Immune Bioactivity in Shellfish toward Serum-free Cultured Human Cell Lines

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The biologically functional effect of eight kinds of hot-water extracts of shellfish on cultured human cell lines was examined in a serum-free medium model. *Meretrix lusoria* and *Simonovacula constricta* extracts enhanced IgM secretion of both hybridoma HB4C5 and SI102 cells when cultured with the respective extracts. The purified principle exhibited remarked activity in the adsorbed fraction in hydroxyapatite and Concanavalin A columns. The extracts of *Corbicula fluminea*, *Crassostreas gigas*, *Meretrix lusoria*, *Anadara granosa*, and *Simonovacula constricta* enhanced in nitroblue tetrazolium (NBT)-reducing ability of macrophage U-M cells. *Meretrix lusoria*, *Anadara granosa*, and *Simonovacula constricta* were specifically cytotoxic to both cultures of MCF-7 breast cancer cells and HuH-6KK hepatoblastoma. These findings imply that the extracts of shellfish that were examined exhibited a differential effect on immune cells and tumor cells.

Key words: bioactivity; shellfish; immunity; functional food; antitumor

The human diet contains a great variety of natural carcinogens, as well as many natural anticarcinogens. The physiological effects of food factors are of particular interest because they may be useful for human cancer prevention. Characterizing and optimizing such defense systems may be an important part of the strategy for minimizing cancer and other age-related diseases.1.2 Mammalian cell lines have been used for studying the physiological effects of food components.3-5 In our previous study, some constituents of vegetables and fruits were found to have significant physiological functions.5-7 Marine resources are an important and potentially rewarding target for pharmacological research into antitumor activity, and the immunological effect of marine algal polysaccharides, especially of fucoidan prepared from *Sargassum thunbergii* of Phaeophyceae,8 omega-3 dietary lipid from a marine source, was to decrease the levels of mono- and polyunsaturated lipids, which may provide additional protection against the age-associated rise in malignancy and autoimmune disorders.9

The present study was to investigate the physiological function of dietary shellfish. In order to clarify performance at the cellular level, human-derived hybridoma, macrophage, and tumor cell lines were cultured and treated in a serum-free system. The proliferation, macrophage activation, and antitumogenicity of cultured cells were used as indicators of function. We elucidate the partially purified fractions from hot-water extracts of different shellfish.

Materials and Methods

Preparation of the hot-water extracts of shellfish. Eight kinds of fresh shellfish were used in this study: fresh water clam, *Corbicula fluminea* (LA); oyster, *Crassostreas gigas* (GY); hard clam, *Meretrix lusoria* (WG); and *Perna viridis* (HG). *Ruditapes philippinarum* (HL), *Anadara granosa* (SG), *Simonovacula constricta* (CO). *Cypraea belofo* (FL). Each was purchased from the fish market in Keelung, Taiwan. Fresh material of each shellfish (500 g) was homogenized and then boiled in water for 20 min around 100°C. After the solution had been centrifugated and filtered, the supernatant was concentrated by vacuum evaporation. The bioactive fraction of the concentrated aqueous extract was obtained by 80% ammonium sulfate precipitation, and dialyzed by ultrafiltration (10KD MW cut off) with a phosphate buffer to remove the low-molecular-weight water-soluble constituents, before the dialyze was concentrated and lyophilized. This dialyze was redissolved in a phosphate-buffered saline solution, sterilized by passing through a membrane filter (0.22 μm) and diluted in a serum-free medium for the assay.

Cell lines and medium. The human-derived cell lines used in this study were tumor cell lines HuH-6KK10 and MCF-7, a U-937-derived macrophage-like cell line (U-M),11 and human hybridomas HB4C5 and SI102.12 These cell lines were obtained from Dr. Murakami of Kyushu University. The HB4C5 and SI102 cell lines were hybridomas producing monoclonal antibodies against lung cancer and breast cancer, respectively. All the cells were cultured in an enriched RDM medium from Kyokuto Pharmaceutical Kogyo Co. (Tokyo), which was supplemented with a 20 mM HEPES buffer, 5 μg/ml of insulin, 10 μg/ml of iron-free human transferrin, 1.53 μg/ml of ethanolamine, and 25 mM sodium selenite. Cells at the log phase were collected and replated at a cell density of 5 x 10⁶ cells/ml for the assay.

Determination of immunoglobulin M (IgM). The monoclonal antibody (IgM) secreted by the cultured hybridoma cells was measured by an enzyme-linked immunosorbent assay with the peroxidase-labeled sheep anti-human IgM antibody.4 The hybridoma cells were treated as described in the proliferation assay, and 50 μl of the supernatant was applied to a microplate for IgM detection.

Macrophage activation assay. The effect of each extracted fraction on macrophage activation was evaluated by an NBT-reducing ability assay. Purple water-insoluble formazan formed in a 96-well microplate was dissolved in DMSO solvent, and the absorbance at 570 nm was measured by a microplate reader.13 Cells were plated at 37°C for 24 h in a humidified 5% CO₂, 95% air atmosphere, and then assay for the NBT-reducing ability was conducted.

