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The cytotoxic effect of *Eucheuma serra* agglutinin (ESA) on cancer cells and its application to molecular probe for drug delivery system using lipid vesicles

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Abstract

Eucheuma serra agglutinin (ESA) derived from a marine red alga, *Eucheuma serra*, is a lectin that specifically binds to mannose-rich carbohydrate chains. ESA is a monomeric molecule, with a molecular weight of 29,000. ESA induced cell death against several cancer cell lines, such as colon cancer Colo201 cells and cervix cancer HeLa cells. DNA ladder detection and the induction of caspase-3 activity suggested that the cell death induced by ESA against cancer cells was apoptosis. ESA bound to the cell surface of Colo201 cells in the sugar chain dependent manner. This means that the binding of ESA to the cell surface is specific for mannose-rich sugar chains recognized by ESA. The binding of ESA to the cell surface of Colo201 cells was slightly suppressed by the high concentrations of serum because of the competition with serum components possessing the mannose-rich sugar chain motifs. On the other hand, a lipid vesicle is a very useful microcapsule constructed by multilamellar structure, and adopted as drug or gene carrier. ESA was immobilized on the surface of the lipid vesicles to apply the lipid vesicles to cancer specific drug delivery system. ESA-immobilized lipid vesicles were effectively bound to cancer cell lines compared with plane vesicles.

Abbreviations: DDS, drug delivery system; EGF, epidermal growth factor; ESA, *Eucheuma serra* agglutinin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline

Introduction

Lectins have been found in various organisms, including bacteria and marine macroalgae. Lectins are proteins specialized to recognize and bind to particular oligosaccharide chains (Sharon et al., 1972). The proteins have many biological activities except for associating property against sugars, such as mitogenic activity against lymphocytes and agglutination activity. Lectins are also very high specific molecular probes against the cell surface carbohydrate chains. Some lectins have been applied to distinguish cancer cells from normal cells, due to the difference in the cell surface carbohydrate side chains between them (Sumi et al., 1999). Because the cell surface carbohydrate chains on cancer cells are complicated compared with those of normal cells.

ESA, a lectin derived from a marine red alga *Eucheuma serra*, recognizes mannose branch structures in oligosaccharide chains (Kawakubo et al., 1997). Molecular weight of ESA is 29,000, and pI is 4.95. ESA is a monomeric protein without carbohydrate side chains. ESA also has hemagglutinating activity, which is stable against a wide range of pH (1.5 to 10.5) and 85 °C treatment.

We introduced ESA as a molecular probe to the surface of lipid vesicle for development of drug delivery system (DDS) toward cancer therapy (Kato et

