A novel polysaccharide derived from algae extract induces apoptosis and cell cycle arrest in human gastric carcinoma MKN45 cells via ROS/JNK signaling pathway

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Abstract. In recent years, interest in biological activities of compounds from marine organisms has intensified. Cancer is the most principal enemy for human life and health. For the first time, to the best of our knowledge, we investigated a novel algae-derived polysaccharide for its role in inducing apoptosis and cell cycle arrest in human gastric carcinoma MKN45 cells. We found that the novel polysaccharide suppressed MKN45 cell proliferation, induced cell apoptosis and arrested the cells at G2/M phase. Furthermore, we observed that the generation of reactive oxygen species (ROS) and the phosphorylation of Jun N-terminal kinase (JNK), p53, caspase-9 and -3 were induced in the polysaccharide-treated MKN45 cells. In addition, pretreatment with N-acetyl-cysteine (NAC) and SP600125, the inhibitor of ROS and JNK, induced MKN45 cell proliferation, prevented the cell apoptosis and released the cells from cycle arrest. Finally, we found that pretreatment with NAC prevented the JNK, p53, caspase-9 and -3 protein phosphorylation induced by the polysaccharide, however, pretreatment with SP600125 did not affect the generation of ROS, suggesting that ROS is upstream of JNK. Taken together, the novel polysaccharide induced cancer cell apoptosis and arrested cell cycle via ROS/JNK signaling pathway.

Introduction

Many studies in recent years focused on the activities of compounds extracted from marine organisms (1). Many such compounds have been investigated, and some have been developed into herbal medicine and made commercially available

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in Japan and even all over the world (2). In the past, we also focused on investigating a novel polysaccharide derived from algae extract for its biological activities (3). The efficiency of the novel algae-extracted polysaccharide was first reported in retinal pigment epithelial (RPE) cells, in which the polysaccharide would protect RPE cells against oxidative damage induced by high-glucose (3). This efficiency of anti-oxidative damage was thought a powerful potential to withdraw the normal cells from some unfavorable circumstance or resist the progress of abnormal cells (cancer cell). However, it is unclear whether this novel polysaccharide could affect cancer cells.

Cancer continue to be the second leading cause of death (4,5). Surgical operation, radiotherapy and chemotherapy are the most common therapies so far, however the side-effects produced by these procedures (especially by chemotherapy) often bring some new problems (6,7).

Due to the limited efficacy of traditional therapy for cancers, we sought to determine a new treatment strategy for cancers. In this study, we for the first time investigated the novel polysaccharide in inducing apoptosis and cell cycle arrest in human gastric carcinoma MKN45 cells.

Apoptosis is an important cause of cell proliferation inhibition (8). There is compelling evidence that excessive reactive oxygen species (ROS) production surmounts cellular antioxidant defenses, triggering apoptosis (9). Apart from apoptosis, cell cycle arrest is another cause of growth inhibition (10,11). Many anticancer agents dampen malignant growth by arresting the cell cycle at the G1, S or G2/M phases (12). It is well known that Jun N-terminal kinase (JNK), a member of mitogen-activated protein kinase (MAPK) family, is associated with cell proliferation inhibition (13,14). The activation of JNK is associated with ROS elevation (13). p-JNK activates downstream tumor suppressor p53, caspase-9 and -3, then leads to apoptosis and cell cycle arrest (15).

In this study, we determined that the novel polysaccharide derived from algae extract induced human cancer cell (MKN45 cell) apoptosis by ROS/JNK signaling pathway and arrested the cell cycle. Our study indicated a novel therapeutic strategy of cancer.
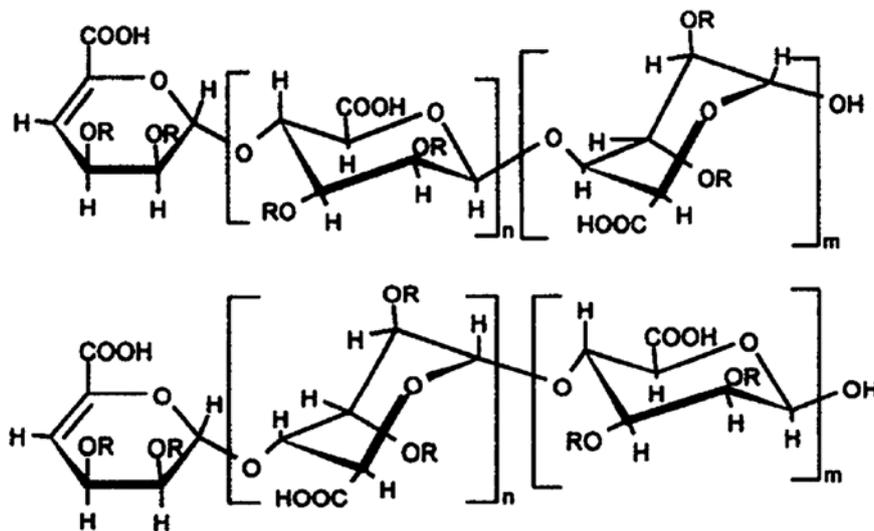
Figure 1. The molecular structure of the novel polysaccharide.

