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Red Sea Cucumber (*Stichopus japonicus*) Suppresses Cancer Progression by Promoting the ROS-Mediated Inhibition of the MAPK Pathway

Jusnseong Kim[1], Eun-A Kim[1], Nalae Kang[1], Youn Kyung Choi[2], Soo-Jin Heo[1,3*]

[1]Jeju Marine Research Center, Korea Institute of Ocean Science and Technology (KIOST), Jeju 63349, Korea

[2]Department of Medicine, School of Medicine, Jeju National University, 102 Jejudaehakno, Jeju 63243, Korea

[3]Department of Biology, University of Science and Technology (UST), Daejeon, South Chungcheong 34113, Korea

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Abstract *Stichopus japonicas* (red sea cucumbers) inhabit the coastal sea surrounding Jeju Island, South Korea, and are thought to have various medicinal properties. In this study, we investigated the anticancer activity of a red sea cucumber (*S. japonicus*) collected from Jeju Island. We obtained the red sea cucumber extract (RSCE), and observed that it inhibited the tumor cell growth and increased reactive oxygen species (ROS) production associated with the induction of apoptosis through the mitogen-activated protein kinase (MAPK) pathway in murine colon carcinoma cells (CT-26). Treatment with RSCE and N-acetylcysteine, which is a ROS scavenger, increased ROS production and apoptosis via the regulation by the MAPK pathway on the ERK and JNK compared with the nontreated group. Therefore, RSCE promotes ROS-mediated suppression of the ERK and JNK activation, and subsequently inhibits cancer progression, suggesting that RSCE may be beneficial in treating colon carcinoma.

Keywords : Red sea cucumber, Cancer, ROS, MAPK pathway, *Stichopus japonicas*

Introduction

Sea cucumbers are echinoderms belonging to the class Holothuroidea. Halothurian are found on the sea floor of deep seas[1], and are characterized by a cylindrical body and rough skin. Sea cucumbers are utilized as a traditional health food and for medicinal purposes in Korea, China, Japan, and other Southeast Asian countries. Previous studies suggest that sea cucumbers are an effective anticancer [2-5], antiangiogenic, antihypertension, anti-inflammatory, and anticoagulant [6-9] agent. Therefore, the sea cucumbers are believed to be a source of high value elements in the functional foods and nutraceutical industry [6]. Among the species of sea cucumbers, *Stichopus japonica* is classified based on it color into three groups, namely red, green, and black. These color types exhibit differences in morphology, physiology, and ecology [10]. Moreover, the color affect the taste and the market price of this species [11]. Red sea cucumber is quantitatively and qualitatively higher, and is the most favored among the other color types in Korea [12, 13]. Red sea cucumbers can be found in abundance offshore of the Jeju Island due to its suitable habitat with average water temperature of 15.7°C−18.7°C and gravel bed offshore topography. Therefore, for this study, we collected a red sea cucumber (*S. japonicus*) from the Jeju Island and investigated its anticancer activity.

Colorectal cancer (CRC) is the most commonly diagnosed cancer [4] and the fourth most common cancer causi

* Corresponding author
Phone: +82 64 798 6101
E-mail: sjheo@kiost.ac.kr

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ng death worldwide [14]. The incidence of obesity and overweight, smoking, stress, lack of exercise, and exces sive alcohol consumption increase the risk of CRC [15]. Although CRC is a treatable cancer, its mortality rate continues to increase. Current treatment options are surgery, chemotherapy, radiotherapy, and targeted thera py. However, these therapies cause secondary complica tions, including metastasis and reduced immune compet ence, and may not prevent the recurrence of tumors [16, 17]. Developing anticancer drugs from natural pro duct materials may improve the effectiveness of these therapies and may eliminate the need for harsh treatmen t measures with concomitant negative effects. In the present study, we confirmed that the red sea cucumber extracts [RSCE] is an effective treatment for CRC [18].

Materials and Methods

1.1 Materials

Red sea cucumber was collected from the sea surroun ding the Jeju Island, South Korea August of 2017. The sample was washed three times with tap water to remov e salt. This was followed by rinsing with fresh water and vacuum drying at 25°C-30°C for 72 h. The vacuum -dried sample was homogenized with a grinder prior to extraction.

