Selective Antitumor Activity *in Vitro* from Marine Algae from Japan Coasts

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***In vitro* selective antitumor activity was tested as a general screening parameter for biologically active substances from a wide range of species of seaweed, 1446 samples of 306 species of marine algae from Japan's coasts. The algae extracts were prepared successively first by phosphate buffered saline (PBS) and then by methanol, and then tested for *in vitro* selective antitumor activity against murine lymphoid leukemia L1210 cells and for low cytotoxic activity against NIH-3T3 normal cells.**

Strong cytotoxic activity against L1210 cells was found in 47 species of algae, also showing similar cytotoxicity to mouse NIH-3T3 normal cells. However, four species of green algae showed strong activity specifically against L1210 cells, with low cytotoxicity to normal cells. Such selective activity was conspicuous in two brown and two green algae extracts. In particular, methanol extracts from the green alga, *Cladophoropsis vaucheriaeformis*, exhibited high viability (86%) to normal cells, showing selective cytotoxicity to tumor cells. This alga extract was not cytocidalic, but cytostatic against L1210 cells. Furthermore, the results of a cytotoxic spectrum test with 9 cell lines including those of L1210 and NIH-3T3 demonstrated that this extract acted strongly only against leukemic cell lines L1210 and P388.

Key words antitumor activity; *in vitro* cytotoxic assay; marine algae extract; cytotoxic spectrum

It has been reported that marine algae contain various biologically active compounds.[1–3] However, screenings of these biological activities have been designed for selected species of algae and screening objects. Although there are over 1500 species of marine algae in Japan, only a few percent of samples have undergone this screening while more than 90% of Japan's marine algae remain untested. To screen for various kinds of biologically active substances on a wider scale, we employed different screening methods for over 300 species of algae which inhabit the country's seashore.

In several other studies which have focused on antitumor activity of marine algae, crude extracts or partially purified polysaccharides from the algae have reportedly been effective against experimental tumors. For example, 107 species of marine algae from the Pacific islands have been tested against P-388 lymphocytic leukemia and Ehrlich ascites tumor, and several extracts were found to possess activity against both tumors.[4] An extract of *Undaria pinnantifida* exhibits antitumor cell activity against implanted tumor, and this antitumor action appears to be due to polysaccharides[5] or to a combination of various components in the extract.[6] A partially purified fucoidan from *Eisenia bicyclis* also exhibits antitumor cell activity against L1210 leukemia.[7] Furthermore, when air-dried algae tissues of 46 species were tested against Ehrlich carcinoma and Meth-A fibrosarcoma, Ehrlich carcinoma was repressed by 7 species (4 brown algae, *Scytosiphon lomentaria*, *Lessonia nigrescens*, *Laminaria japonica* and *Sargassum ringgoldianum*; 2 red algae, *Porphyra yezoensis* and *Eucheuma gelatinae*; 1 green algae, *Enteromorpha prolifera*), and Meth-A fibrosarcoma was repressed by 9 species (5 brown and 4 red algae).[8] These studies concerned water-soluble substances, mostly polysaccharides or glycoproteins. There are no reports on lipid-soluble agents from marine algae which might be very useful for clinical administration. Clinically, several anticancer drugs are effective for cancer treatment, but it is common for these chemotherapeutic agents to have side-effects; thus, compounds with selective antitumor effects that do not have side-effects are urgently required for clinical use.

Since no selective antitumor effects have been noted in the reports and screenings carried out for algae of selected species from limited areas, a wider range of algae species needs to be tested for these effects.

Because of the limited success of the studies described above, screening of *in vitro* selective antitumor activity was conducted in this study on 1446 samples of 306 species of algae from Japan's coasts in an attempt to find methanol-extracts from them which might be different from those in previous reports; mouse lymphocytic leukemia L1210 and normal NIH-3T3 cells were used as controls. Representative algae samples which showed strong *in vitro* antitumor activity were then compared for selective activity against other tumor cells.

MATERIALS AND METHODS

Sample Algae Sample algae were collected by skin-diving from 79 points along the coastline (Fig. 1) between April 1994 and October 1995. Algae were taken to the laboratory and kept either cold or frozen, and were then classified. A total of 1446 samples was obtained comprising 306 species: 93 brown, 151 red, 51 green algae and 11 others.

