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RESEARCH ARTICLE

Carnosol, rosemary ingredient, induces apoptosis in adult T-cell leukemia/lymphoma cells via glutathione depletion: proteomic approach using fluorescent two-dimensional differential gel electrophoresis

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Abstract Adult T-cell leukemia/lymphoma (ATL) is a fatal malignancy caused by infection with human T-lymphotropic virus type-1 and there is no accepted curative therapy for ATL. We searched for biological active substances for the prevention and treatment of ATL from several species of herbs. The ATL cell growth-inhibitory activity and apoptosis assay showed that carnosol, which is an ingredient contained in rosemary (*Rosmarinus officinalis*), induced apoptosis in ATL cells. Next, to investigate the apoptosis-inducing mechanism of carnosol, we applied proteomic analysis using fluorescent two-dimensional differential gel electrophoresis and mass spectrometry. The proteomic analysis showed that the expression of reductases, enzymes in glycolytic pathway, and enzymes in pentose phosphate pathway was increased in carnosol-treated cells, compared with untreated cells. These results suggested that carnosol affected the redox status in the cells. Further, the quantitative analysis of glutathione, which plays the central role for the maintenance of intracellular redox status, indicated that carnosol caused the decrease of glutathione in the cells. Further, *N*-acetyl-L-cystein, which is precursor of glutathione, canceled the efficiency of carnosol. From these results, it was suggested that the apoptosis-inducing activity of carnosol in ATL cells was caused by the depletion of glutathione.

Keywords ATL · Rosemary · Carnosol · Apoptosis · Glutathione depletion

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a fatal malignancy caused by infection with human T-lymphotropic virus type-1 (HTLV-1) [1]. Ten to twenty million people is infected worldwide with HTLV-1 and about 2.5–5 % of

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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

