Protein-bound Polysaccharide K Reduced the Invasive Ability of Colon Cancer Cell Lines

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Abstract. *Background/Aim: A protein-bound polysaccharide, polysaccharide K (PSK), is a non-specific immunological agent used in the treatment of colon cancer, however few studies have investigated the genetic changes in cancer cells treated with PSK. Therefore, we investigated the effect of PSK on cancer cell invasion, which is an indicator for the malignancy of colon cancer cell lines, and performed additional genetic analyses. Materials and Methods: We performed Matrigel invasion assay to examine whether the invasive ability of colon cancer cell lines HT29, HCT116, and LoVo would be impacted upon stimulation with PSK. We used reverse transcription-polymerase chain reaction (RT-PCR) to evaluate for changes in the expression of matrix metalloproteinases (MMP)-2 and -9 upon stimulation of colon cancer cell lines with PSK. Results: The mean number of invasive cells in untreated HCT116, HT29, and LoVo cells was 146, 81, and 65, respectively, while that in PSK-treated cell lines was reduced to 24, 7, and 4, respectively. mRNA levels of MMP2 and MMP9 in PSK-stimulated cell lines were significantly lower than those in unstimulated cell lines. Conclusion: PSK reduced the expression of MMP2 and MMP9 mRNAs and cell invasion of this panel of colon cancer cell lines.*

Polysaccharide K (PSK), a protein-bound polysaccharide obtained from the cultured mycelium of *Coriolus versicolor*, is a non-specific immunotherapeutic agent. PSK is a polysaccharide-protein complex containing about 38% protein (1). The carbohydrate portion consists of β-glucan with a molecular weight of approximately 940,000, and about 75% of this carbohydrate portion is made up of glucose, the rest being made up of other monosaccharide units such as mannose, xylose, and galactose.

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In clinical practice, PSK has been mainly used for the treatment of gastrointestinal cancer, including gastric and colon cancer. The survival rate of PSK-treated patients was reported to be significantly better than the one of patients not treated with it (2-6).

The main mechanism of action of PSK is as follows: Direct effect through apoptosis induction, and enhancement of major histocompatibility complex (MHC) class-I expression; stimulation of immune cells and cells involved in the biological defense against cancer [natural killer (NK) cells, T-cells, and lymphokine-activated killer (LAK) cells], and regulation of cytokine production; inhibition of immunosuppressive agents in tumor-bearing hosts through inhibition of Transforming growth factor (TGF) production and reduction of oxidative stress (7-11). As a biochemical response modifier, PSK has various immune-boosting actions. Previous studies mainly examined the immunological responses in the healthy tissues in mice and humans; few reports provided detailed results of genetic examination on the direct effects of PSK on cancer cells. Therefore, we evaluated the effects of PSK on the invasive ability of colon cancer cells, and investigated the genetic changes caused by PSK in these cells.

Materials and Methods

Cell culture and PSK stimulation. Human colon cancer cell lines, LoVo, HT29 and HCT116 (obtained from the European Collection of Cell Cultures, Salisbury, UK), were cultured at 37˚C in 5% CO_2 in RPMI 1640 medium containing 10% fetal bovine serum (12, 13). Cells were seeded (5×10^5) into 6 cm dishes in triplicate with 100, 300 and 500 μg/ml of PSK(KRESTIN®; Kureha Chemical Industry, Co.Ltd, Tokyo, Japan). for two days.

Cell viability. Cell necrosis and apoptosis were detected by cytometry using the Annexin-V-FLUOS staining Kit (Roche, Mannheim, Germany). Briefly, PSK treated or non-treated cells were incubated with annexin-V-Alexa568 for 15 min. After cells were washed thrice in PBS, we detected red cells under a fluorescence microscope.

Tumor cell invasion assay. Transwells (Biocoat Matrigel 6-well invasion chamber) with filters coated with extracellular matrix (Matrigel) on the upper surface were purchased from BD