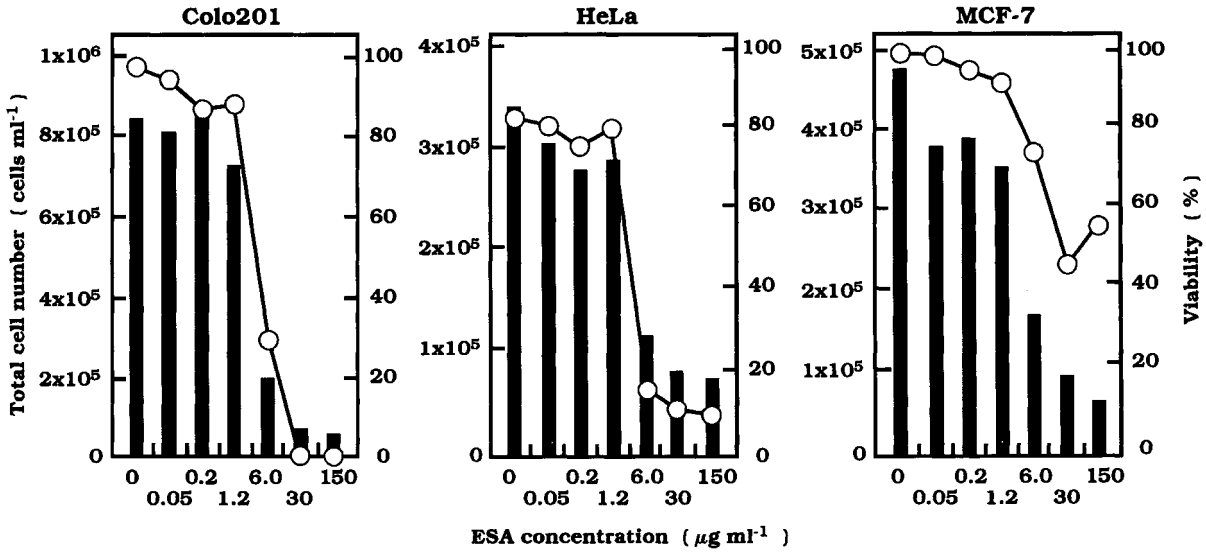

Figure 1. Effect of ESA on cancer cells. Colo201, HeLa, and MCF-7 cells were inoculated at 1.0×10^5 cells ml^{-1} in ERDF medium with 10% FBS and various concentrations of ESA, and cultured for 3 days. Cell number and cell viability were measured by trypan blue dye exclusion test on a hemocytometer. Bars and circles indicate total cell number and cell viability, respectively. The values were means of two separate measurements.

al., 1999a). The lipid vesicle is a microcapsule made by the lipid membranes with multilamellar structure. The lipid vesicles were prepared by a two-step emulsification technique (Kato et al., 1993). The major component of the lipid membrane is Span80 (sorbitan monooleate), artificial amphipathic lipid. The lipid vesicles are very useful microcapsule for drug and gene carriers (Kato et al., 1999b). The structure of the lipid vesicle resembles that of liposome made from natural phospholipids. The advantages of the lipid vesicles over liposomes are the flexibility of the membrane structure and the stability of particles.

Materials and methods

Materials

ESA was extracted and purified from a red alga, *Eucheuma serra* (Kawakubo et al., 1997). ESA is a lectin, which associates with high mannose type carbohydrate chain. ESA was dissolved in 10 mM sodium phosphate buffer (pH 7.4).

Cells and cell culture

Colo201 (human colon adenocarcinoma), HeLa (human cervix adenocarcinoma), MCF-7 (human breast adenocarcinoma) and HB4C5 cells (human hybridoma cell line) were used. These cell lines were cultured in ERDF medium (Kyokuto Pharmaceutical., Tokyo) supplemented with 10% of fetal bovine serum (FBS). MCF10-2A obtained from ATCC (Rockville, MD, USA) is a non-tumorigenic epitherial cell line. MCF10-2A cells were cultured in 10% FBS-ERDF medium supplemented with 500 ng ml^{-1} of hydrocortisone and 20 ng ml^{-1} of epidermal growth factor (EGF). Normal fibroblast cells from the umbilical cord were also cultured in 10% FBS-ERDF medium supplemented with 500 ng ml^{-1} of hydrocortisone and 20 ng ml^{-1} of EGF. Cell number and viability were evaluated by trypan blue dye exclusion test in a hemocytometer.

Quantitative analysis of the association of ESA with Colo201 cells

ESA was directly labeled by fluorescein isothiocyanate (FITC) using FluoroTag FITC conjugation kit purchased from Sigma (St Louis, MO, USA). Following the labeling reaction, FITC-conjugated ESA was separated from unconjugated FITC by using Sephadex G-25 column (Amersham-Pharmacia Biotech, Backinghamshire, England). Colo201 cells were cultured in ERDF medium supplemented with FITC-conjugated ESA for 3 h in a CO_2 incubator at 37 °C. After in-