Materials and methods

Preparation of the novel polysaccharide. The novel polysaccharide (molecular structure was shown as Fig. 1) derived from algae extract was achieved by Toyo Medicine Institute (Ashikita, Kumamoto, Japan). Detailed methods for the preparation of the compound were published elsewhere (16). Briefly, the polysaccharide compound was prepared from a type of phaeophyceae. After being extracted with chloroform, ethyl acetate and n-butyl alcohol, the compound was isolated by column chromatography on silica gel and Sephadex LH-20 columns, was purified on a macroporous absorption resin column, and then sulfonated by sulfuric acid. The structure of the polysaccharide compound is a typical sugar chain structure made of polymeride of disaccharide, which is rich in phenol and sulfate. The average molecular weight of the compound was 11,680. The molecular weight was used for calculation of molar concentration (μM).

Cell culture. Human gastric cancer MKN45 cells expressing the Fucci probes (MKN45-Fucci cells) were purchased from RIKEN BioResource Center (Tokyo, Japan). MKN45 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Tokyo, Japan) supplemented with 10% FBS. 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma), and were cultured in an incubator (Sanyo) with 5% CO_2 at 37˚C.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MKN45 cells were exposed to 100 μg/ml polysaccharide with or without pre-treatment with NAC (5 mM) or SP600125 (5 μM). The viability of normal cells and polysaccharide-treated cells was determined by a colorimetric MTT assay according to the method described previously (17). Absorbance at 550 nm was determined by an MTP-800 microplate reader (Corona Electric, Tokyo, Japan). Absorbance at 690 nm was also measured to compensate for any interfering effects of cell debris and the microtiter plate. Percentage of viable cell number was calculated as: Optical density (OD) of treated sample/OD of untreated control x 100.

Nuclear staining. MKN45 cells were plated in 6-well plates at the density of 1×10^5 cells/well. After 24-h incubation, the cells were treated with the polysaccharide at a concentration of 100 μg/ml and further incubated for another 48 h. Then the cells were washed with PBS, fixed in 4% paraformaldehyde (Sigma) for 30 min and then stained with 20 mg/ml Hoechst 33342 for 15 min at room temperature in the dark. Cells were then assessed by fluorescence microscopy for morphological changes.

Fluorescent ubiquitination-based cell cycle indicator (Fucci) system. MKN45 cells were exposed to 100 μg/ml polysaccharide for 48 h. MKN45 cells expressing two Fucci probes: cells emit red fluorescence (SCF[Skp2]) in G1/G0 phase and green fluorescence (APC[Cdh1]) in S/G2/M phases (18). As described (19), fluorescence and phase contrast images were observed using an FV10i-DOC confocal laser scanning microscope (Olympus, Tokyo, Japan) with a UPLSAPO 60x Wobjective lens.

Detection of intracellular ROS. Intracellular accumulation of ROS was estimated using the fluorescent dye H_2-DCFDA (Life Technologies, Tokyo, Japan), which is converted to a membrane impermeable and highly fluorescent compound, dichlorofluorescin diacetate (DCF), in the cell in the presence of ROS. The MKN45 cells were seeded in a 6-well plate at the density of 1×10^5 cells/well. Following treatment with the polysaccharide or SP600125 (5 μM), MKN45 cells were further incubated for 48 h. The cells were rinsed with a serum-free medium and were incubated in 5 μM H_2-DCFDA for 60 min at 37˚C. The cells were then examined under a fluorescence microscope (C1-T-SM; Nikon, Tokyo, Japan), collected and subjected to a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan) to detect the fluorescence of DCF inside cells (excitation, 488 nm; emission, 521 nm) as described (20).