Preparation of Algae Extracts After washing the sample algae three times with sterilized artificial seawater (ASW, Jamarin Laboratory) and phosphate-buffered saline (PBS), 5 g of each sample (wet weight) was mixed with 20 ml of PBS and homogenized at 8000 rpm for 5 min by a Polytron (Kinematica Inc.). After centrifugation at 3000 rpm for 20 min, supernatants were collected and

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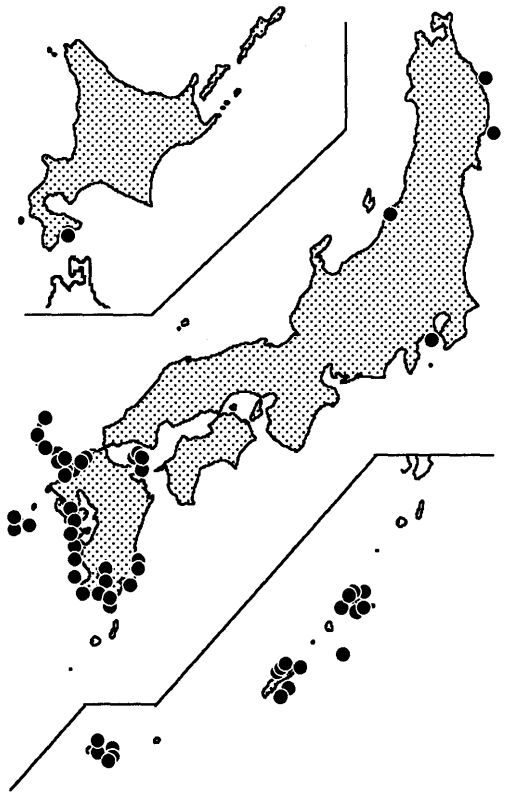

Fig. 1. Collection Locations of Sample Algae in Japan

Sample algae were collected at a total of 79 points along the coastline. ● indicates a sampling point.

filtered with a 0.2 μm-pore-sized filter (Millipore, filter type GV) as the water-soluble extract, and the remaining pellets were mixed with 20 ml of methanol, homogenized and centrifuged as described above. Supernatants were filtered through a 0.2 μm-pore-sized filter (Millipore, filter type GV) as lipid-soluble extracts.

Cells and Culture Condition Mouse lymphoid leukemia L1210 and mouse embryo fibroblast NIH-3T3 were used as the experimental tumor and normal cells, respectively. The cells were kindly provided by the Japanese Cancer Research Resources Bank (JCRB). Cells were cultured in DULBECCO'S modified EAGLE'S medium (Gibco)/F-12 medium (Gibco) (1 : 1, v/v) supplemented with penicillin G 120 μg/ml (Sigma), streptomycin 200 μg/ml (Sigma), ampicillin 25 μg/ml (Sigma), HEPES 3.6 mg/ml (Katayama Chemical Industry), and 10% fetal bovine serum (FBS) for 4 or 6 d at 37 °C in a 5% CO_2-incubator.

***In Vitro* Antitumor Activity Assay** Tumor cells were seeded onto 35 mm tissue-culture dishes (1×10^5 cells/2 ml medium), 10 μl of the sample algae extracts was added to each dish, and the cultures were incubated for 4 d. Under this condition, because these algae samples contained about 3 to 5 mg/ml of extracts in our preliminary experiments, final concentrations of the extracts in these cultures might be expected to be approximately 15 to 25 μg/ml. Cell numbers were then counted by a Coulter Counter (Coulter Electronics Inc. Industrial D). Control cultures were set up with either 10 μl of PBS or MeOH. The growth rate was calculated by dividing the cell number of test samples by the cell numbers of control.

When cytotoxicity was observed in an extract, the selective activity in that extract was subsequently tested against NIH-3T3, normal cells.

Time Course of L1210 and NIH-3T3 Cells Growth with Algae Extract 1×10^5 L1210 and NIH-3T3 cells per dish were prepared and 10 μl of alga extract was added to each dish. As the positive control, an equal dose with the extract of an anti-cancer agent, mitomycin C, was used and compared with the positive algae extracts. During the 10-d incubation, 2 dishes from each culture type were tested for total and viable cell numbers every 2 d, and the cell numbers averaged. Viable cells were estimated by trypan blue staining.

