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Original article

Fucoidan therapy decreases the proviral load in patients with human T-lymphotropic virus type-1-associated neurological disease

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Background: Human T-lymphotropic virus type-1 (HTLV-1) is a human retrovirus that causes HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukaemia (ATL). A higher viral load in individuals with HTLV-1 infection increases their risk of developing HAM/TSP and ATL. Moreover, the high proviral load is associated with the clinical progression of HAM/TSP. Reduction of the number of HTLV-1-infected cells is therefore crucial for preventing and treating HTLV-1-associated diseases. Recently, fucoidan, a complex sulphated polysaccharide derived from marine seaweed, has been demonstrated to exert inhibitory effects on HTLV-1 infection *in vitro*. In this study, we examined the *in vivo* effects of fucoidan on HTLV-1 infection.

Methods: In this single-centre open-label trial, 13 patients with HAM/TSP were treated with 6 g fucoidan daily for 6–13 months. The HTLV-1 proviral DNA load and frequencies of HTLV-1-specific $CD8^+$ T-cells, natural killer cells, invariant natural killer T-cells and dendritic cells in the peripheral blood were analysed. Furthermore, the *in vitro* inhibitory effect of fucoidan on cell-to-cell HTLV-1 infection was examined by using luciferase reporter cell assays.

Results: Fucoidan inhibited the cell-to-cell transmission of HTLV-1 *in vitro*. Furthermore, fucoidan therapy resulted in a 42.4% decrease in the HTLV-1 proviral load without affecting the host immune cells. During the treatment, no exacerbation was observed. Four patients with HAM/TSP developed diarrhoea, which improved immediately after stopping fucoidan administration.

Conclusions: Fucoidan is a new potential therapeutic agent for the prevention and treatment of HTLV-1-associated diseases.

Introduction

Human T-lymphotropic virus type-1 (HTLV-1) is an exogenous human retrovirus that infects 10–20 million people worldwide [1]. Although most of the infected individuals are lifelong asymptomatic carriers, 3–5% of the infected population develop a T-cell malignancy called adult T-cell leukaemia (ATL) and another 0.25–3% develop a chronic progressive inflammatory neurological disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [2–4]. One of the most important pathogenic factors in HAM/TSP is the increased HTLV-1 proviral load in peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid [5–7], which suggests that viral control is inadequate in the affected individuals. Furthermore, a high HTLV-1 proviral load increases the risk of development of HAM/TSP and ATL [5,8]. Therefore, the identification of agents that can reduce the HTLV-1 proviral load is crucial for preventing and treating HTLV-1-associated disorders.

Fucoidan, a complex sulphated polysaccharide derived from marine seaweed, exerts various biological effects on mammalian cells and viral infection [9,10]. Regarding HTLV-1 infection, previous studies have shown that fucoidan inhibits both the adhesion of HTLV-1-infected

T-cells to and their infection of epithelial cells [11]. Furthermore, fucoidan inhibits HTLV-1-induced syncytium formation [12]. Recently, Haneji *et al.* [13] observed *in vitro* that fucoidan extracted from *Cladosiphon okamuranus Tokida* significantly inhibited the growth of PBMCs from patients with ATL and HTLV-1-infected T-cell lines, but not that of normal PBMCs.

We hypothesized that fucoidan therapy can decrease the HTLV-1 proviral load in infected individuals. To validate our hypothesis, 13 patients with HAM/TSP were administered fucoidan orally over the course of 6–13 months. The primary end points were baseline-to-treatment changes in the virological and immunological parameters, which were selected on the basis of evidence that they are potential markers of disease and antiviral activity in HAM/TSP, and included the HTLV-1 proviral DNA load and the frequencies of HTLV-1-specific $CD8^+$ T-cells, natural killer (NK) cells, invariant natural killer T (iNKT)-cells and dendritic cells (DCs). Clinical parameters including standardized neurological grading scores were the secondary end points. The results demonstrate the relevant biological activity of fucoidan in decreasing the proviral load in patients with HAM/TSP by interfering with the cell-to-cell spread of HTLV-1.

Methods

Luciferase reporter gene and cell viability assays

To evaluate the effect of fucoidan on HTLV-1 infection *in vitro*, we used lymphocytic H9 cells that were stably transfected with a plasmid containing the gene encoding luciferase under the control of the HTLV-1 long terminal repeat (H9/K30*luc*; kindly provided by A Adachi) [14]. We cocultured luciferase reporter cells (H9/K30*luc*; 1×10^4 cells/well) in a 24-well flat-bottom plate with an HTLV-1-infected cell line established from a patient with HAM/TSP (HCT-4; 3×10^4 cells/well) [15] at a cell ratio of 1:3. After 72 h, we assessed luciferase activity by using a luciferase assay system (Promega, Madison, WI, USA) and MicroLumat Plus LB96V (Berthold Technologies, Bad Wildbad, Germany); the values were normalized relative to the total protein concentrations. To evaluate the effect of fucoidan on cellular viability, the cell lines (2×10^3 cells/well) were plated into 96-well flat-bottom plates without any mitogenic stimuli. The culture medium used was RPMI-1640 with L-glutamine (Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Grand Island, NY, USA) and Penicillin-Streptomycin solution (Wako Pure Chemical Industries, Ltd). After culturing for 72 h with each concentration of fucoidan, cellular viability was analysed by using a CCK-8 cell proliferation kit (Dojindo, Kumamoto, Japan). Cultures were performed in triplicate for each experiment and the data are expressed as means.