Western blot analysis. Electrophoresis was performed using a vertical slab gel with 12% polyacrylamide content according to the method described previously (21). The transfer of proteins from the SDS polyacrylamide gel to a membrane was performed
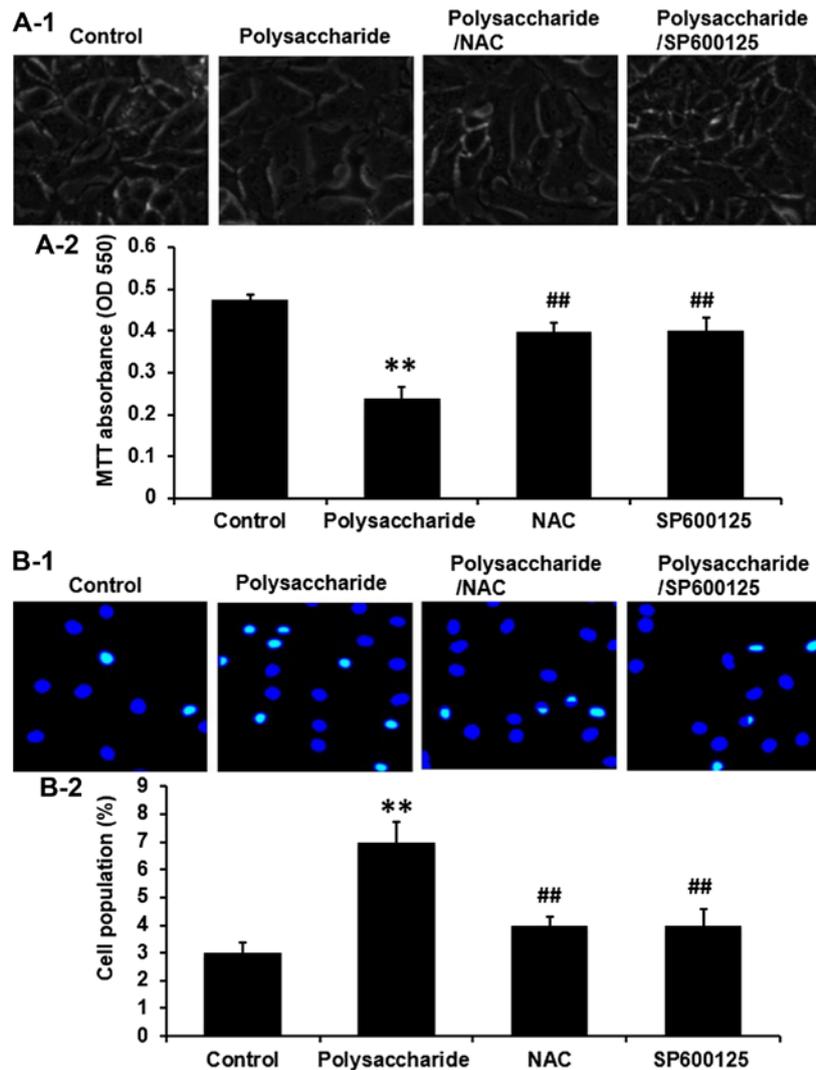
Figure 2. The novel polysaccharide suppressed cell proliferation and induced cell apoptosis in MKN45 cells, NAC and SP600125 inhibited the effects of the novel polysaccharide on cell proliferation and apoptosis. Human gastric cancer MKN45 cells were pretreated with NAC (5 mM) (an inhibitor for ROS) or SP600125 (5 μM) (an inhibitor for JNK) for 1 h prior to the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. (A) The cell viability was determined by MTT assay. (B) The cell apoptosis was determined by nuclear staining. The novel polysaccharide significantly suppressed cell proliferation and induced cell apoptosis. Pretreatment with NAC or SP600125 prevented the polysaccharide-induced cell proliferation and apoptosis in MKN45 cells, significantly. (A-2) The quantification of (A-1). (B-2) The quantification of (B-1). Data are expressed as the mean ± standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (**P<0.01, polysaccharide-treated group vs control group; ##P<0.01, NAC or SP600125 + polysaccharide-treated group vs polysaccharide-treated group).

