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Chemopreventive Effect of Protein Extract of *Asterina pectinifera* in HT-29 Human Colon Adenocarcinoma Cells

Yun-Hee Shon and Kyung-Soo Nam

Department of Pharmacology, College of Medicine and Intractable Disease Research Center, Dongguk University, Gyeongju, Korea

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We investigated the effect of protein extract of *Asterina pectinifera* on the activity of 4 enzymes that may play a role in adenocarcinoma of the colon: quinone reductase (QR), glutathione *S*-transferase (GST), ornithine decarboxylase (ODC), and cyclooxygenase (COX)-2. QR and GST activity increased in HT-29 human colon adenocarcinoma cells increased that had been exposed to 4 concentrations of the protein extract (80, 160, 200, and 240 μg/mL). Additionally, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ODC activity decreased significantly in cells exposed to the extract in concentrations of 160 μg/mL ($p<0.05$), 200 μg/mL ($p<0.005$), and 240 μg/mL ($p<0.005$). TPA-induced COX-2 activity also decreased in cells exposed to extract concentrations of 10, 20, 40, and 60 μg/mL. COX-2 expression was also inhibited in cells exposed to this extract. These results suggest that this protein extract of *A pectinifera* has chemopreventive activity in HT-29 human colon adenocarcinoma cells, and therefore, may have the potential to function as a chemopreventive agent in human colorectal cancer.

Key words: *Asterina pectinifera,* Cyclooxygenase-2, Glutathione *S*-transferase, Ornithine decarboxylase, Quinone reductase

INTRODUCTION

One of the most promising areas of cancer research is chemoprevention, ie, the search for a means of preventing cancer through the administration of chemical or natural substances. Colorectal cancer is one of the most common forms of malignancy in the industrialized world, contributing significantly to cancer mortality and morbidity (Parkin *et al.*, 1993). Because colorectal cancer has a significant environmental component, it is an ideal disease in which to evaluate the potential benefits of chemopreventive agents.

Chemopreventive agents can function by means of a variety of mechanisms of action directed at every major stage of carcinogenesis. One mechanism of particular note involves the induction of phase II (detoxification) enzymes, such as quinone reductase (QR) or glutathione *S*-transferase (GST) (Talaly *et al.*, 1995). Polyamines and the enzymes responsible for their biosynthesis play a significant role in carcinogenesis, tumor promotion, and cellular hyperplasia. Ornithine decarboxylase (ODC), the first and rate-limiting enzyme in the polyamine synthesis pathway, is induced during carcinogenesis in the colon (Tanako *et al.*, 1993). Cyclooxygenase (COX) is also considered important in colon carcinogenesis; COX-2 inhibitors have been effective in the prevention of colon cancer in several animal models (Marnett and DuBois, 2002).

Natural products isolated from marine organisms have become excellent sources of new and effective drugs for patients with such disorders as cancer and inflammatory conditions, including fungal and viral conditions (Ruggieri, 1976). Overall, more than 3000 new substances derived from marine organisms have been developed into anticancer agents during the past 3 decades, demonstrating the great potential of the sea as a source of novel anticancer chemical classes (Rinehart, 2000; Schweitzer *et al.*, 1991).

In this study, the effects of protein extract from the starfish *Asterina pectinifera* on QR and GST, ODC, and COX-2 activity were investigated in human colon adenoma cells.

Correspondence to: Kyung-Soo Nam, Department of Pharmacology, College of Medicine and Intractable Disease Research Center, Dongguk University, Gyeongju, Gyeongbuk 780-714, Korea
Tel: 82-54-770-2412, Fax: 82-54-770-2477
E-mail: namks@dongguk.ac.kr

MATERIALS AND METHODS

Animals

Starfish (*A pectinifera)* were collected during August 2004 off the coast of Pohang, Korea. The specimen (A0014-3) of the starfish was deposited in the Intractable Disease Research Center at Dongguk University in Gyeongju, Korea. The animals were washed with distilled water and stored at -20°C until used.

Preparation of protein extract

The protein extract was prepared from *A pectinifera* following the protocol described by Kishimura and Hayashi (2002). The animals were cut into small pieces and centrifuged in deionized water at 10,000 × g for 30 minutes at 4°C. The supernatant was then fractionated at 0% to 40% (w/v) saturation with ammonium sulfate. Each fraction was dissolved in Tri-HCl buffer (pH 7.4) and dialyzed against the same buffer. The dialyzate was then centrifuged at 10,000 × g for 30 minutes at 4°C. The supernatant (protein extract) was freeze-dried then dissolved in a cell culture medium for use in this experiment.

Determination of QR activity

QR-specific activity was measured in HT-29 human colon adenoma cells grown in 96-well microtiter plates according to the method described by Shon *et al.* (2004). Increase QR activity was calculated based on the ratio of specific enzyme activity in cells exposed to the extract versus a control solvent. Ellagic acid was used as the reference compound in this experiment.

