viral carriers develop ATL after a long latent period (30–50 years) [2]. Once developed, ATL has a poor prognosis with a mean survival time of 13 months, being refractory to currently available combination chemotherapy [3]. Although hematopoietic stem cell transplantation and molecular-targeted drugs have been also tried, there is at present no accepted curative therapy for ATL and the development of new therapeutic and preventive strategies is necessary [4]. Considering that only a part of viral carriers develops ATL after the long latent period, it is speculated that the onset of ATL is influenced by a diet taken daily in a similar manner to that of life-style-related diseases such as diabetes and cancers.

Functional foods and their ingredients are focused as natural resources for the prevention and treatment of life style-related diseases. For example, several polyphenols derived from various fruits and vegetables are suggested to be effective for cancer prevention [5, 6]. In ATL, several groups including us reported that epigallocatechin-3-gallate, capsaicin, and genistein, which are ingredients of green tea, red pepper, and soy, respectively, induce apoptosis in ATL cells and HTLV-1—infected cells [7–10]. These findings support the efficiency of functional foods and these ingredients against ATL and HTLV-1—infection. Thus, in this study, we focused on herbs and their ingredients as the other natural sources, because herbs have been used not only as food but also for medical purposes traditionally [11, 12]. It was found that carnosol, which is a polyphenol contained in rosemary (*Rosmarinus officinalis*), induced apoptosis in ATL cells.

Carnosol has been reported to have the apoptosis-inducing activity [13]; however, its action mechanism is not understood fully. Here, to clarify the mechanism of carnosol-induced apoptosis in ATL cells, we comprehensively examined proteins differentially expressed between the cells treated with the drug and untreated by proteomic analysis based on two-dimensional differential gel electrophoresis (2D-DIGE) and mass spectrometry (MS) [14]. Proteomic analysis of ATL cells suggested that carnosol affected the redox regulation in the cells. Thus, we next focused on glutathione, which plays the central role for the maintenance of intracellular redox status. From the quantitative analysis of glutathione and the experiment using its precursor *N*-acetyl-L-cysteine (NAC), it was suggested that the depletion of glutathione is a cause of carnosol-induced apoptosis in ATL cells.

Materials and methods

Reagents

Carnosol, NAC, and catalase were purchased from Cayman chemical (Ann Arbor, MI), Wako pure chemical industries (Osaka, Japan), and Sigma-Aldrich (St. Louis, MO), respectively.

Cell culture

ATL cell lines (ED and S1T cells), HTLV-1—infected cell line (MT-2 cells), and T-cell acute lymphoblastic leukemia cell line (Jurkat cells) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum. ED cells, S1T cells, and MT2 cells were kindly provided by Dr. Kazuhiro Morishita (Miyazaki University, Japan) [15].

Preparation of herbal extracts

Dried leaves of peppermint (*Mentha x piperita*), rosemary (*Rosmarinus officinalis*), spearmint (*Mentha spicata*), basil (*Ocimum basilicum*), or lemon balm (*Melissa officinalis*) were extracted with 80 % ethanol. After removal of insoluble materials by filtration, the solvent was evaporated. Dry residue was dissolved with dimethyl sulfoxide (DMSO) at the concentration of 200 mg/ml and stored at −20 °C until assay. Similarly, the extract of green tea (*Camellia sinensis*) was also prepared.

Cell viability assay

Briefly, 1×10^4 cells were seeded into a well of 96-well plates, cultured for 24 h, and then allowed to grow in the presence or absence of herbal extracts or carnosol. For the cells untreated with extracts or carnosol, a volume of DMSO equal to that used in the treated cells was added to the medium. After 72 h culture, 10 μl WST-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well followed by 4 h incubation at 37 °C and then the absorbance of each well was measured at 450 nm with reference wavelength at 655 nm using an Emax Precision microplate reader (Molecular Devices Inc., Sunnyvale, CA). Cell viability was calculated as relative index of the untreated cells. Effects of herbal extracts on cell viability were expressed as the 50 % inhibitory concentration (IC50). Cell viability was also examined by trypan blue staining.

Antibodies

For the evaluation of apoptosis, anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology, Beverly, MA) and anti-cleaved caspase-7 (Asp198) antibody (Cell Signaling Technology) were used. Anti-moesin monoclonal antibody, a rabbit polyclonal IgG against annexin A1, and anti-actin monoclonal antibody were purchased from AbD Serotec (Oxford, UK), Aviva Systems Biology (San Diego, CA), and Sigma–Aldrich, respectively. Rabbit

polyclonal antibodies against α-enolase and thioredoxin reductase 1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and mouse immunoglobulins were purchased from Cappel Organon Teknika (West Chester, PA).

Apoptosis assay

Apoptotic cells were detected using the Human annexin V-fluorescein isothiocyanate (FITC) kit (Bender MedSystems, Vienna, Austria). The cells were stained with FITC-conjugated annexin V and propidium iodide (PI) followed by flow cytometric analysis with an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). To detect activated caspases, the cells were extracted with a buffer containing 50 mM Tris–HCl (pH 7.4), 1 % Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, and Protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and then western blotting using antibodies against cleaved caspases was performed.

Western blotting

The protocol for western blotting was modified from the previous report [16]. The samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then the proteins were transferred electrophoretically onto Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). Membranes were blocked and then incubated with primary antibodies in solution 1 of Can Get Signal (Toyobo, Osaka, Japan) for 1 h at room temperature, followed by HRP-conjugated secondary antibodies in its solution 2 for 1 h at room temperature. As primary antibodies, anti-cleaved caspase-3 (Asp175), anti-cleaved caspase-7 (Asp198), and anti-moesin, anti-annexin A1 antibodies were used at 1:1000 dilution. Antibodies against α-enolase, thioredoxin reductase 1, and actin were used at 1:2000, 1:500, and 1:4000 dilutions, respectively. Secondary antibodies were used at 1:10000 dilution. The labeled proteins were visualized with an ECL Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). The band intensity of each protein was measured by NIH image.

Fluorescent 2D-DIGE

Fluorescent 2D-DIGE was performed as described previously [14]. Total cell extracts were obtained by homogenizing the cells harvested in 50 mM phosphate (pH 8) containing the complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) with a polytron homogenizer (Ultra-Turrax T8, IKA-Werke, Staufen, Germany). Fifty microgram of total proteins from carnosol-treated or untreated cells were fluorescently labeled with IC5-OSu (excitation, 640 nm; emission, 660 nm). On the other hand, mixture of equal quantities of both samples was labeled with IC3-OSu (excitation, 550 nm; emission, 570 nm). IC3-OSu—and IC5-OSu—labeled samples were mixed together and then the first dimension isoelectric focusing (IEF) electrophoresis was performed using ReadyStrip IPG strips (pH 3–10 NL, 7 cm; Bio-Rad Laboratories) on Protean IEF cell (Bio-Rad Laboratories) followed by SDS-PAGE using 8 % gel for the second dimensional separation. Fluorescent 2D-DIGE images of IC3-OSu—and IC5-OSu—labeled samples were obtained using appropriate excitation/emission filters equipped on a Proxpress proteomic imaging system (PerkinElmer Life Sciences, Waltham, MA). Because IC3-OSu images obtained and fluorescent intensity of protein spots contained in them are to be theoretically identical between all gels, the IC3-OSu—labeled proteins serve as reference for spot matching and quantification of IC5-OSu—labeled proteins [14]. Thus, after all images were aligned based on IC3-OSu images using SameSpot TT900 S2S (Nonlinear Dynamics, Newcastle, UK), protein spot intensities in IC5-OSu images were calculated using Progenesis Discovery (Nonlinear Dynamics). Each group (carnosol-treated or untreated) was run on triplicate gels twice and the average spot intensities from total 6 gels were expressed as normalized volume ± standard deviation (SD). Statistical differences were determined by the Student t test. Values of $p < 0.05$ were considered significant.

Protein identification

Proteins expressed differentially were identified by in-gel digestion and peptide mass finger printing (PMF) using MS [14]. Briefly, gels were stained with coomassie brilliant blue R-250 and then spots of interest were cut off. The gel pieces were destained in 25 mM NH_4HCO_3 and 50 % acetonitrile and then incubated in reducing solution (25 mM NH_4HCO_3 and 10 mM dithiothreitol) for 1 h at 56 °C followed by further incubation for 45 min at room temperature in alkylation solution (25 mM NH_4HCO_3 and 55 mM iodoacetamide). After dehydrating with acetonitrile, the gel pieces were incubated overnight in digesting solution [50 mM NH_4HCO_3, 10 μg/ml trypsin (Trypsin Gold, mass spectrometry grade; Promega, Madison, WI), and 1 % n-octyl-β-D-glucoside (Dojindo Molecular Technologies)]. The peptides produced were extracted with extraction solution (50 % acetonitrile and 5 % trifluoroacetic acid) and then

spectra were obtained using matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)-TOF–MS, Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany). The data set was entered in an in-house Mascot search engine (Matrix Science, London, UK) to find the closest match with known proteins registered in the database from the Swiss-Prot.