electrophoretically according to the method described previously (22) with certain modifications using a Semi Dry Electroblotter (Sartorius AG, Goettingen, Germany) for 90 min with an electric current of 15 V. The membrane was treated with Block Ace™ (4%) for 30 min at 22°C. The first reaction was performed using rabbit immunoglobulin (IG) G antibodies against JNK, p-JNK, p53, caspase-9 and -3 (Sigma) in PBS containing 0.03% Tween-20 for 1 h at 22°C. Following washing in the same buffer, the second reaction was performed using horseradish peroxidase (HRP)-conjugated anti-rabbit goat IgG (20 ng/ml) for 30 min at 22°C. After washing, the enhanced chemiluminescence (ECL) reaction was performed on the membrane using the ECL Plus Western Blotting Detection System™ (GE Healthcare Life Sciences, UK).

Statistical analysis. Analyses were performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Student's t-test was used and the data are expressed as the mean ± SD. Each experiment was repeated at least 3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

The novel polysaccharide suppresses cell proliferation and induces apoptosis in MKN45 cells. Human gastric cancer MKN45 cells were exposed to 100 μg/ml polysaccharide and incubated for 48 h. The cell viability was determined by MTT assay and the cell apoptosis was determined by nuclear staining. The novel polysaccharide significantly suppressed cell proliferation (Fig. 2A), and induced cell apoptosis (Fig. 2B) in MKN45 cells (P<0.01).

The novel polysaccharide arrests MKN45 cell cycle at G2/M phase. MKN45 cells were exposed to 100 μg/ml polysaccha-
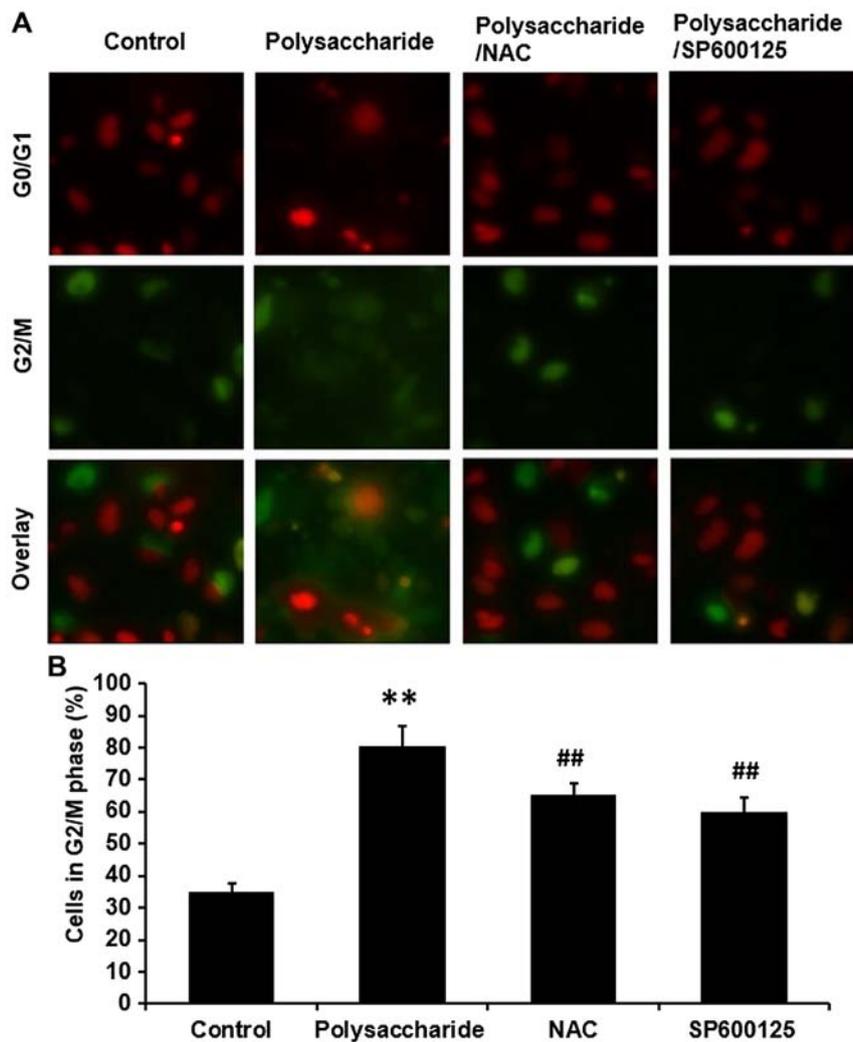
Figure 3. The novel polysaccharide induced cell cycle arrest in MKN45 cells, NAC and SP600125 prevented the polysaccharide-induced cell cycle arrest. MKN45 cells were pretreated with NAC (5 mM) (an inhibitor for ROS) or SP600125 (5 μM) (an inhibitor for JNK) for 1 h prior to the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. The cell cycles were analyzed by the Fucci system. The novel polysaccharide significantly arrested the cell cycle at G2/M phase. Pretreatment with NAC or SP600125 prevented the polysaccharide-induced cell cycle arrest in MKN45 cells significantly. (B) The quantification of (A). Data are expressed as the mean ± standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (**P<0.01, polysaccharide-treated group vs control group; ##P<0.01, NAC or SP600125 + polysaccharide-treated group vs polysaccharide-treated group).