Participants

A total of 17 patients (numbered HAM-1 to HAM-17) with HAM/TSP clinically defined according to World Health Organization criteria [16] were enrolled into the single-arm open-label treatment protocol. Furthermore, six control patients with HAM/TSP (HAM-18 to HAM-23) were not administered fucoidan as per their choice to be included in this group after the protocol was explained to them. The patient profiles are shown in Table 1. Patients with a rapidly progressing clinical course were excluded before enrolment. No medications were changed during the trial. Written informed consent was obtained from each patient. The study complied with the tenets of the Declaration of Helsinki and was part of a clinical protocol reviewed and approved by the institutional ethics committee of Kasumigaseki Urban Clinic, Tokyo, Japan.

Treatment regimen and evaluation

Fucoidan (provided by Kanehide Bio Co., Ltd, Okinawa, Japan) was administered at a dosage of 6 g once daily for a period of 6–13 months. Clinical and laboratory assessments and sample collection were performed at baseline, during therapy and after completion of the therapy at 4-week intervals. The baseline measure consisted of an 8-week 'run-in' period. The patients were observed for at least 4 weeks after the completion of therapy. A standardized neurological rating scale, termed Osame's motor disability scale [17], was used as a measure of disability (Additional file 1).

Determination of HTLV-1 proviral DNA load

The HTLV-1 proviral load was measured with an ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA, USA), as described previously [7,18]. In brief, PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients and DNA was extracted from 2×10^6 PBMCs; 100 ng of the sample DNA solution per well was analysed by this system. All analyses were performed in triplicate. The HTLV-1 proviral DNA load was calculated by the following formula: copy number of HTLV-1 (pX) per 100 cells =([copy number of pX]/[copy number of β-actin/2])×100. To avoid the effect of inter-assay variation of this system, which has previously been reported as 25.8% [5], we measured the viral DNA load of all DNA samples obtained from each patient throughout the treatment course on a single plate. The intra-assay variation determined by this system was 7.0% (Additional file 2).

Flow cytometric analysis of immune cells and identification of virus-specific $CD8^+$ T-cells

PBMCs were stained with monoclonal antibodies against surface markers, including anti-CD3 (UCTH1;

Table 1. Patient demographic data and clinical efficacy of fucoidan treatment

Patient	Age, years	Gender	Motor dysfunction score		Other medication
			Before	After	
HAM-1	61	F	4	4	None
HAM-2	58	F	4	4	None
HAM-3	56	F	6	6	None
HAM-4	67	F	4	4	None
HAM-5	50	F	3	3	None
HAM-6	65	F	8	8	None
HAM-7	75	M	5	5	None
HAM-8	49	M	7	7	None
HAM-9	73	F	5	5	PSL 2.5 mg/day
HAM-10	65	F	8	8	None
HAM-11	47	F	4	4	None
HAM-12	57	F	8	8	None
HAM-13	72	F	7	7	None
HAM-14[a]	53	F	6	6	None
HAM-15[a]	54	F	5	5	None
HAM-16[a]	51	F	10	10	None
HAM-17[a]	72	F	7	7	None
HAM-18[b]	49	M	5	5	IFN-α[c]
HAM-19[b]	55	M	3	3	None
HAM-20[b]	53	M	3	3	PSL 5 mg/day
HAM-21[b]	38	F	4	4	None
HAM-22[b]	39	F	4	4	None
HAM-23[b]	52	F	8	8	PSL 2.5 mg/day

[a]Patient dropped out from the trial within 1 month. [b]Patient included in the control group without fucoidan therapy. [c]Dosage of 1 million IU twice weekly. F, female; IFN, interferon; M, male; PSL, prednisolone.