Glutathione assay

Level of intracellular glutathione (reduced form, GSH; oxidized form, GSSG) was examined using Bioxytech GSH/GSSG-412 assay kit (OxisResearch, Portland, OR). Data were calculated based on standard curves created with known amounts of GSH and expressed as the

Table 1 Leukemia cell growth-inhibitory activity in 80 % ethanol extracts of several herbal species

Herbal species		IC50 value (μg/ml)			
Common name	Botanical name	ED	S1T	MT-2	Jurkat
Green tea	*Camellia sinensis*	204	261	301	284
Peppermint	*Mentha x piperita*	32.5	40.9	56.8	25.1
Rosemary	*Rosmarinus officinalis*	20.6	44.4	22.0	16.7
Spearmint	*Mentha spicata*	43.2	51.3	57.8	46.0
Basil	*Ocimum basilicum*	83.6	47.0	94.2	72.1
Lemon balm	*Melissa officinalis*	303	248	263	366

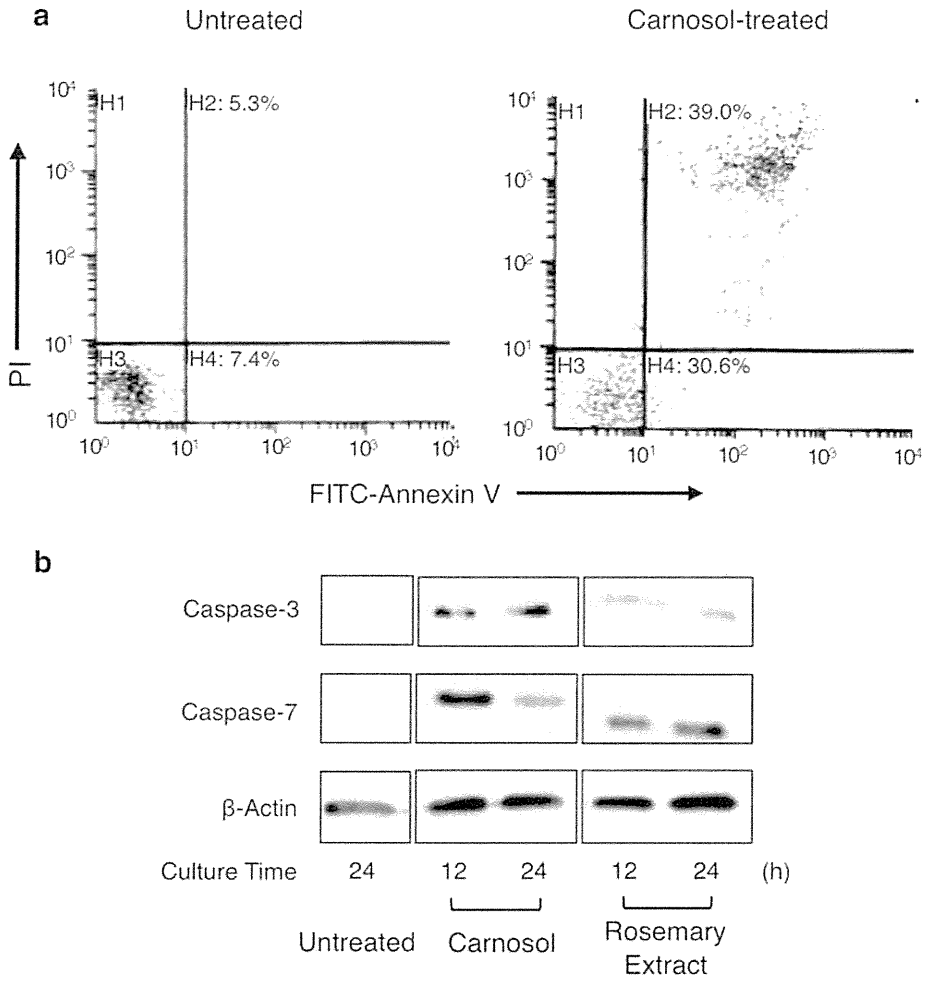

Fig. 1 Carnosol, a rosemary ingredient, induces apoptosis in ATL cells. ED cells were untreated or treated with 40 μM carnosol and then double-stained with PI and FITC-annexin V followed by flow cytometry (**a**). Annexin V-positive cells were increased by carnosol treatment. The activated forms of caspase-3 and caspase-7 were detected by western blotting (**b**)

amount per mg of protein. The ratio of GSH/GSSG was calculated from values of GSH and GSSG. Statistical differences were determined by the Student t test and values of $p < 0.05$ were considered significant.

Results

Rosemary extract and its ingredient, carnosol, induce apoptosis in ATL cells

To screen biological active substances for the prevention and treatment of ATL from herbs, ATL cell growth-inhibitory activity of 5 herbal extracts was examined by WST-8 (Table 1). In ED cells and S1T cells as ATL cell lines, IC50 values of 4 spices (peppermint, rosemary, spearmint, and basil) were lower than that of green tea, which has been reported to be effective for ATL. Similar results were obtained in MT2 cells and Jurkat cells as HTLV-1—infected cells and the other T-cell leukemia cells, respectively. Especially, rosemary extract showed the superior inhibitory activity.

Carnosol is an ingredient of rosemary and a polyphenolic compound having antioxidant activity [17]. To investigate a contributory ingredient present in rosemary extract and its inhibitory form, it was next examined whether carnosol induces apoptosis in ATL cells (Fig. 1). In ED cells treated with carnosol, the annexin V-stained cells were clearly increased, compared with untreated cells (Fig. 1a). Activated caspase-3 and caspase-7, which are both executioners of apoptotic program, were also detected (Fig. 1b). In the cells treated with rosemary extract, we failed to detect the stained cells specifically, maybe owing to the disturbance of staining by some fluorescent substance in the extract (data not shown); however, activated caspases were detected (Fig. 1b). These results suggested that carnosol is a contributory ingredient in ATL cell apoptosis-inducing activity of rosemary.

Investigation of apoptosis-inducing mechanism by proteomic analysis

Proteomic analysis is the powerful tool for clarifying the action mechanism of drugs [14, 18]. Thus, to investigate the mechanism of carnosol-induced apoptosis in ATL cells, ED cells treated with carnosol were subjected to proteomic analysis using fluorescent 2D-DIGE and MS. Proteins extracted from carnosol-treated cells and untreated cells were separated by fluorescent 2D-DIGE and protein spots differentially expressed were examined. Although the spots discernibly different were not found by visual contact of 2D-DIGE images, quantitative analysis revealed that the intensities of 17 spots and 6 spots were significantly increased (Nos. 1–17; arrows in Fig. 2) and decreased (Nos. 18–23; arrowheads in Fig. 2), respectively, in carnosol-

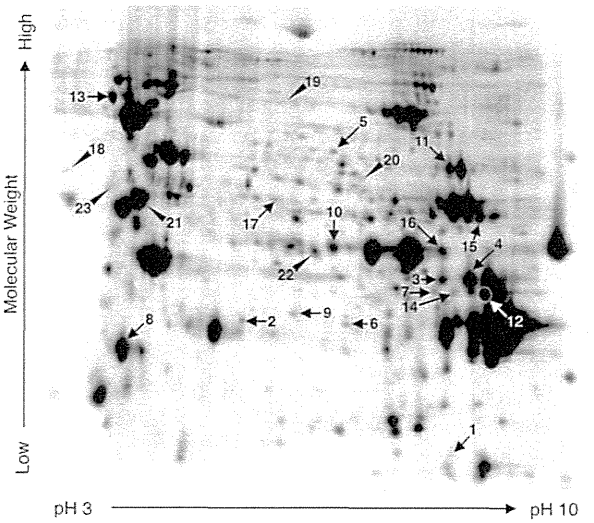

Fig. 2 Protein spots differentially expressed in carnosol-treated ED cells. Protein extracts from the cells that were untreated or treated with 40 μM carnosol for 24 h were fluorescent-labeled and then separated by 2D-DIGE followed by detection of protein spots with fluorescent imager. The fluorescent intensities of detected spots were calculated using Progenesis Discovery software. The image shows a representative pattern of IC3-OSu—labeled proteins as reference sample. In carnosol-treated cells, the spot intensities of 17 spots (Nos. 1–17; *arrows*) were increased as compared with untreated cells. In contrast, those of 6 spots (Nos. 18–23; *arrowheads*) were decreased. *Spot numbers* correspond to those in Table 2

treated cells as compared with untreated cells ($p < 0.05$; 1.3-fold change as cut off). Table 2 shows quantitative values of these spots. Next, proteins derived from these spots were identified by in-gel digestion and PMF using MALDI-TOF-TOF–MS (Table 2). These expression differences of our selected 4 proteins were also confirmed with western blotting (Fig. 3). From the list of proteins whose expression was increased by carnosol (Table 2), we found that these proteins fell mainly into three categories. The first is NADPH-dependent reductases (No. 1, flavin reductase; No. 9, biliverdin reductase A; No. 17, thioredoxin reductase 1). The second is enzymes in glycolytic pathway (Nos. 3 and 4, phosphoglycerate kinase 1; Nos. 10 and 16, α-enolase; No. 12, fructose-bisphosphate aldolase A; No. 15, glucose-6-phosphate isomerase). Although the same proteins were identified from multiple protein spots, this may be due to the post-translational modification and protein processing. The third is enzymes in pentose phosphate pathway (No. 2, transaldolase; No. 11, transketolase). The increase of expression of reductases implies the change of redox-status in the cells treated with carnosol. Moreover, these reductases were NADPH-dependent. Glycolytic and pentose phosphate pathways cooperate to contribute to the production of NADPH [19]. Consequently, it appeared that apoptosis-inducing activity of carnosol is related with NADPH-dependent redox regulation in the cells.

