Original

Pine Cone Antitumor Substances Stimulate Cytotoxic Factor Production in Young Mice, but not in Aged or Tumor-Bearing Mice

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Abstract: Mice produce a cytotoxic factor (CF) against mouse fibroblast L-929 cells. Production of this factor was investigated in young, aged, and tumor-bearing mice. Intravenous administration of pine cone antitumor substances (Fr. VI or VII), followed by OK-432, caused higher amounts of CF to be produced in ICR mouse serum fractions. Significant loss of CF activity by heat or trypsin treatment suggests that the active principle(s) of the induced factor(s) might be proteinaceous substance(s). Tumor-bearing and aged mice produced much lower amounts of CF activity than normal and young mice, when stimulated with pine cone extracts and OK-432.

Key words: pine cone extracts, cytotoxic factor, endogenous production, aging, tumor development

Introduction

There is a folk legend that oral administration of hot water extract of pine cone of *Pinus parviflora* Sieb. et Zucc. is effective against gastric cancer. Partial purification of 10 different fractions of pine cone extracts revealed that the 10 kD Fr. VI and the 70–200 kD Fr. VII of NaOH extract had the most potent antitumor activity.¹⁾ We previously reported that these fractions stimulated the production of differentiation-inducing factor(s) by macrophages,¹⁾ granulocytic cell iodination²⁾ and splenocyte proliferation.³⁾ These fractions also induced antimicrobial activity⁴⁾ and antiparasite activity⁵⁾ in mice, and inhibited the proliferation of herpes simplex virus in various cultured cells.^{6,7)} We have tentatively concluded that the active principle of Fr. VI or Fr. VII might be complex(s) of lignin-like polyphenolic skeleton with molecular matrices of hydrophilic components such as neutral sugars or polysaccharides.⁸⁾

It is believed that endogenous production of tumor necrosis factor (TNF) or cytotoxic factor (CF) by various biological response modifiers is a promising approach to cancer therapy.⁹⁾ We report here, investigation of whether intravenous administration of Fr. VI or VII, followed by elicitation with OK-432, can endogenously induce such soluble factor(s). We also investigated possible fluctuation of CF production by mice after tumor transplant and during aging.

Materials and Methods

Pine cones of *Pinus parviflora* Sieb. et Zucc. were supplied by Mr. S. Matsuda, and Mr. S. Sato. OK-432, a preparation of *Streptococcus pyogenes*,¹⁰⁾ was supplied by Chugai Seiyaku

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Mice. Female ICR mice (5 weeks old, 23–25 g) were obtained from Sankyo Labo. Service Co.

Tumors. Sarcoma-180 was maintained serially in ascites by weekly *in vivo* transfer in ICR mice.

Cell culture. Mouse fibroblast L-929 cells and human myeloblastic leukemic ML-1 cells were maintained at 37°C in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a CO₂-incubator, as described previously.¹¹⁾

Isolation of antitumor substances. Antitumor substances (Frs. VI and VII) were prepared from NaOH extract of pine cone of *Pinus parviflora* Sieb. et Zucc. as described previously.¹¹⁾ In brief, pine cones were extensively washed with methanol, 85% ethanol and boiling water, and then extracted twice with 1% NaOH for 4 hr at room temperature. After centrifuging for 20 min at 10,000×g, the pH of the clarified supernatant was adjusted to 5.0 with acetic acid. The precipitate (Fr. VI) was collected by centrifugation, dissolved in a small volume of NaOH to remove insoluble materials and then neutralized. The remaining supernatant (Fr. VII) was precipitated with one volume of ethanol. All of these fractions were extensively dialyzed against distilled water and lyophilized.

Preparation of cytotoxic serum. Mice were intravenously administered 0.2 ml of the indicated doses of test samples dissolved in 0.9% NaCl, followed 3 hr later by 8 mg/kg of OK-432. After 2 hr, the serum was collected from the exsanguinated mice and stored at –20°C until use.

Cytotoxicity assay. CF activity of the serum samples was tested for cytotoxicity against L-929 cells.¹²⁾ In brief, trypsinized L-929 cells were inoculated at a density of 3×10^4 /0.1 ml of culture medium in an F96 MicroWell (Nunc, InterMed) and incubated for 8 hr at 37°C. After adding 0.1 ml of 2 µg/ml actinomycin D and 50 µl of serially diluted serum preparations, the cells were further incubated for 18 hr. The viable L-929 cells were stained with 0.2% crystal violet in 2% ethanol, and lysed with 1% SDS. Absorbance of the cell lysates was measured at 590 nm with ImmunoReader NJ-2000 (InterMed). One unit of CF activity was defined as the amount necessary to reduce absorbance of the control cells 50%.

Heat stability of CF. The serum sample was heated at 70°C for 30 min, and then immediately cooled in ice water prior to cytotoxicity assay.