ride and incubated for 48 h. The cell cycles were analyzed by Fucci system. The novel polysaccharide significantly arrested the MKN45 cell cycle at G2/M phase (Fig. 3) (P<0.01).

The novel polysaccharide induces ROS generation in MKN45 cells. After MKN45 cells were exposed to 100 μg/ml polysaccharide for 48 h, intracellular accumulation of ROS was estimated using the fluorescent dye H$_2$-DCFDA and flow cytometry was estimated using DCFH-DA. The novel polysaccharide significantly induced ROS generation in MKN45 cells (Fig. 4) (P<0.01).

The novel polysaccharide induces the phosphorylation of p-JNK, p53, caspase-9 and -3 in MKN45 cells. MKN45 cells were exposed to 100 μg/ml polysaccharide and incubated for 48 h. The expression levels of p-JNK, p53, caspase-9 and -3 were determined by western blot analysis. The novel polysaccharide significantly induced the phosphorylation of p-JNK, p53, caspase-9 and -3 in MKN45 cells (Fig. 5) (P<0.01).

NAC and SP600125 inhibit the effects of the novel polysaccharide on cell proliferation and apoptosis in MKN45 cells. MKN45 cells were pretreated with NAC (5 mM) (the inhibitor for ROS) or SP600125 (5 μM) (the inhibitor for JNK) for 1 h prior to the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. The cell viability and cell apoptosis were determined by MTT assay and nuclear staining, respectively. The novel polysaccharide significantly suppressed MKN45 cell proliferation and induced cell apoptosis. Pretreatment with NAC or SP600125 significantly increased the cell proliferation (Fig. 2A) and prevented cell apoptosis (Fig. 2B) in MKN45 cells (P<0.01).