Table 2 Proteins expressed differentially in carnosol-treated ED cells

Spot no.[a]	Spot intensity[b]		Fold change[c]	p value[d]	Protein name[e]	Accession no.[f]	Sequence coverage (%)[g]	MW (kDa)[h]	pI[i]	Protein function[j]
	Untreated	Carnosol								
Increased										
1	1.07 ± 0.35	6.58 ± 4.22	6.16	0.025	Flavin reductase	P30043	28.8	22.1	7.3	NADPH-dependent reductase
2	0.69 ± 0.13	1.53 ± 2.22	2.22	0.011	Transaldolase	P37837	13.9	37.7	6.4	Regulation of pentose-phosphate pathway
3	1.06 ± 0.17	1.69 ± 0.22	1.60	2.5×10^{-4}	Phosphoglycerate kinase 1	P00558	24.8	44.9	8.3	Glycolytic enzyme
4	1.05 ± 0.12	1.66 ± 0.05	1.59	4.9×10^{-7}	Phosphoglycerate kinase 1	P00558	22.1	44.9	8.3	Glycolytic enzyme
5	0.68 ± 0.09	1.03 ± 0.06	1.51	1.2×10^{-5}	Moesin	P26038	18.6	67.8	6.1	Connection of cytoskeleton to the plasma membrane
6	0.76 ± 0.12	1.14 ± 0.08	1.49	8.1×10^{-5}	Annexin A1	P04083	23.8	38.8	6.6	Promotion of membrane fusion in exocytosis
7	0.77 ± 0.11	1.14 ± 0.34	1.48	0.044	26S protease regulatory subunit 10B	P62333	22.6	44.4	7.1	Component of 26S proteasome
8	1.00 ± 0.18	1.44 ± 0.12	1.44	5.6×10^{-4}	Annexin A5	P08758	46.4	35.8	4.9	Anticoaglant in blood coagulation cascade
9	1.00 ± 0.21	1.43 ± 0.32	1.43	0.020	Biliverdin reductase A	P53004	22.0	34.0	6.1	NADH- or NADPH-dependent reductase
10	1.08 ± 0.22	1.51 ± 0.35	1.40	0.029	α-Enolase	P06733	32.6	47.4	7.0	Glycolytic enzyme
11	0.77 ± 0.08	1.08 ± 0.20	1.39	0.006	Transketolase	P29401	12.7	68.5	7.6	Regulation of pentose-phosphate pathway
12	1.04 ± 0.20	1.42 ± 0.17	1.37	0.005	Fructose-bisphosphate aldolase A	P04075	26.2	39.7	8.4	Glycolytic enzyme
13	0.69 ± 0.08	0.95 ± 0.19	1.37	0.012	Endoplasmin	P14625	15.7	92.7	4.8	Molecular chaperone
14	0.95 ± 0.08	1.28 ± 0.21	1.35	0.010	Casein kinase II subunit α	P68400	23.0	45.2	7.3	Signal transduction
15	0.89 ± 0.09	1.18 ± 0.04	1.33	3.5×10^{-5}	Glucose-6-phosphate isomerase	P06744	16.7	63.2	9.1	Glycolytic enzyme
16	0.94 ± 0.04	1.25 ± 0.10	1.33	4.3×10^{-5}	α-Enolase	P06733	30.3	47.4	7.0	Glycolytic enzyme
17	0.80 ± 0.20	1.06 ± 0.03	1.32	0.023	Thioredoxin reductase 1	Q16881	15.4	55.5	6.1	NADPH-dependent reductase
Decreased										
18	1.46 ± 0.68	0.47 ± 0.18	−3.13	0.018	Nuclear autoantigenic sperm protein	P49321	12.4	85.5	4.3	DNA replication
19	0.78 ± 0.17	0.41 ± 0.14	−1.89	0.002	Methionine-tRNA ligase	P56192	9.30	102.2	5.8	Methionylation of tRNA
20	0.88 ± 0.24	0.64 ± 0.04	−1.38	0.040	Stress-induced-phosphoprotein 1	P31948	13.1	63.2	6.4	Mediation of association of HSC70 and HSP90
21	0.95 ± 0.05	0.71 ± 0.12	−1.34	0.001	Tubulin α-1C chain	Q9BQE3	24.3	50.5	5.0	Major constituent of microtubules
22	1.27 ± 0.06	0.94 ± 0.15	−1.34	6.4×10^{-4}	Elongation factor 1-γ	P26641	17.0	50.3	6.3	Elongation of translation
23	0.71 ± 0.09	0.53 ± 0.05	−1.33	0.002	Protein disulfide-isomerase	P07237	16.7	57.5	4.8	Formation of disulfide bonds

[a] Spot numbers correspond to those in Fig. 2

[b] Intensities of spots are shown as normalized volume ± SD (6 gels per group; untreated and carnosol-treated)

[c] Fold changes were calculated using Progenesis Discovery software and expressed as differences of spot intensities in carnosol-treated cells compared with those in untreated cells

[d] Statistical differences were determined by the Student t test. Values of $p < 0.05$ were considered significant

[e] Proteins were indentified using MASCOT with Swiss-Prot database

[f] References for the identified proteins

[g] Percentage cover of the identified peptides in total tryptic digests

[h] Theoretical molecular weight (*MW*) from MASCOT search results

[i] Theoretical isoelectric point (*pI*) from MASCOT search results

[j] The informations about protein functions were obtained by access using accession number from Swiss–Prot knowledgebase

Carnosol-induced apoptosis is caused by glutathione depletion but not extracellular H_2O_2

Glutathione is required for the maintenance of redox-status and plays a central role as antioxidant in the protection against oxidative stress through the cycling of GSH (reduced form) and GSSG (oxidized) [20, 21]. Further, the cycle is regulated by glutathione reductase and its enzymic activity is dependent on NADPH. Thus, to examine the relationship of glutathione to carnosol-induced apoptosis, the amounts of intracellular GSH and GSSG were quantified by commercially available kit (Fig. 4). Amounts of both

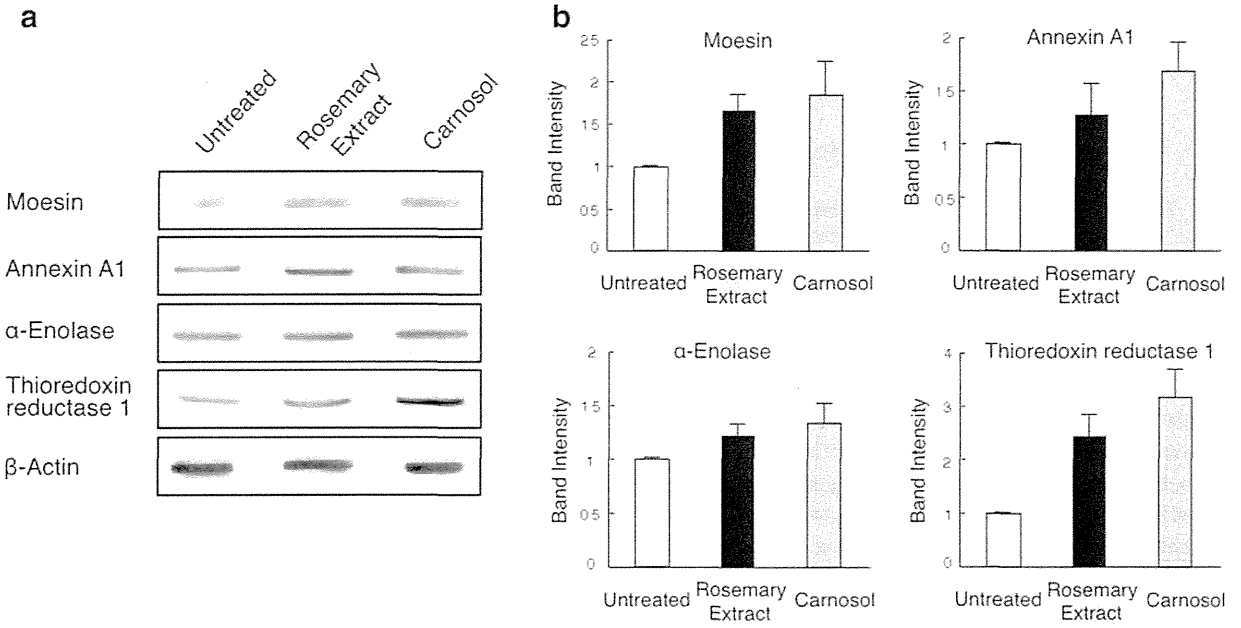

Fig. 3 Confirmation of expression of several identified proteins by western blotting. Protein extracts from the cells that were untreated or treated with 40 mg/ml rosemary extract or 40 μM carnosol for 24 h were electrophoresed and then blotted with antibody against moesin, annexin A1, α-enolase, thioredoxin reductase 1, or β-actin (**a**). The band intensity of each protein was measured by NIH image (**b**). The expression of these proteins was increased in carnosol-treated cells. Data represent the mean ± SD from three experiments

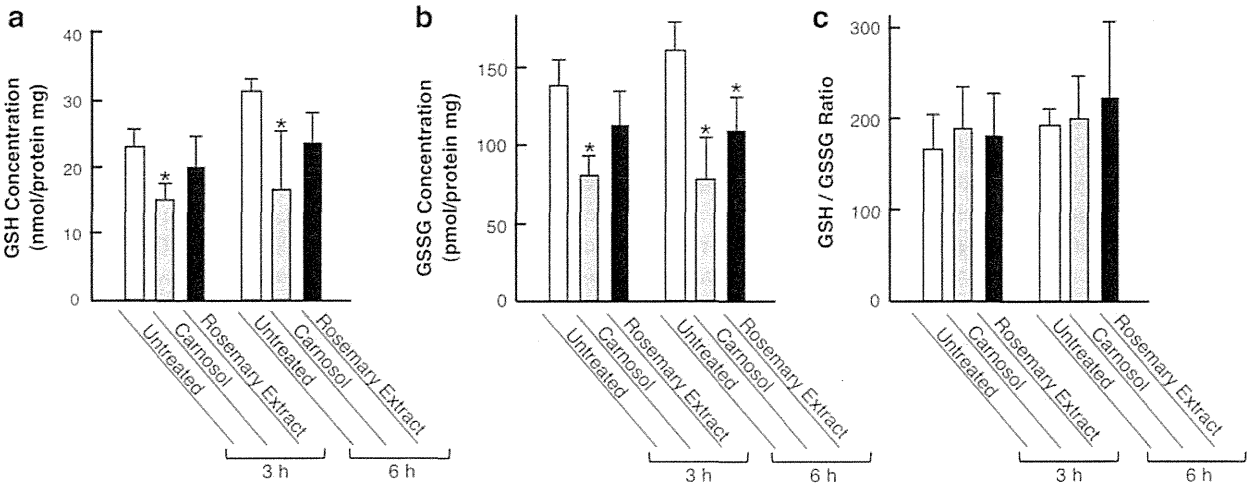

Fig. 4 Low level of intracellular glutathione in carnosol-treated ATL cells. ED cells were untreated (*white columns*) or treated with 40 μM carnosol (*gray columns*) or rosemary extract (*black columns*) for 3 or 6 h. Then intracellular GSH concentration (**a**), GSSG concentration (**b**), and GSH/GSSG ratio (**c**) were examined with GSH/GSSG assay kit. In carnosol-treated cells, the levels of GSH (*gray columns* in **a**) and GSSG (*gray columns* in **b**) were significantly lower as compared with the untreated cells (*white columns* in **a** and **b**). Data represent the mean ± SD from three experiments. * $p < 0.05$ (vs. untreated)

