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RESEARCH PAPER

Cancer Chemopreventive Effects of Starfish Polysaccharide in Human Breast Cancer Cells

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Abstract We investigated the effect of starfish (*Asterina pectinifera*) polysaccharide on the progression and metastasis of human breast cancer cells. At a concentration range of 10 ~ 120 μg/mL the polysaccharide significantly decreased the expression of cyclooxygenase-2 (COX-2) protein induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and of aromatase mRNA. In a wound healing assay, motility of human MDA-MB-231 breast cancer cells was prevented by the polysaccharide in a dose-dependent manner. These results indicate that starfish polysaccharide can prevent breast cancer progression and metastasis by decreasing prostaglandin E_2 and estrogen biosynthesis by COX-2 and aromatase, and by inhibiting cell motility. This report presents information regarding the effectiveness of starfish polysaccharide as a chemopreventive agent against breast cancer.

Keywords: starfish, breast cancer, chemoprevention, polysaccharide, metastasis

1. Introduction

Cancer chemoprevention is defined as the suppression or inhibition of cancer development, progression, and metastasis through the systemic use of natural or synthetic chemical agents. Breast cancer is the most common cause of female mortality worldwide [1]. Cycloxygenases (COXs) are key enzymes in the biosynthesis of prostaglandins from arachidonic acid. Cyclooxygenase-2 (COX-2), an inducible COX isoform activated by extracellular stimuli such as growth factors and cytokines, is a significant factor in tumor progression [2]. Several studies have demonstrated that COX-2 is overexpressed in many types of cancer, including breast, colon, lung, pancreas, etc. [3,4]. Overexpressed COX-2 can enhance metastatic and angiogenic activity, a major cause of increased risk of mortality and poor prognosis, by increasing prostaglandin E_2 (PGE_2) biosynthesis [5,6]. Many COX-2 inhibitors also decrease expression and activation of aromatase (cytochrome P450 19A1), which catalyzes the production of aromatic C_{18} estrogen from C_{19} androgen. Studies in cell systems and animal models showed that newly synthesized estrogen plays a more important role than circulating estrogen in breast cancer promotion and progression [7,8]. Therefore, to prevent breast cancer promotion and proliferation it is necessary to inhibit the expression and activity of these proteins.

Mortality and relapse in cancer patients are primarily the result of metastasis, which involves complex steps including cell adhesion, invasion, and migration. Hence, to successfully prevent and treat cancer, blockage of one or more of these three metastasis steps is very important.

Substances originating from marine organisms can become useful sources of drugs for treatment and prevention of diseases such as cancer and inflammatory disorders [9]. During the past three decades, more than 3,000 new natural materials derived from marine organisms have been confirmed as potential sources for anticancer agents [10]. Consequently, the sea is a repository of natural resources for future medical chemical classes. In a previous report, inhibition of CYP1A1 and ornithine decarboxylase (ODC) activities by starfish polysaccharide was identified [11]. The research suggested that polysaccharide originating from starfish may be a powerful candidate in cancer chemo-

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prevention. Based on this information, we investigated the effects of starfish polysaccharide on the progression and metastasis of MDA-MB-231 human breast cancer cells by studying the changes in COX-2 protein expression, aromatase transcription, and cell motility.

2. Materials and Methods

2.1. Materials

MDA-MB-231 human breast cancer cells were purchased from the Korean Cell Line Bank (Seoul, Korea). RPMI 1640 liquid medium, fetal bovine serum (FBS) and penicillin/streptomycin solution were obtained from WelGENE (Daegu, Korea). COX-2 monoclonal antibody was purchased from Invitrogen (Carlsbad, CA, USA), β-actin monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), biotinylated rabbit anti-mouse IgGAM (H+L) and streptavidin-conjugated alkaline phosphatase from Zymed Laboratories Inc. (San Francisco, CA, USA), and 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate and agarose from Promega (Madison, WI, USA). DNA primers for polymerase chain reaction (PCR) were obtained from Bioneer (Daejeon, Korea).

2.2. Cell culture

Monolayer MDA-MB-231 human breast cancer cells were grown in RPMI 1640 cell culture media containing 10% FBS at 37°C in a 5% CO_2 incubator. The cells were washed with phosphate-buffered saline (PBS, pH 7.4), and culture media were replaced with conditioned media prepared from serum-free media or 1% FBS supplemented-media in each experiment.