***In Vitro* Cytotoxic Spectrum Test** HeLa, KB, P388, Ehrlich ascites, B16 melanoma, sarcoma-180, and myeloma P3x63Ag8U.1 (P3U1) cells were used as comparative tumor cell lines in this test. Each cell type was seeded onto 35 mm tissue-culture dishes (1×10^5 cells/2 ml medium) and 10 μl of algae MeOH-extracts from four representative algae, *Cladopholopsis vaucheriaeformis*, *Halimeda discoidea* (both green algae), *Laurencia okamurae* (red algae), and *Dictyopteris undulata* (brown algae), was added to each dish.

L1210, P388, and P3U1 cell lines (floating cells) were incubated at 37 °C for 4 d and the other cell lines (adhesive cells) for 6 d in a 5%-CO_2 incubator. After incubation, cells were counted and compared.

RESULTS

Screening for Cytotoxic Activity We collected 1446 samples of marine algae from 79 points along Japan's coastline. The total number of species was 306 (brown, red, green algae and others were 93, 151, 51 and 11 species, respectively). Out of 306 seaweed species, 81 exhibited cytotoxic activity against mouse tumor L1210 cells (36 brown, 30 red, 14 green algae and 1 other). Among the four types of sample algae, the highest activity against L1210 was found in brown algae.

Among 93 species of brown algae, cytotoxic activity against L1210 cells was found in 36 species (Table 1). *Sargassum horneri*, *S. thunbergii*, *S. piluliferum*, and *Dilophus okamurae* showed activity in both PBS and methanol extracts. *S. horneri* exhibited particularly high cytotoxic activity, with the cell number of L1210 being less than 10% that of control in both extracts.

Among the brown algae, the activity of Dictyotales algae was found in methanol extracts, but the activities of Laminarials and Scytosiphonales algae were found in PBS extracts. In Fucales algae, activity was present in both extracts, however, more was found in the methanol extracts than in the PBS extracts.

Variations in activity due to seasonal differences was not investigated in detail in this study, but *S. horneri*, which was collected from 15 sampling points, tended to show activity in PBS extracts of spring and summer samples, and in methanol extracts of winter samples (data not shown).

In the results of the NIH-3T3 cell assay to determine cytotoxicity to normal cells, all extracts showed cytotoxicity. However, some brown algae showed relatively low cytotoxicity to normal NIH-3T3 cells. Activity against