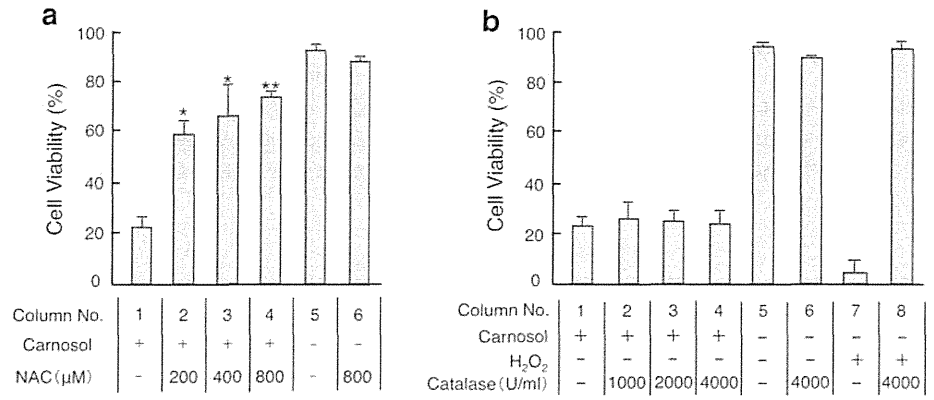

Fig. 5 Effects of exogenous NAC and catalase on efficiency of carnosol. ED cells were un-pretreated or pretreated with indicated concentrations of NAC (**a**) or catalase (**b**), and then 40 μM carnosol, equal volume of vehicle (DMSO), or 40 μM H_2O_2 was further added to media. After culture for 48 h, cell viability was examined by trypan blue staining. While cell viability was decreased by carnosol, it was restored dose-dependently by NAC (**a**) but not catalase (**b**). Data represent the mean ± SD from three experiments. * $p < 0.01$, ** $p < 0.001$ (columns 2–4 vs. column 1)

GSH and GSSG were significantly decreased in carnosol-treated cells as compared to untreated cells (white and gray columns in Fig. 4a, b). Also, in the cells treated with rosemary extract, they tended to be decreased (black columns in Fig. 4a, b). Meanwhile, the ratio of GSH and GSSG was not affected by carnosol (gray columns in Fig. 4c). These results indicated that the decrease of GSH in carnosol-treated cells was due to depletion of glutathione (both of GSH and GSSG), but not the acceleration of the oxidation of GSH to GSSG. Further to confirm the relationship of glutathione depletion, NAC, which is precursor of glutathione and used for the exogenous supplementation [21], was added to culture media of carnosol-treated ED cells (Fig. 5a). Cell viability was restored by NAC in dose-dependent manner (columns 2–4 in Fig. 5a). From these results, it was suggested that the apoptosis-inducing activity of carnosol in ATL cells was caused by the depletion of glutathione.

Apoptosis induced by polyphenols has been reported to be associated with the production of hydrogen peroxide (H_2O_2) in culture media by themselves [22, 23], suggesting that the efficiency may be artifact. Finally, we investigated whether the production of H_2O_2 in culture media is associated to apoptosis-inducing activity of carnosol or not, using catalase, a scavenger of H_2O_2 (Fig. 5b). In carnosol-treated cells, catalase addition did not restore the viability (columns 2–4 in Fig. 5b). The efficiency of carnosol was kept unchanged under the condition that extracellular H_2O_2 was scavenged by catalase. This suggests that carnosol does not act to the cells indirectly via H_2O_2 produced by itself.

Discussion

There has been increased interest in using herbs for the prevention and treatment of cancer [11, 12]. Many studies have reported the efficiency of herbs and their ingredients in various cancers [24, 25]. In this study, we found that rosemary extract and its ingredient carnosol have the activity that induces apoptosis in ATL cells and both action mechanisms are similar in the caspase activation, protein expression, and intracellular glutathione level. This suggests that carnosol is a contributory ingredient in ATL cell growth-inhibitory activity of rosemary, although possibilities of other ingredients such as carnosic acid and rosmarinic acid are still remained [17]. While this study is the first report on apoptosis-inducing activity of carnosol in ATL cells, the activity and cell cycle arrest have been also reported in the other cancer cells [13, 26, 27]. This suggests that the efficiency of carnosol is not specific to ATL, but common in various cancers. However, because this study is limited to the cell line analysis, the therapeutic efficacy and action mechanism of carnosol also need to be examined in xenograft animal models of ATL.

It is of interest why carnosol induced apoptosis in ATL cells. A depletion of intracellular glutathione has been described in a number of different apoptotic systems, with several studies showing that the depletion is the result of accelerated efflux rather than oxidation of GSH [28, 29]. In our system, glutathione depletion occurred in the cells treated with carnosol for short time of only 3 h. This rapid depletion implies the accelerated efflux of glutathione. Although it is still unclear how molecular mechanisms are present between carnosol and the efflux, the over-expression of Bcl-2, anti-apoptotic protein, has been reported to increase intracellular glutathione by suppressing the efflux from the cells [30]. Examining the relationship between carnosol and Bcl-2 might be necessary.

Living cells are always producing reactive oxygen species (ROS) such as H_2O_2 endogenously by the vital activity [29]. Glutathione prevents the oxidation of

intracellular components as a buffer against endogenous ROS by detoxifying H_2O_2. Relationship between oxidative modifications of signaling proteins and apoptosis has been also suggested [31]. Consequently, glutathione depletion by carnosol may increase oxidative damage to the proteins, triggering apoptotic signaling. A possibility is the oxidative damage to mitochondria [29]. Another possibility is the thioredoxin system, which functions as ROS scavenger like glutathione. Apoptosis signal-regulating kinase 1 (ASK1) forms the complex with thioredoxin under the normal condition [32, 33]. When thioredoxin is oxidized by ROS, ASK1 dissociates from the complex and then apoptotic signaling occurs. The increase of endogenous ROS by carnosol may be associated with the activation of apoptosis signal by ASK1. Because thioredoxin reductase 1 regulates redox status of thioredoxin, the increase of its expression in our experiments may reflect this hypothesis.

In several in vitro and in vivo experimental systems, carnosol has been reported to increase the level of glutathione and activity of glutathione-s-transferase (GST) that catalyzes the glutathione conjugation as one of the phase II enzymes [34–37]. Although the stimulation of glutathione metabolism by carnosol is conflicted with its depletion in our experimental system, several possibilities for the underlying mechanism of this confliction are considered. The first is the treatment conditions of carnosol. For example, when neuronal HT22 cells are treated with 5 μM carnosol for 24 h, the expression of phase II enzymes such as glutathione synthesis enzymes and several GST family enzymes is induced through Keap1/Nrf2 pathway [37]. In contrast, we used the eightfold higher concentration of carnosol (40 μM) and glutathione depletion was seen on the treatment for only 3 h. The second is the cell species. While carnosol has been reported to increase the glutathione level and GST activity as antioxidant in HT22 cells, hepatic cell, and rat liver [34–37], it also shows a large variety of action mechanisms against a number of different cell lines and cancer animal models [27]. Collectively, it is implied that concentrations and treatment times of carnosol and characters of cell lines contribute to the superiority of Keap1/Nrf2 and apoptosis pathways. To confirm this, examining the GST activity and expression of glutathione metabolism-related proteins in carnosol-treated ATL cells may be necessary. In addition, GST family enzymes have been also noticed as interesting target molecules for cancer therapy [38–40], supporting the importance of GST assay using not only ATL cell lines but also animal xenograft models of ATL.

Investigation into the action mechanism of carnosol in ATL cells may lead to the development of new therapeutic and preventive strategies for ATL. Studies using ATL cell lines and animal models are in progress, focusing on the target molecule of carnosol, the protein oxidization caused by glutathione depletion, and GST family enzymes.

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Conflict of interest None.

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CASE REPORT

Use of anti-tumor necrosis factor biologics in the treatment of rheumatoid arthritis does not change human T-lymphotropic virus type 1 markers: a case series

Kunihiko Umekita, Kazumi Umeki, Shunichi Miyauchi, Shiro Ueno, Kazuyoshi Kubo, Norio Kusumoto, Ichiro Takajo, Yasuhiro Nagatomo, and Akihiko Okayama

Division of Rheumatology, Infectious Diseases and Laboratory Medicine, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

Abstract

Anti-tumor necrosis factor (anti-TNF) biologics are effective in the treatment of rheumatoid arthritis (RA); however, it is still not clear whether this treatment promotes the development of malignancies such as lymphoma. Human T-lymphotropic virus type 1 (HTLV-1), which is a causative agent of adult T-cell lymphoma (ATL), is prevalent in Japan. Many HTLV-1-positive patients with RA are assumed to exist; however, there have thus far been no reports on the effect of anti-TNF biologics on HTLV-1-positive patients. We analyzed the response to treatment with anti-TNF biologics and change of HTLV-1 markers in two cases of RA. The two cases showed no response based on the European League Against of Rheumatism response criteria 60–96 weeks after administration of anti-TNF biologics (infliximab and etanercept). No signs of ATL were observed and HTLV-1 markers, such as proviral load and clonality of HTLV-1-infected cells, showed no significant change in either of two cases. Therefore, treatment with anti-TNF biologics did not induce activation of HTLV-1, although the effect on RA was not as effective as in HTLV-1-negative patients in this limited study. Further long-term study with a greater number of patients is necessary to clarify the safety and efficacy of anti-TNF biologics in HTLV-1-positive patients with RA.

Keywords

Anti-TNF biologics, Human T-lymphotropic virus type 1, Lymphoma, Rheumatoid arthritis, Viral infection

History

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Introduction

The effectiveness of biologics, which target inflammatory cytokines, has revolutionized the treatment of rheumatoid arthritis (RA); however, there are many concerns regarding potential adverse effects to be resolved. It is still not clear whether this treatment promotes the development of malignancies such as lymphoma, and epidemiological studies on this matter are ongoing [1]. RA has been considered a risk factor for the development of lymphoma. The most common lymphoma associated with RA is diffuse large B-cell type non-Hodgkin lymphoma [1, 2].