NAC and SP600125 prevent the novel polysaccharide-induced MKN45 cell cycle arrest. MKN45 cells were pretreated with NAC (5 mM) (the inhibitor for ROS) or SP600125 (5 μM) (the inhibitor for JNK) for 1 h prior to the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. The cell cycles were determined by the Fucci system.
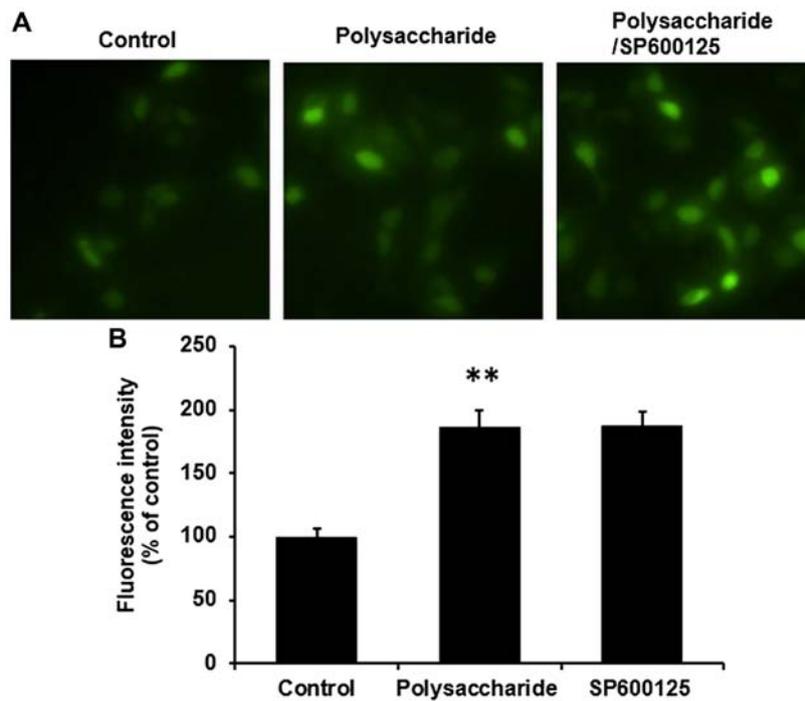
Figure 4. The novel polysaccharide induced the generation of ROS, SP600125 did not affect the novel polysaccharide-induced ROS generation in MKN45 cells. MKN45 cells were pretreated with SP600125 (5 μM) (an inhibitor for JNK) for 1 h before the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. Intracellular accumulation of ROS was estimated using the fluorescent dye H$_2$-DCFDA and flow cytometry using DCFH-DA. The novel polysaccharide significantly induced the ROS generation while SP600125 did not affect the novel polysaccharide-induce ROS generation in MKN45 cells. (B) The quantification of (A). Data are expressed as the mean ± standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (**P<0.01, polysaccharide-treated group vs control group; ##P<0.01, SP600125 + polysaccharide-treated group vs polysaccharide-treated group).
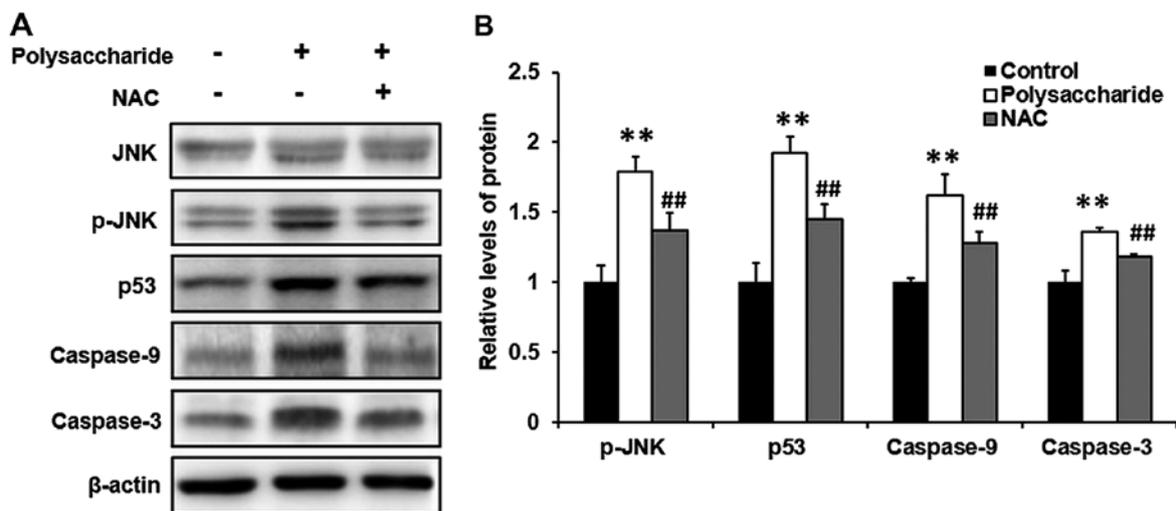
Figure 5. The novel polysaccharide induced phosphorylation of JNK, p53, caspase-9 and -3, NAC prevented the novel polysaccharide-induced protein phosphorylation in MKN45 cells. MKN45 cells were pretreated with NAC (5 mM) (an inhibitor for ROS) for 1 h before the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. The phosphorylation of JNK, p53, caspase-9 and -3 were determined by western blot analysis. The novel polysaccharide significantly induced phosphorylation of p-JNK, p53, caspase-9 and -3. The novel polysaccharide-induced protein phosphorylation was significantly prevented by the pretreatment with NAC. (B) The quantification of (A). Data are expressed as the mean ± standard deviation (n=3). P<0.05 was considered to indicate a statistically significant difference (**P<0.01, polysaccharide-treated group vs control group; ##P<0.01, NAC + polysaccharide-treated group vs polysaccharide-treated group).

