ANTITUMOR AND ANTIMETASTATIC ACTIVITY OF AN ANTIBIOTIC, ASCOFURANONE, AND ACTIVATION OF PHAGOCYTES

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Ascofuranone demonstrated antitumor activity against FM3A murine mammary carcinoma, implanted in the peritoneal cavity of syngeneic mice, C3H/He. It was more effective by treatment prior to implantation than by that after implantation. Treatment with ascofuranone also increased splenic cytotoxicity and phagocytic activity of host animal cells. Moreover, ascofuranone induced inflammatory cells in the peritoneal cavity which are mainly composed of polymorpho-nuclear leukocytes and macrophages. These cells are more potent in cytotoxicity against FM3A cells than with resident peritoneal cells. The antitumor activity of ascofuranone was suppressed by ip administration of silica, just prior to tumor implantation. These results suggest that the prophylactic antitumor activity of ascofuranone is expressed through the activation of phagocytes.

Ascofuranone also suppressed pulmonary metastasis of B16 melanoma and Lewis lung carcinoma. Treatment after tumor implantaion failed to suppress the metastasis. Single treatment of ascofuranone 4 days prior to implantation decreased the metastasis of Lewis lung carcinoma but not that of B16, whereas single treatment of ascofuranone 24 hours prior to the tumor implantation decreased the metastasis of B16 but not that of Lewis lung carcinoma.

Ascofuranone is an isoprenoid antibiotic, originally isolated as a hypolipidemic substance from a culture broth of a phytopathogenic fungus, *Ascochyta viciae*[1]. We found it to have antitumor activity against murine experimental tumors[2]. The characteristics of the antitumor activity are that ascofuranone is effective by treatment prior to tumor implantation as well as by that after tumor implantation and that prophylactic antitumor activity needs no vaccination, suggesting that it directly stimulates nonspecific defense mechanisms against tumor development. In fact, treatment of normal mice with ascofuranone enlarges solid lymphoid organs[2] and enhances spontaneous cytotoxic activity of splenocytes[3].

In vitro study of ascofuranone[4] shows that it is suppressive to lymphoid cell functions such as lectin-induced proliferative responses, interleukin 2 (IL-2) production and formation of IL-2 receptors. It also suppressed natural killer (NK) activity of splenocytes. In contrast, it stimulates glycolysis, interleukin 1 (IL-1) production and tumoricidal activity of macrophages. These results suggest that the antitumor activity of ascofuranone is expressed through activation of macrophages. This paper describes *in vivo* activation of phagocytes by ascofuranone in relation to the antitumor and antimetastatic activities.

Materials and Methods

Mice

Six-week-old male C3H/He and BDF1 mice were purchased from Shizuoka Experimental Farm (Hamamatsu). Commercial pellet diet (CE-2, Clea, Japan, Ltd., Tokyo) and tap water were fed *ad libitum*. Mice were used no later than 10 weeks of age.

Ascofuranone

Purified ascofuranone was supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo). For administration, ascofuranone was suspended in 0.5% Tween 80 in phosphate-buffered saline (PBS, 0.8% NaCl, pH 7.4) with the aid of a Teflon homogenizer.

Development of FM3A Ascites Tumor

FM3A cells, freshly isolated from the peritoneal cavity, were suspended in PBS and cell number was adjusted just before implantation. The tumor cells (10^5/mouse) were implanted in the peritoneal cavity. Antitumor activity was assessed by average survival time or the number of survived mice.

Preparation of Splenocytes and Peritoneal Cells

A single cell suspension of splenocytes, erythrocytes of which were ruptured by ammonium chloride, was prepared as described previously[2]. Splenocytes were suspended in RPMI-1640 medium supplemented with 50 μg/ml kanamycin and 5×10^{-5} M 2-mercaptoethanol and 10% fetal bovine serum (FBS). Peritoneal cells were collected with MEM supplemented with 5×10^{-5} M 2-mercaptoethanol and 50 U/ml heparin. The cells were washed twice with MEM and suspended in RPMI-1640 medium. Cell type was determined morphologically under a microscope using Mäy-Grünwald Giemsa staining of cytocentrifuged cells.