1.2 Extraction of red sea cucumber

Dried red sea cucumber powder was extracted with 70% methanol at room temperature until its color was lost. A liquid layer was obtained via filtration, and the filtrate was concentrated using an evaporator under red uced pressure. The concentrated filtrate was dissolved in dimethylsulfoxide (DMSO) and used in experiments.

1.3 Cell cultures

The murine colon cancer cells CT26 and normal cells RIE-1 were purchased from the Korean Cell Line Bank (Seoul, South Korea). The cells were cultured in RPMI medium (Welgene) with 10% fetal bovine serum and 1% antibiotics. The CT26 and RIE-1 cells were culture d every 2 days, and incubated in an atmosphere of 5% CO_2 at 37°C.

1.4 Cell viability

Cell viability was measured using the MTT assay. The CT26 cells and RIE-1 cells were seeded on 24-well plates at a concentration of 1.5×10^5 cells/ml. After 16 h, the cells were treated with different concentrations of RSCE (50, 100, and 200 μg/ml), and then incubated for 48 h at 37°C in an incubator in a humidified atmosp here of 5% CO_2. MTT solution (5 mg/ml) was added at 50 μl per well for 3 h at 37 °C. Formazan crystals in each well were dissolved in DMSO. The intensity of purple formazan was determined by measuring the absorbance at 540 nm using a microplate reader.

1.5 Measurement of ROS production

The CT26 cells were seeded in 60-mm dishes and co-treated with RSCE (50, 100, and 200 μg/ml) and H2DCFDA dye in the absence at 37°C. After 1 h, the cells were harvested and ROS production was detected using a flow cytometer (BD Accuri C6; BD Bioscience s, San Diego, CA, USA). The data were analyzed using a flow cytometer with measurement in the FL1 channel. For the inhibition of ROS production, the cells were pretreated with *N*-acetyl-L-cysteine (NAC, 2.5 mM, Sig ma-Aldrich, St. Louis, MO, USA) for 1 h before RSCE and H2DCFDA co-treatment.

1.6 Measurement of apoptosis

The CT26 cells were seeded in 60-mm dishes and trea ted with RSCE (50, 100, and 200 μg/ml) for 48 h, in the absence at 37°C. Thereafter, the cells were stained with annexin V-FITC (BD Bioscience, San Jose, CA, USA), and placed in the dark at room temperature for 15 min, followed by incubation with 7AAD in the dark at room temperature for 15 min. Annexin V- and 7AAD -positive cells were detected using a flow cytometer (BD Accuri C6; BD Biosciences, San Diego, CA, US A). The data were analyzed using a flow cytometer wit h measurements in the FL1 and FL3 channels.

1.7 Western blotting

The CT26 cells were seeded in 6-well plates at a conc entration of 1.5×10^5 cells/ml. After 16 h, the cells were treated with various concentrations of RSCE (50, 100,

and 200 μg/ml), and then incubated for 24 h or 15 min at 37 °C in an incubator in a humidified atmosphere of 5% CO_2. After incubation, the cells were harvested and washed twice with cold PBS. The cells were lyzed with RIPA buffer, and equal amounts of protein in total cell lysate were run on 8%-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked and blotted with the relevant primary antibodies. Anti-actin, -Bcl2, -p-ERK, -ERK and -p-JNK antibodies were purchased from Santa Cruz Biotechnology (Santa, CA, USA). Anti-AKT, -JNK1, -p-AKT, -PARP, p-STAT3, and -STAT3 antibodies were obtained from Cell Signaling (Danvers, MA, USA). The protein bands were visualized using the ECL Western blotting detection kit (BIO-RAD, USA) and Olympus FV10i Self-Contained confocal laser system.

1.8 Statistics

All data are expressed as means ± S.D. Significant differences among the groups were determined using the unpaired Student's t-test. The P-values of <0.5, <0.1, < 0.001 were considered statistically significant.

Results

2.1 Effects of RSCE induced apoptosis in cancer cells

The CT26 and RIE-1 cells were treated with different concentrations (0, 50, 100, and 200 μg/ml) of RSCE for 48 h. RSCE significantly suppressed growth in the CT26 cells, but did not affect RIE-1 cells' viabilities (Figure 1A). To examine if RSCE inhibited cell growth by inducing apoptotic cell death, we investigated the expression of the extent of annexin V staining and apoptosis-related proteins using flow cytometry and Western blot, respectively. RSCE dose-dependently increased the presence of apoptotic cells as shown by annexin V and 7AAD double-positive cell staining (Figure 1B). Moreover, RSCE increased the expression of PARP and decreased the expression of Bcl2 (Figure 1C). Therefore, RSCE decreased the growth of cancer cells by increasing apoptosis.