Table 1. *In Vitro* Antitumor Activity from Brown Algae

Name of algae species		Cytotoxicity[a]		MW[b]
Scientific	Japanese	L1210	3T3	
Chordariales				
Chordaria flagelliformis (O. F. MUELLER) C. AGARDH	Nagamatsumo	++	−	L
Ishige sinicola (SETCHELL *et* GARDNER) CHIHARA	Iroro	(+++)	(+++)	(L)
Leathesia difformis (LINNAEUS) ARESCHOUG	Nebarimo	+	−	L
Nemacystis decipiens (SURINGAR) KUCKUCK	Mozuku	++	++	S
Dictyotales				
Dictyopteris membranacea (De CANDOLLE) LAMOUROUX	Uraboshiyahazu	(++)	(++)	(L)
Dictyopteris undulata HOLMES	Shiwayahazu	(++)	(++)	(S)
Dictyota dichotoma (HUDSON) LAMOUROUX	Amijigusa	(++)	(+)	(S)
Dictyota spinulosa HARVEY	Hariamiji	+, (+++)	+, (+++)	(L)
Dilophus okamurae DAWSON	Hukurin-amiji	+, (++)	−, (−)	S, (L)
Pachydictyon coriaceum (HOLMES) OKAMURA	Sanadagusa	(++)	(++)	(S)
Padina arborescens HOLMES	Umiuchiwa	+	−	L
Stypopodium zonale (LAMOUROUX) PAPENFUSS	Jigamigusa	(++)	(++)	(S)
Zonaria stipitata TANAKA *et* K. NOZAWA	Etsukishimaoogi	(+)	(−)	(L)
Fucales				
Myagropsis myagroides (MERTENS *et* TURNER) FENSHOLT	Joromoku	(++)	(++)	(S)
Sargassum sp.	Hondawara sp.	(++)	(−)	(L)
Sargassum confusum C. AGARDH	Hushisujimoku	(++)	(+)	L
Sargassum fulvellum (TURNER) C. AGARDH	Hondawara	+, (++)	−, (+)	L, (S)
Sargassum horneri (TURNER) C. AGARDH	Akamoku	++, (++)	+, (+)	L, (L)
Sargassum macrocarpum C. AGARDH	Nokogirimoku	++	+	S
Sargassum micracanthum (KUTZING) ENDLICHER	Togemoku	(+)	(++)	(S)
Sargassum oligocystum	—	(++)	(++)	(S)
Sargassum piluliferum (TURNER) C. AGARDH	Mametawara	(++)	(+)	(L)
Sargassum thunbergii (MERTENS *et* ROTH) Kuntze	Umitoranoo	+, (++)	+, (++)	L, (L)
Sargassum yendoi OKAMURA *et* YAMADA	Endomoku	(++)	(−)	(S)
Coccophora langsdorfii (TURNER) GREVILLE	Sugimoku	+	−	L
Hizikia fusiformis (HARVEY) OKAMURA	Hijiki	+	−	L
Sargassum ringgoldianum (J. AGARDH) YOSHIDA	Yanagimoku	(++)	(+)	(L)
Sargassum siliquastrum (TURNER) C. AGARDH	Yoremoku	(+++)	(+++)	(L)
Turbinaria ornata (TURNER) J. AGARDH	Rappamoku	+	+	L
Laminariales				
Ecklomia cava KJELLMAN	Kajime	++	−	L
Eisenia bicyclis (KJELLMAN) SETCHELL	Arame	++	−	L
Ecklonia kurome OKAMURA	Kurome	++	+	L
Laminaria religiosa MIYABE	Hosomekonbu	++	+	L
Undaria pinnatifida (HARVEY) SURINGAR	Wakame	++	++	L
Scytosiphonales				
Colpomenia sinuosa (MERTENS *et* ROTH) DERBES *et* SOLIER	Hukuronori	+	−	L
Scytosiphon lomentaria (LYNGBYE) LINK	Kayamonori	++	−	L

a) +++, Growth rate of L1210 in the presence of PBS-extracts (parentheses indicate the results from cultures with MeOH-extracts) was less than 0.03; ++, 0.03—0.1; +, 0.1—0.3; −, 0.3—0.8. *b*) Molecular weight was roughly estimated by dialysis using 3500-MW cut-off membrane; L, >3500; S, <3500.

L1210 cells might be proportional to the cytotoxicity against NIH-3T3 cells.

Among 151 species of red algae, activity against L1210 cells was found in 30 species (Table 2). Three red algae showed activity in both PBS and methanol extracts. The methanol extract of *P. telfairiae*, in particular, exhibited relatively low cytotoxicity to NIH-3T3 cells, although it showed high activity against the tumor cell. Nine red algae showed not only high activity against the tumor cells, but against NIH-3T3 cells as well. No brown or red algae extracts showed selective cytotoxicity, which was highly toxic (more than ++) to L1210 tumor cells and much less toxic (−*) to NIH-3T3 normal cells.

Green algae and most of the active algae were collected from the southern area of Japan. Among 51 species of green algae, activity against L1210 cells was found in a total of 14 (Table 3). Three green algae showed activity in both PBS and methanol extracts. High activity against tumor cells was found in methanol extracts from 8 green algae. Interestingly, *Halimeda discoidea* and *Caulerpa racemosa* var. *laete-virens* showed markedly low cytotoxicity to normal NIH-3T3 cells. *Cladophoropsis vaucheriaeformis* also exhibited highly selective cytotoxicity to L1210 tumor cells, but the lowest activity against NIH-3T3 normal cells in algae extracts which showed high activity against L1210 tumor cells. The viability of NIH-3T3 cell culture with MeOH extract from *C. vaucheriaeformis* was 86% (data not shown). The selective cytotoxicity was found at a final concentration of 12.5 μg/ml of MeOH extract from *Halimeda discoidea*, and at 25 to 100 μg/ml of MeOH extract from *Cladophoropsis vaucheriaeformis* (data not shown). These results differed markedly from the brown or red algae mentioned above.