Carbon Clearance Test

Experiments were carried out as described by GOTOH *et al.*[5]. Drawing ink (Rotring Werke, West Germany) was 10-fold diluted with PBS and 0.2 ml of the suspension was injected into mice *via* tail vein. Three minutes after the injection, 50 μl of blood sample was removed from retro-orbital venous plexus by the use of capillary pipettes. The blood samples were immediately discharged into 3 ml of 0.5% ammonium chloride solution to disrupt erythrocytes and centrifuged at 3,000 rpm for 5 minutes. The amount of carbon in the supernatant was measured by a spectrophotometer at 620 nm, using blood samples from the mice without carbon injection as a blank.

Cytotoxicity Assay

One million splenocytes or 4×10^5 peritoneal cells in 200 μl of medium on a flat-bottomed microplate well were cultured for 4 hours with 1×10^4 ^{51}Cr-labeled target cells prepared as described previously[4]. At the termination of the culture, 100 μl of the supernatant were removed and the radioactivity was determined by a γ-counter. The spontaneous release was determined by incubating ^{51}Cr-labeled target cells alone and the maximum release was determined by incubating ^{51}Cr-labeled target cells with 0.5% sodium dodecyl sulfate. The following formula was used to compute percent lysis.

$$\text{Lysis (\%)} = 100 \times (\text{test cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$$

Metastasis

Lewis lung carcinoma and B16 melanoma cells were maintained *in vitro*, using RPMI-1640 medium. Cells were detached from culture vessels by trypsin-treatment and suspended in MEM. After washing twice with MEM, 1×10^6 cells (Lewis lung carcinoma) or 4×10^5 cells (B16 melanoma) were inoculated through tail vein of BDF1 mice. Antimetastatic activity was determined by comparison of lung weights of the ascofuranone-treated mice with the control mice.

Results

Antitumor effect of ascofuranone on syngeneic mammary carcinoma, FM3A, is shown in Table 1. When mice were implanted with 10^5 FM3A cells, they died within 30 days after the tumor implantation.

Table 1. Survival time of ascofuranone-treated mice inoculated with FM3A.

Treatment (day)	Survival time (day)[a]	40 day-survivors
None	23.0±7.3	0/5
+1	34.4±6.5*	2/5
−1	25.0±13.7	2/5
−4	40.0±0.0**	5/5
−7	34.4±12.5	4/5
−7, −5, −3	40.0±0.0**	5/5

C3H/He mice (5 mice/group), treated ip with or without ascofuranone (200 mg/kg) as the indicated schedule, were inoculated ip with FM3A cells (10^5/mouse) on day 0.

[a] Survival time on day 40 (mean±SD).

* $P<0.05$, ** $P<0.01$.

Table 2. Activation of cytotoxicity of spleen cells by ascofuranone-treatment.

Treatment (day)	^{51}Cr-release (cpm)	Lysis (%)
None	337±26[a]	10.6
−1	556±3*	26.0
−4	714±54*	37.0
−7	396±29	14.3

Spleen cells from three C3H/He mice, treated with or without ascofuranone (200 mg/kg) as indicated schedule were incubated with ^{51}Cr-labeled YAC-1 cells for 4 hours at E/T ratio, 50 : 1, on day 0. Maximum release and spontaneous release were 1,471±157 and 246±18, respectively.

[a] Mean±SD ($n=3$).

* $P<0.01$.

Table 3. Cytotoxicity of fractionated spleen cells treated with ascofuranone.

Cell population	Lysis (%)[a]
Nonadherent cells	49.1±4.1
Unfractionated cells	64.1±5.7

Ascofuranone (200 mg/kg) was administered ip to three C3H/He mice. 4 days after the administration, spleen cells were pooled and cells (10^6/well) were cultured on a flat-bottomed microtiter well for 2 hours at 37°C. Nonadherent cells were separated by vigorous pipetting. Nonadhernt cells and unfractionated cells were further cultured with ^{51}Cr-labeled YAC-1 cells (10^4/well) and radioactivity in the supernatant was determined after 4 hours.