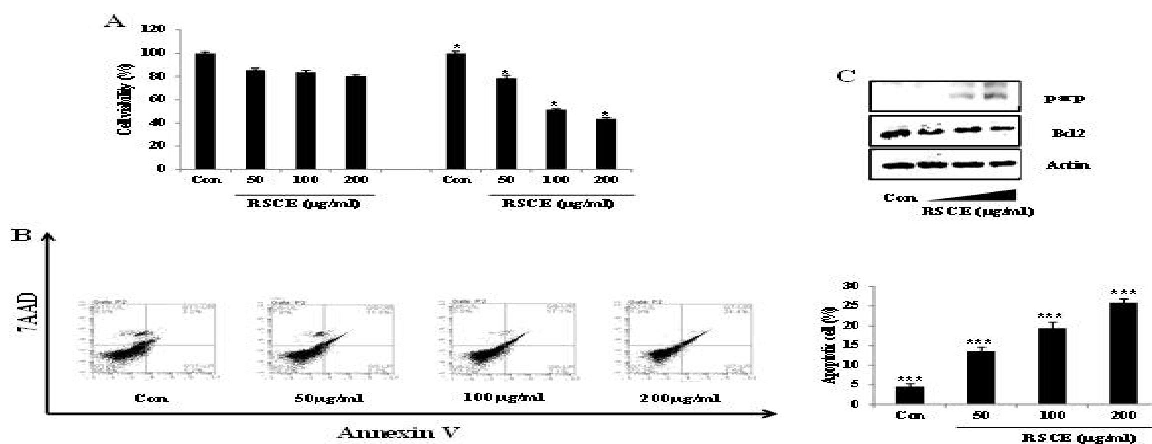

Figure 1. RSCE induced apoptotic cell death: (A) Cells were seeded in 24-well plates and then treated with different concentrations of RSCE (0, 50, 100, and 200 μg/ml) and DMSO for 48 h. Significant differences among the groups were identified using the unpaired Student's t-test. * P <0.5, ** P <0.1, and *** P < 0.001 were considered statistically significant. (B) CT26 cells were treated with RSCE (0, 50, 100, and 200 μg/ml) for 24 h, and then harvested. Cells were stained with annexin V and 7AAD in a binding buffer at room temperature in the dark. The stained cells were detected using FACSCalibur. The graph shows the examples of annexin V only-positive cells (early apoptotic cells) and annexin V and 7AAD double-positive cells (late apoptosis cells) from the total stained cells. * P <0.5, ** P <0.1, and *** P < 0.001 were considered statistically significant. (C) The CT26 cells were treated with RSCE (0, 50, 100, and 200 μg/ml) for 24 h, and whole lysates were analyzed using Western blot with PARP and Bcl2 antibodies. Actin was used as the internal control.

2.2 RSCE induces apoptosis via MAPK pathway

To investigate the anticancer effects of RSCE, we identified which intracellular signaling pathways were involved. The CT26 cells were treated with different concentrations (0, 50, 100, and 200 μg/ml) of RSCE for 15 min and then subjected to Western blotting. RSCE considerably decreased the phosphorylation of the AKT, ERK, JNK and STAT3 in a dose-dependent manner (Figure 2).