GST activity

GST activity was measured using a modified version of the procedure developed by Habig *et al.* (1974), in which 1-chloro-2,4-dinitrobenzene served as the substrate. The protein content of the solution obtained by this method was measured in a duplicate plate using a bicinchoninic acid protein assay kit (Sigma, St Louis, Mo, U.S.A.), with bovine serum albumin (BSA) serving as the standard. GST activity was expressed as the slope/min·mg^{-1} protein. The data derived from cells exposed to the extract were compared with the values obtained for the solvent control. Ellagic acid was used as the reference compound.

ODC activity

ODC activity in the HT-29 cells was evaluated according to the method developed by Shon and Nam (2003), in which the amount of $^{14}CO_2$ released from L-[1-^{14}C]ornithine is measured.

COX-2 expression (western blotting)

HT-29 cells were plated in 12-well plates and grown to subconfluency. They were then washed and transferred to a serum-free medium containing 0.1% BSA for 24 h. Incubation was continued in 12-*O*-tetradecanoylphorbol-13-acetate (TPA), with or without the *A pectinifera* protein extract, for 30 h. The cells were then harvested and lysed using a method described previously (Liu *et al.*, 1993). The protein obtained from cell lysates underwent electrophoresis on a 9% SDS-polyacrylamide gel and was then transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was treated with 5% nonfat milk for 1 h to prevent nonspecific binding and probed with primary antibodies to COX-2 and actin at a final dilution of 1:1000. Primary antibodies were detected using biotin-rabbit anti-mouse immunoglobulin GAM (H+L) and alkaline phosphate-conjugated streptavidin, then visualized using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as a substrate (Promega, Madison, Wisc, U.S.A.).

Assay of COX-2 activity

Lysates of HT-29 cells were prepared as described above. COX-2 activity was measured using the prostaglandin (PG) E_2 Biotrak enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's protocol. This assay is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2-specific antibody. The anti-PGE_2 antibody has 25% and 0.04% cross-reactivity with PGE_1 and PGE_2, respectively. The determination range for this assay is 50 to 6400 pg/mL.

Statistical analysis

The data were analyzed for statistical significance using Student's *t*-test. p value lower than 0.05 was considered evidence of significance.

RESULTS AND DISCUSSION

QR activity increased in a dose-dependent manner in HT-29 human colon adenoma cells exposed to the protein extract we obtained from *A pectinifera* within a concentration range of 80 to 240 μg/mL, with a 1.7-fold induction in enzyme activity at the highest extract concentration tested (Fig. 1). GST activity also increased significantly ($p < 0.01$) in HT-29 cells exposed to the extract in culture (Fig. 2). An increase in the activity of these detoxification enzymes could lead to a reduction in the carcinogen burden in the colon and a subsequent decrease in carcinogenesis in the colon (Joseph *et al.*, 1994; Talalay *et al.*, 1995). The increase in QR and GST activity in cells exposed to the *A pectinifera* protein extract supports the hypothesis that this extract prevents the initiation of colon

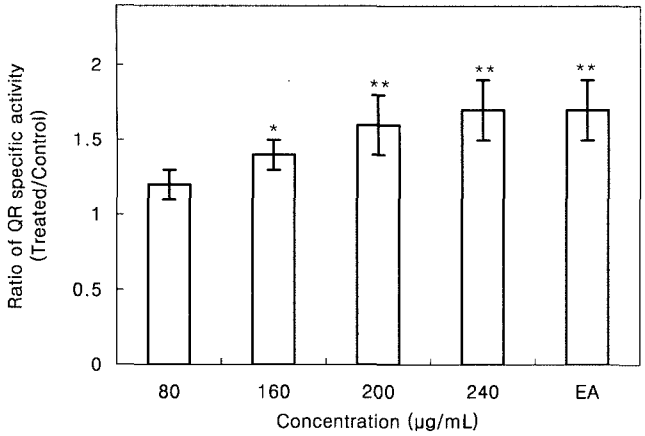

Fig. 1. Effect of protein extract obtained from *A pectinifera* on QR activity. EA, 15 μg/mL ellagic acid. Data shown are mean values, with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 compared with the control.

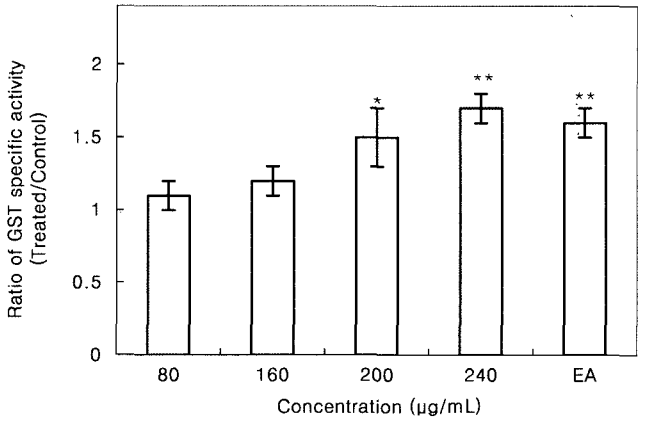

Fig. 2. Effect of protein extract obtained from *A pectinifera* on GST activity. EA, 15 μg/mL ellagic acid. Data shown are mean values, with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 compared with the control.

carcinogenesis by increasing phase II enzyme activity.