Trypsin digestion. The serum sample was incubated for 30 min at 37°C with 3 mg/ml trypsin (DIFCO), and 5.5 mg trypsin inhibitor (Sigma) was added to stop the digestion.

Results

Stimulation of CF production by Frs. VI and VII.

The amount of cytotoxic factor (CF) produced in the serum of the ICR mice was measured by L-929 cell cytotoxicity assay in the presence of 1 µg/ml actinomycin D (Table 1). Single or double intravenous administration of Fr. VI (10 mg/kg) induced relatively weak CF activity. However treatment with OK-432 (8 mg/kg) induced higher amounts of CF. Administration of Fr. VI (10 mg/kg) or Fr. VII (20 mg/kg), 3 hr before OK-432 treatment, further increased CF production to a level almost equal to that attained by double OK-432 injection. The stimulation effect of Fr. VI was detectable at 1–20 mg/kg, but decreased at higher concentrations (Fig. 1). The 50% lethal doses of intravenously administered Fr. VI, Fr. VII and OK-432 were about 60, 80 and 16 mg/kg, respectively. We found that near lethal doses

Fig. 1. Dose-dependent effect of Fr. VI on OK-432-elicited cytotoxic factor production. Mice (6 weeks old) were administered i.v. either saline (control) or indicated doses of Fr. VI, followed 3 hr later by OK-432 (8 mg/kg). Two hr thereafter, CF activity in the serum was determined. Each value is mean $\pm$ S.E. of 3 mice.

Table 1. Induction of endogenous CF production by sequential administration of Fr. VI or Fr. VII and OK-432.

Treatment		CF activity (units/ml of serum)
1st	2nd	
Saline	Saline	$< 8 \pm 2$
Saline	Fr. VI	20 ± 11
Saline	Fr. VII	7 ± 3
Fr. VI	Fr. VI	23 ± 1
Saline	OK-432	488 ± 232
Fr. VI	OK-432	45762 ± 23642
Fr. VII	OK-432	22083 ± 6305
OK-432	OK-432	30476 ± 12597

Mice (6 weeks old) (3 mice/group) were intravenously administered saline, 10 mg/kg Fr. VI, 20 mg/kg Fr. VII or 8 mg/kg OK-432, and 3 hr later injected with the same concentrations of the indicated samples. Two hr later, the serum was collected and CF activity in the serum fraction was determined.

Each value is mean $\pm$ S.E. of 3 mice.

(2–8 mg/kg) of OK-432 were required to elicit sufficient CF production (data not shown).

Table 2 shows that most of the CF activity induced in the mouse serum after treatment with Fr. VI and OK-432 was destroyed by heat treatment (70°C, 30 min) or by trypsin digestion. This suggests that the active principle(s) in the stimulated serum might be proteinaceous substance(s).

Table 2. Stability of serum CF activity induced by Fr. VI and OK-432.

Pretreatment	CF activity (units/ml of serum)	(% of control)
Exp. I		
0°C 30 min	9766	100
37°C 30 min	7813	80
70°C 30 min	3	0.03
100°C 30 min	20	0.2
Exp. II		
None	6104	100
Trypsin (3 mg/ml) 37°C 30 min	40	0.7

Three mice (7 weeks old) were intravenously administered 10 mg/kg Fr. VI and after 3 hr 8 mg/kg OK-432. Two hr thereafter, the sera of the exsanguinated mice were obtained. Aliquots of the pooled sera were heated (Exp. I) or digested with trypsin (Exp. II) under the indicated conditions, and the remaining CF activity was determined.

Table 3. Effect of tumor implantation on endogenous CF production.

	Treatment		CF activity (units/ml of serum)	Stimulation index ^b (n-fold)
	1st	2nd		
Exp. I (Ascites tumor)				
Day 0	Saline	OK-432	204 ± 111	(1)
	Fr. VI	OK-432	1009 ± 411	(4.9)
	Fr. VII	OK-432	4462 ± 2663	(21.9)
Day 8	Saline	OK-432	305 ± 126	(1)
	Fr. VI	OK-432	2794 ± 2090	(9.2)
	Fr. VII	OK-432	3888 ± 2941	(12.7)
Day 13	Saline	OK-432	24 ± 0	(1)
	Fr. VI ^a	OK-432	27 ± 20	(1.1)
	Fr. VII	OK-432	22 ± 8	(0.9)
Exp. II (Solid tumor)				
Day 0	Saline	OK-432	338 ± 147	(1)
	Fr. VI	OK-432	2697 ± 854	(8.0)
Day 13	Saline	OK-432	640 ± 465	(1)
	Fr. VI	OK-432	625 ± 248	(1.0)

Mice (8 weeks old) were inoculated intraperitoneally (Exp. I) or subcutaneously (Exp. II) with 10⁶ sarcoma-180 cells. At indicated days after inoculation, the mice were intravenously administered saline (control), 10 mg/kg Fr. VI, or 20 mg/kg Fr. VII, and after 3 hr 8 mg/kg OK-432. Two hr thereafter, sera were obtained, and CF activity determined. Each value is mean ± S.E. of 3 mice.