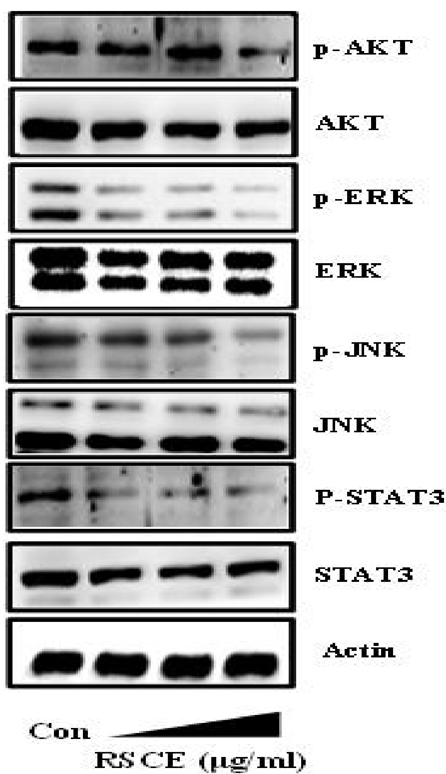

Figure 2. RSCE inhibits the cell signaling pathway: (A) The CT26 cells were treated with RSCE (0, 50, 100, and 200 μg/ml) for 15 min, and the whole lysates were analyzed using Western blot with anti-p-AKT, -AKT, -p-ERK, -ERK, - p-JNK, -JNK, -p-STAT3 and -STAT3 antibodies. Actin was used as the internal control.

2.3 RSCE increases ROS production in cancer cells

The regulation of ROS production plays an important role in cell proliferation and apoptosis. The CT26 cells were treated with different concentrations (0, 50, 100, and 200 μg/ml) of RSCE to confirm ROS production. RSCE increased ROS production by 4.5%, 8.2%, and 18.0% in 50, 100, and 200 μg/ml respectively, compared with the control (Figure 3A). Further, we used co-treatment with RSCE and NAC to confirm ROS production. NAC, which is well-known ROS scavenger suppressed RSCE-induced ROS production as shown in Figure 3B. Thus, RSCE regulates ROS production in cancer cells.

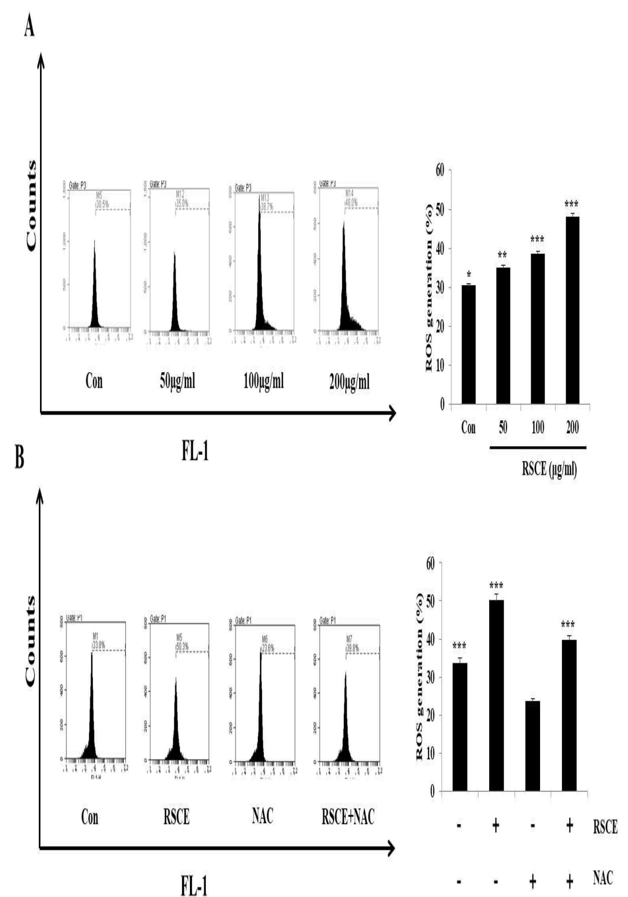

Figure 3. RSCE increases ROS production in cancer cells: (A) The CT26 cells were co-treated with (0, 50, 100, and 200 μg/ml) of RSCE and H2DCFDA dye for 1 h at 37°C. ROS production was detected using FACSCalibur. The graph shows H2DCFDA-positive cells from the total cells. (B) The cells were pretreated for 1 h with or without NAC, followed by exposure to 200 μg/ml of RSCE and H2DCFDA dye for 1 h at 37°C. * P <0.5, ** P <0.1, *** P < 0.001 were considered statistically significant.

2.4 RSCE induces ROS generation causes MAPK-kinase pathway and apoptosis

ROS production inhibits the MAPK pathway. Therefore, we investigated the MAPK expression in cells after co-treatment with RSCE and NAC. We observed that RSCE-mediated reduction in the phosphorylated MAPK pathway kinases (ERK and JNK) was restored after treatment with NAC (Figure 4A). Therefore, RSCE induces apoptosis through ROS production by the MAPK pathway.

