フィージー産,群体ボヤDidemnum variansから調製した高分子量画分の抗腫瘍活性について

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Antitumor Activity of the Macromolecular Fraction from a Fijian Tunicate *Didemnum varians*[*1]

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The high-molecular weight fraction from the aqueous exract of the tunicate *Didemnum varians* was found to have antitumor activity against Sarcoma 180 solid tumors in ICR mice, The assocaite toxicity with antitumor activity in the extract disappeared by the pronase E digestion. The pronase digest with molecular weight over 10,000 Da caused complete disappearance of tumor when injected into lesion, in the dose of 250 mg/kg, The inhibition ratio observed with iv injection was less than the high values achieved by local injection, but still significant. The cytocidal activity of the pronase digest was found to reduce when compared with those of the intact specimen. Lower-molecular weight fractions which were obtained by the enzyme digestion and successive ultrafiltration gave high cytocidal activity against Sarcoma 180 tumor cells *in vitro*, but had only weak antitumor activity *in vivo*. These results suggested that the main antitumor component in *D. valians* was high-molecular weight and the activity was mainly due to its carbohydrate portion. The aqueous extract prepared from the different lots of *D. varians* showed high antitumor activity but no toxicity.

Marine organisms have been attracting attention as potential sources of bioactive compounds with pharmaceutical interest. Many investigators, for example, have found that marine animals and plants contain antitumor compounds.[1–5] Most of the antitumor substances isolated are fat-soluble and small to medium-size molecules of low molecular weight. On the other hand, water-soluble compounds, biopolymers in particular seem to receive only limited attention. Our recent investigation has revealed that the high molecular weight substances with potent antitumor effect distributed widely in marine animals, especially in marine invertebrates and protochordata.[6,7] Here we report the antitumor effect of the macromolecular fraction of the Fijian tunicate *Didemnum varians*.

Materials and Methods

Collection of Didemnum varians

Tunicate *Didemnum varians* was collected by the last author from the Makaluva Reef, Suva, Fiji in October 1983, and in August or October 1985. Specimens gathered were frozen immediately and stored at −20°C.

Extraction and Fractionation of Antitumor Substances

The specimens were homogenized with 2 volumes of 0.85% saline three times and then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was dialyzed, lyophilized, and kept at 4°C until used. The digestion of *Didemnum* extract with pronase E was carried out at 37°C overnight in 0.01 M phosphate buffer, pH 7.2, at a ratio of enzyme to substrate of 1: 700 (w/w). The pronase E digest was dialyzed against distilled water for 48 h, and the retentate was centrifuged to remove insolubles. The filtrate was lyophilized and then fractionated by ultrafiltration using Diaflo UM-05 membrane (molecular cut 500 Da).

Tumor Cells

Sarcoma 180 was initially supplied by the Sloan-

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Kettering Institute, New York, NY, and has been maintained in National Cancer Center Research Institute in ascites form.

Assay of in vivo Antitumor Activity

A 50 μl (about 2×10^6 cells) of 7-day-old ascites tumors was transplanted subcutaneously into the right flank of female ICR-JCL mice (6 week-old). Test samples at a suitable concentration in 0.1 m*l* of physiological saline were sterilized using an Acrodisc disposable filter assembly (Gelman Sciences, Ann Arbor, MI) and then injected into the lesion (il) or intravenously (iv) at days 5, 7, and 9 after tumor inoculation, by which time the tumors had reached an average diameter of 4 mm. Mice received similar treatment with physiological saline served as controls. At the end of the 5th week, the mice were killed and the tumors were removed and weighed. Inhibition ratios were calculated by: Inhibition ratio (%)=[(A−B)/A] $\times$100, where A is the average tumor weight of the control group, and B is that of the treated group. Complete regression indicates the ratio of the number of mice with complete tumor regression over the total number of mice tested.

In vitro Cytocidal Test

Ascites tumor cells (10^5 cells) of Sarcoma 180 in 0.5 m*l* of RPMI 1640 medium with 10% heat inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY) and 50 μg/m*l* of kanamycin containing various concentrations (10–200 μg/m*l*) of test samples were incubated at 37°C for 48 h in moist air containing 5% CO_2. The viability of these cells incubated with or without the samples, assessed by exclusion of trypan blue, was over 95%. The number of cells was counted using a phase contrast microscope. Inhibition ratios were calculated by: Inhibition ratio (%)=[(A−B)/A]$\times$100, where A is the average number of cells incubated without the samples, and B is that of cells with the samples.

Protein and Carbohydrate Determination

Protein was measured by the procedure of Lowry *et al.*[8] using egg albumin as a standard. Carbohydrate was determined by the phenol-sulfuric acid method[9] using glucose as a reference.

Statistical Analysis

We used Student's *t*-test to study statistical significance. All p value below 0.05 were taken out to be significant.

Results and Discussion

This is the first observation that the tunicate contains macromolecular antitumor agent(s). The high-molecular weight fraction (DV-1) from the extract of *Didemnum varians* (collected in 1983) was found to have antitumor activity as shown in Table 1. The fraction DV-1 had the tumor inhibition ratio of 68.1% on Sarcoma 180 solid tumors in ICR mice by il injection three times at the dose of 250 mg/kg. Complete disappearance of the tumor was observed in 2 out of 3 mice. However, it also caused death in 3 out of mice administered.