In addition, one species of Cyanophyta showed non-selective cytotoxic activity (Table 3).

Time Course of L1210 and NIH-3T3 Cell Growth in the

Table 2. *In Vitro* Antitumor Activity from Red Algae

Name of algae species		Cytotoxicity[a]		MW[b]
Scientific	Japanese	L1210	3T3	
Ceramiales				
Chondria crassicaulis HARVEY	Yuna	+, (++)	−, (−)	L, (L)
Digenea simplex (WULFEN) C. AGARDH	Makuri	(+)	(−)	(L)
Laurencia okamurae YAMADA	Mitsudesozo	(++)	(++)	(L)
Laurencia papillosa (C. AGARDH) GREVILLE	Papirasozo	(++)	(++)	(L)
Laurencia sp.	Sozo sp.	(+)	(−)	(S)
Martensia denticulata HARVEY	Ayanishiki	++	++	L
Cryptonemiales				
Amphirosa dilatata LAMOUROUX	Kaninote	(+)	(−)	(L)
Chondrococcus homemannii (LYNGBYE) SILVA	Hosobanaminohana	(++)	(++)	(S)
Corallina pilulifera POSTELS *et* RUPRECHT	Pirihiba	(+)	(+)	(L)
Grateloupia acuminata HOLMES	Oomukadenori	+	−	L
Grateloupia filicina (LAMOUROUX) C. AGARDH	Mukadenori	+	−	L
Grateloupia sparsa (OKAMURA) CHIANG	Hijirimen	+	−	L
Grateloupia turuturu YAMADA	Tsurutsuru	+	−	L
Kallymenia perforata J. AGARDH	Tsukasaami	+	−	L
Gelidiales				
Gelidiella acerosa (FORSSKAL) FELDMANN *et* HAMEL	Shimatengusa	(+)	(−*)	(S)
Gelidium amansii KUTZING	Makusa	(++)	(++)	(L)
Gigartinales				
Ahnfeltiopsis flabelliformis (HARVEY) MASUDA	Okitsunori	(++)	(++)	(S)
Chondrus occellatus HOLMES	Tsunomata	++	−	L
Hypnea charoides LAMOUROUX	Ibaranori	(++)	(++)	(L)
Hypnea saidana HOLMES	Saidaibara	+	−	L
Mazzaella japonica (MIKAMI) HOMMERSAND	Akabaginnansou	+	−	L
Plocamium telfairiae (HARVEY) HARVEY	Yukari	+, (++)	++, (−)	L, (S)
Nemaliales				
Actinotrichia fragilis (FORSSKAL) BOERGESEN	Sodegarami	(+)	(++)	(S)
Asparagopsis taxiformis (DELILE) TREVISAN	Kagikenori	(++)	(++)	(L)
Galaxaura fastigitata (ELLIS *et* SOLANDER) HUISMAN *et* BOROWITZKA	Garagara	+, (+)	−, (−)	L, (S)
Liagora fariosa LAMOUROUX	Kekonahada	+	−	L
Liagora japonica YAMADA	Yogorekonahada	+	−	L
Scinaia moniliformis J. AGARDH	Juzuhusanori	++	++	S
Trichogloeopsis mucosissima (YAMADA) ABBOTT *et* DOTY	Nuruhada	+	+	L
Rhodymeniales				
Lomentaria cataenata HARVEY	Fushitsunagi	++	+	L

a) ++, Growth rate of L1210 in the presence of PBS-extracts (parentheses indicate the results from cultures with MeOH-extracts) was less than 0.03; +, 0.03—0.1; +, 0.1—0.3; −, 0.3—0.8, *, more than 0.8. *b*) Molecular weight was roughly estimated by dialysis using 3500-MW cut-off membrane; L, >3500; S, <3500.