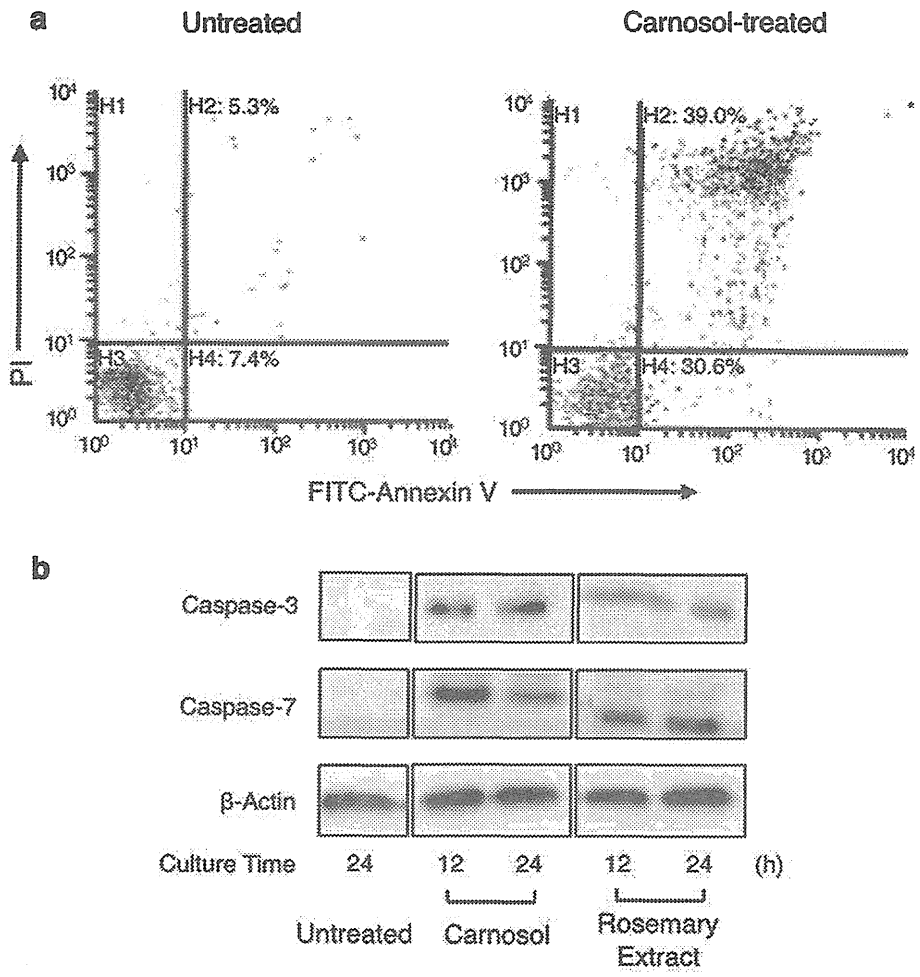

Fig. 1 Carnosol, a rosemary ingredient, induces apoptosis in ATL cells. ED cells were untreated or treated with 40 μM carnosol and then double-stained with PI and FITC-annexin V followed by flow cytometry (**a**). Annexin V-positive cells were increased by carnosol treatment. The activated forms of caspase-3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

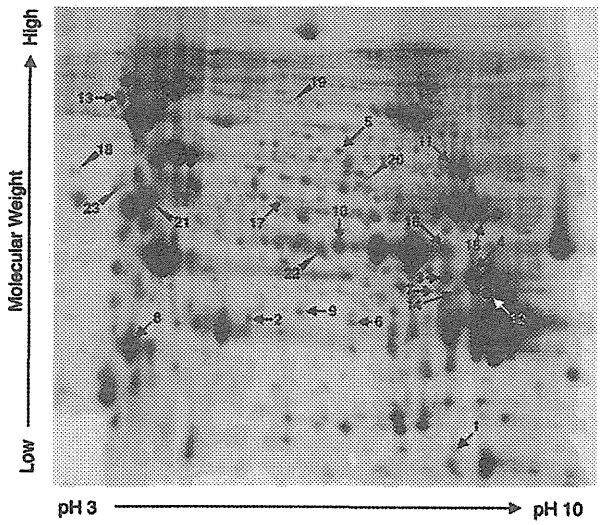

Fig. 2 Protein spots differentially expressed in carnosol-treated ED cells. Protein extracts from the cells that were untreated or treated with 40 μM carnosol for 24 h were fluorescent-labeled and then separated by 2D-DIGE followed by detection of protein spots with fluorescent imager. The fluorescent 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