Cell proliferation assay. The effect of each extracted fraction on cell growth was evaluated by an MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.14 Cells were plated at 96-well microplates and mixed with different amounts of each shellfish extract. In the control experiment, phosphate was used in place of the extracts. Each experimental plate was incubated at 37°C for 48 h in a humidified 5%
CO₂, 95% air atmosphere, and the MTT assay was then conducted. All experiments were carried out in triplicate. The water-insoluble formazan formed in the microplate was dissolved in HCl-isopropanol, and the absorbance at 570 nm was measured by a microplate reader (Dynatech Co.).

**Hydroxypatite column chromatography.** The bioactive fraction obtained by ammonium sulfate precipitation was then applied to a hydroxypatite gel column (2 × 30 cm) that had been pre-equilibrated with a 20 mM phosphate buffer (pH 7.2). The non-adsorbed fraction was washed out with the same buffer and eluted with 0.5 M NaCl, before being finally eluted with 0.5 M phosphate. The eluate was measured for its absorbance at 280 nm, and the fraction eluted just around the peak was dialyzed prior to being freeze-dried.

**Concanavalin A affinity column chromatography.** After the hydroxypatite gel, the bioactive fraction was purified in a Concanavalin A-Sepharose 4B column (1.5 × 25 cm) by eluting with 0.5 M α-D-mannopyranosides. Each fraction was monitored for its absorbance at 280 nm.

**Results**

**Effect of the shellfish fractions on IgM secretion**

IgM secretion was used as a bioactive marker of cell immune response. Figure 1 shows the increase in secreted IgM after both HB4C5 and SI102 hybridoma cells were cultured with each extract. The absorbance generally tended to increase in a dose-dependent manner when treated with the shellfish extracts. A greatest increase in the amount of IgM was observed in the case of the *Meretrix lusoria* and *Sinonovacula constricta* extracts. The profile of a glycoprotein purified from *Sinonovacula constricta* is shown in Fig. 2, while Fig. 3 shows the SI102 cell growth and aggregation after being treated with the *Meretrix lusoria* extract for 12 h. The eluate from the hydroxypatite gel column (HA2 fraction) of the *Sinonovacula constricta* extract enhanced IgM secretion, and the Concanavalin A-Sepharose 4B column eluate (Con2 fraction) showed the strongest activity (Fig. 4). No marked activity was detected in the non-adsorbed fractions (HA1 and Con1). These findings suggest

**Concentration (μg/ml)**

Fig. 1. Effects of Extracts of Some Shellfish on the Production of IgM by Hybridoma Cells.

The IgM produced by HB4C5 (left) and SI102 (right) cells cultured with different concentrations of each shellfish extract for 48 h was measured by ELISA. CG (*Sinonovacula constricta*, ●), WG (*Meretrix lusoria*, □), HL (*Raditapex philippinorum*, ▲), FL (*Cypara helvola*, Δ), LA (*Conchula fluminea*, □), OY (*Ctenostrea gigas*, △), SG (*Anadara granosa*, ○), HG (*Perna viridis*, △). Each value represents the result of triplicate determinations.

**Whole Sinonovacula constricta** boiled at around 100°C for 20 min

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Crude extract centrifuged for 20 min at 6,000rpm

Supernatant added with 80% AS

Hydroxypatite column chromatography (40x20cm)

**HA1, 20mM NaP**

Phenyl-Toyopearl hydrophobic column (16x30 cm)

PT1 30% AS elute

PT2 20% AS elute

PT3 0% AS elute

Dialyzed against NaP

Concanavalin A-Sepharose chromatography (adsorbed fraction. Con2)

Mono Q anion-exchange column (5x 50mm)

Lyophilized, 43kDa M.W. glycoprotein

**Fig. 2.** Procedure for preparing the Tumoricidal Fractions from Hard Clam.

NaP, 10 mM phosphate buffer at pH 7.2; AS, ammonium sulfate.
that the principle in the Sinonovacula constricta extract was a Concanavalin A-binding glycoprotein, and that this promoted IgM secretion.

Effect of the shellfish fractions on macrophage activation

NBT-reducing ability provides a unique assay for evaluating macrophage activation. The extracts of Crassostrea gigas, Meretrix lusoria, and Sinonovacula constricta significantly enhanced the formazan formation of macrophage (U-M cells) by the NBT assay (Table I). We also found that the extract of Meretrix lusoria treated U-M cells had higher relative viability (Table I), implying that this shellfish extract may have been involved in cell-growth stimulation.