In Japan, human T-lymphotropic virus type 1 (HTLV-1), which is a causative agent of adult T-cell lymphoma (ATL), is prevalent, with the number of HTLV-1 carriers estimated to be 1.08 million individuals [3]. Therefore, the number of patients in Japan with RA who are also infected with HTLV-1 can be estimated at approximately 10,000. Moreover, a cohort study in Nagasaki prefecture, one of the highest areas of HTLV-1-prevalence in Japan, showed the rate of HTLV-1 infection in patients with RA to be higher than that of healthy blood donors [4]. HTLV-1 has been reported to be associated not only with ATL, but also with chronic inflammatory diseases, such as HTLV-1-associated myelopathy (HAM), uveitis, arthropathy, Sjogren syndrome (SS), and myositis [5, 6]. These chronic inflammatory diseases are often treated with corticosteroids and immunosuppressive agents. An important question is whether these treatments adversely affect HTLV-1 infection. In fact, progression to ATL in HTLV-1 carriers treated with the immunosuppressive agent tacrolimus after liver transplant has been reported [7]. Therefore, it is important to clarify whether treatment with biologics increases the risk of ATL in HTLV-1-positive patients. Higher proviral load (PVL), advanced age, family history of ATL, and first opportunity for HTLV-1 testing during treatment for other diseases have been reported as risk factors in the progression of ATL [8]. The clonal evolution of HTLV-1-infected cells has also been reported to occur before the onset of ATL [9]. In this study, we investigated the change of HTLV-1 markers (HTLV-1 proviral load and clonality of HTLV-1-infected cells) in two patients with RA before and after treatment with anti-TNF biologics. In addition, we evaluated RA disease activity to clarify the response to anti-TNF biologics for 60–96 weeks.

Patients and methods

Patients

Case 1

A 52-year-old woman with polyarthralgia, rheumatoid factor and anti-cyclic citrullinated peptide antibody (ACPA) was diagnosed with RA based on the 1987 American College of Rheumatology (ACR) classification criteria for RA 3 years prior to the present study [10]. She had pneumonitis and was treated with bucillamine

Correspondence to: Kunihiko Umekita, MD, PhD, Division of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan. Tel. +81-985-85-7284. Fax: +81-985-85-4709. E-mail: kumekita@fc.miyazaki-u.ac.jp

and salazosulfapyridine, but not with methotrexate (MTX). As these medicines proved inefficacious, we recommended biologics. She had dry mouth and tested positive for anti-Ro/SSA antibody. She was diagnosed with SS based on the American-European consensus criteria for SS [11]. She tested positive for HTLV-1 antibody. Her disease activity score in 28 joints (DAS28) based on the European League Against Rheumatism (EULAR) response criteria was 4.05. After obtaining informed consent, she was started on treatment with etanercept (ETN). Her RA seemed to respond at 12 weeks after treatment with ETN. However, after this time point, DAS28, the levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) gradually increased despite treatment with ETN (Figure 1-A). The number of swollen and tender joints and her modified health assessment questionnaire (mHAQ) did not improve significantly (Figure 1-A). As a result, she was judged to have had no response by EULAR response criteria [12].

Case 2

A 32-year-old woman with polyarthralgia, rheumatoid factor, and ACPA was diagnosed with RA based on the 1987 ACR classification criteria for RA [10] when she was pregnant 4 years prior to the present study. She tested positive for HTLV-1 antibody. After giving birth, treatment with MTX was started; however, she complained of worsening arthralgia. As MTX dosage could not be increased because of the adverse effect, she accepted our recommendation to use biologics. She also had dry eyes and dry mouth. She tested positive for anti-Ro/SSA antibody and was diagnosed with SS based on the American-European consensus criteria for SS [11]. Her DAS28 was 5.17. After obtaining informed consent, she was started on treatment with infliximab (IFX) in addition to MTX. Her DAS28 remained unchanged and high disease activity continued. For this reason, we changed IFX to ETN and the dosage of MTX was escalated; however, DAS28 and mHAQ at 60 weeks after the beginning of biologics remained high. Therefore, she was judged to be non-responsive to anti-TNF agents (Figure 2-A) [12]. The levels of serum CRP gradually decreased; however, the levels of CRP and ESR remained high, in this case. DAS28 after the IFX and ETN treatments was judged not to have changed in this case based on the absence of significant improvement in the number of swollen joints, the number of tender joints, the levels of inflammatory markers and her mHAQ.

Change of HTLV-1 PVLs and clonality of infected cells in peripheral blood

HTLV-1 PVLs and clonality of HTLV-1-infected cells in peripheral blood in these two cases were analyzed. Written informed consent was obtained, and the study protocol was approved by the institutional review board of University of Miyazaki.

The methods for measuring HTLV-1 PVL and clonality of HTLV-1-infected cells are described in detail elsewhere [13, 14]. In brief, peripheral blood mononuclear cells (PBMCs) were obtained from both cases and genomic DNA was isolated. Real-time polymerase chain reaction (PCR) using primers and probe for HTLV-1 *pX* regions and human RNase P gene were performed to evaluate PVL (HTLV-1 copies per 100 PBMCs).

Inverse-long PCR (IL-PCR) was performed with slight modification to determine the clonality of HTLV-1-infected cells in each case [14]. In brief, the genomic DNA was digested with *EcoR* I, and then self-ligated by T4 ligase following digestion with *Mlu* I. The resultant DNA was amplified using the LA Taq Hot start version (Takara Bio, Shiga, Japan) in triplicate. PCR products were analyzed using 0.6% agarose gel, and each band represented the individual HTLV-1-infected clone.

The PVL of Case 1 before ETN therapy was low at 0.2 copies per 100 PBMCs and continued at the same level until 96 weeks after the beginning of treatment (Figure 1-B). Analysis using IL-PCR in this case showed many bands with different sizes, suggesting oligoclonal expansion of HTLV-1-infected cells before ETN therapy, which did not change thereafter (Figure 1-C). The PVL of Case 2 before anti-TNF biologics was also low at 0.3 copies per 100 PBMCs. It increased slightly after the beginning of IFX; however, it had returned to nearly the same level by 60 weeks after the beginning of treatment (Figure 2-B). Analysis using IL-PCR also showed oligoclonal expansion of HTLV-1-infected cells before anti-TNF therapy, which remained unchanged to the end of observation (Figure 2-C). In addition, there were no signs, symptoms, or laboratory abnormalities, suggesting ATL or HAM during treatment with anti-TNF agents.

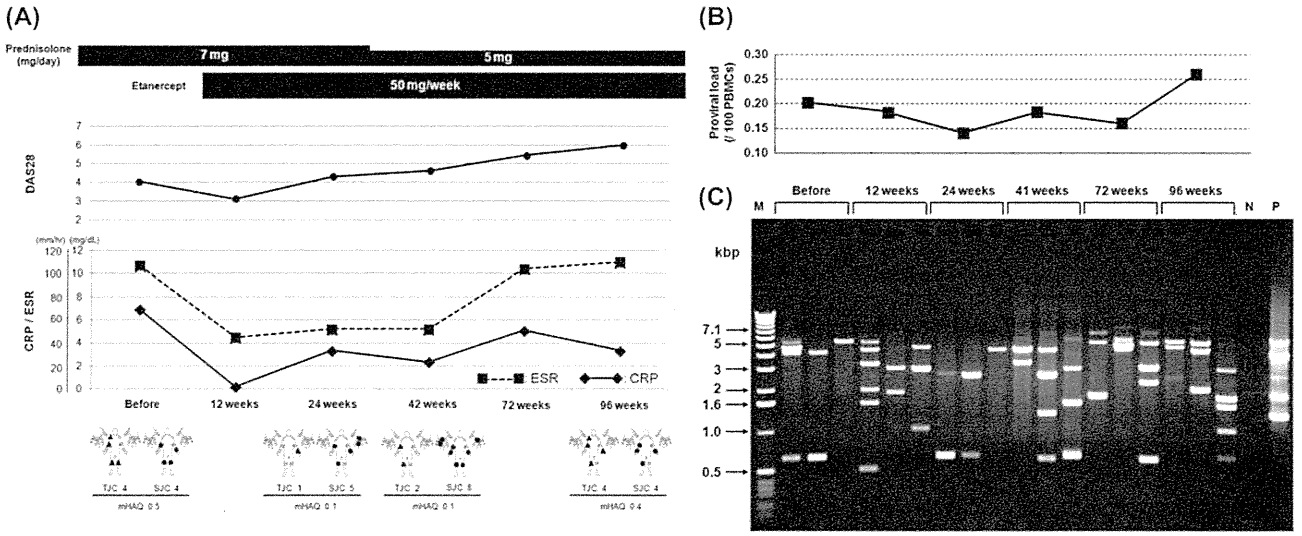

Figure 1. Case 1. Clinical course (A); time sequential analysis of HTLV-1 proviral loads (B) and detection of clonalities of HTLV-1-infected cells in Case 1 by IL-PCR (C). IL-PCR assays were performed in triplicate. ESR: erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score in 28 joints; TJC, tender joint counts (▲); SJC, swollen joint counts (●); and mHAQ, modified health assessment questionnaire. M, molecular weight marker; N, PBMCs from HTLV-1-negative subject as negative control; P, HTLV-1-infected cell line; and HUT102, as positive control.