eBioscience, Inc., San Diego, CA, USA), anti-CD4 (RPA-T4; eBioscience, Inc.), anti-CD8 (OKT8; eBioscience, Inc.), anti-CD25 (BC96; eBioscience, Inc.); lineage cocktail of monoclonal antibodies against CD3, CD14, CD16, CD19, CD20 and CD56 (BD Bioscience, San Diego, CA, USA); and anti-HLA-DR (LN3; eBioscience, Inc.), anti-CD123 (9F5; BD Bioscience), anti-CD11c (3.9; eBioscience, Inc.), anti-CD16 (CB16; eBioscience, Inc.), anti-CD56 (B159; BD Bioscience) and anti-Vα24Jα18 (6B11; BD Bioscience). Each cell phenotype was defined as follows: myeloid DCs (mDCs), $Lin^-HLA\text{-}DR^+CD11c^+$; plasmacytoid DCs (pDCs), $Lin^-HLA\text{-}DR^+CD123^+$; NK cells, $CD3^-CD16^+CD56^+$; and iNKT-cells, $CD3^+V\alpha24J\alpha18^+$. The cells were stained with saturating concentrations of antibody (4°C for 30 min) in the dark and washed twice before analysis by using a FACS Calibur (BD Bioscience). Fluorescein isothiocyanate-conjugated anti-human HLA-A2 (BB7.2; BD Bioscience) and fluorescein isothiocyanate-conjugated anti-human HLA-A24 (17A10; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) monoclonal antibodies were used to screen participants with HLA-A2 and A24. PBMCs from $HLA\text{-}A2^+$ patients and $HLA\text{-}A24^+$ patients were stained with phycoerythrin-conjugated *Tax*11–19 peptide-loaded HLA-*0201 tetramers and with phycoerythrin-conjugated *Tax*301–309 peptide-loaded HLA-*2402 tetramers (Medical & Biological Laboratories, Co., Ltd), respectively, for the detection of virus-specific $CD8^+$ cells, as described previously [6,7]. The data were processed with FlowJo software (TreeStar, San Carlos, CA, USA).

Statistical analyses

Comparisons of the baseline-to-treatment changes and luciferase assays were made by using a generalized linear model with repeated measures analysis of variance and evaluated by the Student's paired *t*-test.

Results

Inhibitory effect of fucoidan on cell-to-cell HTLV-1 transmission

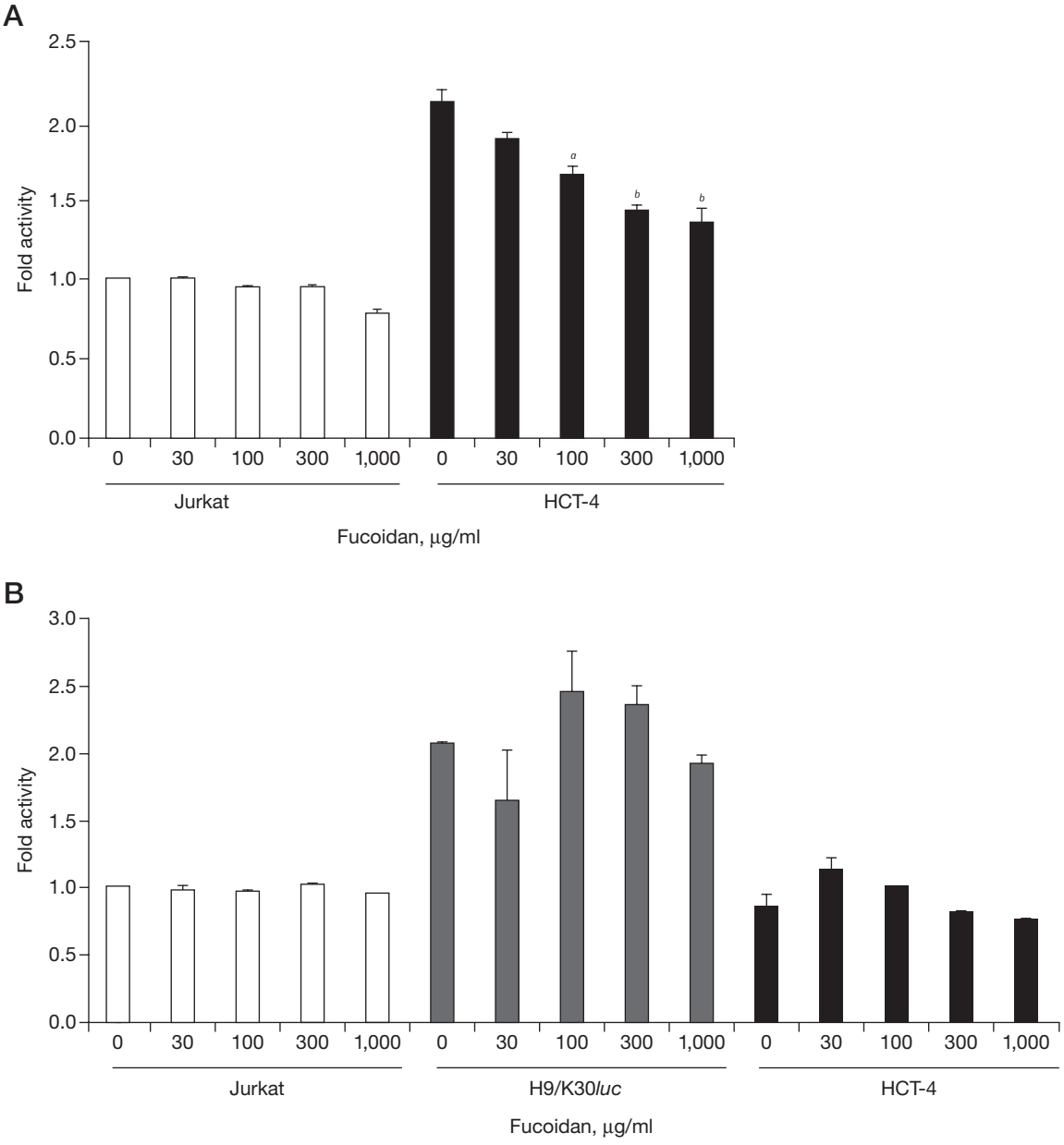
HTLV-1 is known to spread by cell-to-cell transmission [19]; therefore, we examined whether fucoidan can inhibit the spread of HTLV-1 infection. Luciferase reporter cells (H9/K30*luc*) were cocultured with HCT-4 cells [15] or an HTLV-1-uninfected T-cell line (Jurkat cell line) under different concentrations of fucoidan for 72 h, and luciferase activity was measured. As shown in Figure 1A, fucoidan inhibited cell-to-cell HTLV-1 transmission in a dose-dependent manner. To test whether this dose-dependent inhibition