^a Due to the cytotoxicity of Fr. VI, the amount of Fr. VI administered was reduced from 10 mg/kg to 5 mg/kg.

^b Stimulation index represents ratio of CF activity in Fr. VI (or Fr. VII)-stimulated serum vs CF activity in control serum.

Table 4. Effect of aging on endogenous CF production.

Age (weeks)	Body weight (g)	Treatment		CF production (units/ml)	Stimulation index (n-fold)
		1st	2nd		
7	27	Saline	OK-432	567 ± 280	(1)
7	27	Fr. VI	OK-432	6381 ± 1517	(11.3)
7	27	Fr. VII	OK-432	10148 ± 1918	(17.9)
15	50	Saline	OK-432	580 ± 218	(1)
15	50	Fr. VI	OK-432	1324 ± 604	(2.3)
15	50	Fr. VII	OK-432	836 ± 364	(1.4)

Mice at indicated ages were intravenously administered saline (control), 10 mg Fr. VI or 20 mg/kg Fr. VII, and after 3 hr 8 mg/kg OK-432. Two hr thereafter, sera were obtained and CF activity determined. Stimulation index was determined as in Table 3. Each value is mean ± S.E. of 3 mice.

Fig. 2. Reduced endogenous CF production by aged mice.

Mice at indicated ages (3 mice/group) were administered saline (control), 10 mg/kg Fr. VI (●) or 20 mg/kg Fr. VII (○), and after 3 more hr, 8 mg/kg OK-432. Two hr thereafter, the serum was obtained, and stimulation index determined as in Table 3.

Effect of tumor implantation on endogenous CF production.

We next investigated the endogenous CF production by tumor-bearing mice. Mice intraperitoneally transplanted with 10^6 sarcoma-180 cells all died within 21 days. Their mean survival time was 16.8 days.¹⁾ During the late stage of tumor development, the stimulation effect of Fr. VI or Fr. VII on OK-432-induced CF production (judged by the stimulation index) declined significantly from 9.2–12.7 on day 8 to 0.9–1.1 on day 13 (Table 3, Exp. I).

Mice subcutaneously transplanted with sarcoma-180 cells also responded less to Fr. VI. No stimulation effect of Fr. VI was observed 13 days after solid tumor transplantation (Table 3, Exp. II).

Effect of aging on endogenous CF production

We next investigated endogenous CF production by aged mice. Table 4 shows that treatment with Fr. VI or VII stimulated OK-432-induced CF production 11.3–17.9 times in younger mice (7 weeks old, 27 g). The same treatments stimulated production only 1.4–2.3

times in the older mice (15 weeks, 50 g). Fig. 2 shows that the stimulation effect of Fr. VI or VII rapidly declined after the age of 11 weeks.

Discussion

This study revealed that intravenous administration of the pine cone antitumor substances, Fr. VI and VII, could endogenously produce proteinaceous CFs in mouse serum, upon subsequent treatment with OK-432. A previous report described the OK-432-induced factor(s) as TNF.⁹⁾ It has recently been reported that a cytolytic factor secreted from BCG-activated macrophages is serologically related to necrosin,¹³⁾ the amino acid composition of which is analogous to that of TNF.¹⁴⁾ These data suggest that the CF identified in the present paper might be identical to TNF. As expected from the identity of TNF with differentiation-inducing factor,¹⁵⁾ the serum obtained from mice stimulated with Fr. VI (or Fr. VII) and OK-432 induced monocytic differentiation in human myelogenous leukemic ML-1 cells (data not shown).

When the mice, which were inoculated with sarcoma-180 cells 2 weeks before, were sequentially treated with Fr. VI (or Fr. VII) and OK-432, growth of the transplanted tumor was significantly suppressed, and there was occasional necrosis (data not shown). This suggests the possibility that the induction of antitumor activity with either Fr. VI or Fr. VII might be mediated by CF production. However, the response of the mice to Fr. VI or Fr. VII was significantly reduced after they were implanted with tumor cells (Table 3). This supports a previous report that the capacity for TNF production broadly paralleled the survival rate in mice that received transplants of Ehrlich tumors.¹⁶⁾ We also found that the response of the mice to pine cone extracts declined when they aged (Table 4, Fig. 2). It is necessary to further investigate whether the reduced response of the tumor-bearing or aged mice might be due to the action of immunosuppressive substances.¹⁷⁾

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