Selective cytotoxicity of marine algae extracts to several human leukemic cell lines

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Abstract

Extracts from 8 species of marine algae which showed selective cytotoxicity in our previous screening program, were further examined for cytotoxic spectra to five human leukemic cell lines. The extract from a red alga, *Amphiroa zonata* exhibited strong cytotoxicity to all human leukemic cell lines tested and murine leukemic cells L1210 at the final concentrations from 15 to 375 $\mu\text{g ml}^{-1}$. Then the cytotoxicity was not found in normal human fibroblast HDF and murine normal cells NIH-3T3. The active extract fraction from this alga was soluble in higher polar organic solvents and water and heat-stable. The extract from a brown alga *Dilophus okamurai* with weak selective cytotoxic activity to L1210 cells exhibited not only strong cytotoxicity to L1210, but also to human leukemic cells, HL60 and MOLT-4 at 50 $\mu\text{g ml}^{-1}$. While, the extract from a green alga, *Cladophoropsis vaucheriaeformis* with most selective cytotoxic activity, did not show cytotoxicity to any human leukemic cell lines tested at 50 $\mu\text{g ml}^{-1}$. However, this extract showed strong cytotoxicity to two human leukemic cell lines and NIH-3T3 at 100 $\mu\text{g ml}^{-1}$. Thus, it was considered that a red alga, *Amphiroa zonata* might be suitable natural source for development of anti-cancer agents without side-effect.

Introduction

It has been reported that marine algae contain various biologically active compounds (Rinehart, 1981; Lincoln *et al.*, 1991; Cannell *et al.*, 1993). To date, several studies have focused on water soluble antitumor active substances from various marine algae (Kashiwagi *et al.*, 1980; Noda *et al.*, 1990). For examples, these include one of marine algae, Wakame (Furusawa *et al.*, 1985; Ohigashi *et al.*, 1992), crude extracts and partially purified polysaccharides from brown algae (Yamamoto *et al.*, 1984). In these reports, marine algae have exactly active substances against experimental tumor, but such as anticancer agents derived from marine algae still have not been applied for clinical use. Only a few applications of some active substances from marine algae are seen in health food diets. Clinically, several anticancer agents are effectively used for cancer treatment, but serious side-effects of these chemotherapeutic agents are commonly observed in patients because of nonselective cytotoxic effect

to both tumor and normal cells. Therefore, selective antitumor compounds without side-effects are urgently required for clinical use.

Thus, development of anticancer agents is attempted based on new concept of selective cytotoxic activity against only tumor cells in this study. Furthermore, because such an antitumor activity is not enough researched on wide-ranged species of marine algae in previous other reports, marine algae species more than hundreds which were not attempted previously must be screened to obtain selective antitumor activity.

From the above reason, our previous screening of *in vitro* antitumor activity from marine algae was initiated to evaluate the cytotoxicity to murine leukemic cells L1210 and normal cells NIH-3T3 with the PBS and MeOH extracts from in total 306 species of marine algae from Japan coasts, and the selective cytotoxicity to only tumor cells L1210 was found in some particular marine algae extracts. Among these positive algae extracts, the cytotoxic spectrum was further examined to six mouse- and two human-derived tumor cell lines,

and the extracts from such a green alga *Cladophoropsis vaucheriaeformis* and a red alga *Amphiroa zonata* were previously noted to have most prominent selective cytotoxicity to murine leukemic cell lines (Harada *et al.*, 1997).

In this study, we further examined *in vitro* antitumor activity of these marine algae extracts against five human leukemic cell lines, K-562, WIL2-NS, HL60, MOLT-4 and Raji, comparing with murine leukemic cells L1210. These cytotoxic spectra will be discussed in this paper.

Materials and methods

Sample algae extracts

The marine algae extracts which showed selective cytotoxicity to only murine leukemic cells L1210 in our previous study were selected to investigate the cytotoxic spectra to human leukemic cell lines. The number of algae samples were 8 (5 brown, 1 red and 2 green algae), and these crude MeOH or phosphate buffered saline (PBS) extracts were evaporated by freeze-drying, and redissolved in PBS or MeOH to give the concentrations of 4, 10 and 20 mg ml⁻¹.