Table 3. *In Vitro* Antitumor Activity from Green Algae and Other Seaweed

Name of algae species		Cytotoxicity[a]		MW[b]
Scientific	Japanese	L1210	3T3	
Green algae				
Halimeda macroloba DECAISNE	Hirohasabotengusa	(++)	(−*)	(S)
Caulerpa cupressides	Byakushinzuta	(++)	(++)	(S)
Caulerpa racemosa var. laete-virence	Surikogizuta	(++)	(−*)	(L)
Caulerpa serrulata f. lata (WEBER *van* BOSSE) TSENG	Yorezuta	(+)	(−)	(S)
Caulerpa sertularioides f. longipes (J. AGARDH) COLLINS	Takanohazuta	(++)	(+)	(S)
Cladophoropsis vaucheriaeformis (ARESCHOUG) PAPENFU	Kitsunenoo	+, (++)	+, (−*)	L, (L)
Codium adhaerens (CABRERA) C. AGARDH	Haimiru	+	−*	L
Codium coarctatum OKAMURA	Nezashimiru	(+)	(++)	(S)
Codium fragile (SURINGAR) HARIOT	Miru	+, (++)	−*, (++)	S, (L)
Codium intricatum OKAMURA	Motsuremiru	+	−	L
Codium tenue OKAMURA	Itomiru	+, (+)	+, (++)	L, (S)
Enteromorpha intestinalis (LINNAEUS) NEES	Boaonori	(++)	(++)	(L)
Halimeda discoidea DECAISNE	Uchiwasabotengusa	(++)	(−*)	(S)
Halimeda incrassata (ELLIS) LAMOUROUX	Mitsudesabotengusa	++	++	L
Other seaweed				
Cyanophyceae sp.	Ranso sp.	(++)	(++)	(S)

a) ++, Growth rate of L1210 in the presence of PBS-extracts (parentheses indicate the results from cultures with MeOH-extracts) was less than 0.1; +, 0.1—0.3; −, 0.3—0.8; *, more than 0.8. *b*) Molecular weight was roughly estimated by dialysis using 3500-MW cut-off membrane; L, >3500; S, <3500.

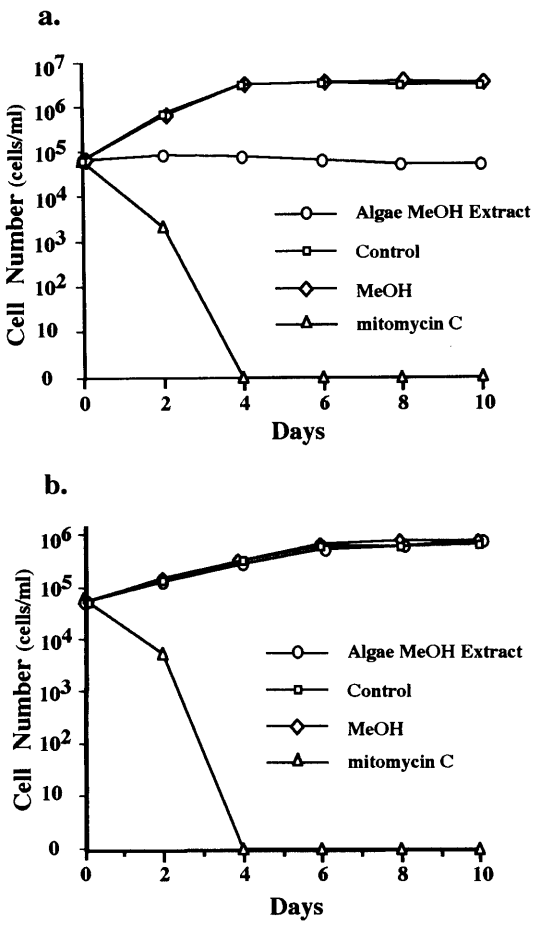

Fig. 2. Growth Curve of L1210 Cells (a) and NIH-3T3 Normal Cells (b) Cultured with MeOH Extract from a Marine Green Alga, *Cladophoropsis vaucheriaeformis*, and Commercial Anti-cancer Agent Mitomycin C

Ten μl of algae extract and mitomycin C (final concentration, 20 μg/ml) was added to 2 ml of culture per dish, and compared to non-additive and MeOH-additive control culture. Initial cell number was 5.0×10^4 cells/ml. After incubation at 37 °C for 10 d in a 5% CO_2 incubator, the cells were counted and calculated for viability every 2 d during a 10-d incubation period.