The novel polysaccharide significantly arrested the MKN45 cell cycle at G2/M phase. Pretreatment with NAC or SP600125 prevented the polysaccharide-induced cell cycle arrest significantly (Fig. 3) (P<0.01).

NAC prevents the novel polysaccharide-induced phosphorylation of p-JNK, p53, caspase-9 and -3 in MKN45 cells. To further determine the potential signaling pathway involved in the process, MKN45 cells were pretreated with NAC (5 mM)

Polysaccharide

↓

NAC ——| ROS

↓

SP600125 —|JNK

↓

p53, caspase-9 and caspase-3

↓

Apoptosis
Cell cycle arrest
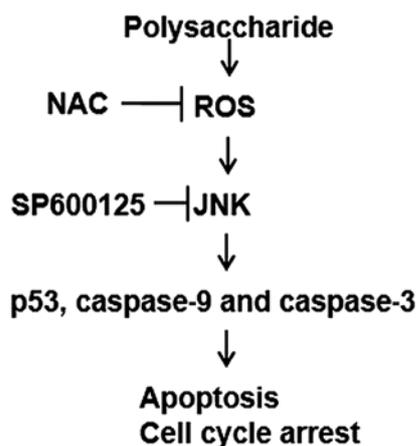
Figure 6. Mechanism of the inhibitory process. The novel polysaccharide derived from algae extract induces the generation of ROS, then enhances the phosphorylation of JNK, and activates the downstream cascades p53, caspase-9 and -3, finally suppresses cancer cell proliferation, induces cell apoptosis and arrests the cell cycles.

(an inhibitor for ROS) for 1 h before the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. The phosphorylation of JNK and p-JNK were determined by western blot analysis. The novel polysaccharide significantly induced the p-JNK phosphorylation, and the novel polysaccharide-induced phosphorylation of p-JNK was significantly prevented by the pretreatment with NAC (Fig. 5) (P<0.01).

SP600125 does not affect the novel polysaccharide-induce ROS generation in MKN45 cells. MKN45 cells were pretreated with SP600125 (5 μM) (an inhibitor for JNK) for 1 h prior to the polysaccharide (100 μg/ml) treatment. Then the cells were further incubated for 48 h. Intracellular accumulation of ROS was estimated using the fluorescent dye H_2-DCFDA and flow cytometry using DCFH-DA. The novel polysaccharide significantly induced ROS generation in MKN45 cells (P<0.01), however, pretreatment with SP600125 did not affect the polysaccharide-induce ROS generation in MKN45 cells (Fig. 4). These data suggested that ROS is upstream of JNK.

Discussion

In recent years, as elevating of therapeutic quality of cancer chemotherapy was recognized, researchers focused on the application of marine organisms in molecular treatment for diseases (1,23). Many such marine organism extracted-compounds have been investigated, and some have been developed into herbal medicine and made commercially available in Japan and even all over the world (2). In the past, we focused on investigating a novel polysaccharide derived from algae extract for its biological activities (3) (molecular structure is shown in Fig. 1). In this study, we investigated the activity of the novel polysaccharide on cancer cell apoptosis and cell cycle arrest. The pre-experiments were performed in a dose-dependent manner (1, 10, 100 and 1,000 μg/ml) and a time-dependent manner (12, 24 and 48 h). We found significant difference when the concentration reached 100 μg/ml and the

treatment-time reached 48 h, so we decided to use 100 μg/ml of polysaccharide and 48 h of treatment for our experiments.

Cancer is the principal enemy for human life and health (4), a leading cause of mortality worldwide (24). Chemotherapy and radiotherapy are the most common therapy in the patients when surgical operation is not possible, however the side-effects are hard to bear and always bring suffering, physically and psychologically (6,7). In consideration of the unwanted side-effects (25), it is crucial to develop new treatment strategies for cancers.