2.3. Preparation of polysaccharide

Starfish polysaccharide was prepared from *Asterina pectinifera* as follows: 500 g of *A. pectinifera* collected from the coast of Pohang in Korea was cut into small pieces, and crude extracts containing water-soluble components were prepared by boiling in 10 volumes of water (10 L) for 3 h at 100°C. The extracts (approximately 2.5 L) were centrifuged at 12,000 × *g* for 20 min and debris was removed by filtration. To prepare the precipitates containing polysaccharide from the filtrates, 3 volumes of 95% (v/v) ethanol were added to the extract and the mixture was incubated at 4°C overnight. The precipitates were collected by centrifugation (18,000 × *g*, 30 min), dissolved in distilled water, and then centrifuged for 20 min at 12,000 × *g*. The supernatant was carefully collected and lyophilized. The carbohydrate content in the supernatant was measured using a phenol-sulfuric acid reaction. The final weight of the lyophilized-polysaccharide was approximately 70 mg. Mineral levels in the polysaccharide powder were measured with an ICPS-7510 sequential plasma spectrometer (Shimadzu, Kyoto, Japan). For use in the experiments, the freeze-dried polysaccharide was dissolved in serum-free cell culture medium (RPMI 1640) and filtered through a 0.2 μm sterile membrane filter.

2.4. Cell viability assay

MDA-MB-231 human breast cancer cells were seeded in 96-well plates at 1×10^4 cells/well and suspended in RPMI 1640 medium supplemented with 10% FBS and grown for 24 h. The cells were treated with starfish polysaccharide at various concentrations (0 ~ 120 μg) and cultured for 24, 48, and 72 h. The cell proliferation assay was performed using 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, USA).

2.5. Western blot analysis of COX-2

To confirm the change of COX-2 protein expression induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 3×10^5 cells/well of MDA-MB-231, human breast cancer cells, were seeded on 6-well culture plates. After culturing for 24 h at 37°C in a 5% CO_2 atmosphere, the media was replaced with conditioned media containing defined concentrations (from 10 to 120 μg/mL) of starfish polysaccharide. Cells were incubated for an additional 24 h and cell lysates were prepared using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, MA, USA). The lysates were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. A primary antibody reaction for COX-2 was performed for 2 h at room temperature and confirmed using biotinylated rabbit anti-mouse IgGAM (H+L) and streptavidin-conjugated alkaline phosphatase. Bands were visualized by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) of aromatase

MDA-MB-231, human breast cancer cells (4×10^5 cells/well), were seeded on 6-well culture plates. After culturing for 24 h at 37°C in a 5% CO_2 atmosphere, the media was replaced with conditioned media containing various concentrations of polysaccharide. Cells were incubated for an additional 24 h and harvested by trypsinization. Total RNA was extracted with an easy-BLUE^{TM} Total RNA Extraction Kit (iNtRON Biotechnology, Sungnam, Korea) according to the manufacturer's protocol. RT-PCR was performed with an RNA PCR Kit (AMV) ver. 3.0 (Takara, Shiga, Japan) using 1 μg of total RNA. Primer sequences for the

aromatase transcript were 5'-GAATATTGGAAGGATGC-ACAGACT-3' and 5'-GGGTAAAGATCATTTCCAGCAT-GT-3' [12], and the beta-actin primers used for the control were 5'-CAAGAGATGGCCACGGCTGCT-3' and 5'-TCCTTCTGCATCCTGTCGGCA-3' [13]. The PCR reaction conditions were 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and 72°C for 10 min. PCR products were subjected to 1.2% agarose gel electrophoresis and stained with ethidium bromide. Bands were densitometrically quantified using Scion Image software.

2.7. Wound healing migration assay

MDA-MB-231 breast cancer cells were seeded on 6-well culture plates coated with collagen (20 μg/mL) and grown in culture media. Wells covered by confluent cells were gently scratched with a pipette tip and washed with PBS to remove cellular debris. After 24 h, the cells were treated with various concentrations of polysaccharide dissolved in conditioned media containing 1% FBS. Cells were then cultured for 48 h with 5% CO_2 at 37ºC. Photographs were taken of scratched areas for comparison at 0 and 48 h.

3. Results and Discussion

3.1. Mineral components of starfish polysaccharide powder

Of the minerals measured, sodium ion was present in the greatest amount (6.39 μg/mg of polysaccharide) and potassium ion was not detected in the prepared starfish polysaccharide powder (Table 1). Trace amounts of four other mineral ions were also identified in the polysaccharide powder. Protein moiety was undetectable according to the protein assay results (data not shown).