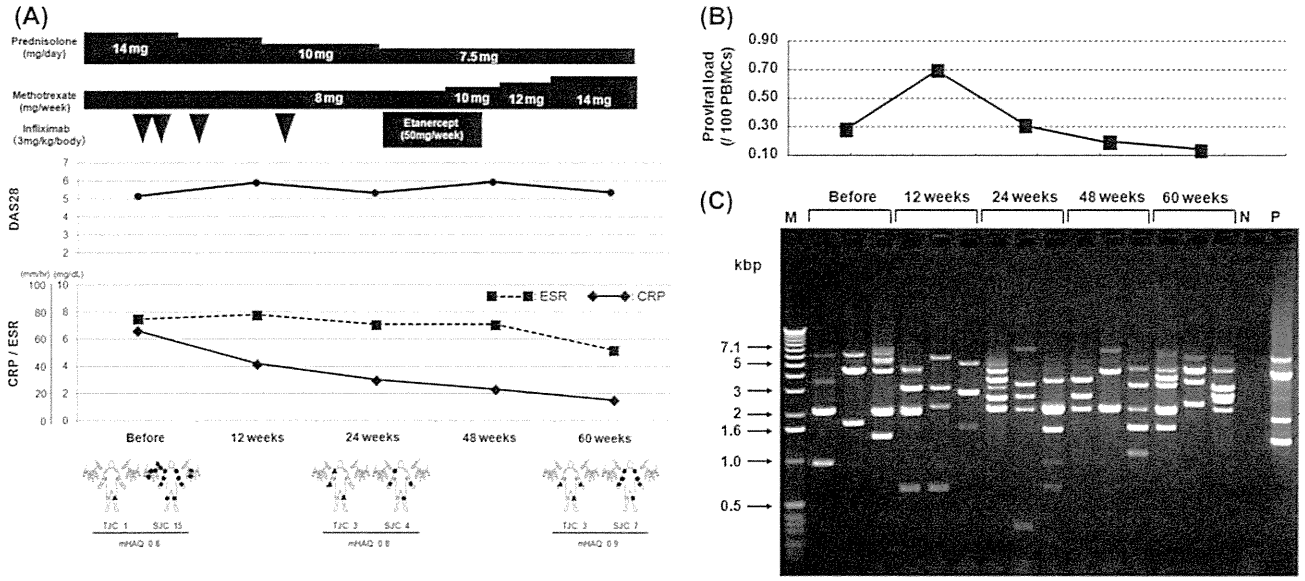

Figure 2. Case 2. Clinical course (A); time sequential analysis of HTLV-1 proviral loads (B) and detection of clonalities of HTLV-1-infected cells in Case 2 by IL-PCR (C). IL-PCR assays were performed in triplicate. The abbreviations used in this figure were same as those in Figure 1.

Discussion

We experienced two cases of RA associated with SS in HTLV-1 carriers. A high incidence of arthritis and SS in HTLV-1carriers has been reported in Japan [4, 15]. Sato et al. reported oligo-arthritis in the shoulders, wrists, and knees in HTLV-1 carriers in Japan [16]. The reported patients tended to have high inflammation and extra-joint symptoms such as SS. Both cases in the present study had features similar to the patients reported by Sato et al. At the same time, the present cases were ACPA positive and fulfilled both the definition of ACR (1987) and ACR/EULAR criteria for RA in 2010 [17].

As conventional disease-modifying anti-rheumatic drugs proved inefficacious, these two HTLV-positive cases were treated with anti-TNF therapy. According to the RECONFIRM study, 84.5% of Japanese patients with RA showed good or moderate response to treatment with IFX by EULAR response criteria [18]. On the basis of post-marketing surveillance, approximately 80% of Japanese patients with RA showed good or moderate response to treatment with ETN [19]. However, the two HTLV-1-positive cases of RA in the present study showed no response to anti-TNF agents.

It has been reported that patients with advanced RA and long disease histories showed low response rates to anti-TNF treatments; however, the duration of RA in the present study was only 3–4 years, and neither case was advanced (Steinbrocker's Classification stage II, data not shown). Both of the cases in the present study had SS in addition to RA; however, association of SS was not always a factor in RA resistance to anti-TNF therapy [20, 21].

Thus far, there have been no reports on the effectiveness of anti-TNF agents or other biologics in HTLV-1 carriers with RA. In an animal model experiment, transgenic mice carrying the HTLV-I genome showed strong expression of mRNA of IL-1 and IL-6, but not TNF [22]. There is a possibility that cytokines such as IL-1 and IL-6, but not TNF, are more important to RA activity in HTLV-1-positive patients than in HTLV-1-negative patients. In fact, one of the two patients in the present study was treated with IL-6 inhibitor (tocilizumab), thereafter, and showed a better response, although the period of observation was not sufficient for a definite conclusion to be reached (data not shown).

There have been no studies on the risk of progression to ATL in HTLV-1 carriers receiving biologics for the treatment of RA. Patients with RA are considered to be at high risk of lymphoma, mainly B-cell type [2]. Thus far, anti-TNF therapy has not been reported to be associated with lymphoma [23, 24]. However, re-activation of Epstein–Barr virus has been reported to be associated with MTX-related lymphoproliferative diseases in RA [2]. In addition, progression to ATL in HTLV-1 carriers who received the immunosuppressive agent tacrolimus after liver transplant has been reported [7]. Therefore, it is important to clarify whether treatment of RA with the biologics increases the risk of ATL.

High HTLV-1 PVL has also been reported in patients with various connective tissue diseases [25]. High PVL, greater than 4–5 copies per 100 PBMCs, has been reported to be associated with progression to ATL in carriers [8, 9]. Therefore, HTLV-1 PVL was monitored during treatment with anti-TNF reagents in the present study. HTLV-1 PVL was low at less than 0.5 copies per 100 PBMCs before treatment with anti-TNF agents in both the present cases. In fact, we thought that the two cases in the present study were not in the high-risk group for the development of ATL and could choose anti-TNF agents for their treatment. Fortunately, even after the beginning of treatment, the levels of PVL showed no significant increase.

In addition, the clonal evolution of HTLV-1-infected cells has also been reported to occur before the onset of ATL [9]. We also monitored the clonality of HTLV-1-infected cells, and neither case showed significant change. In addition, there were no signs (lymphadenopathy, eruption), symptoms or laboratory abnormalities (abnormal lymphocytes on blood smear) related to the progression of ATL. Therefore, no data suggesting the progression of HTLV-1-related diseases such as ATL were observed for 60–96 weeks after anti-TNF therapy in the present cases.

This study has a number of limitations. The number of patients was small, and the observation period was short. Generally, expansion into ATL from HTLV-1 exposure requires a period of 50–60 years. Therefore, we have to follow patients over a longer period of time to see the actual incidence of ATL among them. Because RA patients with high PVL were not included in this study, we could not say whether such patients would show the same course. From this point of view, future study should include RA patients with various levels of PVL ranging from low to high.

In conclusion, two HTLV-1-positive patients with RA were treated with anti-TNF agents. They had high RA disease activity and did not exhibit a good response to anti-TNF agents. Virological study on HTLV-1 infection showed no data suggesting that progression of ATL was promoted by these treatments. Further study including a greater number of patients is necessary to clarify whether these results can be generalized or whether HTLV-1 screening is necessary for RA patients before treatment with biologics.

Conflict of interest

None.

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INTERNAL MEDICINE

□ CASE REPORT □

Development of Adult T-cell Leukemia in a Patient with Rheumatoid Arthritis Treated with Tocilizumab

Hideki Nakamura[1], Yukitaka Ueki[2], Shigeki Saito[3], Yoshiro Horai[1], Takahisa Suzuki[1], Tomoki Naoe[3], Katsumi Eguchi[4] and Atsushi Kawakami[1]

Abstract

Tocilizumab (TCZ) was administered from 2004 to 2008 in a 52-year-old woman with rheumatoid arthritis (RA) refractory to methotrexate (MTX) as a clinical trial. TCZ therapy with MTX was resumed in March 2009 due to exacerbation of RA. The patient was an human T-lymphotropic virus type I (HTLV-I) carrier, and, in April 2011, a peripheral blood smear showed many atypical lymphocytes, thus leading to a diagnosis of adult T-cell leukemia (ATL). Complete remission of ATL was achieved with a standard therapeutic regimen.

Key words: adult T-cell leukemia, rheumatoid arthritis, tocilizumab

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Introduction

The treatment of rheumatoid arthritis (RA) has drastically changed over the past decade with the introduction of biological agents (1). A humanized anti-interleukin 6 receptor monoclonal antibody, tocilizumab (TCZ), has been verified to be effective for treating RA (2). In general, treatment with TCZ is reported to be safe, although the most frequent adverse event observed in TCZ-treated patients is infection (3, 4). In addition, increased incidences of malignancy in patients treated with TCZ compared with that observed in conventional RA patients or the general population has not been reported in clinical trials (3, 4). On the other hand, there may be endemic problems among RA patients treated with biological agents. Nagasaki Prefecture, located in the western part of Kyushu Island in Japan, is an area in which human T-cell leukemia virus type 1 (HTLV-I) is endemic (5). Therefore, one such endemic problem may be the development of adult T-cell leukemia (ATL) in HTLV-I carriers with RA treated with biological agents. Iwanaga et al. demonstrated that both a high baseline proviral load and the presence of HTLV-I infection during treatment of other diseases are independent risk factors for the development of ATL (6), the latter being described in the present case. We herein report the first case of ATL developing during treatment for RA with TCZ.