Cell lines

Mouse lymphoid leukemia L1210 cells and embryo fibroblast NIH-3T3 cells were used as control. These cells were cultured in Dulbecco's modified Eagle's medium (Gibco)/F-12 medium (Gibco) (1:1, v/v) supplemented with 120 µg ml⁻¹ of penicillin G (Sigma), 200 µg ml⁻¹ of streptomycin (Sigma), 25 µg ml⁻¹ of ampicillin (Sigma), 3.6 mg ml⁻¹ of HEPES (Katayama Chemical Industry), and 10% fetal bovine serum (FBS).

Human leukemic cell lines, MOLT-4, HL60, K-562, WIL2-NS and Raji were used as the comparative cells for cytotoxic spectrum test. These cell lines were kindly provided by the Japanese Cancer Research Resources Bank (JCRB). These cells were cultured in E-RDF medium (Kyokuto Pharmaceutical Industrial Inc.) with 10% FBS.

Cytotoxic spectrum assay

Each cell line was seeded into 35 mm tissue-culture dishes (1 × 10⁵ cells/2 ml), and incubated for 4 or 6 days at 37 °C in humidified 5%-CO₂ incubator. Then,

each algal extract was added in the cell cultures. The final concentrations of these extracts were 20, 50 and 100 µg ml⁻¹.

After incubation, the cells were counted by cell counter (Coulter Electronics Inc. Industrial D), and the growth rate was calculated by dividing the test cell number by the cell number in control. In order to determine the cell death treated with algal extracts, the cells were further stained with Hoechst 33342 (Molecular Probes, Inc.) and confirmed apoptotic cell death.

Dose response of *Amphiroa zonata* extract to cells

The extract from a red alga, *Amphiroa zonata* was further investigated to confirm the dose response of cytotoxic activity to the human leukemic cell lines described above and normal fibroblast HDF cells (human dermal fibroblast, Morinaga Institute of Biological Science). Each cell was seeded 1 × 10⁴ cells/200 µl into 48 well plate and the 5-fold serial diluted extracts were added to give the final concentrations from 0.6 to 375 µg ml⁻¹. These cells were incubated in condition described above. The cell growth was evaluated with MTT assay kit (Boehringer Mannheim). The cytotoxicity was estimated by dividing the absorbance of test group by that of control.

Characterization of the active extract fraction from *Amphiroa zonata*

Water, MeOH, acetonitrile, ethyl acetate, chloroform and hexane (all organic solvents were obtained from Katayama Chemical Industry) were used for solubility test of the active extract fraction from a red alga, *Amphiroa zonata*. A freeze-dried extract from this alga was dissolved in each test solvent, then the supernatant was taken out to new tube and further dried. The dried sample was redissolved in MeOH and adjusted to initial volume. These extracts were tested for the cytotoxic activity against several tumor cell lines described above. Solubility was indirectly confirmed by evaluating the recovery of cytotoxic activities of these extracts.

The active extract fraction from *Amphiroa zonata* was treated at 60 °C for 10 min in water bath. Then, the extract was tested for the selective cytotoxicity as described above. Roughly estimation of the molecular weight of the cytotoxic substances in the MeOH extract was conducted by ultrafiltration technique with molecular weight of 3000, 5000, and 10000 cut-off membrane tubes (Millipore). After centrifugation at 13000 rpm for 10 min, each supernatant was assayed

for cytotoxic activity to confirm whether or not the activity was ultra filtered.

Results

Selective cytotoxic spectrum of marine algal extracts

Cytotoxicity of marine algal extracts was evaluated by growth inhibition. When the growth inhibited cells were stained with Hoechst 33342, apoptotic cell death was observed in all cultures (data not shown). These results suggested that algal extracts caused irreversible cell damages in cultured cells. Table 1 shows results of the cytotoxic spectra of marine algal extracts to several human leukemic cell lines. The sensitivity of the human leukemic cell lines to the algal extracts were generally weaker than murine-derived cell lines.

Prominent selective cytotoxic activity was found in the MeOH extract from a red alga, *Amphiroa zonata*. This algal extract showed strong cytotoxicity to all leukemic cell lines tested including murine leukemic cells L1210 at $20 \mu\text{g ml}^{-1}$, and then such a cytotoxicity was not observed in murine normal cells NIH-3T3.