It is often reported, especially in marine organisms that collections of the same species, either from a relatively small geographical region or the same site at different seasons, vary greatly in activity. Then, we inquired the antitumor activity of the high molecular weight fraction from the extract of the other *Didemnum* specimens. The results are summarized in Table 1. DV-2 prepared from the specimen collected in August 1985 was non toxic. It inhibited 58.7% of the tumor growth and caused complete tumor regression in 4 out of 7 mice. DV-3 gave the high inhibitory activity. Complete disappearance of the tumor was observed by a single il injection of DV-3 at the dose of 400 mg/kg (Table 1). The result indicated that the different lots of the macromolecular fractions from *Didemnum* specimens had almost the same as the inhibitory activity.

DV-1 contained 56.5% of proteins and 10.8% of carbohydrates by weight. In order to see whether or not the active site of the antitumor agent was protein portion, DV-1 was digested with pronase E. Interestingly, the toxicity of DV-1 disappeared after the enzyme digestion in which animals were absolutely healthy and gained weight. Pronase E digest (DV-1P, molecular weight over 10,000 Da) of DV-1 showed the inhibition ratios of 100 and 90.0% when given il at the dose of 250 and 50 mg/kg, respectively.

One of the most important problems concerning the antitumor action of DV-1P is whether or not systemic injection is effective. This fraction showed tumor suppression at the dose of 25 mg/kg, given an inhibition ratio of 68.2%, when injected iv three times. The inhibition ratio observed with systemic injection was somewhat less than the high values achieved by local injection, but it was still significant.

On the other hand, cytocidal activity of this fraction was found to reduce markedly when

Table 1. Antitumor activity of the fractions from *Didemnum varians* on Sarcoma 180 solid tumors in ICR mice

Fraction	Dose (mg/kg × days)	Administration route	Average tumor weight (g) (treated/control)[a]	Tumor inhibition ratio (%)	Complete regression[b]
DV-1[c]	250×3	il[f]	2.14±3.03/6.70±2.52	68.1	2/3
DV-2[d]	250×3	il	1.90±2.90[h]/4.60±1.65	58.7	4/7
DV-3[e]	400×1	il	0[i]/5.04±3.21	100	5/5
Autoclave-treated DV-1	250×3	il	1.15±0.98[i]/6.70±2.52	82.8	1/5
DV-1P	25×3	iv[g]	2.10±1.26[h]/6.60±2.94	68.2	0/6
(over 10,000 Da)	50×3	il	0.66±0.54[i]/6.60±2.94	90.0	1/6
	250×3	il	0[i]/6.70±2.52	100	5/5
DV-1P-R (500–10,000 Da)	100×3	il	6.28±4.50/6.42±4.30	2.2	1/6
DV-1P-F (below 500 Da)	100×3	il	5.14±4.62/6.24±4.30	19.9	0/5

a. Values are average±S.D.
b. Numbers of mice with complete tumor regression/total number of mice tested.
c. Collected in October 1983.
d. Collected in August 1985.
e. Collected in October 1985.
f. Lesional injection.
g. Intravenous injection.
h. $p<0.05$ compared to control group.
i. $p<0.01$ compared to control group.

compared with those of the intact DV-1. Pronase E digest (DV-1P) showed only weak cytocidal effect on Sarcoma 180 cells (inhibition ratio, 8.4% at 10 μg/ml), while the intact DV-1 inhibited 66.1% of the *in vitro* growth of Sarcoma 180 cells at 10 μg/ml. On the other hand, lower-molecular weight fractions, which were obtained by pronase E digestion and successive ultrafiltration using UM-05 membrane (molecular cut 500 Da), gave high cytocidal activity. Thus, DV-1P-R (molecular weight in the range of 500–10,000 Da) inhibited 54.9% of Sarcoma 180 cells growth at 10 μg/ml *in vitro*, which was comparable to those of the intact DV-1. The filtrate of UM-05 membrane (DV-1P-F) also had moderate cytotoxic activity (14.2% at 10 μg/ml). In spite of these highly cytocidal activity, these fractions had only weak antitumor activity *in vivo*. These results suggested that the main antitumor agent(s) in *D. varians* was high-molecular weight and the activity was mainly due to its carbohydrate portion. The preservation of the initial antitumor activity after autoclaving of DV-1 at 120°C for 25 min at 1 kg/cm^2 supported this possibility (Table 1).

We studied whether the mice, which showed complete regression of Sarcoma 180 by administration of DV-1P, maintained an ability to reject the reinoculation of Sarcoma 180 cells. Sarcoma 180 cells (2×10^6 cells/mouse) were transplanted sc into the right flank of ICR mice. The mice were injected il with 250 mg/kg of DV-1P on days 5, 7, and 9 after tumor inoculation. The mice which showed complete regression of Sarcoma 180 on the day 35 after the tumor inoculation were reinoculated with the same tumor cells into their left flank (2×10^6 cells/mouse). All of the mice could reject the rechallenged Sarcoma 180 tumor cells completely. This result and the result that DV-1P showed almost no direct cytocidal action on tumor cells strongly suggest that the antitumor activity of DV-1P would be host-mediated.

Recently, a Caribbean tunicate, *Tridemnum* sp. was found to contain a new class of depsipeptides with highly antiviral and antitumor activities.[10] The major active principle termed didemnin A has a molecular weight of 942.[11] The active component which we detected in the present study was quite different from didemnins in their physicochemical properties and also in biological activity. Purification of the active principle is under progress.

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