[a] Mean±SD ($n=3$).

Table 4. Activation of phagocytes by ascofuranone-treatment (carbon clearance test).

Treatment (day)	A_{660}
None	0.500±0.029[a]
−1	0.377±0.035**
−4	0.395±0.047*
−7	0.508±0.057

Carbon clearance test was carried out on day 0 as described in Materials and Methods, using C3H/He mice. Ascofuranone (200 mg/kg) was administered ip on an indicated day.

[a] Mean±SD ($n=3$).

* $P<0.05$, ** $P<0.01$.

Treatment with ascofuranone expanded survival time of the mice and some of them survived on day 40. Although ascofuranone was effective both by pretreatment and by posttreatment, more evident effect was obtained by the former. All the mice treated once on day −4 or three times on days −7, −5 and −3 were alive for 40 days.

Ascofuranone activates cytotoxic activity of mouse splenocytes *in vivo*[3]. Significant activation was observed as early as 24 hours after the treatment and reached maximum level on day 4 (Table 2). Seven days later, the activation disappeared. Thus, the kinetics of splenic cytotoxic activity were in parallel with that of antitumor activity. Effectors mediating the cytotoxicity in the ascofuranone-treated splenocytes, belonged to the plastic dish-nonadherent population (Table 3).

Since ascofuranone activates macrophages *in vitro*[4], phagocytic activity of ascofuranone-treated mice was assessed by carbon clearance test (Table 4). As expected, ascofuranone-treatment significantly activated phagocytic activity *in vivo*. The activation reached maximum level 24 hours after the treatment and disappeared after 7 days. Thus, the activation of phagocytes preceded the expression of antitumor activity or the activation of cytotoxic activity of splenocytes.

In addition, ip treatment with the antibiotic induced phagocytes in the peritoneal cavity which

Table 5. Effect of ascofuranone-treatment on the cell population of spleen and peritoneal cavity.

	Untreated	Ascofuranone-treated	
		Day −1	Day −4
Spleen			
Total number (10^6/mouse)	91.8±11.7[a]	100.0±0.71	87.7±13.8
Population (%)			
Macrophage	6.0±0.0	11.0±1.4	8.0±2.81
Lymphocyte	87.5±2.1	78.5±4.9	63.0±7.10**
PMN	7.0±2.8	10.0±2.8	29.5±3.52**
Peritoneal cavity			
Total number (10^6/mouse)	2.15±0.46	9.93±1.50**	7.59±2.54*
Population (%)			
Macrophage	57.0±15.6	27.0±9.9	58.0±18.4
Lymphocyte	42.0±14.1	6.5±0.7*	6.5±3.01*
PMN	1.0±1.4	67.0±11.3**	35.5±17.8*

Spleen cells and peritoneal cells from C3H/He mice treated ip with ascofuranone (200 mg/kg) were prepared as described in Materials and Methods. Cell population was determined on day 0 by May-Grünwald Giemsa staining.

[a] Mean±SD (n=3).

* $P<0.05$, ** $P<0.005$.

Table 6. Cytotoxic activity of ascofuranone-induced peritoneal cells.

Treatment	^{51}Cr-release (cpm)		
	FM3A	P388	YAC-1
None	445±35[a]	232±19	558±47
	(17.6)	(14.3)	(39.6)
Ascofuranone			
On day −1	394±21	314±8**	641±10*
	(13.2)	(28.8)	(50.8)
On day −4	868±53**	475±29**	677±18
	(53.8)	(57.2)	(55.7)

Peritoneal cells (2×10^5/well) from C3H/He mice were incubated with ^{51}Cr-labeled target cells (5×10^3/well) for 4 hours. Ascofuranone (200 mg/kg) was administered ip on the day indicated.

[a] Mean±SD (n=3). Numbers in parentheses; lysis (%).