by fucoidan was caused by a cytotoxic effect on the cell lines, cellular viability was examined under different concentrations of fucoidan. Fucoidan had no effect on the cellular viability and growth of these cell lines (Figure 1B).

Clinical outcomes

On the basis of the data on the *in vitro* effects of fucodian, HAM/TSP patients were orally administered fucoidan. The patients were aged between 49 and 75 years, and their disability ranged from mild to severe (Table 1).

Figure 1. *In vitro* effects of fucoidan



(A) Inhibitory effect of fucoidan on the cell-to-cell transmission of human T-lymphotropic virus type-1 (HTLV-1). H9/K30*luc* cells (1×10^4 cells) were cocultured with Jurkat or HTLV-1-infected HCT-4 cells (3×10^4 cells) for 72 h in the presence or absence of various concentrations of fucoidan, and cell lysates were prepared for luciferase assays. The luciferase activity values were normalized relative to the total protein concentrations. The inhibitory effect of each concentration of fucoidan was evaluated statistically versus the effect of 0 μg/ml ($^aP<0.01$, $^bP<0.001$). **(B)** The effects of fucoidan on cellular viability and growth. H9/K30*luc*, Jurkat and HCT-4 cells were cultured for 72 h with various concentrations of fucoidan, and the cellular viability was evaluated by using a CCK-8 cell proliferation kit (Dojindo, Kumamoto, Japan). The data are presented as the mean ±SE.

Overall, 13 of the 17 patients (HAM-1 to HAM-13) reached the maintenance dose of fucoidan 6 g daily. Four patients (HAM-14 to HAM-17) dropped out from the trial because they developed diarrhoea within 1 month of fucoidan administration, which improved immediately after stopping the therapy. The motor disability scale scores of the 13 patients (HAM-1 to HAM-13) who completed the full course of therapy remained unchanged after therapy (Table 1). There were no severe side effects and abnormalities in the blood cell count and conventional biochemical examination.

Reduction of HTLV-1 proviral DNA load after fucoidan treatment

At baseline, the patients had increased HTLV-1 proviral DNA loads [5–7]. The mean baseline HTLV-1 proviral DNA load for each patient ranged from 3.8 to 100.3 copies/100 cells. The overall mean HTLV-1 proviral DNA load was 36.5 copies/100 cells at baseline (Figure 2A). After fucoidan therapy, there was a significant reduction in the mean HTLV-1 proviral DNA load of the 13 treated patients (21.1 copies/100 cells [range 2.7–55.9]) who completed the full course of therapy (P=0.00037), whereas the mean HTLV-1 proviral DNA load of the 9 patients who did not receive fucoidan therapy (HAM-14 to HAM-16, and HAM-18 to HAM-23) showed no significant change after an interval of >6 months (Figure 2A). We could not measure the HTLV-1 proviral load of HAM-17 because this patient dropped out from the trial. The changes in the HTLV-1 proviral load of the 13 patients with HAM/TSP during fucoidan therapy are plotted in Figure 2B, and the results indicated that a significant reduction was obtained approximately 6 months after treatment was initiated.

Changes in activated CD4$^+$ and CD8$^+$ T-lymphocyte counts and virus-specific CD8$^+$ T-cells during fucoidan treatment

To examine the effect of fucoidan therapy on immune cells, we analysed the ratio of CD4$^+$ and CD8$^+$ T-lymphocytes: the ratio remained stable during the therapy (mean ±SD 2.60 ±1.26 versus 2.72 ±1.37; P=0.64557; Figure 3A). Furthermore, there was a slight statistically significant reduction in the frequencies of CD4$^+$ T-cells expressing CD25 (α-subunit of interleukin-2 receptor), which is a marker of cell activation (mean ±SD 47.7% ±13.2% versus 44.1% ±13.3%; P=0.01460), whereas the frequencies of CD8$^+$ T-cells expressing CD25 remained unchanged during the treatment period (mean ±SD 6.92% ±4.80% versus 7.59% ±5.70%; P=0.32987; Figure 3B and 3C). As HTLV-1-specific CD8$^+$ T-cells are known to be important for the control of HTLV-1-infected cells [20], the frequency of HTLV-1-specific CD8$^+$ T-cells was measured by using tetrameric peptide/HLA class I complexes to label HTLV-1 *Tax*-specific CD8$^+$ T-cells that recognize the immunodominant HTLV-1 *Tax*11–19 peptide bound to HLA A*0201 or HTLV-1 *Tax*301–309 peptide bound to HLA A*2402. Analysis of the patients with the appropriate HLA phenotype (HAM-2 to HAM-9) showed that HTLV-1 *Tax*-specific CD8$^+$ T-cells constituted 0.5–6.4% of the total CD8$^+$ T-cell population in these patients at the baseline. Fucoidan therapy had no significant effect on the frequency of HTLV-1 -specific CD8$^+$ T-cells (percentage of *Tax*/A2 tetramer [P=0.97808] and *Tax*/A24 tetramer [P=0.14482] in CD8$^+$ cells; Figure 3D and 3E, respectively).

