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Technical paper

Water-soluble Component in Dried Chrysanthemum Flower Stimulates Tumor Necrosis Factor-α Production by Mouse Macrophage-like Cell Line RAW264.7

Satomi KOBAYASHI[1], Naoto OGAWA[1], Yoshinori FUJIMURA[1], Hirofumi TACHIBANA[1,2] and Koji YAMADA[1]*

[1] *Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan*
[2] *Bio-Architecture Center, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan*

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To clarify immunoregulatory activity of "Shiranui Himekiku" *(Chrysanthemum indicam×Erigeron annus),* we examined the effect on tumor necrosis factor α (TNF-α) production by mouse macrophage-like cell line RAW264.7. The dried chrysanthemum flower (CF) petals were extracted with water, and the cells were cultured in the presence of the extract. CF extract significantly enhanced TNF-α production of the cells by the dose-dependent manner. Diluted CF solution did not significantly affect the cell number and viability; however, non-diluted solution suppressed the cell proliferation and decreased the cell viability. Heating CF extract at 100°C for 30 min enhanced the activity of water extract markedly, and freeze-thawing only moderately. The TNF-α production enhancing activity of the extract was observed within 3 h. These results suggest that the TNF-α production enhancing activity of CF extract can be recovered efficiently by hot water extraction and preserved stably by freezing.

Keywords: chrysanthemum flower, TNF-α, macrophage

Introduction

Chinese medicine has been used widely in East Asia (Jeong *et al.,* 2003; Jeong *et al.,* 2004; Staniforth *et al.,* 2004). Among these, various biological effects such as anti-bacterial, anti-cancer and anti-inflammatory effects have been reported in chrysanthemum family plants (Rajic *et al.,* 2001; Ukiya *et al.,* 2002; Hou *et al.,* 2003; Wang *et al.,* 2003). Shiranui Himekiku belongs to the chrysanthemum family, which is cultivated in a narrow district of Kyushu Island in Japan. This flower is different from other known members of the chrysanthemum family and the registration of its species was only recently accepted in 2005. The species was set for *Chrysanthemum indicam×Erigeron annus,* but a formal academic name has not yet been given. This dried petal extract has been used as a folk medicine for cancer, digestive diseases, and diabetes. However, the information on active components present in this folk medicine is very limited. To clarify the biological effect of the folk medicine, we studied the effect of cancer cell proliferation of PBS extract of the dried flower and discovered that the extract exerted strong cytotoxicity against human liver cancer cell lines (Aramaki *et al.,* 2003). In addition, we found that the extract suppressed production of immunoglobulin (Ig) and interferon γ by mouse spleen lymphocytes (Aramaki *et al.,* 2004). These results suggest that the extract contains some active components, which regulate cancer cell proliferation and immune functions. Since this crude extract may also contain many other different active components, we examined its possible activity.

Tumor necrosis factor α (TNF-α) has been reported to participate to the induction of cell death in cancer cells (Carswell *et al.,* 1975; Monick *et al.,* 2003). It is also reported that TNF-α is one of inflammatory cytokine and regulate immune functions (Wen *et al.,* 2004). Although excess production of TNF-α may cause various diseases such as blood poisoning, its optimal production is useful for removal of bacteria or cancer cells (Carswell *et al.,* 1975; Monick *et al.,* 2003; Staniforth *et al.,* 2004). Since Shiranui Himekiku was reported to suppress the proliferation of cancer cell and IFN-γ production, it may also regulate TNF-α production. In the present study, we examined the effect of this chrysanthemum flower (CF) extract on TNF-α production by mouse macrophage cell line RAW 264.7 to clarify the biological effects of "Shiranui Himekiku".

Materials and Methods

Reagents Dried petals of Shiranui Himekiku were obtained from Shiranui Himekiku Institute (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from Biofluid (Rockville, MD), RPMI1640 medium from Nissui (Tokyo, Japan), Block Ace from Dainippon Seiyaku (Osaka, Japan), and recombinant TNF-α from Pepro Tech (Rocky Hill, NJ). Rabbit anti-mouse TNF-α Ig was purchased from Endogen (Woburn, MA), biotin-conjugated rabbit anti-mouse TNF-α Ig from Biosource (Camarillo, CA), horse-

* To whom correspondence should be addressed.
E-mail: yamadako@agr.kyushu-u.ac.jp

radish peroxidase (HRP)-conjugated streptabidin from Zymed (San Francisco, CA), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) from Wako Pure Chemicals (Osaka, Japan).

Preparation of extract Water-soluble components of chrysanthemum flower (CF) extract were extracted with deionized water, according to the method of Aramaki *et al.* (2003). Dried petals of Shiranui Himekiku (0.4 g) were suspended in 20 mL water, and then stored for 4 h at 37℃. After filtration with a sheet of gauze, the filtrate was passed through a 0.2 μm filter (Sartorius, Gettingen, Germany) for sterilization. Water extract was dried under vacuum and redissolved in PBS. Heat-treated CF extract was obtained by boiling micro tube containing 1 mL water extract for 30 min.