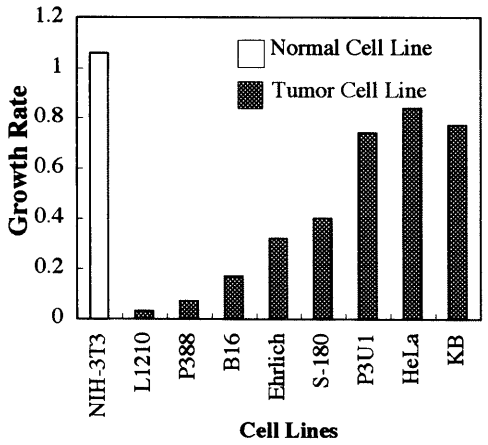

Fig. 3. Selective Cytotoxic Activity of MeOH Extract from a Marine Green Alga, *Cladophoropsis vaucheriaeformis*, against Several Tumor Cell Lines

This alga extract was strongly active against mouse leukemia cell lines L1210 and P388, but weakly active against B-16 melanoma, Ehrlich, and sarcoma 180.

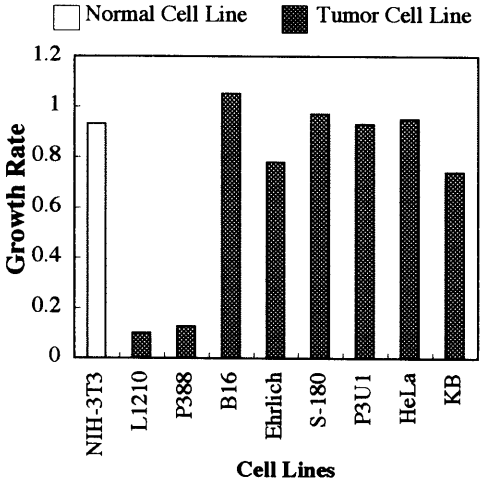

Fig. 4. Selective Cytotoxic Activity of MeOH Extract from a Marine Green Alga, *Halimeda discoidea*, against Several Tumor Cell Lines

This alga extract was active only against mouse leukemia cell lines L1210 and P388.

Presence of Algae Extract Since the methanol extract from *C. vaucheriaeformis* exhibited good selective activity against L1210 tumor cells, the effect of the extract on tumor cell growth was further investigated. Growth peaks of L1210 cells in non-additive and 10 μl methanol additive controls were reached 6 d after the start of incubation, and maximum cell numbers were about 3.5×10^6 cells/ml. Cytotoxicity of methanol itself was not observed at this concentration. The number of L1210 cells with 10 μl of algae extract did not increase during the first day (the final cell number was 5.1×10^4 cells/ml on day 10) (Fig. 2a). L1210 cells at day 4 stained by trypan blue indicated that the active substance in this alga extract was effective as a growth inhibitor against these cells. Conversely, NIH-3T3 normal cells were not affected by this extract during the 10 d of incubation (Fig. 2b). Furthermore, a commercial antitumor agent, mitomycin C, added to the cultures to compare with the effect of the extract showed strong activity at 20 μg/ml. Survival of both L1210 cells and NIH-3T3 normal cells was 0% by the fourth day based on the trypan blue staining technique (Fig. 2a and 2b). However, when the concentration was lower, the survival of both cells increased, indicating no selective activity different from *C. vaucheriaeformis* extract.

***In Vitro* Cytotoxic Spectrum** Extract from a marine green alga *C. vaucheriaeformis* exhibited strong cytotoxicity against two mouse leukemia cell lines, L1210 and P388, and weak cytotoxicity against B16 melanoma, Ehrlich, and sarcoma 180, while normal NIH-3T3 cells were not affected by this extract (Fig. 3). Similarly, extracts from the marine green alga *Halimeda discoidea* were only active against mouse leukemia cell lines L1210 and P388 (Fig. 4).

However, extracts from the marine red alga *Laurencia okamurae* and brown algae *Dictyopteris undulata* exhibited cytotoxicity to all mouse-derived cell lines tested including normal NIH-3T3 cells (data not shown).