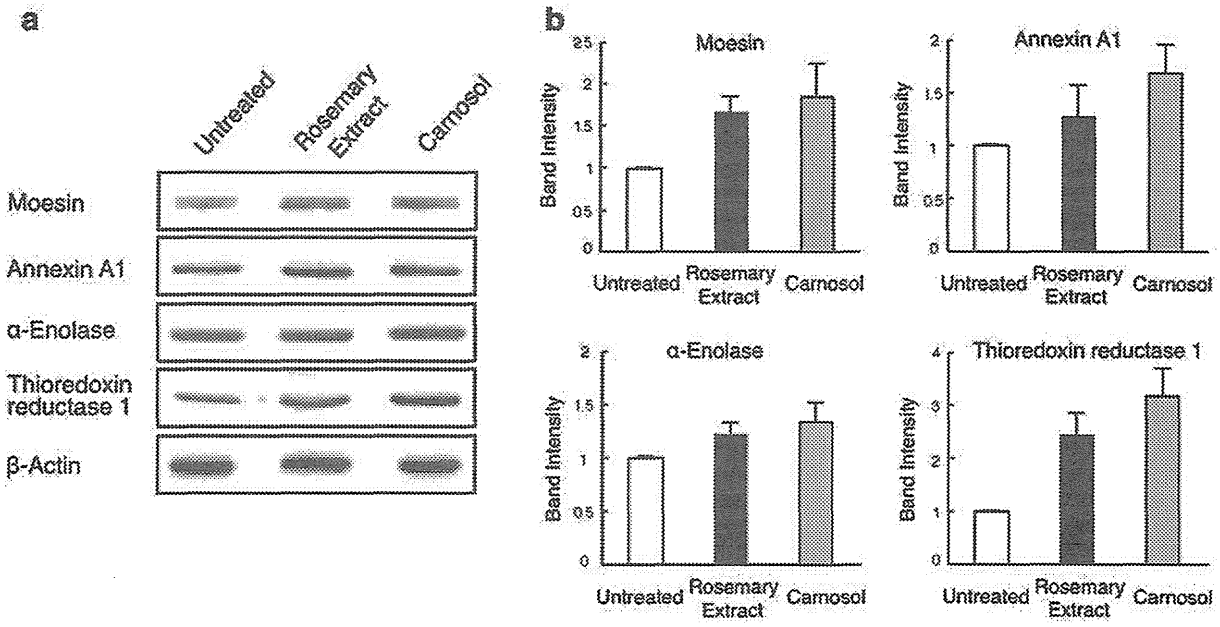

Fig. 3 Confirmation of expression of several identified proteins by western blotting. Protein extracts from the cells that were untreated or treated with 40 mg/ml rosemary extract or 40 μM carnosol for 24 h were electrophoresed and then blotted with antibody against moesin, annexin A1, α-enolase, thioredoxin 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

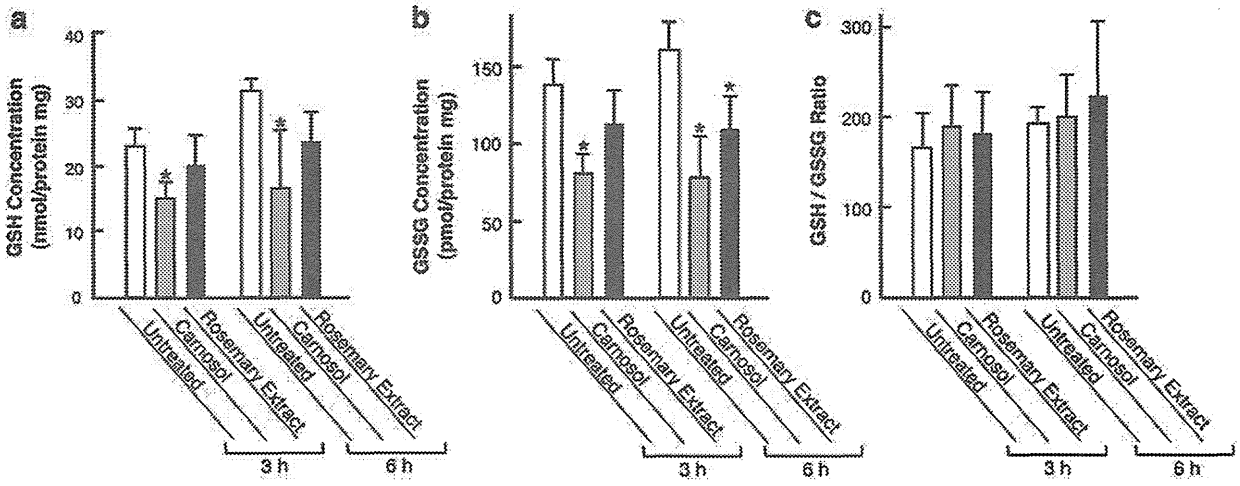

Fig. 4 Low level of intracellular glutathione in carnosol-treated ATL cells. ED cells were untreated (*white columns*) or treated with 40 μM carnosol (*gray columns*) or rosemary extract (*black columns*) for 3 or 6 h. Then intracellular GSH concentration (**a**), GSSG concentration (**b**), and GSH/GSSG 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)