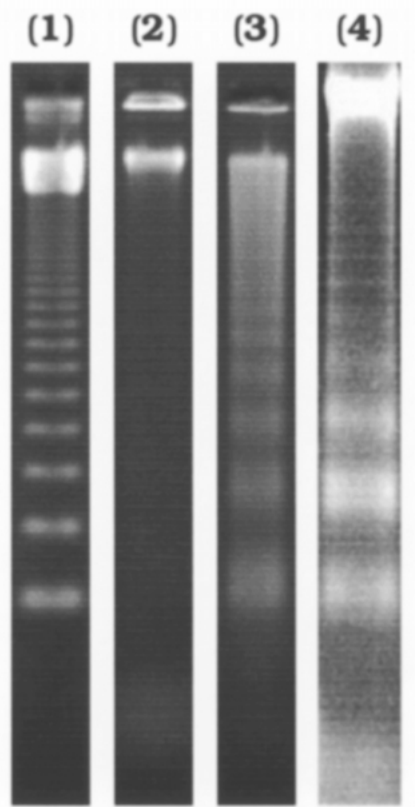

Figure 2. Determination of DNA fragmentation in Colo201 cells treated with ESA. Colo201 cells were inoculated in 10% FBS-ERDF medium supplemented with 64.0 μg ml^{-1} of ESA or 1 μM staurosporine, and cultured for 24 h. Following cell wash, DNA extraction was performed. Lane 1: ladder marker; lane 2: control Colo201 cells; lane 3: treated with ESA; lane 4: treated with staurosporine.

cubation, Colo201 cells were washed with cold PBS and analyzed by flow cytometry using FACSCalibur (Becton Dickinson, Mansfield, MA, USA).

Detection of DNA laddering on agarose gel electrophoresis

Colo201 cells were washed with PBS twice, and resuspended in lysis solution containing 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 200 μg ml^{-1} of protease K (Ogata et al., 1998). After incubation for 1 h at 56 °C, same volume of 2-propanol was added. After centrifugation at 16,000xg for 20 min, the pellet was resuspended in TE buffer containing 100 μg ml^{-1} of RNase A and incubated for 1 h at 37 °C. Electrophoresis was performed in 2% agarose gel. DNA was visualized by UV illuminator after staining with 0.5 μg ml^{-1} of ethidium bromide.

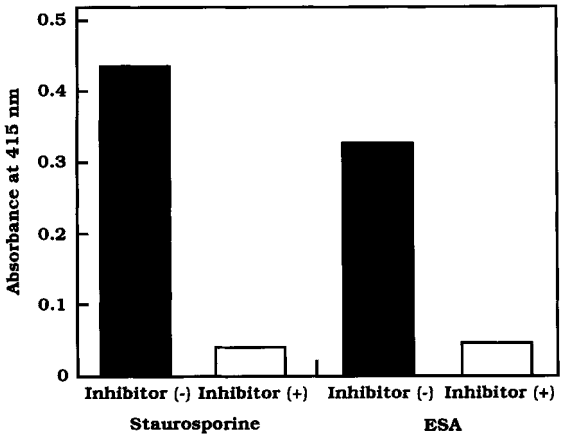

Figure 3. Determination of caspase-3 activity in Colo201 cells treated with ESA. Colo201 cells were inoculated in 10% FBS-ERDF medium containing 10.8 μg ml^{-1} of ESA or 1 μM staurosporine as an inducer of the apoptosis. Z-VAD-FMK, caspase-3 inhibitor, was also added to the medium at 50 μM for inhibition of the apoptosis. Cells were cultured for 16 h. Following cultivation, caspase-3 activity in Colo201 cells was determined. The values were means of two separate measurements.

Caspase-3 assay

Colo201 cells were inoculated in 10% FBS-ERDF medium containing 10.8 μg ml^{-1} of ESA or 1 μM staurosporine (Sigma, St Louis, MO, USA) as an inducer of apoptosis, and cultured for 16 h. Following cultivation, caspase-3 activity in Colo201 cells was determined by using CaspACE assay system (Promega, Madison, WI, USA).

Preparation of the lipid vesicles

The major component of the lipid vesicles was Span80 (sorbitan monooleate) purchased from Wako Pure Chemistry (Osaka, Japan). The lipid vesicles were prepared by the two-step emulsification technique (Kato et al., 1996). Span80 in hexan was mixed with PBS and Tween80 (Polyoxyethylene(20) sorbitan monooleate) by using homomixer. Following evaporation, ESA dissolved in PBS was added and mixed by ultrasonication for 3 min.