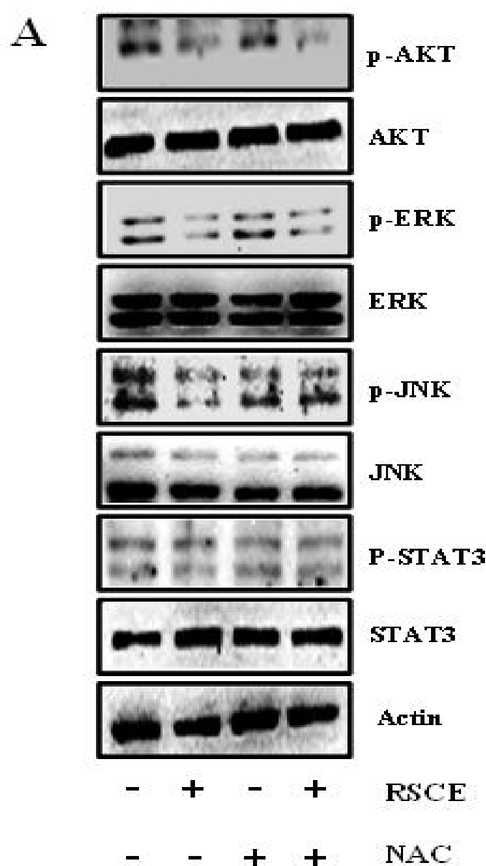

Figure 4. RSCE-induced ROS production causes the MAP kinases' inhibition and apoptosis: (A) The CT26 cells were pretreated with NAC (2.5 mM) for 1 h and then exposed to RSCE (200 μg/ml) for 15 min. p-AKT, AKT, p-ERK, ERK, p-JNK, JNK, p-STAT3 and STAT3 protein expression levels were analyzed using Western blot. Actin was used as the loading control. * P <0.5, ** P <0.1, and *** P < 0.001 were considered statistically significant.

Discussion

In this study we confirmed the potential anticancer properties of red sea cucumber from the Jeju Island, and showed that the components of red sea cucumber produce cell death in the CT26 colorectal cancer cells. Advancements in the treatment of colorectal cancer using various methods are ongoing, and currently the focus is on isolates from natural products because of their anticancer properties (2, 19). Further, it is necessary to develop new anticancer drugs with high stability and excellent anticancer effects (20). Many natural compounds appear to inhibit the development of cancer cells via ROS production (21). ROS damages DNA and leads to gene mutations, and once damaged, the cells that are not removed become stem cells and finally cancer cells (22, 23). Therefore, inhibiting the production of ROS can prevent cancer cell proliferation. Our results showed that RSCE can regulate ROS levels and enhance apoptosis in cancer cells. ROS production, which causes mitochondrial oxidative stress, involves and regulates intracellular signaling pathways. Higher levels of ROS contribute to apoptosis through impaired receptors and mitochondrial oxidative stress pathways, mitochondria dysfunction, and impaired cerebral energy metabolism (24-26). The phosphorylation of specific proteins (MAPK) in the cell affects cancer cell death (27, 28). RSCE led to ROS-mediated apoptosis by down-regulation of the ERK and JNK phosphorylation. The MAPKs are serine/threonine protein kinases that play a major role in signal transduction in the cell nucleus (29). Studies have shown that ROS is involved in the regulation of various signaling pathways, including the MAPK pathway and transcription factors. The ERK signaling pathway modulates various processes, such as survival, differentiation, proliferation, and migration, depending on the particular cell type (30, 31). The JNK inhibitors have been considered for cancer treatment because they can interfere with DNA repair in response to genotoxic drugs (32). Both ERK and JNK can affect cell survival in response to oxidant damage (33, 34). RSCE inhibits the activation of the ERK and JNK proteins associated

with apoptosis, suggesting its involvement in the signali ng initiation of mitochondrial-mediated cell death in the CT26 cells (Figure 2). Further, NAC inhibited RSCE induced apoptosis through the MAPK pathway.

Conclusion

Thus, RSCE induces apoptosis via the ROS-mediated MAPK pathway in CRC. Therefore, RSCE, a vacuum-dried extract of a red sea cucumber from the Jeju Islan d, may provide an important option for future treatment of CRC.

Acknowledgements

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