Cells and cell culture Mouse mocrophage-like cell line RAW264.7 was cultured in RPMI1640 medium supplemented with 5% FBS. The cells were inoculated into 24 well plates at a cell density of 1×10^5 cells/mL and cultured for 24 h in the presence of CF extract. After a trypsinization, cell number and viability was estimated by trypan blue staining. Culture supernatant was isolated by a centrifugation at 300×g for 5 min to estimate TNF-α content.

Determination of tumor necrosis factor TNF-α content in culture supernatant was measured according to the method of Yamasaki *et al.* (2001). Rabbit anti-mouse TNF-α Ig was diluted 500 times with PBS and then 100 μL of the solution was added to each well of 96 well plates. After incubating for 2 h at 37℃, the antibody solution was removed and each well was rinsed three times with PBS containing 0.05% Tween 20 (TPBS). After blocking with 300 μL Block Ace diluted four times with deionized water for 2 h at 37℃, each well was rinsed three times with TPBS. Then, wells were treated with 50 μL of culture supernatants or standard TNF-α solutions for 1 h at 37℃. After rinsing three times with TPBS, wells were treated with 100 μL of biotin-conjugated Ig diluted 1000 times with ten times diluted Block Ace for 1 h at 37℃. After rinsing three times with TPBS, wells were treated with HRP-conjugated streptavidin diluted 1000 times with ten times diluted Block Ace for 1 h at 37℃. After rinsing three times with TPBS, 100 μL of a 10: 9: 1 mixture of 0.06% H_2O_2 in 0.1 M citrate buffer (pH 4.0), water and 6 mg/mL of ABTS was added to each well, which were then stored at room temperature for coloring. The coloring reaction was stopped by the addition of 100 μL of 1.5% oxalic acid solution and then absorbance at 405 nm and 490 nm was measured with Immuno-Mini NJ-2300 microplate reader (Roskilde, Denmark).

Results and Discussion

Effect of CF extract on tumor necrosis factor production by RAW264.7 cells It has been reported that production of TNF-α by RAW264.7 cells is strongly stimulated by lipopolysaccarides (LPS). Thus, we have previously examined dose-dependent effect of PBS extract of CF with or without LPS. This result suggested that PBS extract of CF enhances TNF-α production irrespective of LPS stimulation; however, the stimulation rates of TNF-α in the absence of LPS were higher than those in the presence of LPS (Data not shown). Next, we examined the effects of water extract on TNF-α production of RAW 264.7 cells without LPS. As shown in Table 1, water extract of the CF enhanced TNF-α production by the cells in a dose-dependent manner. Medium TNF-α level was much higher than control culture, approximately 1.6 times higher with ten times diluted CF extract, and 1.9 times higher with non-diluted CF extract. High TNF-α level in the presence of CF extract suggests that it contains some TNF-α production enhancing factor(s).

Effect of CF extract on proliferation and viability by RAW264.7 cells To clarify the effect of CF extract on RAW264.7 cells, we examined the effect of CF extract on proliferation and viability of the cells (Table 2). While no significant effect of cell number was observed in the presence of diluted solution, non-diluted solution significantly decreased the cell number. Viability of the cells was somewhat greater in the presence of 10 to 1000 times-diluted solutions than in the presence of non-diluted solution. The decrease of viability and cell number in the cells treated with non-diluted solution suggests that CF extract has the cytotoxic effect at the highest dose.

Effect of heating and freeze and thawing on the enhancement of tumor necrosis factor production To examine the participation of proteinaceous components on the stim-

Table 1. Dose-dependent effect of CF extract on TNF-α production by RAW264.7 cells.

Dilution rate	TNF-α (ng/mL)
None	0.75 ± 0.00
10^{-4}	0.78 ± 0.03*
10^{-3}	0.78 ± 0.76
10^{-2}	0.82 ± 0.08**
10^{-1}	1.17 ± 0.08***
10^{0}	1.42 ± 0.16***

Data are means ± SD (n=3, *p<0.05, **p<0.01, ***p<0.001).

Table 2. Dose-dependent effect of CF extract on proliferation of RAW264.7 cells.

Dilution rate	Cell number (x 10^4 cells/well)	Viability (%)
None	64 ± 17	86.5
10^{-4}	60 ± 14	88.9
10^{-3}	71 ± 11	90.4
10^{-2}	84 ± 11	90.5
10^{-1}	69 ± 10	93.6
10^{0}	19 ± 4***	71.6

Data are means ± SD (n=3, ***p<0.001).

ulation of TNF-α production, CF extract was heated at 100℃ for 30 min. As shown in Table 3, the heated extract exerted a stronger activity than unheated sample shown in Table 1. These results suggest that the active component in the extract is heat stable and that heating of the extract induces the enhancement of activity or reduction of suppressive effect. Although the activity of CF extract was not only heat-stable, but also heat-activated, preservation as solution may lead to the decrease of activity. To maintain biological activity, freeze preservation is often effective. Thus, the effect of freeze-thawing on the TNF-α production enhancing activity of CF extract was examined. In this experiment, freeze and thawing also enhanced the activity, but the enhancing effect was much weaker than that observed after heating. These results suggest that the activity of CF extract is enhanced strongly by heating and weakly by freeze-thawing.