3.2. Effect of starfish polysaccharide on cell viability of MDA-MB-231 human breast cancer cells

The effect of starfish polysaccharide on cell viability of MDA-MB-231 human breast cancer cells was evaluated by MTT assay. Starfish polysaccharide did not exhibit significant cytotoxicity at the concentration range assessed (Fig. 1). Hence, based on this result, all studies were performed using non-toxic concentrations of starfish polysaccharide.

Table 1. Mineral components in starfish polysaccharide powder

Mineral	Na	Mg	Ca	P	Fe	Cu	K
Amount (μg/mg polysaccharide)	6.39	0.62	5.12	0.02	1.04	1.28	ND

ND: not detected.

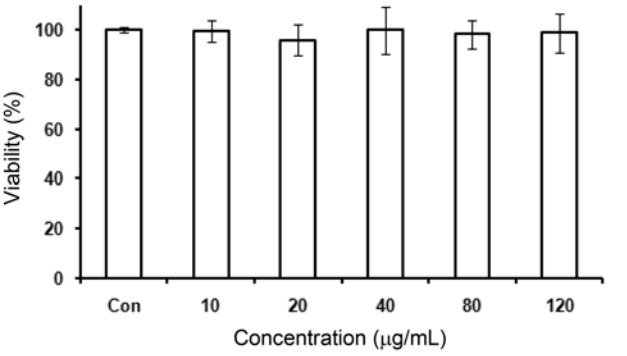

Fig. 1. Effect of starfish polysaccharide on cell viability of MDA-MB-231 human breast cancer cells. Cells were grown in different concentrations of starfish polysaccharide for 24 h and viability was assessed by MTT assay. The values indicate means ± standard deviation in each group (n = 6).

3.3. Inhibition of COX-2 protein expression by starfish polysaccharide

COX is a key enzyme in PGE_2 synthesis from arachidonic acid. High PGE_2 levels are observed in many human breast cancers [5], and COX-2 is closely involved in tumor growth, invasion, and metastasis [14]. Consequently, COX-2 is considered a target for breast cancer therapy and chemoprevention strategies. Actually, inhibition of tumor induction and growth by COX-2 inhibitors has been reported in previous studies. Masferrer *et al*. (2000) and Kundu and Fulton (2002) demonstrated antiangiogenic, antimetastatic, and antitumor activities of COX-2 inhibitors [7,15]. Another study found that silencing COX-2 in MDA-MB-231 cells

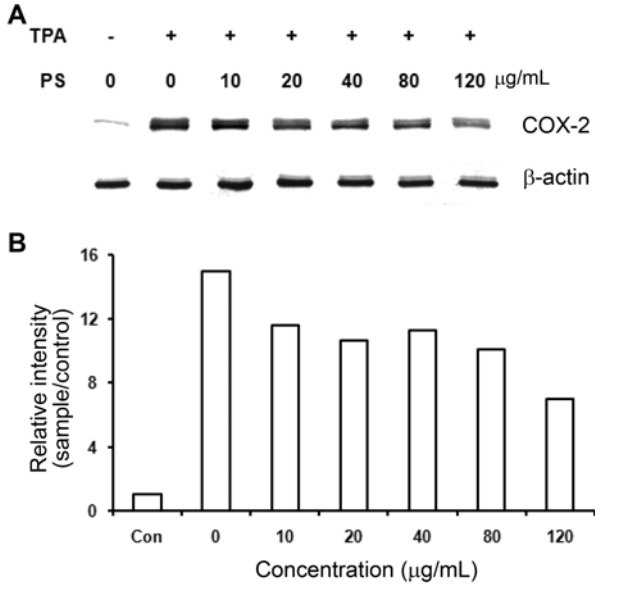

Fig. 2. Effect of starfish polysaccharide on cyclooxygenase-2 (COX-2) expression induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA: 100 nM) in MDA-MB-231 human breast cancer cells. Graph indicates relative intensity of each sample compared to control; each band is normalized to beta-actin. PS indicates starfish polysaccharide.