* $P<0.05$, ** $P<0.005$.

are mainly composed of polymorpho-nuclear leukocytes (PMNs) and macrophages (Table 5). Peak of the induction of PMNs was 24 hours after the treatment, while that of macrophages was 4 days after the treatment. PMNs in splenocytes also increased 4 days after the treatment, without affecting the total number of splenocytes. These kinetic studies shown in Tables 1, 2, 3 and 4 were reproducible in several additional experiments.

Cytotoxic activity of the peritoneal phagocytes induced by ascofuranone was significantly enhanced (Table 6). The peritoneal cells which were induced 4 days after the treatment, acquired the ability to kill NK-resistant cell lines, FM3A and P388, as well as a standard NK-sensitive cell line, YAC-1. Effectors mediating the cytotoxicity against NK-resistant and sensitive tumors belonged to the plastic dish-nonadherent population as in the case of splenic cytotoxicity (Table 7). The cytotoxic activities by 20 hours-assay were also determined because tumoricidal activities mediated by macrophages and PMNs require long term co-culture with target cells[6,7]. Results were same as in the case of 4 hour-assay.

Table 7. Cytotoxicity of fractionated peritoneal cells treated with ascofuranone.

Cell population	Lysis (%)[a]	
	4 hour-assay	20 hour-assay
Adherent cells	2.8±0.4	2.7±2.8
Nonadherent cells	11.5±0.2	40.2±10.7
Unfractionated cells	25.8±2.8	44.1±5.5

Ascofuranone (200 mg/kg) was administered ip to three C3H/He mice. 4 days after the administration, peritoneal cells were pooled and cells (2×10^5/well) were cultured on a flat-bottomed microtiter well for 2 hours at 37°C. Nonadherent cells were separated by vigorous pipetting. Residual adherent cells, nonadherent cells and unfractionated cells were further cultured with ^{51}Cr-labeled FM3A cells (5×10^3/well) and radioactivity in the supernatant was determined after an indicated cultivation.

[a] Mean±SD ($n=3$).

Table 8. Inhibition of ascofuranone-induced antitumor activity by silica-treatment.

Treatment	80 day-survivors	Survival time[a] (day)
None	0/5	25.6±1.8
Ascofuranone	4/5	68.0±26.8*
Ascofuranone+silica	0/5	24.3±8.5

C3H/He mice were implanted ip with FM3A cells (10^5/mouse). Ascofuranone (200 mg/kg) was administered ip 4 days prior to the implantation. Silica (20 mg/kg) was injected ip just before the implantation.

[a] Survival time on day 80 (mean±SD, $n=5$).

* $P<0.05$.

Table 9. Suppression of pulmonary metastases by ascofuranone.

Treatment (day)	Lung weight (mg)	
	B16 melanoma	Lewis lung carcinoma
None	509±227[a]	385±97
+1	662±264	384±92
−1	247±84*	398±84
−4	556±229	269±20*

BDF1 mice were inoculated with Lewis lung carcinoma cells (10^6/mouse) or B16 melanoma cells (4×10^5/mouse) *via* tail vein on day 0. Ascofuranone (200 mg/kg) was administered ip on the day indicated. Lung weight was determined on day 18 (Lewis lung carcinoma) or on day 16 (B16 melanoma). Lung weight of normal mice, 238±47 mg.

[a] Mean±SD ($n=5$).

* $P<0.05$.

The above results imply the role of phagocytes in the antitumor activity of ascofuranone. Therefore, mice treated with ascofuranone 4 days prior to tumor implantation were injected ip with silica immediately before tumor implantation. The result is shown in Table 8. Four of the five ascofuranone-treated mice survived 80 days after the tumor implantation, whereas all of the tumor bearing mice without treatment died as a result of the tumor incidence. Silica-treatment completely diminished the prophylactic antitumor activity of ascofuranone, confirming the role of phagocytes in the antitumor activity.