Changes in the frequencies of NK cells, iNKT-cells, and DCs among the PBMCs during fucoidan treatment

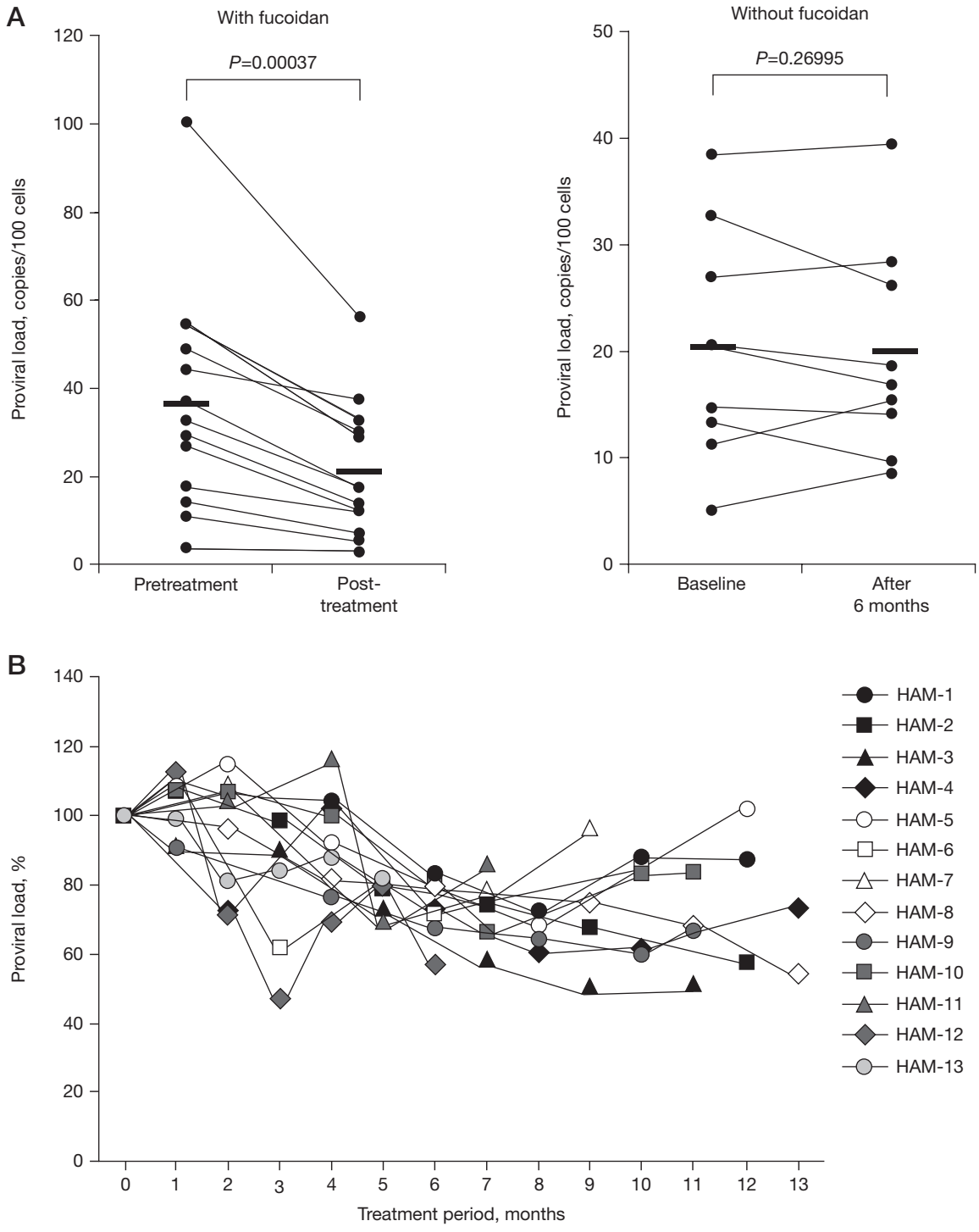
The importance of cellular components responsible for innate immunity in the control of HTLV-1 infection has been demonstrated [21,22]. Therefore, we examined the effect of fucoidan on the frequency of cell subsets of innate immunity by analysing the frequencies of NK cells, iNKT-cells, mDCs and pDCs in the peripheral blood before and after treatment. The frequencies of NK cells (P=0.95066), iNKT-cells (P=0.76289), mDCs (P=0.08053) and pDCs (P=0.39218) did not significantly change during the treatment (Figure 4).