Dose response of the extract from Amphiroa zonata

Since the MeOH extract from a red alga, *Amphiroa zonata* showed excellent selective cytotoxicity to all leukemic cell lines tested as described above, the dose response of the algal extract was further examined to the human leukemic cell lines and one normal fibroblast (Figure 1). At the concentrations more than $15 \mu\text{g ml}^{-1}$, these leukemic cells showed significant low growth rate, indicating higher cytotoxicity of the extract to these cell lines. However, both normal murine NIH-3T3 and human HDF cells were not affected their growth rates.

MeOH extract from a brown alga, *Dilophus okamurae* showed not only strong cytotoxicity to L1210 cells, but also to two human leukemic cells, HL60 and MOLT-4 cells at $50 \mu\text{g ml}^{-1}$. Then, the growth rate of NIH-3T3 was relatively high, indicating low cytotoxicity to normal NIH-3T3 cell line (Figure 2a).

Figure 2b shows the cytotoxic spectrum of MeOH extract from a green alga *Cladophoropsis vaucheriaeformis* to the leukemic cell lines. At a concentration of $50 \mu\text{g ml}^{-1}$, strong cytotoxicity was particularly found in L1210 cells, showing low cytotoxicity to NIH-3T3 cell line. While, no cytotoxicity was observed in all human leukemic cell lines tested. At a higher con-

centration of $100 \mu\text{g ml}^{-1}$, strong cytotoxicity was not only found in leukemic cells L1210, HL60 and MOLT-4, but also in normal cells NIH-3T3. Conspicuously selective cytotoxic activity was not observed at over $100 \mu\text{g ml}^{-1}$, as did at lower concentration of this alga extract. However, other algal extracts did not show such a selective cytotoxic activity to human leukemic cell lines at any concentrations tested.

Chemical properties of the active extract fraction from Amphiroa zonata

Solubility of the active extract fraction from *Amphiroa zonata* was investigated with several organic solvents and water by indirectly estimating the recovery of the cytotoxic activity. High solubility was found in higher polar solvents, acetonitrile and ethyl acetate, and water (Figure 3). Furthermore, the active extract fraction showed heat-stable property. The molecular-sieving study also indicated that the molecular weight of this cytotoxic substance might be less than 3000.

Discussion

It has been reported that water-soluble extracts from marine algae show *in vivo* antitumor activity, and it is considered that these active substances may be polysaccharides (Kashiwagi *et al.*, 1980; Yamamoto *et al.*, 1984; Furusawa *et al.*, 1985; Noda *et al.*, 1990; Ohigashi *et al.*, 1992). In these reports, marine algae have active antitumor substances against experimental tumor, but such as anticancer agents derived from marine algae still have not been applied for clinical use.

In our previous screening program, 306 species of marine algae were examined for *in vitro* selective antitumor activity against murine leukemic cells L1210, comparing with murine normal cells NIH-3T3. Among them, only 17 species of marine algae extracts showed the selective cytotoxicity to murine leukemic cells L1210, but not to normal cells NIH-3T3. Furthermore, a tendency which the extracts exhibited cytotoxicity to only leukemic cell lines was obtained in cytotoxic spectrum test using several tumor cell lines (Hara-da *et al.*, 1997). Therefore, in this study, these algae extracts were further examined for cytotoxic spectra to additional five human leukemic cell lines and normal fibroblast.

A remarkably selective cytotoxic activity was found in MeOH extract from a red alga, *Amphiroa zonata* in

Table 1. Cytotoxic spectra of marine algal crude extracts to several human leukemic cell lines

Algae name	Concentration ^a ($\mu\text{g ml}^{-1}$)	Murine cells ^b		Human leukemic cells				
		L1210	3T3	K-562	HL60	MOLT-4	Raji	WIL2-NS
Brown algae								
<i>Dilophus okamurae</i>	50	++ ^c	—	—	++	++	—	—
<i>Eisenia bicyclis</i> ^d	20	+	—	—*	—	—*	—	—
<i>Ecklonia cava</i> ^d	20	++	+	—	—	—	—	—
<i>Ecklonia kurome</i> ^d	20	++	+	—*	—	—	—	—*
<i>Myagropsis myagroides</i>	20	+	—	—	+	—	—*	—*
Red algae								
<i>Amphiroa zonata</i>	20	++	—*	++	++	++	++	++
Green algae								
<i>Cladophoropsis vaucheriaeformis</i>	50	++	—	—*	—*	—*	—*	—
<i>Halimeda discoidea</i>	20	+	—	—*	—	—	—*	—