Case Report

A 52-year-old woman with polyarthritis visited Sasebo Chuo Hospital in September 2000 with a diagnosis of RA. Since methotrexate (MTX) and prednisolone (PSL) were not effective in attaining a low disease activity, the patient was enrolled in a clinical trial (MRA012JP; SAMURAI followed by the long-term MRA214JP trial) of an anti-IL-6 receptor monoclonal antibody in the beginning of January 2004 (Fig. 1). The patient's laboratory findings at the time of enrollment showed a hemoglobin level of 10.6 g/dL, a total leukocyte count of 4,400/mm^3 with no abnormal lymphocytes and a platelet count of 22.3×10^4/mm^3. Rheumatoid factor (22.4 IU/mL: normal range <14) was positive without elevation of anti-double-stranded DNA antibodies or anti-SS-A/Ro antibodies. The patient exhibited a high level of disease activity with a disease activity score (DAS) 28-ESR of 6.47 points. TCZ monotherapy (MRA012JP; SAMURAI

[1]Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University Graduate School of Biomedical Sciences, Japan, [2]Rheumatic and Collagen Disease Center, Sasebo Chuo Hospital, Japan, [3]Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Japan and [4]Department of Internal Medicine, Sasebo City General Hospital, Japan
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Correspondence to Dr. Hideki Nakamura, nhideki@nagasaki-u.ac.jp

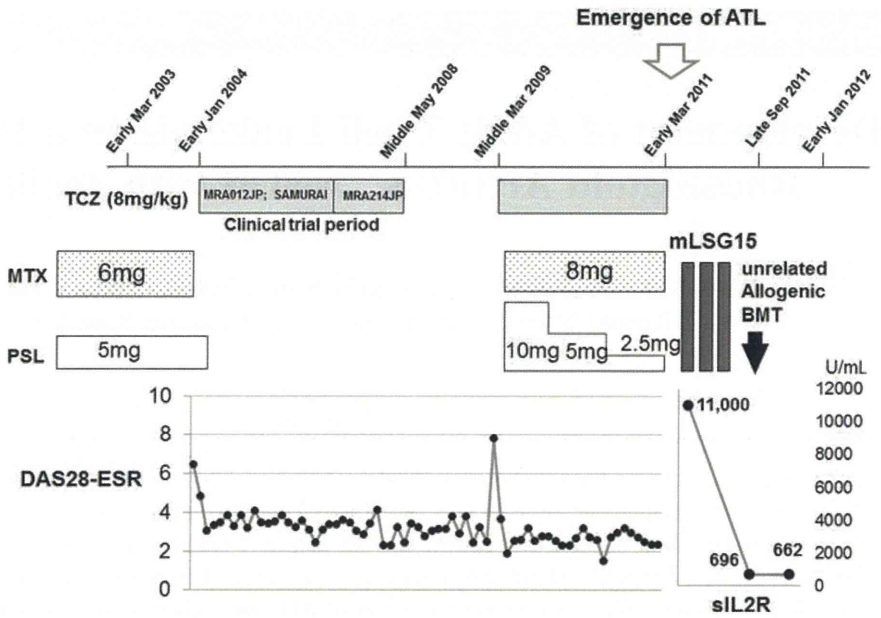

Figure 1. **The patient's clinical course following the clinical trial of tocilizumab (TCZ). When the diagnosis of rheumatoid arthritis (RA) was determined, treatment with 6 mg of methotrexate (MTX) and prednisolone (PSL) was initiated. The patient was then enrolled in a clinical trial of TCZ. Although MTX and PSL were discontinued after a low level of disease activity was attained, 8 mg/kg of TCZ coupled with 8 mg of MTX and PSL was administered again, as of the exacerbation of RA in March 2009. Since adult T-cell leukemia (ATL) appeared in March 2011 after MTX and TCZ were discontinued, the mLSG chemotherapy protocol was introduced, followed by unrelated allogenic bone marrow transplantation. Although a few opportunistic infections were observed following the administration of these regimens, no recurrence of ATL was detected. BMT: bone marrow transplantation**

followed by the long-term MRA214JP trial) at a dose of 8 mg/kg was effective for achieving a low level of disease activity, according to DAS28-ESR, and remained effective until the completion of the above clinical trial in the middle of May 2008 (Fig. 1). In March 2009, 8 mg/kg of TCZ was restarted with the administration of MTX and PSL due to exacerbation of polyarthritis. At that time, the patient was initially screened for anti-HTLV-I antibodies and found to be positive (10.0 C.O.I.). Following the readministration of TCZ for two years, many ATL-like cells with 44% atypical lymphocytes without obvious signs of ATL were detected on a peripheral blood smear performed at the administration of TCZ late in April, 2011. The patient was diagnosed with chronic-type ATL with monoclonal integration of HTLV-I proviral DNA in peripheral blood mononuclear cells verified by a Southern blot analysis (data not shown); however, a tendency toward elevation of lactose dehydrogenase indicated conversion to acute-type disease, regardless of the absence of skin rashes and lymph node enlargement. Because elevation of the total leukocyte count to 14,100/mm^3 with 66% atypical cells was observed (Fig. 2, left panels) with double-positive findings for CD4 and CD25 in the peripheral blood on flow cytometry (Fig. 2, right panel), TCZ and MTX were discontinued. Following the administration of a modified version of the Lymphoma Study Group 15 chemotherapy protocol (7) containing three regimens described in the Japan Clinical Oncology Group (JCOG) 9303 in addition to allogeneic bone marrow transplantation (BMT) from an human leukocyte antigen (HLA)-matched unrelated donor, remission was achieved followed by normalization of the soluble interleukin-2 receptor (sIL-2R) level. No recurrence of ATL has since been observed (Fig. 1). Tacrolimus was administered as an immunosuppressant after transplantation following the discontinuation of glucocorticoids. Subsequently, no exacerbation of the rheumatoid arthritis disease activity or elevation of the C-reactive protein (CRP) level was observed.

Discussion

In the present case, ATL emerged following treatment with MTX for 11 years and TCZ for six years. The use of immunosuppressive agents may be related to the development of ATL. For example, ATL was detected in a systemic lupus erythematosus patient treated with steroids and cytotoxic agents (8). Another case of adult T-cell leukemia/lymphoma (ATLL) was reported under the administration of MTX in a patient with disseminated psoriasis (9). The incidence is low; however, it is known that the reactivation of hepatitis B virus occurs during immunosuppressive therapy

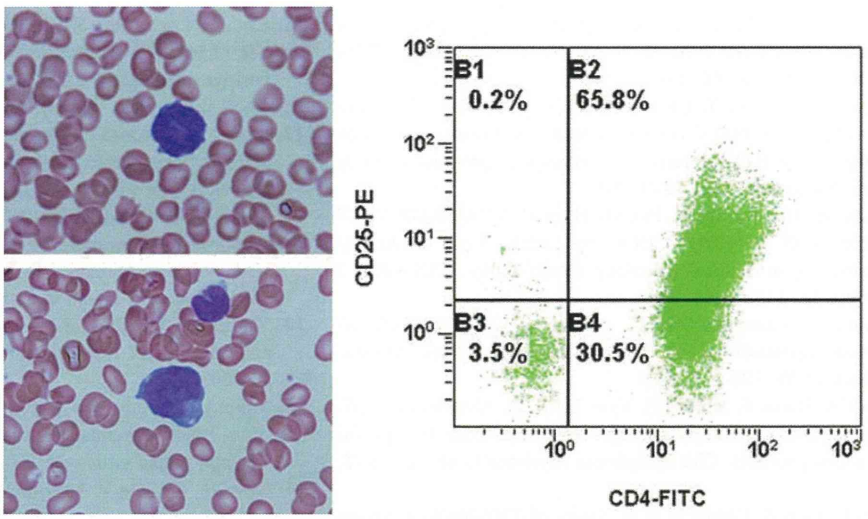

Figure 2. Presence of abnormal lymphocytes on a peripheral blood smear. When adult T-cell leukemia (ATL) was diagnosed, abnormal lobulated lymphocytes were detected in the peripheral blood. The neoplastic cells co-expressed CD4 and CD25 (Left: high amplification of the peripheral blood smear; right: flow cytometry dot plots in the peripheral blood).

for rheumatic diseases, including the administration of biological agents to treat RA (10, 11). Therefore, although we are not able to show direct evidence that the proliferation of HTLV-I-infected cells observed in this case was induced during treatment, it can be speculated that the administration of TCZ in conjunction with MTX accelerated the increase in the copy number of HTLV-I, resulting in ATL. Although it has been previously shown that leukemic cells freshly isolated from patients with ATL have the potential to produce high levels of IL-6 (12), there have been no reports of a direct relationship between the inhibition of IL-6 and the administration of TCZ with respect to the emergence of ATL until now. Rather, HTLV-I infection *per se* has been reported to have the potential to cause opportunistic infections, such as strongyloidiasis (13), suggesting that immunological impairment related to HTLV-I infection is accelerated by the administration of biologic agents.

The majority of HTLV-I carriers remain asymptomatic throughout their lives, with an estimated lifetime risk of developing ATL of approximately 2.5% to 5% (14, 15). Therefore, prospective and/or retrospective studies are needed to explore the risk of the development of ATL/ATLL during therapy with biological agents and/or immunosuppressive drugs, including MTX, in patients with RA who are HTLV-I carriers, especially in HTLV-I-endemic areas.

Another important point is the influence of BMT on the disease activity of RA. The efficacy of BMT in a murine model mimicking RA has been previously reported (16), demonstrating the inhibition of joint destruction and bone absorption by BMT. In human RA patients, the efficacy of autologous transplantation has also been reported (17). However, clinicians should allow for the use of premedication, such as high-dose cyclophosphamide, that may modify the RA disease activity. In that report (17), the application of BMT in patients with RA was shown to be limited to cases refractory to ordinary therapies, including biologics. Additionally, the administration of tacrolimus may have exerted an influence on the clinical course of RA in the present case.

This is the first report of the emergence of ATL during treatment with TCZ and MTX in a patient with RA. The medium-term efficacy and safety of TCZ have been reported, and, in general, no increases in the incidence of malignancy, including hematologic malignancy, have been found. However, in some specific situations, such as HTLV-I carriers, the pharmacologic actions of TCZ and MTX may affect the life cycle of HTLV-I, inducing reactivation. Accumulating further ATL/ATLL cases occurring in association with treatment using immunosuppressive agents is therefore necessary.

The authors state that they have no Conflict of Interest (COI).

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ORIGINAL ARTICLE

HTLV-I virological and histopathological analysis in two cases of anti-centromere-antibody-seropositive Sjögren's syndrome

Hideki Nakamura · Yoshiro Horai · Ayuko Tokuyama · Shunsuke Yoshimura · Hideki Nakajima · Kunihiro Ichinose · Satoshi Yamasaki · Tatsufumi Nakamura · Tomayoshi Hayashi · Atsushi Kawakami

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Abstract
Introduction The aim of this study was to show the clinical and pathological characteristics of anti-centromere-antibody (ACA)-seropositive Sjögren's syndrome (SS) in two anti-human T-cell leukemia virus type I (HTLV-I)-seropositive patients.
Methods One patient was an HTLV-I carrier whereas the other was diagnosed with HTLV-I-associated myelopathy (HAM). Background data including serum HTLV-I titers, viral loads, and cytokine profiles were recorded. Azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry of the labial salivary glands (LSGs) and a muscle biopsy specimen from the HAM patient were performed.
Results Serum transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), and HTLV-I viral load were high in the HAM-SS patient compared with the HTLV-I carrier. Fibrous change in LSG was prominent in the HAM-SS patient. Although TGF-β expression was similar in the two patients, expression of HTLV-I-related proteins including p12, p28, group-specific antigen (GAG), and nuclear factor kappa-B (NF-κB) in the LSG were dominantly detected in the HAM-SS patient. Frequency of TGF-β staining in HTLV-I-seropositive SS patients without ACA, HTLV-I-seronegative SS patients with ACA, and HTLV-I-seronegative SS patients without ACA was lower than that of the previous two patients.
Conclusion A high HTLV-I viral load in situ is supposed to promote the production of cytokines, especially TGF-β, resulting in the fibrous change of LSG in ACA-seropositive SS patients.