Discussion

The clinical progression of HAM/TSP is usually subtle, and it is difficult to quantify the effect of therapy by using only the clinical parameters, even over the course of 1 year [17,23]. Several virological and immunological parameters have been identified as potential markers of disease activity in HAM/TSP [5,7,24,25]. The HTLV-1 proviral DNA load is one of the most important pathogenic factors in HAM/TSP, and is correlated with the risk of HAM/TSP and ATL in asymptomatic carriers of HTLV-1 infection [25–27]. Therefore, the major purpose of this study was to examine the potential of fucoidan for decreasing the HTLV-1 proviral load in HTLV-1-infected individuals. Surprisingly, oral administration of fucoidan decreased the HTLV-1 proviral load by approximately 42.4%, and the therapy was relatively safe and well-tolerated. Because CD4$^+$CD25$^+$ T-cells constitute the predominant viral reservoir [28,29], the reduction in the number of CD4$^+$CD25$^+$ T-cells after fucoidan therapy (Figure 3B) might also reflect the effect of fucoidan on reducing HTLV-1 proviral DNA load.

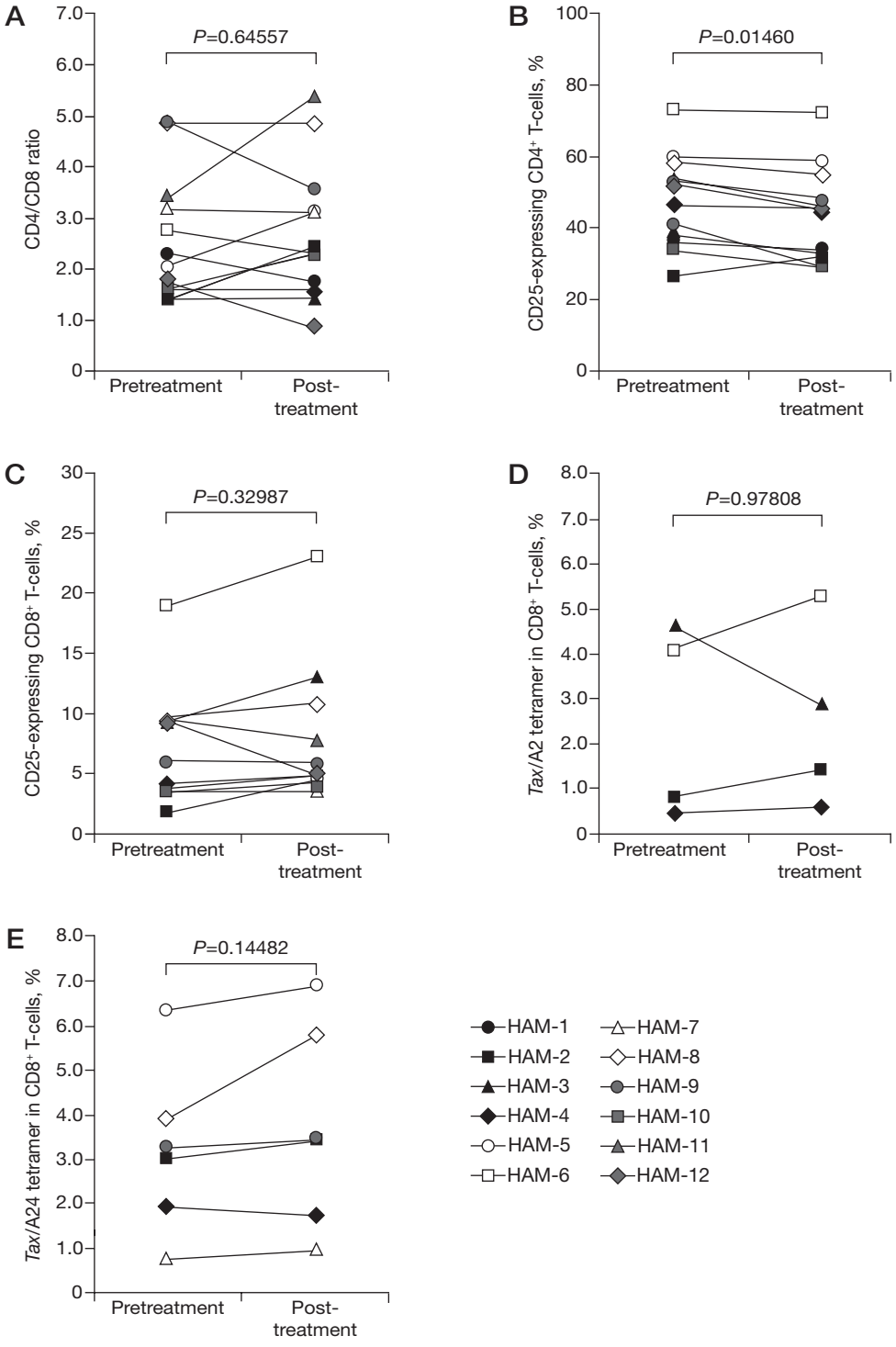
With regard to the mechanism underlying the decrease in HTLV-1 proviral load by fucoidan therapy, the following possibilities must be considered: inhibition of cell-to-cell infection, inhibition of HTLV-1-infected cell growth, increased frequency of HTLV-1-specific CD8$^+$ cytotoxic T-lymphocytes that