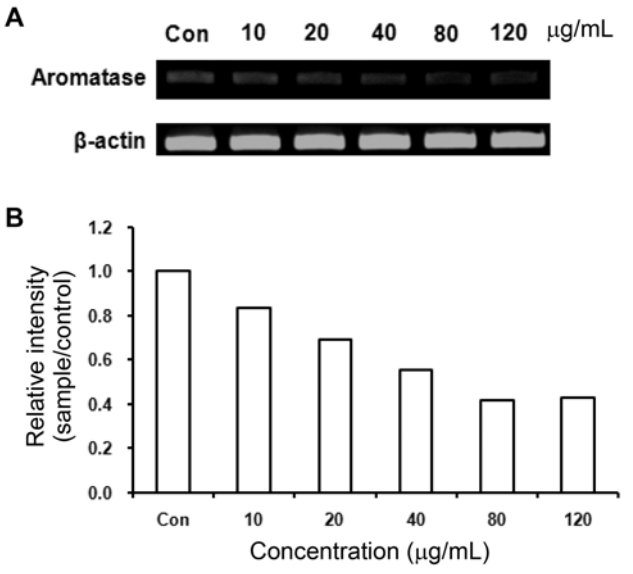

Fig. 3. Effect of starfish polysaccharide on aromatase transcription in MDA-MB-231 human breast cancer cells. Graph indicates relative intensity of each sample compared to control; each band is normalized to beta-actin.

led to the induction of antiangiogenic gene expression and the reduction of prometastatic transcription [14]. In the present research, starfish polysaccharide effectively inhibited TPA-induced COX-2 expression in MDA-MB-231 breast cancer cells in a dose-dependent manner (Fig. 2). This result indicates that starfish polysaccharide should lessen the aggravation of breast cancer by delaying cancer promotion and preventing metastasis.

3.4. Inhibition of aromatase transcription by starfish polysaccharide

Aromatase is another target for therapy and chemoprevention associated with human breast cancer. The protein is the key enzyme in estrogen biosynthesis. The level of newly synthesized estrogen is more important than the level of circulating estrogen in tumor growth and metastasis [8]. Accordingly, cancer progression and metastasis could be effectively prevented by inhibition of estrogen synthesis. The transcriptional level of aromatase was dose-dependently decreased by starfish polysaccharide (Fig. 3). Because down-regulation of aromatase transcription leads to inhibition of estrogen synthesis, promotion and metastasis mediated by estrogen in breast cancer may be prevented. Hence, this result suggests that starfish polysaccharide could be used as an aromatase inhibitor.

3.5. Suppression of TPA-induced cell motility by starfish polysaccharide

Cancer cell motility is necessary for metastasis to tissues distant from the primary tumor. In many cases, the death of a cancer patient is closely related to metastasis. Because metastasis progresses via cell adhesion, invasion, and motility, interference with one or more of these steps is the best method for cancer therapy. In the wound healing migration assay, cell motility was successfully controlled with various concentrations of starfish polysaccharide (Fig. 4). The present result shows that starfish polysaccharide may decrease the anti-metastatic activity in breast cancer cells by inhibiting cell migration.

4. Conclusion

Here, we investigated the effect of starfish polysaccharide on progression and metastasis in MDA-MB-231 human breast cancer cells. The results have demonstrated for the first time that starfish polysaccharide suppressed cancer progression and metastasis. The decrease of COX-2 and aromatase expression and the inhibition of motility were involved in this suppression. In conclusion, the results suggest that starfish polysaccharide may have value as a chemopreventive agent for human breast cancer. To confirm this suggestion, further investigations including animal studies and clinical trials are necessary.

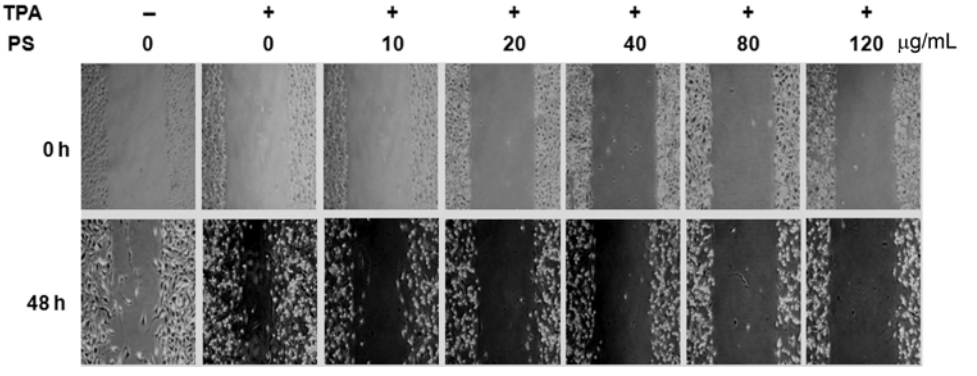

Fig. 4. Effect of starfish polysaccharide on cell motility induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA: 100 nM) in MDA-MB-231 human breast cancer cells. Cell motility is demonstrated by wound healing analysis for 48 h. PS indicates starfish polysaccharide.

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