Since activated macrophages and NK cells are possible candidates for suppression of tumor metastasis[6,7], the above results prompted us to study the effect of ascofuranone on tumor metastasis (Table 9). Pulmonary metastasis was evaluated by the increase of lung weight. In the case of Lewis lung carcinoma, a single treatment of ascofuranone 4 days before the inoculation significantly suppressed increase of lung weight. More remarkable suppression was observed, when using B16 melanoma cells. A single treatment 1 day before the inoculation as well as multiple treatment significantly suppressed increase of lung weight. It should be noted that ascofuranone showed no antimetastatic activity against B16 melanoma cells by a single treatment 4 days before the inoculation, while the same treatment gave most striking antitimor activity against FM3A or antimetastatic activity against Lewis lung carcinoma. Posttreatment also showed no metastatic activity, as in the case of Lewis lung carcinoma. Two additional experiments showed similar kinetics.

Discussion

Intraperitoneal treatment with ascofuranone enhances splenic cytotoxic activity and the plausible mediator of the cytotoxicity is suggested to be NK cells[3]. However, *in vitro* study shows that the antibiotic inhibits functions of lymphocytes including NK cells, while it activates macrophages[4]. Here, we showed that ascofuranone also activates phagocytes *in vivo*. The intraperitoneal treatment induced inflammatory cells mainly composed of macrophages and PMNs, and these induced peritoneal cells were activated to kill tumor cells nonspecifically. Since NK cells[8,9], PMNs[10] and macrophages[9,11,12] are important in the protection of host animal from tumor incidence, present results showing significant activation of those cells by ascofuranone are consistent with the prophylactic antitumor activity of the antibiotic.

Activation of splenic and peritoneal cytotoxicity and induction of peritoneal macrophages reached maximum level 4 days after the treatment. The kinetic were consistent with that of antitumor activity. On the other hand, maximum activation of phagocytes, when determined by carbon clearance test, was observed as early as 24 hours after the treatment, suggesting that ascofuranone primarily activates phagocytes, which subsequently enhances cytotoxic activity of the spleen cells or peritoneal cells. Complete abrogation of the antitumor activity by silica treatment, also suggests an essential role of phagocytes in the expression of antitumor activity of ascofuranone.

A time course study demonstrated that the peak of induction of macrophages in the peritoneal cavity was 4 days after the ascofuranone-treatment, whereas that of PMNs was 24 hours after the treatment. Similar kinetics have been reported with inflammatory agents[6,12,13], although the inflammatory cells have no cytotoxic activity without appropriate stimulation of lectin-treatment[6,7,14,15], and such inflammation never induced host-mediated antitumor activity[12,13]. Our preliminary experiments suggested that ascofuranone-induced effector cells with cytotoxicity in the peritoneal cavity and spleen were not adherent to the plastic dish. Since ascofuranone-induced peritoneal cells were mainly composed of macrophages and PMNs, the most probable candidates are PMNs. However, induction of PMNs reached a maximum level 24 hours after the treatment, whereas that of cytotoxicity 4 days after the treatment. Thus, early induced PMNs may be qualitatively different from late induced ones. In fact, the latter had more segmented nuclears than the former.

Both NK cells and activated macrophages[16~20] are important populations which suppress metastasis. As was expected, ascofuranone significantly suppressed pulmonary metastasis of Lewis lung carcinoma and B16 melanoma. However, suppressive effect of ascofuranone on these two metastatic models was somewhat different. Metastasis of Lewis lung carcinoma was suppressed only when ascofuranone was administered 4 days before the tumor implantation. On the other hand, that of B16 melanoma was not suppressed by the same treatment, although high efficacy was obtained by the other schedule. Two explanations may be possible. One is difference of the effector population. The other is difference of locus where tumor cells are attacked by effector cells since there has been reported that local activity of effectors is important in the suppression of metastasis[18,20].

Although further study is necessary to elucidate the effector population responsible for the suppression of metastasis or cytotoxic activity in the spleen and peritoneal cavity, present results suggest that ascofuranone exhibits its antitumor and antimetastatic activity through activation of phagocytes and subsequent induction of cytotoxic effectors which eliminate tumor cells.

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