Proliferation and apoptosis are very important physiological processes for cancer cells (26). Apoptosis is an important cause of cell proliferation inhibition (8). Apoptosis (programmed cell death), is an essential mechanism through which many types of chemotherapeutic agents inhibit tumor growth (27). This study demonstrated that the novel polysaccharide suppressed cell proliferation (Fig. 2A) and induced apoptosis (Fig. 2B) in MKN45 cells. Apart from apoptosis, cell cycle arrest is another cause of growth inhibition (10,11). Many anticancer agents dampen malignant growth by arresting the cell cycle at the G1, S or G2/M phases (12). Deregulation of cell cycle has been linked with cancer initiation and progression (28). Arresting the cell cycle is an effective method to regulate cell cycle progression, and contribute to malignant cell proliferation (11). In this study, by using the Fucci system, we confirmed these theories and showed that the novel polysaccharide arrested MKN45 cells at G2/M phase (Fig. 3). These results suggested the potential of the novel polysaccharide as an anti-cancer agent.

ROS, which is the byproduct of normal cellular oxidative processes, has been suggested to regulate the process involved in the initiation of apoptotic signaling (29) and has been implicated in several oncogenic pathways. There is compelling evidence that ROS production surmounts cellular antioxidant defenses, triggering apoptosis (9), and cancer cells are more sensitive to rapid increases in ROS levels than normal cells. ROS-mediated cytotoxicity has also been identified as an important mechanism in some anticancer agents (30). Accumulating evidence indicates that many anticancer agents destroy tumor cells by raising the level of ROS above a toxic threshold (31). Oncogenic transformation elevates basal ROS levels significantly so that any further acute increases can trigger reactivation of the apoptotic program in cancer cells (32). To investigate whether the novel polysaccharide-induced MKN45 cell apoptosis and cell cycle arrest is promoted through an increase in ROS production, we measured ROS levels. Our results showed that the novel polysaccharide induced ROS generation in MKN45 cells significantly (Fig. 4).

It is well known that JNK, a member of mitogen-activated protein kinase (MAPK) family, is associated with cell proliferation inhibition (13,14). Various apoptotic stimuli can rapidly activate MAPKs, which include p-JNK (33). The activation of JNK is associated with ROS elevation (13). p-JNK activates a downstream tumor suppressor (15). p53, caspase-9 and -3, then leads to apoptosis and cell cycle arrest. p53, a tumor suppressor, is well known for coordinating apoptosis to preserve genomic stability and prevent tumor formation (34). p53 can induce the expression of several factors involved in apoptosis, such as caspase-9 and -3 (35). The activation of

caspase-9 and -3 damage the cell structure and cause functional disorder by proteolysis (36). In addition to cell cycle arrest, p53 also triggers cell cycle arrest to provide time for self-mediated apoptosis through transcriptional activation of cyclin-dependent kinase inhibitor (37). p-JNK activated p53, caspase-9 and -3, and finally leads to apoptosis and cell cycle arrest (15). Our study showed that the novel polysaccharide induced the phosphorylation of p-JNK, p53, caspase-9 and -3 (Fig. 5), suggesting the involvement of p-JNK, p53, caspase-9 and -3 in this process.

To better understand the mechanism, we used NAC, an inhibitor for ROS, and SP600125, an inhibitor for JNK. Pretreatment with NAC or SP600125 significantly increased the cell proliferation inhibited by the polysaccharide (Fig. 2A), prevented the polysaccharide-induced cell apoptosis (Fig. 2B) and cell cycle arrest significantly (Fig. 3) in MKN45 cells. Furthermore, we found that pretreatment with NAC prevented the protein phosphorylation induced by the novel polysaccharide (Fig. 5), however, pretreatment with SP600125 did not affect the generation of ROS (Fig. 4), suggesting that the novel polysaccharide induces apoptosis and cell cycle arrest in MKN45 cells via ROS/JNK signaling pathway, and ROS is upstream of JNK.

According to the mechanism described in Fig. 6, the novel polysaccharide derived from algae extract induces the generation of ROS, then enhances the phosphorylation of JNK, and activates the downstream cascades p53, caspase-9 and -3, finally suppresses cancer cell proliferation, induces cell apoptosis and arrests the cell cycles. The potential of the novel polysaccharide in cancer cells is expected to provide insight into the development of novel clinical treatments for cancers.

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