Results

Cytotoxic effect of ESA on cancer cells

Colo201, HeLa and MCF-7 cells were inoculated at 1.0×10^5 cells ml^{-1} in ERDF medium supplemented with 10% FBS and various concentrations of ESA,

and cultured for 3 days. As shown in Figure 1, ESA inhibited the cell growth of Colo201 cells at the concentrations above 1.2 μg ml^{-1} and conducted the complete cell death at 30 μg ml^{-1}. HeLa cells were also inhibited the cell growth above 1.2 μg ml^{-1} of ESA. MCF-7 cells had relatively high tolerance for ESA, and the cell viability was maintained above 40% even at the high doses. These facts reveal that the cytotoxic effect of ESA depends on target cell lines.

The cytotoxicity of ESA against MCF10-2A cells was investigated. MCF10-2A cells were inoculated in each culture medium supplemented with 10 μg ml^{-1} of ESA. In contrast to the cytotoxic effects on cancer cell lines, ESA did not have cytotoxicity against MCF10-2A cells that do not have tumorigenecity (data not shown).

Induction of apoptosis by ESA

As indicated above, ESA had cytotoxic effects on several cancer cell lines. Then, the mode of the cell death induced by ESA was investigated. At first, DNA degradation in Colo201 cells treated with 64.0 μg ml^{-1} of ESA was observed. Staurosporine was employed as a positive control of the apoptotic cell death. As shown in Figure 2, ESA treatment caused DNA degradation in Colo201 cells.

Moreover, induction of caspase-3 activity was measured in confirmation of the apoptosis. As indicated in Figure 3, ESA induced expression of caspase-3 in Colo201 cells in a similar way as staurosporine. These results suggest that the cell death conducted by ESA is apoptosis.

Interaction between ESA and Colo201 cells

The binding of ESA to Colo201 cells was investigated. FITC-conjugated ESA was supplemented in the culture medium of Colo201 cells at 6.3 μg ml^{-1} and the cells were cultured for 3 h. Following wash with cold PBS three times, the flow cytometric analysis was performed. As indicated in Figure 4, ESA was bound to Colo201 cells cultured in control medium containing 10% FBS. To investigate the effect of FBS on the binding of ESA to the cells, FBS concentration was reduced from 10% to 1%. In 1% FBS-ERDF medium, the amount of ESA bound to Colo201 cells was increased than that in 10% FBS-ERDF medium (Figure 4–3). It is expected from this result that FBS contains the suppressive substances possessing mannose branch structures.