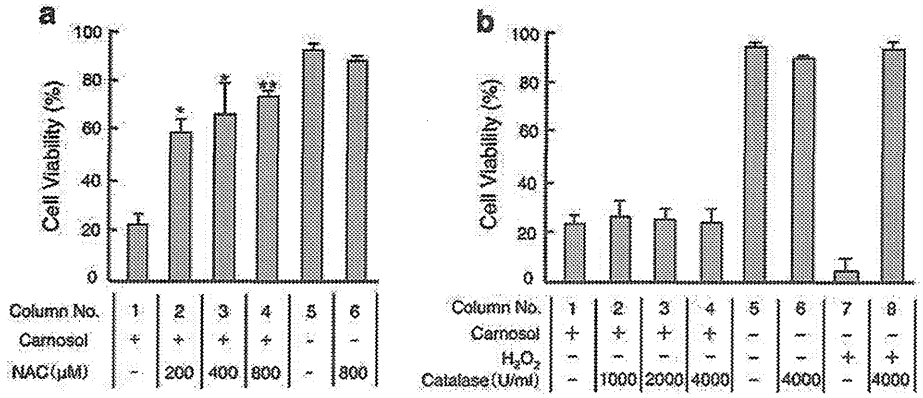

Fig. 5 Effects of exogenous NAC and catalase on efficiency of carnosol. ED cells were un-pretreated or pretreated with indicated concentrations of NAC (**a**) or catalase (**b**), and then 40 μM carnosol, equal volume of vehicle (DMSO), or 40 μM H_2O_2 was further added to media. After culture for 48 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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Brief Report

Treatment with anti-tumor necrosis factor biologics in human T-lymphotropic virus type 1 positive patients with rheumatoid arthritis

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Abstract

Objectives: To investigate the response to and safety of anti-tumor necrosis factor (TNF) therapy in human T-lymphotropic virus type 1 (HTLV-1) positive patients with rheumatoid arthritis (RA).

Methods: Therapeutic response was evaluated in 10 HTLV-1 positive and 20 negative patients with RA (sex- and age- matched) at three months after the beginning of anti-TNF therapy using the European League Against Rheumatism improvement criteria. As secondary endpoints, discontinuation rate of anti-TNF therapy and safety, especially the development of adult T-cell leukemia (ATL), were evaluated over a 2-year period.

Results: Significantly higher baseline levels of C-reactive protein (CRP) were observed in HTLV-1 positive patients than in HTLV-1 negative patients (P= 0.0003). Response rate to anti-TNF therapy was lower in HTLV-1 positive patients than in HTLV-1 negative patients. The median levels of CRP, erythrocyte sedimentation rate, and DAS28 at 3 months after anti-TNF treatment in HTLV-1 positive patients were significantly higher than those in HTLV-1 negative patients (P= 0.003, P= 0.03 and P= 0.003, respectively). Discontinuation rate due to insufficient response was

significantly higher in HTLV-1 positive patients than in HTLV-1negative patients (P= 0.013). During the 2-year observation period, no patients developed ATL.

Conclusion: These data suggested that HTLV-1 positive patients with RA have higher inflammation and greater resistance to anti-TNF treatment than HTLV-1 negative patients. Further study is necessary to determine whether HTLV-1 infection should be measured when anti-TNF agents are administrated to patients with RA, especially in endemic areas.

Significance and Innovations

It is important to know whether pre-existing infection may influence the effect of treatment with biologics in rheumatoid arthritis (RA). To date, however, few studies have been published on this point.

We hypothesized that human T-lymphotropic virus type 1 (HTLV-1), which has been known to modify the function of T-cells, would influence the effect of anti-tumor necrosis factor (TNF) biologics.

The present study showed that inflammatory markers were higher in HTLV-1 positive patients with RA than in HTLV-1 negative patients. Moreover, anti-TNF biologics revealed lower efficacy in HTLV-1 positive patients than in HTLV-1 negative patients.

These results raised the important question of whether we should test HTLV-1 when we begin anti-TNF treatment in HTLV-1 endemic areas.

Introduction

Rheumatoid arthritis (RA) is characterized by systemic inflammation with proliferation of synovial cells and destruction of joint bone. The effectiveness of biologics, which target proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL) -1 and IL-6, has revolutionized the treatment of RA; however, reports have noted a less efficient response to biologics in approximately 30 percent of patients with RA (1). Patients with advanced and active RA tended to be resistant to biologics; however, the mechanism remains unclear.

Human T-lymphotropic virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia (ATL). The number of HTLV-1 carriers within the global population is estimated at 20 million. HTLV-1 is endemic in Japan, and a recent study reported the number of carriers to be one million (2). Chronic inflammatory diseases such as myelopathy, uveitis, Sjögren syndrome, arthritis, brocho-alveolitis, and polymyositis have been reported to be related to HTLV-1 infection (3,4). A study in Nagasaki, Japan showed the HTLV-1 positive rate in patients with RA to be higher than in blood donors (5). There have been reports of HTLV-1-associated arthropathy,

which had unique clinical characteristics such as the involvement of large joints, marked inflammation, and extra-articular symptoms (4,6). Recently, we reported two HTLV-1 positive patients with RA treated with anti-TNF agents showing lower effectiveness (7).

