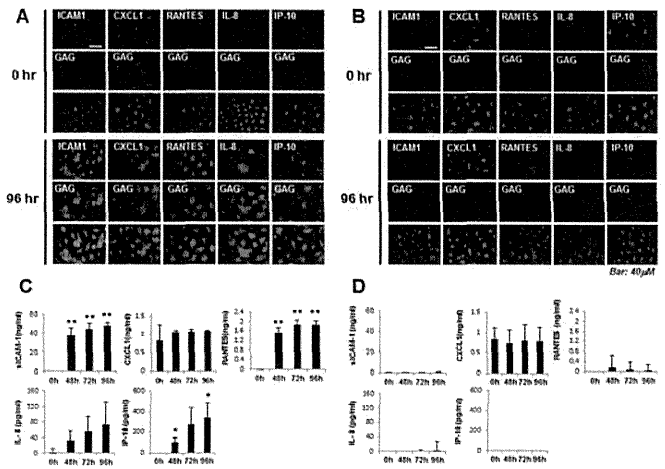

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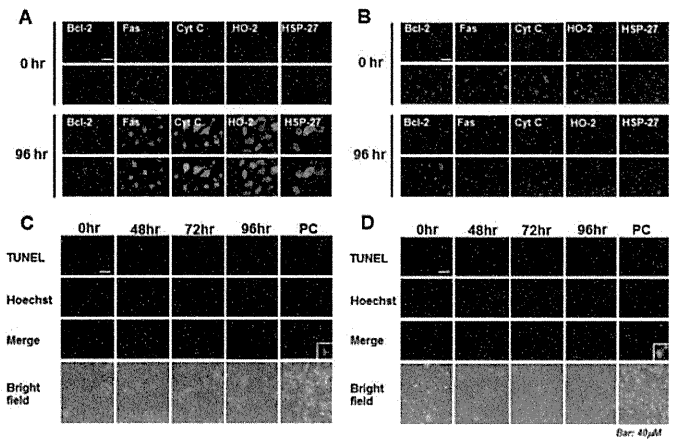

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ORIGINAL ARTICLE

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 (αLβ2)/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 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

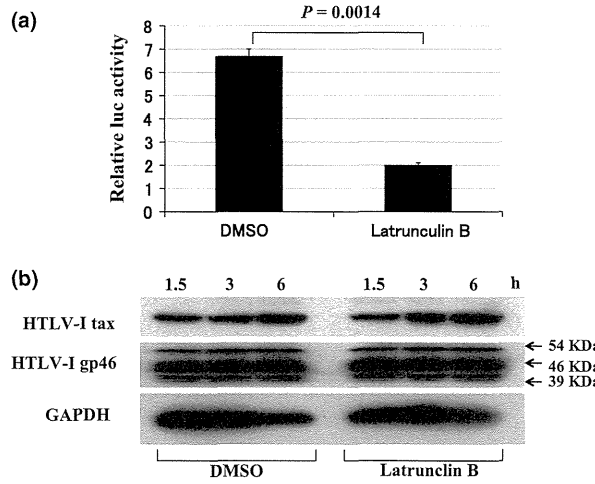

Figure 1 Effect of latrunculin B treatment on HCT-5 cells. HCT-5 cells treated with 1.25 μmol/L latrunculin B for 90 min (5×10^5 cells) were co-cultivated for 6 h with H9/K30 *luc* cells (3.5×10^5 cells) in a 24-well culture plate at 37°C in 5% CO_2. (a) Treatment with latrunculin B significantly suppressed (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).

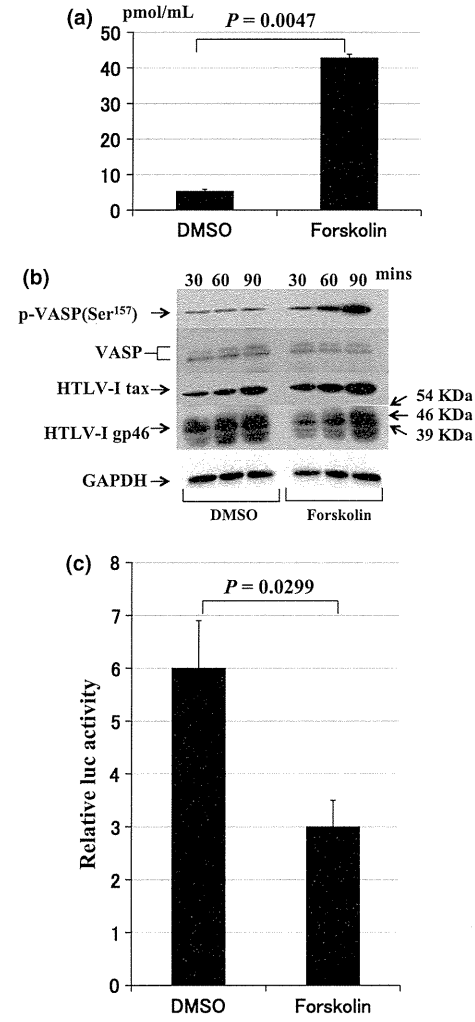

Figure 2 Effect of forskolin treatment on HCT-5 cells. (a) Treatment with 15 μmol/L forskolin for 90 min induced a significant increase in the intracellular cyclic adenosine monophosphate concentration (dimethyl sulfoxide [DMSO] treated, forskolin treated: 5.37 ± 0.47, 42.90 ± 0.95 pmol/mL, respectively). (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.