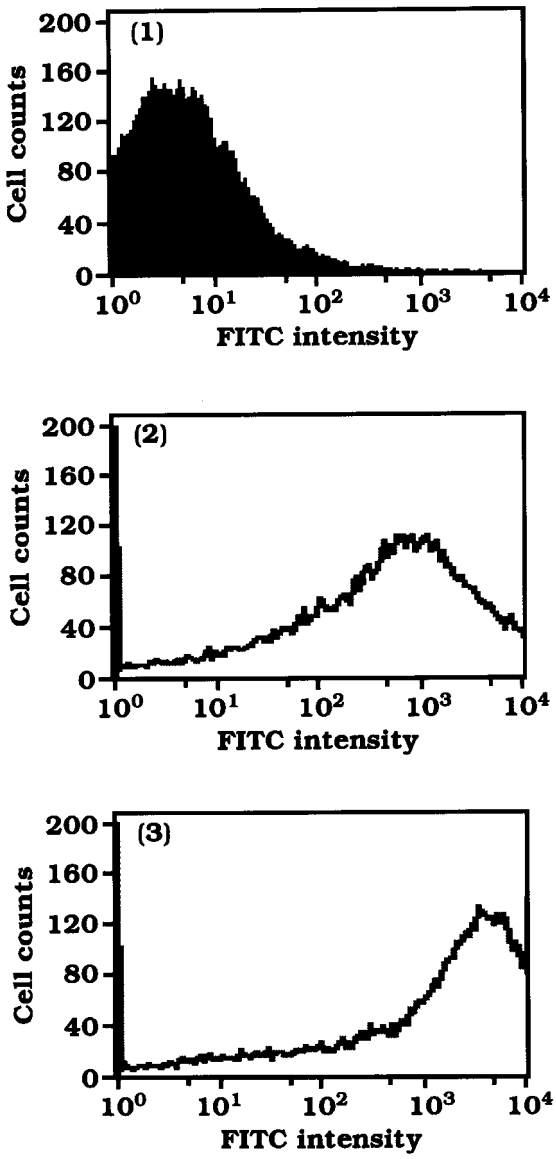

Figure 4. Flow cytometry analysis of the interaction between ESA and Colo201 cells. Colo201 cells were inoculated in 10% or 1% FBS-ERDF medium containing 6.3 μg ml^{-1} of FITC-labeled ESA. Following 3 h-cultivation, cells were washed three times with cold PBS and analyzed. (1) Colo201 cells cultured in 10% FBS-ERDF medium without ESA as control, (2) 10% FBS-ERDF medium with FITC-ESA, (3) 1% FBS-ERDF medium with FITC-ESA.

Then, the mode of interaction between ESA and Colo201 cells was investigated. Colo201 cells were treated with α-mannosidase, β-mannosidase, and endoglycosidase H to cleave cell surface high mannose type sugar chains prior to ESA treatment. After each glycosidase treatment of Colo201 cells, FITC-labeled ESA was added to the culture medium, and the cells