Figure 2. Reduction of the HTLV-1 proviral load in PBMCs of the patients with HAM/TSP following fucoidan treatment



(A) A total of 13 patients (HAM-1 to HAM-13) were treated with 6 g fucoidan daily for 6–13 months. Total cellular DNA prepared from peripheral blood mononuclear cells (PBMCs) was subjected to quantitative PCR analyses to measure the number of human T-lymphotropic virus type-1 (HTLV-1) proviral copies in the peripheral blood before the treatment and 6–13 months after treatment (left panel). The proviral DNA load decreased by approximately 42.4% (P=0.00037, Student's paired t-test). As a negative control, we measured the HTLV-1 proviral DNA load of nine patients with HAM/TSP (HAM-14 to HAM-16, and HAM-18 to HAM-23) after an interval of >6 months (right panel). **(B)** The plot of the HTLV-1 proviral DNA load in the PBMCs during fucoidan treatment is shown. The proviral load before treatment is defined as 100%, and the HTLV-1 proviral DNA load in each plot is presented as the percentage of the HTLV-1 proviral DNA load before treatment. The proviral load gradually decreased in almost all of the patients during the treatment.

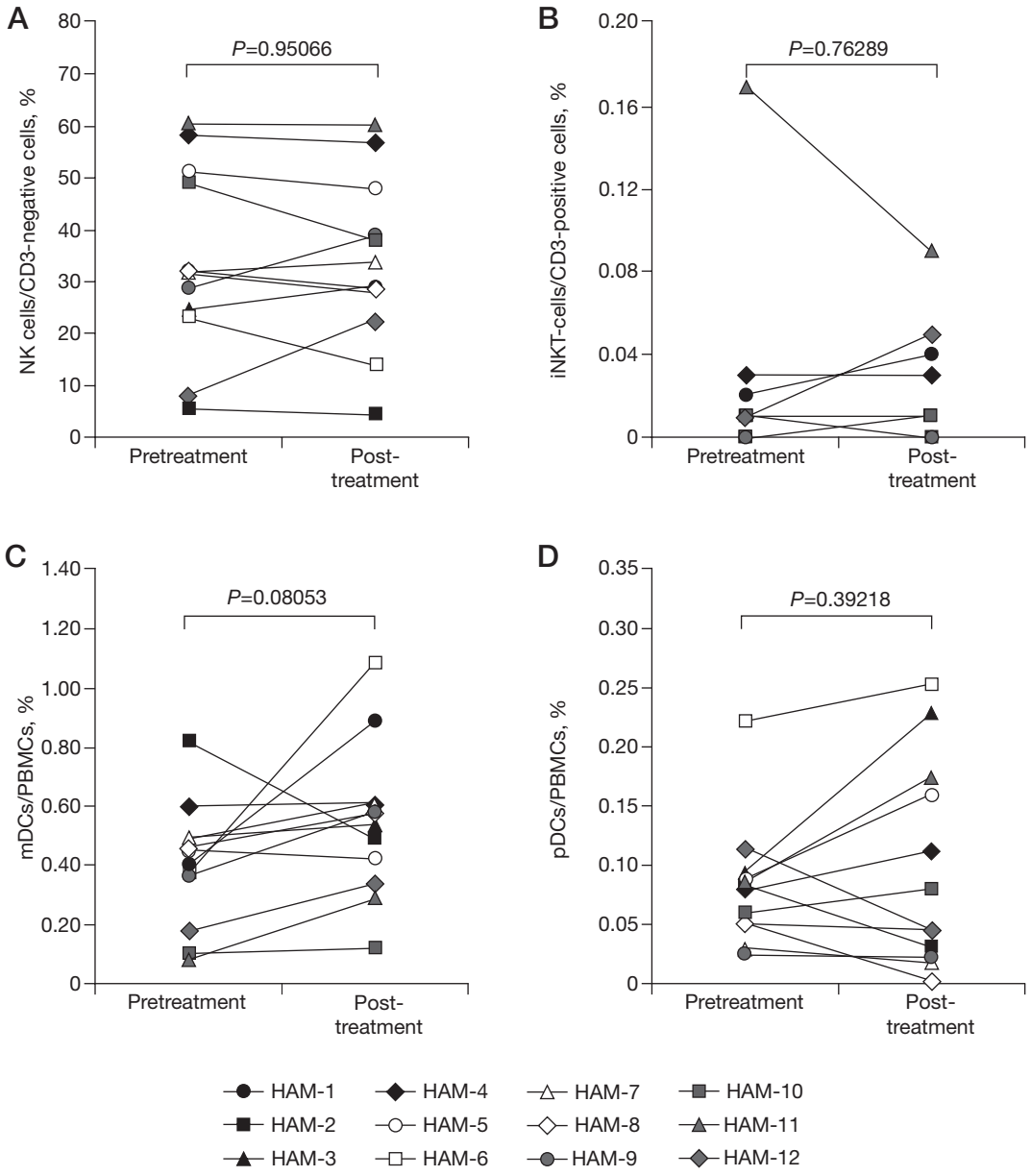
Figure 3. Effect of fucoidan treatment on the activated CD4+ and CD8+ T-lymphocyte counts and virus-specific CD8+ T-cells



