

# The anti-tumor effect of *Eucheuma serra* agglutinin on colon cancer cells *in vitro* and *in vivo*

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*Eucheuma serra* agglutinin (ESA) is a lectin derived from a marine red alga *E. serra* and binds specifically to mannose-rich sugar chains. Previous reports have indicated that ESA associates with several cancer cells via sugar chains on cell surfaces and induces apoptotic cell death. In this study, we investigated the effect of ESA on Colon26 mouse colon adenocarcinoma cells both *in vitro* and *in vivo*. ESA induced cell death against Colon26 cells *in vitro*, and the expression of caspase-3 and the translocation of phosphatidylserine in ESA-treated Colon26 cells suggested that this cell death was induced through apoptosis. An intravenous injection of ESA significantly inhibited the growth of Colon26 tumors in BALB/c mice; moreover, DNA fragmentation was detected in tumor cells following ESA treatment. These results indicated that ESA is effective as an anti-cancer drug not only *in vitro* but also *in vivo*. The side-effects of ESA were not considered to be serious because the decrease in body weight of the mice injected with it was negligible. These

observations suggest that ESA has the potential to be an effective anti-tumor drug. *Anti-Cancer Drugs* 17:943–947  
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## Introduction

We previously demonstrated that *Eucheuma serra* agglutinin (ESA), which is a lectin derived from a marine red alga *E. serra*, specifically recognizes mannose-rich motifs in oligosaccharide chains and binds to several cancer cells via sugar chains. ESA is a monomeric protein without carbohydrate side-chains and has a molecular weight of 29 000. It is extremely stable over a wide range of pH values (1.5–10.5) and against treatment at temperatures as high as 85°C [1]. We demonstrated ESA-induced cell death in several human cancer cells such as human colon adenocarcinoma Colo201 cells, human cervix adenocarcinoma HeLa cells and human breast adenocarcinoma MCF-7 cells [2]. In contrast, ESA did not affect normal cells such as human normal mammary gland fibroblasts MCF10-2A cells, normal human fibroblasts derived from the umbilical cord or human peripheral blood lymphocytes [2]. Moreover, ESA induced caspase-3 activity and DNA fragmentation in Colo201 cells. Several reports have documented lectin-induced cell apoptosis. Hostanska *et al.* [3] revealed that recombinant mistletoe lectin induces apoptosis in E1A/T24 H-*ras*-transformed mouse embryo fibroblasts.

In this study, colon adenocarcinoma Colon26 cells from BALB/c mice were used as target cells for ESA because these cells are able to form tumor tissue in BALB/c mice. The objective of this study was to investigate the anti-tumor effect of ESA on Colon26 cells, both *in vitro* and *in vivo*, and hypothesize the mode of action of the anti-cancer effect.

## Materials and methods

### Materials

ESA was extracted by ethanol precipitation from a red alga, *E. serra*, and purified by gel filtration as outlined in a previous study [1]. It was then dissolved in a 10 mmol/l sodium phosphate buffer (pH 7.4). The caspase inhibitor Z-VAD-FMK was purchased from Calbiochem (San Diego, California, USA). The Annexin V-PE Apoptosis Detection Kit I containing annexin V-PE and 7-amino-actinomycin D (7-AAD) was obtained from BD Biosciences (Franklin Lakes, New Jersey, USA).

### Cells and cell culture

Colon26 colon adenocarcinoma cells derived from BALB/c mice were kindly provided by the Institute of Development, Aging and Cancer, Tohoku University (Sendai,

Japan) [4,5]. Colon26 cells were subcultured in an ERDF medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) supplemented with 5% of fetal bovine serum (FBS) at 37°C in a humidified atmosphere consisting of 5% CO<sub>2</sub>.

#### Cytotoxic assay

Colon26 cells were inoculated in 24-well culture plates at a cell density of  $1.0 \times 10^5$  cells/ml suspended in the ERDF medium with 5% FBS and various concentrations of ESA. After cultivation for 48 h, cell viability was determined by the trypan blue dye exclusion test.

#### Caspase-3 assay

Caspase-3 activity in Colon26 cells treated with ESA was determined using the CasPACE Assay System (Promega, Madison, Wisconsin, USA). For the assay, Colon26 cells were inoculated in 24-well culture plates at a cell density of  $1.0 \times 10^5$  cells/ml in a 5% FBS-ERDF medium containing 50 µg/ml of ESA and cultured for 48 h.