DISCUSSION

Screening of *in vitro* antitumor cell activity was carried out as part of a program on biologically active compounds

from marine algae. To determine selective cytotoxicity, experimental tumor L1210 and normal NIH-3T3 cells were used for *in vitro* assay. Cytotoxic activity against L1210 cells was found in 81 of 306 species of marine algae (36 brown, 30 red, 14 green and 1 other algae species); a particularly high incidence of activity (38.7%) was found in brown algae.

Brown and red algae are known to contain antitumor substances, mainly polysaccharide fucoidan and arginate, *etc.* For example, an edible alga, *Undaria pinnantifida*, exhibits antitumor activity against implanted Lewis lung carcinoma and the active substance is thought to be a polysaccharide.[5] In our *in vitro* assay, this alga showed weak cytotoxic activity against mouse L1210 leukemia cells. Crude or partially purified polysaccharides from various brown algae showed antitumor activity against experimental tumor. A partially purified fucoidan from *Eisenia bicyclis* exhibits antitumor activity against L1210 leukemia.[7] In our study, PBS extract from *Eisenia bicyclis* also showed activity against L1210 cells and relatively low cytotoxicity to normal NIH-3T3 cells. In the report of Noda *et al.*,[8] the brown algae, *Scytosiphon lomentaria* and *Sargassum ringgoldianum* and the red alga, *Porphyra yezoensis*, showed antitumor activity against implanted Ehrlich carcinoma. In the present study using L1210 cells, these brown algae showed results which confirmed those findings; however, these algae also exhibited cytotoxicity to NIH-3T3 cells (Table 1). The antitumor effect of polysaccharides is thought to be based on the increase of phagocytosis in the reticuloendothelial system.[9]

A few antitumor lipids are also known: 10-hydroxy-2-decenoic acid,[10] linoleic and linolenic acids,[11,12] palmitoleic acid,[13] amino-fatty acid salts[14] and glyceryl esters.[15] Cytotoxic activity against L1210 cells was frequently found in methanol extracts in this study. Antitumor substances which are to be used as chemotherapeutic agents at the clinical stage should be of low molecular weight and free of cytotoxicity to avoid side-effects. Thus, methanol-extracts from these marine algae samples might be suitable in this regard.

We found that most algae extracts showed cytotoxic activity not only to L1210 cells, but also to NIH-3T3 normal cells. However, among the active extracts, MeOH extracts from green algae, *Caulerpa racemosa* var. *laete-virens*, *Cladophoropsis vaucheriaeformis*, *Halimeda discoidea* and *H. macroloba* showed selective cytotoxic activity to L1210 tumor cells. In particular, *C. vaucheriaeformis* exhibited markedly selective cytotoxicity to these cells. The results of the time course study of L1210 cell growth in *C. vaucheriaeformis* extract-added culture showed that the cytotoxicity had a cytostatic effect only on the tumor cells (Fig. 2). The positive control, mitomycin C, on the other hand, which inhibits DNA synthesis of cells, strongly inhibited the growth of both L1210 and NIH-3T3 cells, indicating a non-selective cytotoxicity.

In the test of the *in vitro* cytotoxic spectrum, the green algae extract from *C. vaucheriaeformis* also had a strong effect against mouse P388 cells (Fig. 3). It appears that the active substance in this extract may act specifically against leukemic cell lines. Similarly, extracts from the other green alga, *Halimeda discoidea* also had a strong effect on mouse leukemic cell lines (Fig. 4). In contrast, red and brown algae extracts did not exhibit such selective cytotoxic activity. Extracts from the red alga, *Laurencia okamurae*, were active against only mouse-derived cell lines which included both tumor and normal cell lines, and extracts of the brown alga *Dictyopteris undulata* showed strong cytotoxicity to all cell lines tested. These results showed that some species of green algae might possibly have antitumor substances against leukemic cell lines. In other reports, antitumor active substances have been mainly found in brown and red algae, which showed cytotoxicity to normal NIH-3T3 cells in this study, but not in green algae. Therefore, these results indicate that some particular green algae, such as *C. vaucheriaeformis*, appear to have novel and valuable antitumor active substances which have no clinical side-effects.

We are in the process of purifying and characterizing the antitumor substances from *C. vaucheriaeformis*.

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