(A–C) Alteration of the CD4/CD8 ratio and percentage of CD25+ T-cells among the CD4+ or CD8+ T-cells following fucoidan treatment. The CD4/CD8 ratio (A) and percentage of CD25-expressing cells among the CD4+ (B) or CD8+ (C) T-cells in the peripheral blood before and 6–13 months after fucoidan treatment were measured using flow cytometry. Although the percentage of CD25-expressing CD4+ T-cells (B) was slightly reduced after fucoidan treatment (P=0.01460, Student's paired t-test), no statistically significant change was observed in the CD4/CD8 ratio and the percentage of CD25-expressing CD8+ T-cells (A&C). Furthermore, fucoidan treatment had no effect on the frequency of human T-lymphotropic virus type-1 (HTLV-1)-specific cytotoxic T-lymphocytes. **(D&E)** Alteration of the frequency of HTLV-1-specific cytotoxic T-lymphocytes during fucoidan treatment. The frequency of HTLV-1-specific cytotoxic T-lymphocytes in the peripheral blood before and 6–13 months after fucoidan treatment was measured in human leukocyte antigen (HLA)-A2+ (D) or -A24+ (E) patients with HTLV-1-associated myelopathly/tropical spastic paraparesis by flow cytometric analysis. No statistically significant change was observed by Student's paired t-test.

kill HTLV-1-infected cells [20] and increased frequency of NK cells and iNKT-cells with anti-HTLV-1 activity [21,22]. Although these possibilities are not mutually exclusive, there was no change in the frequencies of HTLV-1-specific $CD8^+$ cytotoxic T-lymphocytes, NK cells, iNKT-cells and DCs during fucoidan treatment (Figures 3 and 4), suggesting that fucoidan has less effect on the immune system. Rather, fucoidan demonstrated the ability to inhibit the cell-to-cell spread of HTLV-1 (Figure 1A), without a significant effect on the cellular growth and viability of the HTLV-1-infected cell line (Figure 1B). Thus, the major mechanism by which fucoidan decreases HTLV-1 proviral load might involve the inhibition of HTLV-1 spread *in vivo*.

Figure 4. Frequencies of cells imparting innate immunity during fucoidan treatment



(A) Natural killer (NK) cells, **(B)** invariant natural killer T (iNKT)-cells, **(C)** myeloid dendritic cells (mDCs) and **(D)** plasmacytoid dendritic cells (pDCs) were measured in the peripheral blood during the course of the fucoidan treatment. No statistically significant change was observed by Student's paired *t*-test.

Daily intramuscular injection of interferon-α [30], daily intravenous injection of prosultiamine [31] and daily oral intake of green tea extract [32] reportedly have the potential to decrease the HTLV-1 proviral load. In a randomized double-blind placebo-controlled study of zidovudine plus lamivudine therapy, both of which inhibit the reverse transcriptase of HTLV-1 *in vitro*, no significant decrease in the HTLV-1 proviral load of HTLV-1-infected individuals was observed, suggesting that the inhibition of reverse transcriptase is not effective in decreasing the number of HTLV-1-infected cells [33]. Although the mechanism responsible for the decreased viral load by interferon-α is still not clear, prosultiamine and green tea extract induce apoptosis of HTLV-1-infected cells [29,34]. The present study has demonstrated for the first time that inhibition of cell-to-cell HTLV-1 infection is a potentially important target of therapeutic interventional strategies to decrease the HTLV-1 viral load in infected individuals. Because the present study was open and uncontrolled, a larger randomized double-blind placebo-controlled study of HTLV-1-infected asymptomatic carriers with high viral load is required.

In conclusion, oral administration of fucoidan decreased the HTLV-1 viral load in patients with HAM/TSP through the inhibition of cell-to-cell transmission without the activation of the host immune system. Fucoidan is, therefore, a new potential therapeutic agent for the prevention and the treatment of HTLV-1-associated diseases.

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Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: A standardized neurological rating scale that was used as a measure of motor disability can be found at http://www.intmedpress.com/uploads/documents/AVT-10-OA-1580_Araya_Add_file1.pdf

Additional file 2: A figure showing the intraassay variation determined by calculating the proviral DNA load for 10 samples from one patient can be found at http://www.intmedpress.com/uploads/documents/AVT-10-OA-1580_Araya_Add_file2.pdf

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