#### Flow cytometry analysis

The induction of apoptosis was analyzed by using the Annexin V-PE Apoptosis Detection Kit I according to the manufacturer's instructions [6,7]. Briefly, Colon26 cells were inoculated at a cell density of  $1.0 \times 10^5$  cells/ml in a 5% FBS-ERDF medium containing 50 µg/ml of ESA and cultured for 48 h. After cultivation, these cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then resuspended in a binding buffer [10 mmol/l HEPES/NaOH (pH 7.4), 140 mmol/l NaCl, 2.5 mmol/l CaCl<sub>2</sub>] at a cell density of  $1.0 \times 10^6$  cells/ml. The cell suspension (100 µl) was transferred to a 5-ml culture tube to which 5 µl of annexin V-PE and 5 µl of 7-AAD were added as fluorescent dyes. After incubation for 15 min at 25°C in the dark, 400 µl of binding buffer was added to the solution. Flow cytometry analysis was performed within 1 h according to the manufacturer's instructions.

#### Animal experiment

Five-week-old female normal BALB/c mice, purchased from Clea Japan (Tokyo, Japan), were used in this study and maintained under extremely clean conditions. The animal experiment was carried out according to the guidelines for animal experimentation established by Ehime University. To establish tumors in the BALB/c mice, the Colon26 cells were subcutaneously injected into the mid-dorsal region of the mice at a cell density of  $4 \times 10^6$  cells suspended in 100 µl of PBS. Tumors were allowed to grow until the tumor volume was increased to approximately 0.2 cm<sup>3</sup>. After tumor growth, the mice were intravenously injected with 400 µg of ESA in 200 µl PBS or the same volume of PBS as the vehicle via their tail veins every 3 days after the first injection. Tumor volume ( $V$ ) was calculated using the following

formula [4]:

$$V = (\text{longest tumor diameter}) \times (\text{shortest tumor diameter})^2 \times 1/2.$$

#### Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay

This assay measures DNA strand breaks and is therefore used to detect cells undergoing apoptosis. Subcutaneous tumors were harvested from sacrificed BALB/c mice 15 days after the first ESA injection. The tumor tissues were fixed with 10% paraformaldehyde at room temperature for 24 h. The paraffin-embedded specimens were cut into sections with a thickness of 3 µm. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using the In-situ Apoptosis Detection Kit (TAKARA Bio, Shiga, Japan) according to the manufacturer's instructions. A coloring reaction was performed using 3-amino-9-ethylcarbazole.

#### Statistical analysis

The results have been expressed as means ± standard deviations (SDs). Tukey's test was used to assess the statistical significance of the difference obtained between the treatment and control groups. A  $P$  value of less than 0.01 was used as the criterion for statistical significance.

## Results

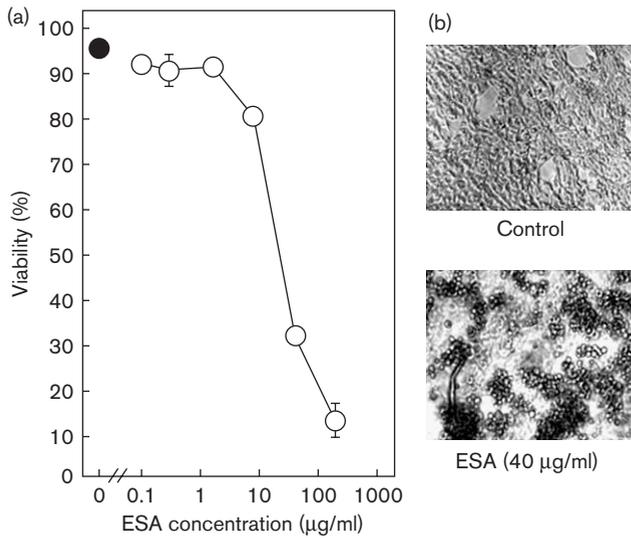
#### Cytotoxic effect of *Euchema serra* agglutinin on Colon26 cells

Our previous report revealed the cytotoxic effect of ESA on Colo201, HeLa and MCF-7 cells. Before the examination of the anti-tumor effect of ESA on the mice, we evaluated its cytotoxic activity against transplantable mouse cancer cells *in vitro*. Subsequently, the effect of ESA on Colon26 cells derived from BALB/c mice was investigated. Colon26 cells were inoculated in 24-well culture plates at a cell density of  $1.0 \times 10^5$  cells/ml and cultured for 48 h. As indicated in Fig. 1(a), ESA treatment at concentrations higher than 8 µg/ml induced apparent cell death. Microscopic images of Colon26 cells treated with or without 50 µg/ml of ESA are shown in Fig. 1(b). These results reveal the dose-dependent cytotoxic effect of ESA on Colon26 cells. This means that Colon26 cells could be adopted for the examination of the anti-cancer effect of ESA *in vivo*.