Effect of the shellfish fractions on the growth of cultured cells

In Table II, the effects of each shellfish extract on the relative viability of hepatoblastoma (HuH-6KK) cells are listed. The dose-dependency of growth inhibition of the cultured HuH-6KK cells varied with the kind of shellfish. The results of the MTT assay on HB4C5 cells for each shellfish extract are shown in Fig. 5. The relative viability increased when the extracts of Meretrix lusoria, Anadara granosa, and Sinonovacula constricta were added to the cell cultures, suggesting the existence of growth-promoting constituents in the eluate from the hydroxyapatite gel column (HA2 fraction) of those shellfish. Similar results

![Image: Microphotograph showing the Aggregation of S1102 Cells](image)

Table 1. Effect of Extracts of Some Shellfish on NBT-reducing Activity and on the Growth of U-Macrophage Cells

<table>
<thead>
<tr>
<th>Treat (μg/ml)</th>
<th>WG</th>
<th>LA</th>
<th>SG</th>
<th>CG</th>
<th>OY</th>
<th>HG</th>
<th>FL</th>
<th>HL</th>
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<td>Blank</td>
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<td>0.19 ± 0.02</td>
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<td>0.18 ± 0.03</td>
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<td>0.20 ± 0.04</td>
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<td>0.23 ± 0.03</td>
<td>0.23 ± 0.04</td>
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<tr>
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* Each value represent the mean ± S.D. of three determinations.

** Numbers in parentheses indicate the relative viability expressed as a percentage. Relative viability (%) = absorbance by the MTT assay compared to that of blank. Each value represents the mean of three determinations.

![Image: Effect of the Partially Purified Extracts of Sinonovacula constricta on the Production of IgM by HB4C5](image)
Table II. Effect of Extracts of Some Shellfish on the Growth of HuH-6KK Cells

HuH-6KK cells were cultured in an enriched RDF medium at a density of $5 \times 10^4$ cells/ml. CG. S. constricta; WG. M. lusoria; HG. R. philippinarum; HL. R. philippinarum; FL. C. helvola; LA. C. fluminea; OY. C. gigas; SG. A. granosa.

<table>
<thead>
<tr>
<th>Treat (µg/ml)</th>
<th>Viability (%) by an antitumor assay</th>
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<tr>
<td></td>
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</table>

* Relative viability (%)= absorbance by the MTT assay compared to that of the blank. Each value represents the mean of three determinations.

Fig. 5. Effect of Extracts of Some Shellfish on the Growth of HB4C5 Cells.

The growth of HB4C5 cells was measured by an MTT assay. HA2, eluted fraction by hydroxyapatite chromatography. Each value represents the result of triplicate determinations. CG. S. constricta; WG. M. lusoria; SG. A. granosa.

were also obtained with SI102 cells (data not shown). In contrast, as shown in Fig. 6, the relative viability decreased when the extracts of M. lusoria, A. granosa, and S. constricta were added to the cultures of MCF-7 breast cancer cells. These findings suggest that those extracts may have had growth-inhibition activity toward MCF-7 cells, especially the eluate from the hydroxyapatite gel column (HA1 fraction) of the shellfish extracts.

Discussion

The results of the present study demonstrate that eight kinds of hot-water extracts of shellfish had a specific effect on the growth of several kinds of human-derived cells by the MTT assay, a macrophage-activating effect by the NBT-reducing ability assay, and an enhancing effect on the antibody production of hybridomas by the ELISA method. To prevent cancer and age-related diseases, the physiological effects of food components in the human diet should be investigated and clarified. Marine resources are an important and potentially rewarding source for Chinese pharmacological research, especially in the case of shellfish. Macrophages and antibody-secreting cells in the immune system are important in phagocytosis, antigen-presentation, and tumor cytotoxicity. We have reported here that the nondialyzable fraction of several shellfish extracts showed macrophage-activating ability, and cell-aggregation activity with cultured macrophage cell and hybridoma cells, which suggests that a specific binding site was involved in cellular surface molecules. Several shellfish fractions inhibited the growth of cultured hepatoma and breast tumor cells, while promoting the growth of cultured macrophage cells and hybridoma cells. In a previous study, we found that mannos and N-acetylgalactosamine residues were rich on the cell membrane of U-M cells, but were absent on U-937 cells. The extracts of shellfish also induced cell-aggregation with U-M cells, but not with U-937 cells (data not shown). The binding ability and immune effects of the shellfish fractions were directed toward specific cells, indicating the existence of a counter receptor on the surface of the cultured cells. We also found that partially purified fractions of the extracts significant enhanced the production of antibodies in cultured human cells. Some lectin-like proteins were purified from the boiled shellfish extracts. The causative compound in M. lusoria and S. constricta was confirmed to be a mannos-binding glycoprotein, and we found triggering apoptosis in tumor cells (data not shown). However, we are not sure whether all the glycoprotein structure is needed for activation or not. These results reveal that the content in dietary shellfish affected cell activity in vitro. Further purification and characterization of the novel bioactive substances is necessary to evaluate their physiological performance in the diet.

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References