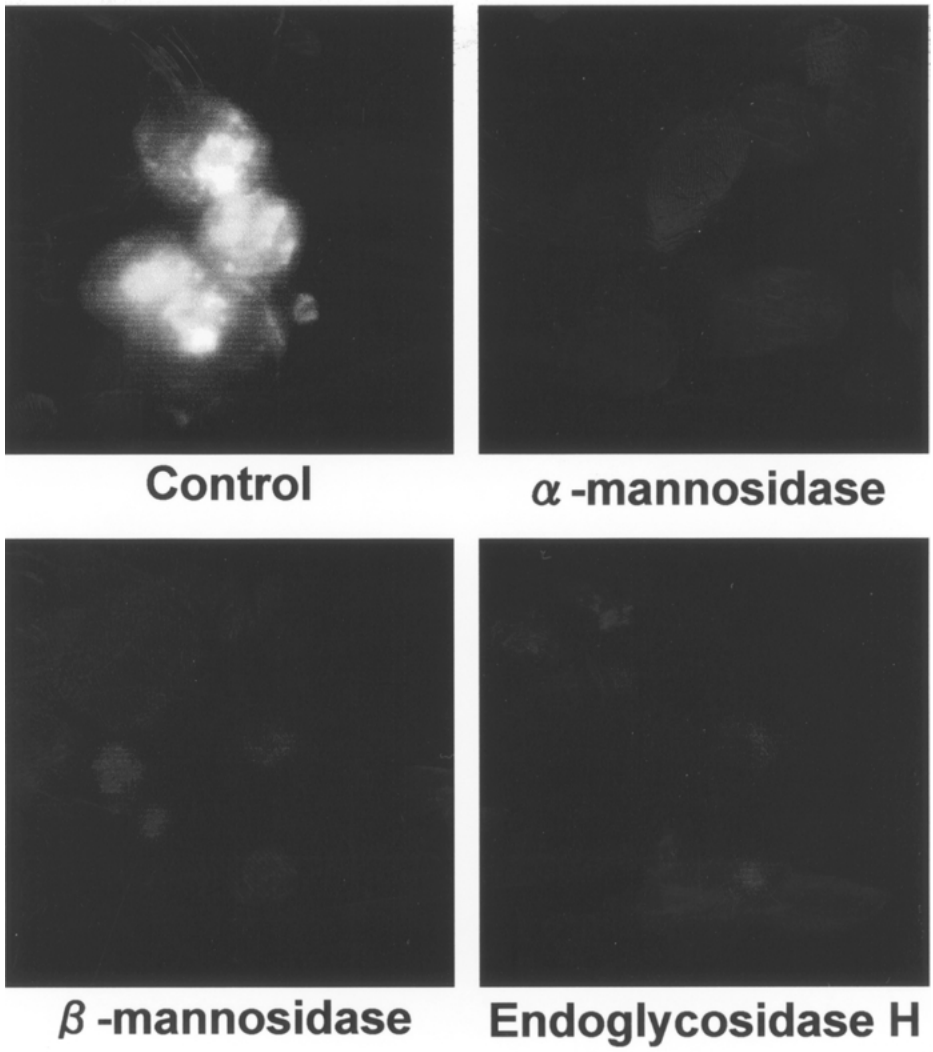

Figure 5. Interaction of ESA and Colo201 cells. Colo201 cells were treated with 0.5 unit ml^{-1} of each glycosidase (α-mannosidase, β-mannosidase, and endoglycosidase H) for 2.5 h, and inoculated in the medium supplemented with 1,275 μg ml^{-1} of FTIC-conjugated ESA. Cells were visualized by a laser scanning confocal microscope.

were incubated for 6 h. Afterward washing with cold PBS three times and fixation with 95% ethanol, the cells were observed with a laser scanning confocal microscope equipped with an argon ion laser tuned at 488 nm as excitation source. As shown in Figure 5, ESA associated with the control cells that were not treated with glycosidase. However, glycosidase treatment obviously decreased the amount of ESA attached to the cells. This result clearly demonstrates that ESA interacts with Colo201 cells through the cell surface mannose branches.

Cancer-specific binding of ESA-immobilized lipid vesicles

Then, we immobilized ESA on the surface of lipid vesicles for the purpose of target-specific drug delivery toward the cancer therapy. ESA was anchored on the lipid vesicle surface during the step of the second-stage emulsification. In this experiment, we prepared ESA-immobilized lipid vesicles containing FITC inside of them. Colo201, HB4C5, MCF10-2A, and normal fibroblasts from the umbilical cord were cultured in the medium supplemented with ESA-immobilized lipid vesicles for 3 h. The fluorescent intensity of each cell derived from FITC delivered