#### Expression of caspase-3 activity induced by *Euchema serra* agglutinin

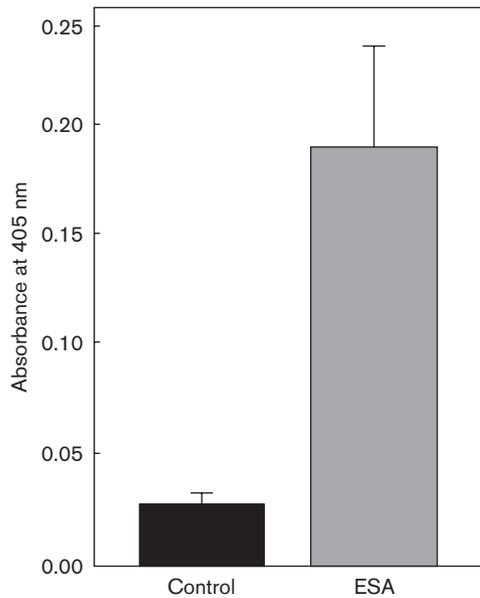
As indicated above, the cytotoxic effect of ESA on Colon26 cells was observed at a concentration of 50 µg/ml. Following such an observation, the molecular mechanism of the cell death induced by ESA at this concentration was investigated. First, investigation of the effect of ESA on the induction of caspase-3 activity was undertaken. As indicated in Fig. 2, caspase-3 activity in

**Fig. 1**



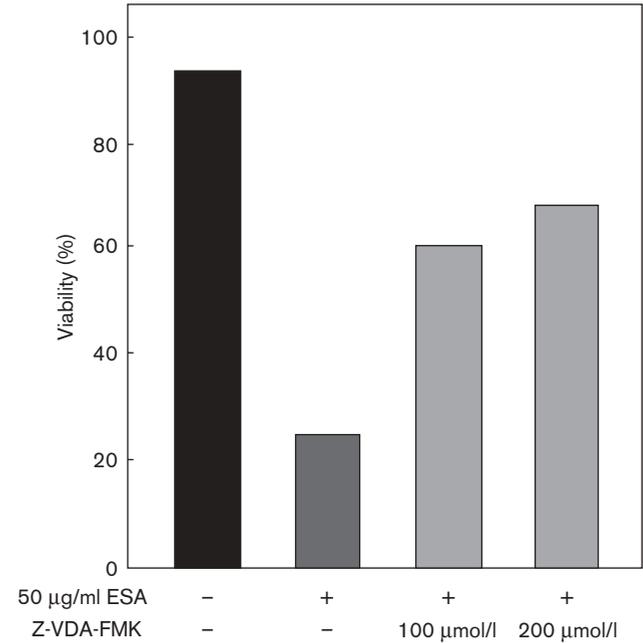
Effect of ESA on Colon26 cells. (a) Colon26 cells were inoculated in 24-well culture plates at a cell density of  $1.0 \times 10^5$  cells/ml in ERDF medium with 5% fetal bovine serum and various concentrations of ESA, and cultured for 48 h. Phosphate-buffered saline was added to the medium instead of ESA as a control (closed circle). Cell viability was determined by the trypan blue dye exclusion test. The values were means of two separate measurements. (b) The morphological changes of cells were examined by microscopy. ESA, *Euchema serra* agglutinin.

**Fig. 2**



Determination of caspase-3 activity induced by ESA. Colon26 cells were inoculated at a cell density of  $1.0 \times 10^5$  cells/ml in 5% fetal bovine serum-ERDF medium containing 50 µg/ml of ESA and cultured for 48 h. After cultivation, caspase-3 activity in Colon26 cells was determined. The values were means of two separate measurements. ESA, *Euchema serra* agglutinin.

**Fig. 3**



Effect of caspase-3 inhibitor on ESA-induced cell death. Colon26 cells were inoculated at a cell density of  $1.0 \times 10^5$  cells/ml in 5% fetal bovine serum-ERDF medium containing 50 µg/ml of ESA. Z-VAD-FMK was also added to the medium at 100 or 200 µmol/l. After cultivation for 48 h, cell viability was determined by the trypan blue dye exclusion test. ESA, *Euchema serra* agglutinin.