^a Final concentrations of the marine algal crude extracts in the cell cultures. ^b These cell lines were used in our previous screening program. ^c Growth rate: ++, <0.1; +, 0.1 to 0.3; —, 0.3 to 0.8; —*, >0.8. ^d Result from PBS extract and others are that from MeOH extract.

Figure 1. Dose response of a MeOH extract from a red alga, *Amphiroa zonata*. This algal extract showed selective cytotoxicity to all leukemic cells tested, and then no cytotoxicity was observed in two normal cells, murine NIH-3T3 and human HDF. The selective cytotoxicity was found at 15 to 375 $\mu\text{g ml}^{-1}$. Solid lines show the growth rates of leukemic cell lines and broken lines show those of normal cell lines in the presence of the extract in the cultures.

Figure 2a–b. Cytotoxic spectra of MeOH extracts from marine algae to human leukemic cell lines. a: the cytotoxic spectra of a brown alga, *Dilophus okamurae*. b: the cytotoxic spectra of a green alga, *Cladophoropsis vaucheriaeformis*. The brown alga, *Dilophus okamurae* showed the selective cytotoxicity to only HL60 and MOLT-4 cells at a final concentration of 50 $\mu\text{g ml}^{-1}$. The extract from a green alga, *Cladophoropsis vaucheriaeformis* showed highly selective cytotoxicity to murine leukemia at a same concentration. However, it did not show such selective cytotoxicity to any human leukemic cell lines, when these were compared to control cell lines used in our previous screening program.

this study. At a concentration of 20 $\mu\text{g ml}^{-1}$, this extract showed the selective cytotoxicity to L1210 and all human leukemic cell lines tested. Then, the growth rate of NIH-3T3 was significantly high. Such a selective cytotoxic spectrum was not found in the other algae extracts in current other reports. A few chemical

properties of the active substance present in the extract was soluble in relatively higher polar organic solvents and water and heat-stable. The molecular weight might be less than 3000 which would be different from the substances such as polysaccharides from other reports.

Figure 3. Solubility of the active crude extract fraction from a red alga, *Amphiroa zonata* to several solvents. Solubility was indirectly evaluated by estimating the activity ratio which growth rate in each solvent groups was divided by that in MeOH group. This active fraction was soluble in higher polar solvents and water.

In the extract from a brown alga, *Dilophus okamurae*, selective cytotoxicity was found in HL60 and MOLT-4 cultures at a concentration of $50 \mu\text{g ml}^{-1}$. It has been reported that members of the family Dictyotaceae including *Dilophus okamurae* contains many unique structural compounds (Fattorusso *et al.*, 1976; Finder *et al.*, 1979; Ochi *et al.*, 1979, 1980; Enoki *et al.*, 1982; Ishitsuka *et al.* 1988). Most of them have been researched only on their chemical structures, however, their bioactivities have not been enough investigated. Furthermore, though Ochi *et al.* (1982) reported some chemical structures of compounds from this alga, *Dilophus okamurae*, there are no description of cytotoxic spectrum against several human-derived cell lines.

MeOH extract from a green alga, *Cladophoropsis vaucheriaeformis* which showed most selective cytotoxicity in our previous screening program did not show similarly selective cytotoxicity to human

leukemic cell lines tested. Strong cytotoxicity was found to two human leukemic cell lines and murine leukemic and normal cell lines at $100 \mu\text{g ml}^{-1}$. However, there have been no reports on the cytotoxic activity against those human-derived cell lines from these marine green algae. Other algal extracts did not show such a selective cytotoxicity to human leukemic cell lines at any concentrations tested.

From above results, a marine red alga, *Amphiroa zonata* might be the most suitable for further research to deal with selective antitumor active substances to human leukemia. Thus, purification and characterization of the active substance from this alga and the mechanism of selective cytotoxicity are also needed on further studies.

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