Time dependent effect of water extract on tumor necrosis factor production by RAW264.7 cells Figure 1 shows the time-dependent effect of CF extract. In the absence of the extract, increase in TNF-α production was negligible for the first 12 h and then some increase was detected at 24 h. In the presence of water extract, rapid increase of TNF-α level was observed within 3 h after the addition of the extract. The accumulation of TNF-α slowed down thereafter, followed by a second increase of TNF-α level observed at 24 h. These results suggest that the enhancing effect of dried chrysanthemum flower can be induced within 3 h.

Table 3. Effect of heating and freeze and thawing on the enhancement of TNF-α production by CF extract.

Dilution rate	TNF-α (ng/mL)	
	Heated	Frozen
None	0.75 ± 0.00	0.75 ± 0.00
10^{-4}	0.82 ± 0.04*	0.82 ± 0.04
10^{-3}	0.83 ± 0.03**	0.82 ± 0.02**
10^{-2}	1.07 ± 0.03***	1.02 ± 0.07**
10^{-1}	3.80 ± 0.08***	2.65 ± 0.36***
10^{0}	11.76 ± 1.37***	1.91 ± 0.31**

Data are means ± SD (n=3, *p<0.05, **p<0.01, ***p<0.001).

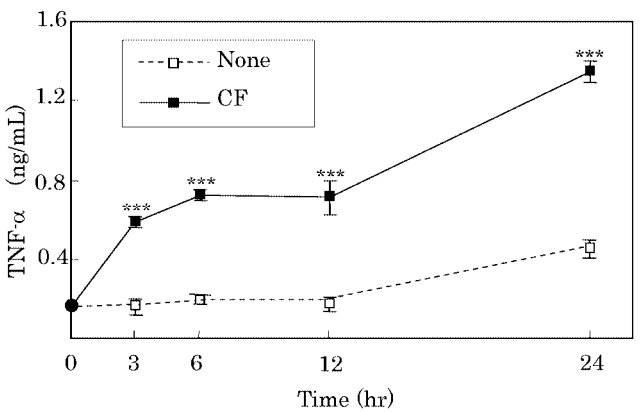

Fig. 1. Time dependent effect of CF extract on TNF-α production by RAW264.7 cells. Cells (1×10^5 cells/mL) were precultured for 24 h, and then treated with 10 times diluted CF extract. Culture supernatant was collected after 3, 6, 12, 24 hr-cultured, and measured by ELISA. Values are means±SD for 3. Data containing asterisk marks are significantly different from control group (None) for same collection time at $p<0.001$***.

In the present study, we demonstrated that water extract of the dried flower contain a TNF-α production enhancing component. Similar activation of TNF-α production is reported in some food extracts (Hsu *et al.,* 2001; Wakabayashi *et al.,* 1997). It was reported that TNF-α is cytokine produced by immune cells such as macrophage, B cells and T cells, and it exhibits anti-cancer and immunoregulatory effects (Aggarwal and Natarajan, 1996). Thus, the strong activation of TNF-α production in mouse macrophage line RAW264.7 in the presence of the extract may lead to the expression of anti-cancer activity. This CF extract was indeed reported to decrease proliferation of various cancer cells (Aramaki *et al.* 2003). However, the results of both of these past studies and this study were obtained using crude CF extract containing many different compounds, and the active components have not yet been cleared. We believe that in the future we should examine what enhances the TNF-α production and whether the effect causes the decrease of cancer cells.

Foods contain various biologically active substances (Yamada *et al.,* 1999). Among these, unsaturated fatty acids and proteins easily lose their activities by heating. Since Shiranui Himekiku dried flower is usually extracted with hot water or hot Japanese wine, the active component in the folk medicine should be heat stable. Moreover, we found that heating significantly enhanced the activity. This suggests that the active component in the dried flower is not only heat stable, but also activated by heating. Sugahara *et al.* (2002) reported that the immunoglobulin production stimulating activity of lysozyme is strongly stimulated by heating. Thus, the activation of TNF-α production by heating may not exclude the possibility that the active component is protein.

In addition, the TNF-α production enhancing activity of the water extract was weakly enhanced by freeze-thawing, thus suggesting that the extract can be frozen without decrease of biological activity. The results of this study indicated that hot water extraction of dried chrysanthemum flower and freezing is useful for the activation of its biological effect and preservation of the extract.

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