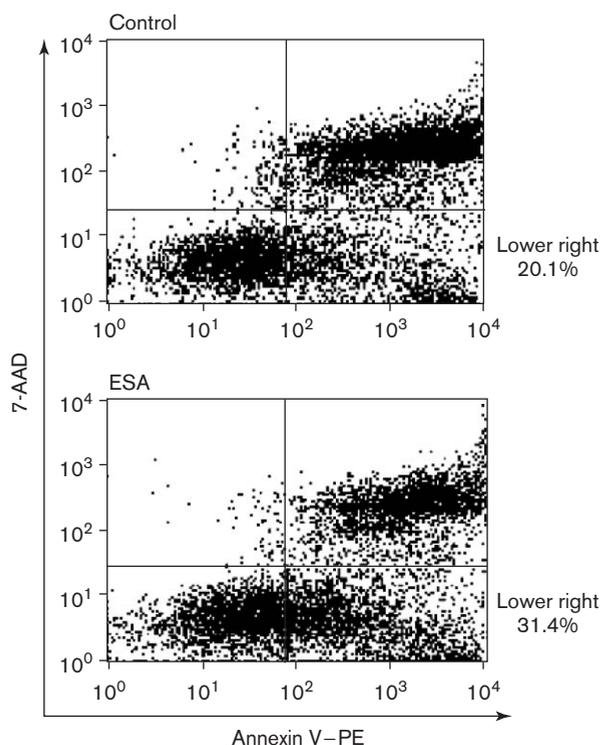
Colon26 cells were remarkably induced by the ESA treatment.

As the expression of caspase-3 activity was triggered by the ESA treatment, the effect of a caspase inhibitor on ESA-induced cell death was determined. The caspase inhibitor Z-VAD-FMK was added at a concentration of 100 or 200 µmol/l to the 5% FBS-ERDF medium containing 50 µg/ml of ESA and the Colon26 cells were cultured for 48 h. As shown in Fig. 3, the cell death induced by ESA was partially, but not completely, inhibited by Z-VAD-FMK.

**Flow cytometry analysis of *Euchema serra* agglutinin-treated Colon26 cells**

Flow cytometry analysis of the apoptosis induced by ESA was carried out by performing annexin V-PE and 7-AAD staining [6,7]. As shown in Fig. 4, the annexin V-PE<sup>+</sup>/7-AAD<sup>-</sup> cell population (lower right quadrant) was markedly increased from 20.1 to 31.4% by ESA treatment. The lower right quadrant represents the early stage of apoptosis. This result also suggests that ESA induces apoptosis; however, the induction was slightly weak. Thus, this result is consistent with that obtained following the caspase inhibitor assay.

Fig. 4



Flow cytometry analysis of ESA-treated Colon26 cells. Colon26 cells were inoculated in 5% fetal bovine serum-ERDF medium containing 50  $\mu\text{g/ml}$  of ESA and cultured for 48 h. Cells were stained with annexin V-PE and 7-amino-actinomycin D, and analyzed by FACSCalibur (DB Biosciences, Franklin Lakes, New Jersey, USA). ESA, *Euchema serra* agglutinin.

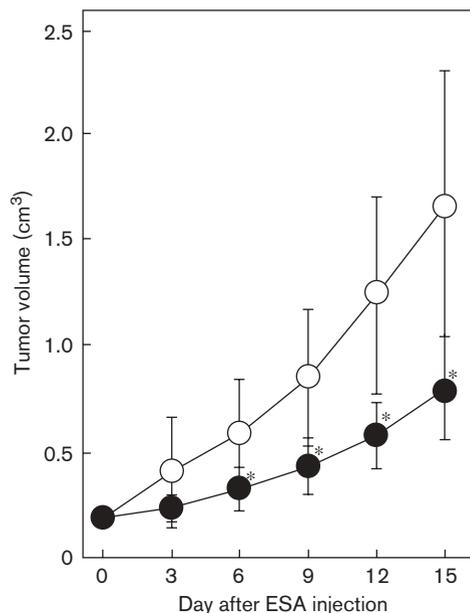
#### Anti-tumor effect of *Euchema serra* agglutinin *in vivo*

We examined the effects of ESA on the growth of subcutaneous Colon26 tumors. As indicated in Fig. 5, the growth of the tumors in the BALB/c mice intravenously injected with ESA via the tail vein was significantly delayed when compared with those of the control mice injected with PBS. In addition, physical condition of the mice, such as decrease in body weight, was not affected by ESA (data not shown), and no death of mice caused by ESA injection was observed. These results reveal the anti-tumor effect of ESA not only *in vitro* but also *in vivo*.

#### Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay for subcutaneous tumors treated with *Euchema serra* agglutinin

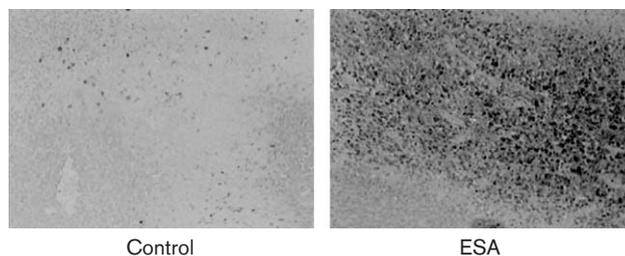
To investigate the mechanism of the anti-tumor effect of ESA *in vivo*, subcutaneous tumors were analyzed after ESA treatment by an in-situ TUNEL assay, which detects DNA fragmentation and is commonly used to detect apoptosis. As indicated in Fig. 6, ESA-treated tumors had widespread TUNEL-positive cells in contrast to the staining of control tumors treated with PBS. These results demonstrate that ESA induces apoptotic cell death in Colon26 tumors *in vivo*.

Fig. 5



Effect of ESA on tumor growth in the BALB/c mice. BALB/c mice were subcutaneously injected with Colon26 cells and tumors were allowed to grow up to tumor volume of about 0.2  $\text{cm}^3$  before treatment with ESA (closed circles) or phosphate-buffered saline (as control) (open circles). Values were means  $\pm$  SD ( $n=10$ ). \* $P<0.01$ . ESA, *Euchema serra* agglutinin.

Fig. 6



Detection of ESA-induced apoptosis in Colon26 tumor. Subcutaneous tumors were harvested from sacrificed BALB/c mice after ESA or phosphate-buffered saline (as control) treatment and analyzed by TUNEL assay. Original magnification:  $\times 400$ . ESA, *Euchema serra* agglutinin.

## Discussion

Lectins have been found in various organisms, including bacteria and marine macroalgae. They are specialized proteins that recognize and bind to particular oligosaccharide chains [8]. Lectins have many biological activities apart from their property of associating with sugars, such as mitogenic activity against lymphocytes and agglutination activity. In addition, lectins are very highly specific molecular probes against 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 these cells [9], the chains on the cancer cells being more complicated than those of normal cells. In recent years, the number of patients suffering from colon cancer has been increasing in Japan [10,11]. As indicated in our previous report, ESA induces cell death in several human cancer cells, particularly colon cancer cells [2]. ESA, however, does not associate with and induce cell death in normal cells such as peripheral blood lymphocytes or normal fibroblasts from the umbilical cord.

The major purpose of this study was the evaluation of the anti-cancer effect of ESA *in vivo*. Before the *in-vivo* investigation, we began the study by examining the *in-vitro* cytotoxic effect of ESA on the mouse colon cancer cell line Colon26 derived from BALB/c mice. ESA was observed to induce cell death in Colon26 cells in a dose-dependent manner. The expression of caspase-3 activity and the translocation of phosphatidylserine on cytoplasmic membranes were observed in the ESA-treated Colon26 cells. These findings suggested that the mode of the cell death induced by ESA is via the apoptosis pathway. On the other hand, ESA-induced cell death was not completely inhibited by the caspase inhibitor Z-VAD-FMK, as indicated in Fig. 3. This indicates a possibility that the cell death induced by ESA is triggered by necrosis or caspase-independent apoptosis such as the apoptosis-inducing factor pathway [12]. Many studies have reported on the anti-cancer effects of polysaccharides derived from algae, such as fucoidan [13,14]. Few reports, however, exist on the induction of apoptosis in cancer cells by lectins extracted from marine algae. Hence, the anti-tumor effect of ESA observed in this study is very important. The detailed pathway of cell death induced by ESA is currently under investigation.

ESA also revealed the anti-tumor effects on Colon26 tumors established in BALB/c mice, demonstrating that the effect of ESA is manifest not only *in vitro* but also *in vivo*. The cell death induced by ESA in the Colon26 tumors *in vivo* was also due to apoptosis, as demonstrated by the *in-situ* TUNEL assay.

In addition, the administration of ESA every 3 days did not cause death in the mice. This means that the side-effects of ESA such as anaphylactic shock may not be so serious. These desirable properties suggest that ESA has the potential to be a novel drug for cancer therapy. ESA,

however, will induce immune response in humans upon repeated administration. To overcome the immunogenicity of ESA, it is necessary to obtain a low-molecular-weight ESA possessing cancer recognition property and anti-tumor effects by cloning and manipulation of the ESA coding gene.

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