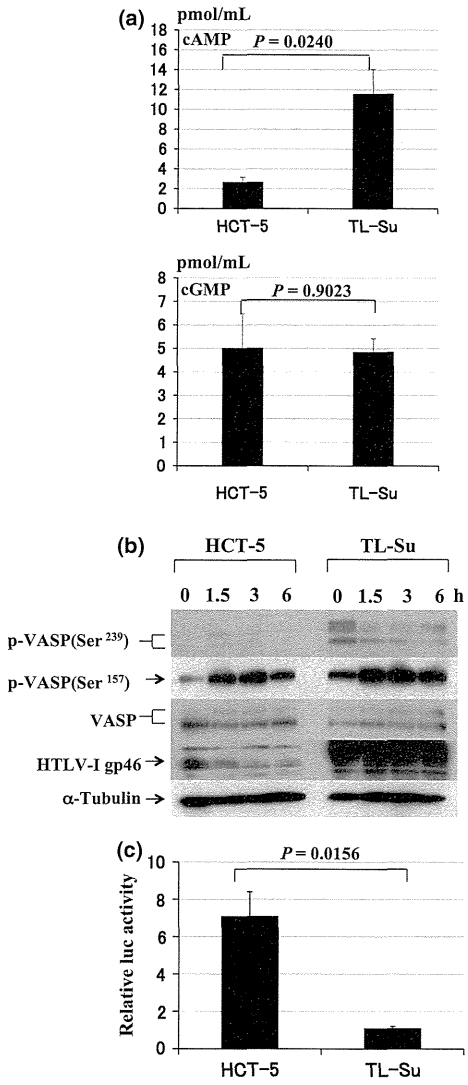

Figure 3 Comparative analysis of HCT-5 and TL-Su. (a) The intracellular cyclic adenosine monophosphate (cAMP) level was significantly lower in HCT-5 (2.67 ± 0.50 pmol/mL) than in TL-Su (11.58 ± 2.47 pmol/mL; $P = 0.0240$), although there was no significant difference in the intracellular cyclic guanosine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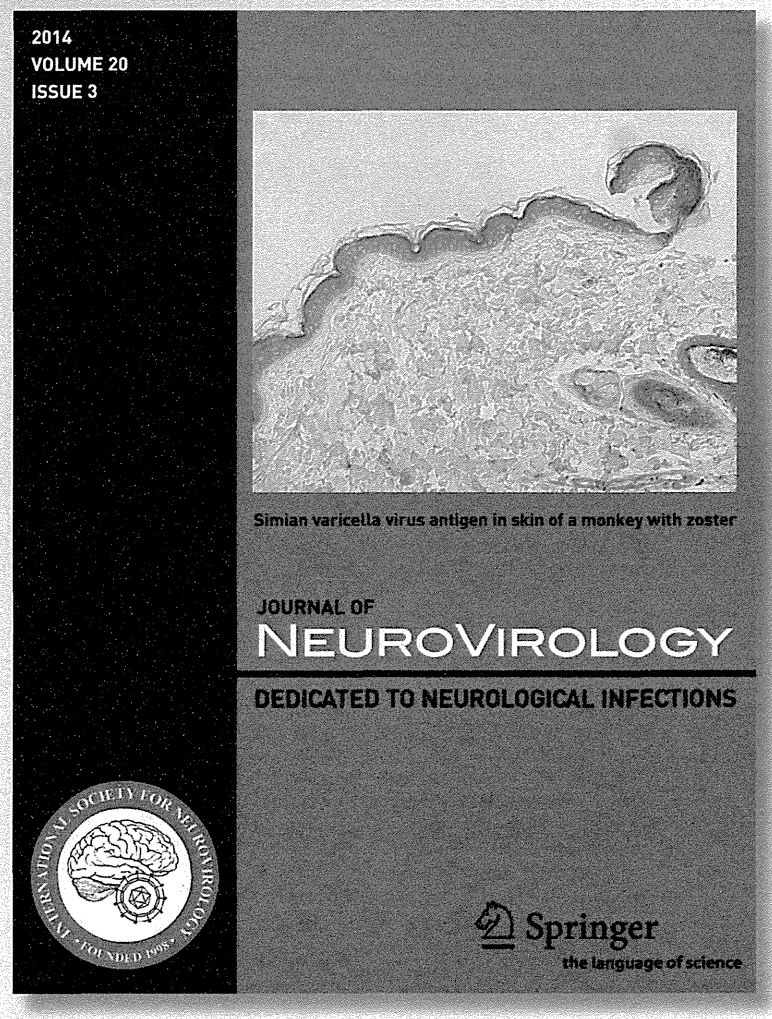

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Pentosan polysulfate treatment ameliorates motor function with increased serum soluble vascular cell adhesion molecule-1 in HTLV-1-associated neurologic disease

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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.