H. Nakamura (✉) · Y. Horai · A. Tokuyama · K. Ichinose · S. Yamasaki · A. Kawakami
Unit of Translational Medicine,
Department of Immunology and Rheumatology,
Nagasaki University Graduate School of Biomedical Sciences,
1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8501, Japan
e-mail: nakamura_hideki911@yahoo.co.jp;
nhideki@nagasaki-u.ac.jp

S. Yoshimura · H. Nakajima
Unit of Translational Medicine, Department of Neurology,
Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

T. Nakamura
Department of Molecular Microbiology and Immunology,
Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

T. Hayashi
Department of Pathology, Nagasaki University Hospital,
Nagasaki, Japan

Keywords HTLV-I infection · Anti-centromere antibody · Sjögren's syndrome · Cytokine

Abbreviations

ACA	Anti-centromere antibody
ANA	Anti-nuclear antibody
CSF	Cerebrospinal fluid
HAM	HTLV-I-associated myelopathy
HTLV-I	Human T-cell leukemia virus type I
IFN-γ	Interferon gamma
MNC	Mononuclear cell
LSG	Labial salivary gland
SS	Sjögren's syndrome
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha

Introduction

Human T-cell leukemia virus type I (HTLV-I) is known to be one of the causative agents of Sjögren's syndrome (SS) [1, 2]. Our previous epidemiologic studies show a close association between HTLV-I and SS [3, 4]. In addition, we found a significantly high prevalence of SS in patients with HTLV-I-associated myelopathy (HAM) [3, 5]. On the other hand, anti-centromere antibody (ACA) is known as a second class of autoantibodies in SS patients [6, 7]. Our previous report revealed that ACA is detected in only 4 % of HTLV-I-seropositive SS cases, demonstrating that HTLV-I might not be involved in the pathogenesis in ACA-seropositive SS patients [8]. However, if HTLV-I infection coincidently occurs in ACA-seropositive SS patients, the influence of ACA on HTLV-I-associated SS might become obvious. In this study, we report two cases of ACA-seropositive SS patients who were also seropositive for anti-HTLV-I antibody. One patient was complicated with HAM, whereas the other was an HTLV-I carrier. The variation in HTLV-I viral road in these patients appears to explain the differences in labial salivary gland (LSG) histopathology and cytokine profile.

Patients and methods

Patients

Case 1

This was a 61-year-old female patient who complained of sicca symptoms. Both ACA and anti-HTLV-I antibody measured by chemiluminescent enzyme immunoassay (CLEIA) were highly positive, as shown in Table 1. As no other symptoms or signs, including in the neuromuscular systems, were found in this patient, she was classified as an HTLV-I carrier.

Case 2

A 57-year-old female patient who complained of sicca symptoms and myalgia was diagnosed with HAM based on the diagnostic guidance for HAM determined by the Ministry of Health, Labour and Welfare. She had slowly progressive and symmetrical pyramidal tract damage with positive anti-HTLV-I antibody in both serum and cerebrospinal fluid (CSF). Antibodies against gp46, p53, p24, and p19 of HTLV-I in CSF were all positive. Serum ACA was also positive at a high titer (Table 1). She also suffered from inflammatory myopathy as evidenced by the elevation of muscle enzymes and by magnetic resonance imaging and muscle biopsy findings.

Both patients were diagnosed with SS according to the revised criteria [9], as proposed by the American–European Consensus Group. In both cases, HTLV-I viral roads in sera and serum cytokines including tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and transforming growth factor beta (TGF-β) were measured. For comparison, we studied the three groups of patients: (1) HTLV-I-seropositive SS patients without ACA, (2) HTLV-I-seronegative SS patients with ACA, and (3) HTLV-I-seronegative SS patients without ACA with respect to TGF-β immunostaining of LSG (four patients each in three groups).

LSG biopsy

LSG biopsy from the lower lip was performed under local anesthesia in SS patients. Informed consent to use biopsy samples was obtained from all participating patients at the commencement of the study. The study was conducted with the approval of the human ethical committee of our institution. The classifications of Chisholm and Mason [10] were used to determine the severity of mononuclear cell (MNC) infiltration.

Azan–Mallory staining and immunohistochemistry of labial salivary glands

Formalin-fixed, paraffin-embedded sections (3-μm thick) from the LSGs of these ACA-seropositive SS patients were used for azocarmine with aniline blue (Azan)–Mallory staining and immunohistochemistry. The sections were then stained using the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan) with mouse anti-human CD4, CD8, CD20, and CD68 antibodies (DakoCytomation, Glostrup, Denmark), mouse anti-HTLV-I [p19, p28, and group-specific antigen (GAG)] antibody (Chemicon International Inc., Temecula, CA, USA), mouse anti-nuclear factor kappa B (NF-κB) p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse anti-TGF-β antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA). Briefly, endogenous peroxidase was inactivated in a 3 % hydrogen peroxide (H_2O_2) solution after microwave epitope retrieval. These sections were then blocked with 5 % normal horse serum, followed by incubation with monoclonal and polyclonal antibodies in a humid chamber for 60 min at room temperature. After incubation, all sections, including the negative control sections, were treated with peroxidase-conjugated secondary antibodies for 30 min. The color was developed by soaking the sections in 3.3′-diaminobenzidine (DAB) and H_2O_2 for 10 min, followed by counterstaining by soaking the sections in hematoxylin solution. Negative

Table 1 Background information and serum data of the human T-cell leukemia virus type I (HTLV-I)-associated anti-centromere antibody (ACA)-seropositive patients

	Case 1 HTLV-I carrier with ACA-seropositive SS	Case 2 HAM with ACA-seropositive SS
Age and gender	61 years old, female	57 years old, female
Xerostomia	Positive	Positive
Xerophthalmia	Positive	Negative
Schirmer test (right/left mm; <5 mm: positive)	5/4	11/11
Saxon test (g/2 min; <2 g: positive)	1.47	2.7
ANA: pattern	160×, centromere	640×, centromere
Anti-SS-A antibody: normal 10–30 U/ml	0.7	0.9
Anti-SS-B antibody: normal 15–25 U/ml	0.9	0.5
ACA: normal <16 index	172.8	165.0
IgG: normal 870–1,700 mg/dl	1,712	1,623
Rheumatoid factor: normal <15 IU/ml	11.4	17.0
Sialography[a] (Rubin and Holt)	Stage 1	Stage 2
Lip biopsy grade[b] (Chisholm and Mason)	3	3
LST (cpm)	105,936/617	184,859/19,319
PHA(+)/no stimulation		
LST (cpm)	160,934/617	102,299/19,319
ConA(+)/no stimulation		
Serum anti-HTLV-I antibody: normal <1.0 COI	>45	>45
Serum viral load (copies/10^4 cells)	<53	373
Serum TNF-α: normal 0.6–2.8 pg/ml	1.0	2.9
Serum IFN-γ: normal <0.1 IU/ml	<0.1	<0.1
Serum TGF-β: normal 1.56–3.24 ng/ml	2.76	12.6

Anti-SS-A Ab and anti-SS-B Ab (Mesacup SS-A/Ro test and SS-B/La test; Medical and Biological Laboratories, Nagoya, Japan) and ACA (Mesacup-2 test CENP-B; Medical and Biological Laboratories, Nagoya, Japan) were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Serum anti-HTLV-I antibody was measured by chemiluminescent enzyme immunoassay, and HTLV-I viral load was measured by the FastStart DNA Master Hybridization probe method. Serum TNF-α and TGF-β were measured by ELISA. Serum IFN-γ was measured by enzyme immunoassay. Data shown represent the period before treatments with agents such as glucocorticoids or immunosuppressive agents

SS Sjögren's syndrome, *ANA* anti-nuclear antibody, *COI* cutoff index, *ConA* concanavalin A, *cpm* count per minute, *HAM* HTLV-I-associated myelopathy, *Ig-G* immunoglobulin G, *LST* lymphocyte stimulation test, *PHA* phytohemagglutinin, *TNF* tumor necrosis factor, *IFN* interferon *TGF* transforming growth factor

[a] Sialography grading was determined by Rubin and Holt. Stages 1 and 2 represent punctate and globular patterns, respectively

[b] Grading defined by Chisholm and Mason: the presence of at least one focus of mononuclear cells per 4 mm^2 section = grade 3

control sections were treated with mouse immunoglobulin (Ig)G1.

Results

Clinical and serological data with cytokine profile

As shown in Table 1, a high ACA titer was detected in both patients. Serum IgG was almost normal, which is characteristic in ACA-seropositive SS patients [6]. As patient 2 was diagnosed with HAM, spontaneous proliferation of MNCs was significantly higher than in patient 1. Serum HTLV-I viral load was 373 copies/10^4 cells in patient 2, which is obviously higher than in patient 1 (<53 copies/10^4 cells). Serum TNF-α and TGF-β levels in patient 2 were increased compared with those in patient 1, although serum IFN-γ in both patients was within normal limits.

Azan–Mallory staining and immunohistochemical analysis

MNC infiltration was similar in both patients; however, Azan–Mallory staining showed a stronger fibrosis in patient 2 than in patient 1 (Fig. 1). In patient 2, TGF-β was highly stained in infiltrating MNCs and vessels, except in ductal and acinar cells. TGF-β staining, although weaker than MSG, was also performed in the muscle in patient 2. Accordingly, infiltration of CD4+ lymphocytes, which were dominant compared with CD20 and CD68, was shown in the LSGs of both patients (Fig. 2). Although CD8+ lymphocytes were also scattered in LSGs, CD4+