These data suggested the possibility that RA patients with HTLV-1 infection may have clinical features and responses to anti-rheumatic treatment that differ from HTLV-1 negative RA patients. Therefore, we performed a small retrospective study to evaluate the clinical response of 10 HTLV-1 positive and 20 negative RA patients treated with anti-TNF agents. Moreover, as secondary endpoints, discontinuation rate of anti-TNF therapy and its safety (development of HTLV-1 associated diseases, especially ATL) were also evaluated over a 2-year period.

Patients and methods

Patients

We retrospectively evaluated 124 Japanese patients with RA, who were treated with one of the following anti-TNF therapies as first biologic agents: infliximab (IFX), etanercept (ETN), or adalimumab (ADA). The initial diagnosis of RA was based on the 1987 diagnostic criteria of the American College of Rheumatology (ACR). Three of the patients were already known to be positive for HTLV-1 antibody before the beginning of anti-TNF treatment. Serum samples from the other 121 patients were tested for HTLV-1 antibody using Lumipulse HTLV-1 (FUJIREBIO INC. Tokyo, Japan) after obtaining informed consent, and seven of these tested positive. Therefore, a total of ten patients with RA were positive for HTLV-1 antibody. Then, two age (within 5 years) and anti-TNF agent matched HTLV-1 negative patients were selected for each HTLV-1 positive patient as controls in this cohort. Therefore, ten HTLV-1 positive and 20 HTLV-1 negative patients with RA were subjects of this study. The study protocol was approved by the institutional review board of University of Miyazaki.

The characteristics of these patients before anti-TNF therapy are

summarized in the Table. All patients were female. Only one HTLV-1 positive patient was negative for anti-citrullinated protein antibody (ACPA); however, she had poly-arthritis, and X-ray showed progressive bone erosion. Her clinical features fulfilled 1987 ACR criteria. In HTLV-1 positive patients, the anti-TNF agents, IFX, ETN, and ADA were administrated in three, six, and one patient, respectively.

Methods

Differences in background characteristics and clinical outcomes after anti-TNF treatment were evaluated between HTLV-1 positive and negative patients. The European League Against Rheumatism (EULAR) improvement criteria were used to evaluate clinical responses and disease activity. The patients were categorized into high, moderate, and low disease activity, and remission when the disease activity scores in 28 joints (DAS28) calculated by erythrocyte sedimentation rate (ESR) were >5.1, 3.2 to 5.1, 2.6 to 3.2, and <2.6, respectively. At three months after the beginning of anti-TNF therapy, DAS28 scores were evaluated and categorized into good, moderate or no-responders based on changes in DAS28 and the level of

DAS28 reached. Good responders were defined as patients who had a decrease in DAS28 from a baseline (ΔDAS28) of >1.2 and a DAS28 at 3 months of < 3.2; moderate responders had either a ΔDAS28 of >1.2 and a DAS28 at 3 months of ≧3.2 or a ΔDAS28 of 0.6 to 1.2 and a DAS28 at 3 months of < 5.1; and non-responders were those who had either a ΔDAS28 of < 0.6 or a DAS28 at 3 months of ≧5.1.

As secondary endpoints, discontinuation rate of anti-TNF therapy and safety (development of HTLV-1 associated diseases, especially ATL) were also evaluated during the 2-year period.

Statistical analysis

Results are expressed as median with interquartile range (IQR). A nonparametric test (Mann–Whitney U test) was used to compare disease activity markers, such as C-reactive protein (CRP), elevated ESR, tender joint counts in 28 joints (TJC28), swollen joint counts in 28 joints (SJC28), and DAS28 between HTLV-1 positive and negative patients with RA at baseline and after anti-TNF treatment. Fisher's exact test was used to compare the positive rates of rheumatoid factor (RF), those of ACPA, and