In homogenates of HT-29 cells, TPA-induced ODC activity decreased with increasing concentrations of the protein extract in a dose-dependent manner. A comparable effect was observed with difluoromethylornithine, a traditional inhibitor of ODC (Fig. 3). Other studies have provided convincing evidence for the roles of polyamine and ODC in colon cancer cell growth and in the biological response of tumor promoters and growth factors to these substances (Marton and Pegg, 1995; Rao *et al.*, 1993). Hence, the use of inhibitors that alter normal polyamine levels by inhibiting ODC may serve as another approach to colon cancer chemotherapy. Overinduction of ODC activity results in the stimulation of tumor promotion. Inhibition of ODC activity may be one of several mechanisms by which this protein extract inhibits the development of colon cancer.

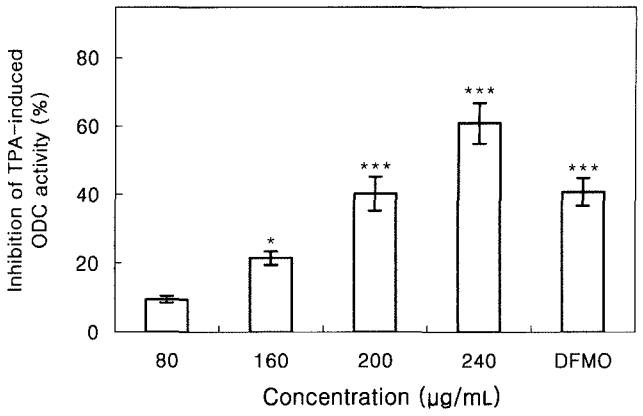

Fig. 3. Effect of protein extract obtained from *A pectinifera* on TPA-induced ODC activity in HT-29 human colon adenoma cells. The ODC activity of controls is 512 ± 47 pmol $^{14}CO_2/h \cdot mg^{-1}$ protein. DFMO, 0.01 mM difluoromethylornithine. Data shown are mean values, with bars indicating the SD of the mean (n=3). *p<0.05, ***p<0.005 compared with the control.

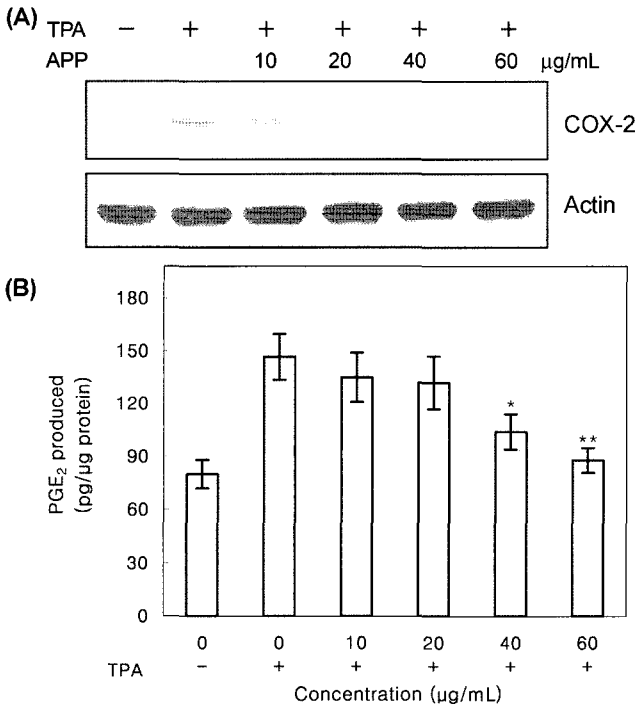

Fig. 4. Effect of protein extract obtained from *A. pectinifera* (APP) on TPA-induced protein expression (A) and activity (B) of COX-2. Data shown are mean values, with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 compared with the control.

Exposure of HT-29 cells to TPA was accompanied by an increase in COX-2 levels, while TPA-induced expression of COX-2 was inhibited in cells exposed to the protein extract (Fig. 4A). We monitored PGE_2 levels in these cells to determine the relationship between COX-2 expression and activity. Figure 4B shows that the HT-29 cells produced a low basal PGE_2 level that decreased

substantially when these cells were exposed to TPA for 30 h. This TPA-induced COX-2 activity decreased on exposure to the protein extract in a dose-dependent manner (Fig. 4B). Because PGs mediate inflammation and chronic inflammation predisposes to carcinogenesis (Kawamori *et al.*, 1998), it has been suggested by an increasing number of investigators that inhibitors of COX-2 activity are effective antiinflammatory agents and, therefore, may prevent colon cancer and be useful in the treatment of patients with this disorder. Consequently, agents that can inhibit COX-2 activity might be useful for inhibiting carcinogenesis in the colon.

The results of our study suggest that the protein extract we obtained from *A pectinifera* acts as a chemopreventive agent in colon cancer because of its specific ability to increase phase II enzyme (QR and GST) activity and inhibit COX-2 and ODC activity. Therefore, the data derived from this study may be useful in the development of this extract into a chemopreventive agent of colon cancer in animal studies and later in human clinical trials.

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