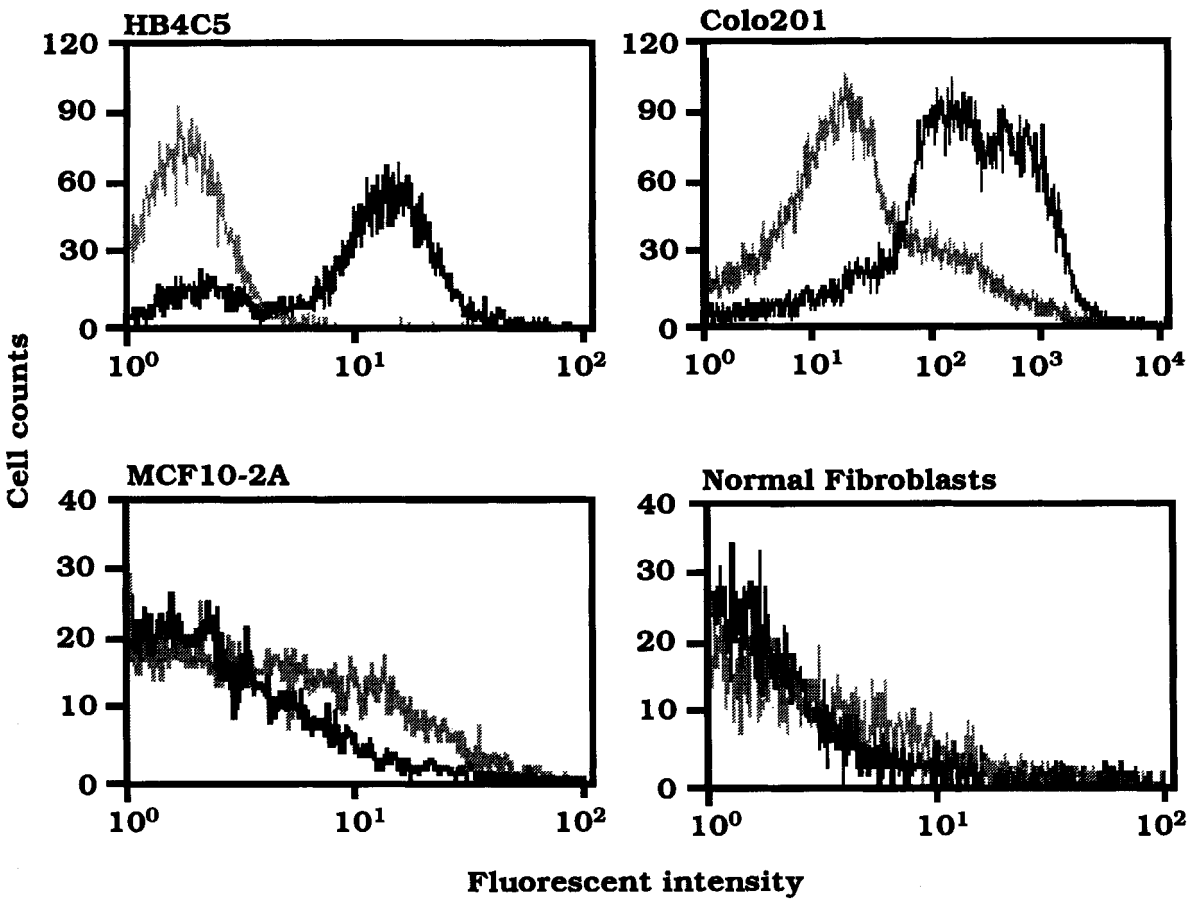

Figure 6. Cancer-specific binding of ESA-immobilized lipid vesicles. ESA-immobilized lipid vesicles (black line) and plane lipid vesicles (gray line) encapsulating FITC were added to the culture medium of each cell line (HB4C5, Colo201, MCF10-2A and normal fibroblasts), and cultured for 3 h. Following wash with cold PBS, FITC intensity in cell was statically analyzed by the flow cytometry.

by ESA-immobilized lipid vesicles was analyzed by flow cytometer. As the result of that ESA vesicles associated with Colo201 cells and HB4C5 cells and FITC encapsulated in the lipid vesicles was introduced into these cells (Figure 6). However, the significant transfer of FITC from ESA-immobilized lipid vesicles to MCF10-2A cells and human normal fibroblasts was not observed. These results mean that the ESA-immobilized lipid vesicles selectively combine with cancer cells and transfer the encapsulated materials, such as drugs.

Discussion

It is well-known that lectins are very useful proteins exhibiting the specific affinity to carbohydrate chains. Because of this feature, lectins have been employed in various provinces, such as affinity chromatography and glycobiochemistry. Many of lectins have the ability to agglutinate erythrocytes and normal or transformed cells. Some of the lectins also stimulate mitogenesis in lymphocytes. These activities of lectins are triggered by the binding to the carbohydrate chains on cell surface. ESA has binding affinity to mannose branching structures in high mannose type sugar chains (Kawakubo et al., 1997). The addition of ESA to the culture medium suppressed the cell growth and induced the cell death of the several cancer cell lines. Human colon adenocarcinoma cell line, Colo201 cells were the most sensitive to ESA stimulation, compared with other cell lines evaluated here. The cell death induced by ESA was suggested as the apoptosis on the ground of induction of DNA fragmentation and caspase-3 activity. On the other hand, ESA did not affect non-tumorigenic epitherial cell line, MCF10-2A

cells and normal fibroblasts (data not shown).

The association property of ESA against Colo201 cells was depressed by glycosidase treatment of the cells. This result reveals that the binding of ESA to cancer cells is strictly restricted by the cell surface mannose related carbohydrate chains.

The final purpose of this study is utilization of ESA as a molecular probe for the target-specific drug delivery aiming cancer therapy (Kato et al., 1999a). ESA-immobilized lipid vesicles were prepared and evaluated the target specificity. As indicated in Figure 6, ESA-immobilized lipid vesicles were attached to caner cells and the contents in the vesicles were transferred to the target cells. However, ESA-immobilized lipid vesicles ignored normal cells. This fact implies that ESA-immobilized lipid vesicles become one of the effective means for drug delivery to tumor *in vivo*.

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