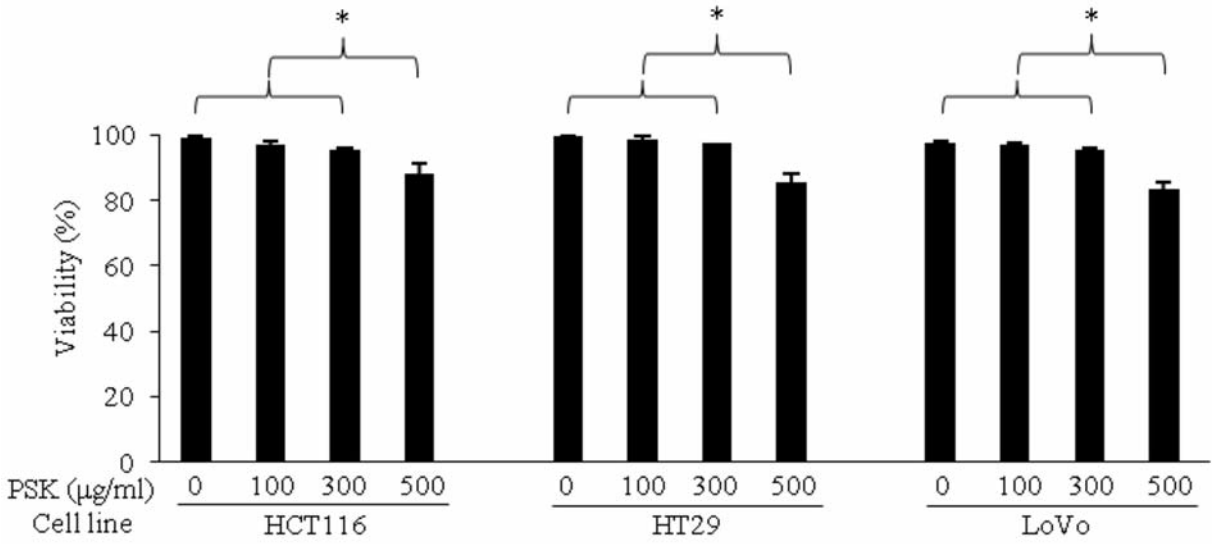

Figure 1. *Evaluation of viable cell count in polysaccharide K (PSK)-stimulated colon cancer cells. Cell viability was detected by flow cytometry using the Annexin V Detection kit. The viability of colon cancer cells was no significant different between the colon cancer cell lines not stimulated with PSK and those stimulated with 100 and 300 μg/ml of PSK. Results are presented as the mean±SD. *p<0.05.*

Biosciences (San Jose, CA, USA). Complete medium was added to the bottom chamber to induce the invasion of the cells through the Matrigel. Serum-free medium was added to the cells (2×10^5) seeded to the top chamber. The Matrigel invasion chamber was incubated for 48 hour incubation at 37˚C with 5% CO2. Non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. The invasive cells were determined by counting the stained cells. Cell numbers were counted with a hemocytometer (12).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. The total RNA was extracted from the colon cancer cells using ISOGEN (Wako, Japan). Single-strand cDNA was prepared from 3 μg of total RNA using Prime Script RT reagent kit (Takara Bio Inc. Ohtsu, Japan). The primers for PCR amplification of the matrix metalloproteinase (MMP2) gene-coding regions were as follows: 5' primer: *MMP2*-AX, ACCCATTTAC ACCTACACCAAG, 3' primer: *MMP2*-BX, GTATACCGCATCAATCTTTTCCG. The primers for PCR to amplify *MMP9* gene-coding regions were as follows: 5' primer: *MMP9*-AX, TGGGCTACGTGACCTATGACAT; 3' primer: *MMP9*-BX, GCCCAGCCCACCTCCACTCCTC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal PCR control with 5'-GGGGAGCCAAAAGGGTCA TCATCT-3' as the sense primer and 5'-GACGCCTGC TTCACCACCTTCTTG-3' as the antisense primer. A total of 30 cycles of denaturation (94˚C, 1 min), annealing (50˚C, 1.5 min) and extension (72˚C, 2 min) were carried out in a thermal cycler (PTC-100, Programmable Thermal Controller; MJ Research Inc., Waltham, MA, USA). The PCR products (10 μl) which demonstrated relevant bands in RT-PCR analysis were sequenced by electrophoresis in 1.2% agarose gel. Ethidium bromide staining of the gels identified a band of MMP2 and MMP9 mRNA. To ensure reproducibility, all PCR amplifications were performed in triplicate. Densitometric analysis of the photographic negatives was used for band quantification (12, 14).

Statistical considerations. Characteristics of the two treatment arms were compared using Student *t*-test or chi-square test. Values of *p* less than 0.05 were considered as statistically significant.

Results

Evaluation of viable cell count in colon cancer cell lines after PSK exposure. No significant difference was observed in cell viability between the colon cancer cell lines not stimulated with PSK and those stimulated with 100 and 300 μg/ml of PSK. Only after stimulation with 500 μg/ml PSK, was cell viability reduced by approximately 15% (Figure 1).

Investigation of the invasive ability of colon cancer cell lines. Figures 2 and 3 show the results regarding invasion by colon cancer cell lines. The mean number of invading colon cancer cells was 146 for HCT116, 81 for HT29, and 65 for LoVo.

Investigation of the effects of PSK exposure on the invasive ability of colon cancer cell lines. Figures 2 and 3 show the results regarding invasion by colon cancer cell lines cultured with PSK. The mean number of invading colon cancer cells was 24 for HCT116, 7 for HT29, and 4 for LoVo. The number invading PSK-treated colon cancer cells significantly decreased ($p<0.05$).

Investigation of *MMP2* and *MMP9* mRNA expression in colon cancer cell lines. Figure 4 shows the results concerning *MMP2* and *MMP9* mRNA expression in PSK-stimulated colon cancer cell lines. All three colon cancer cell lines

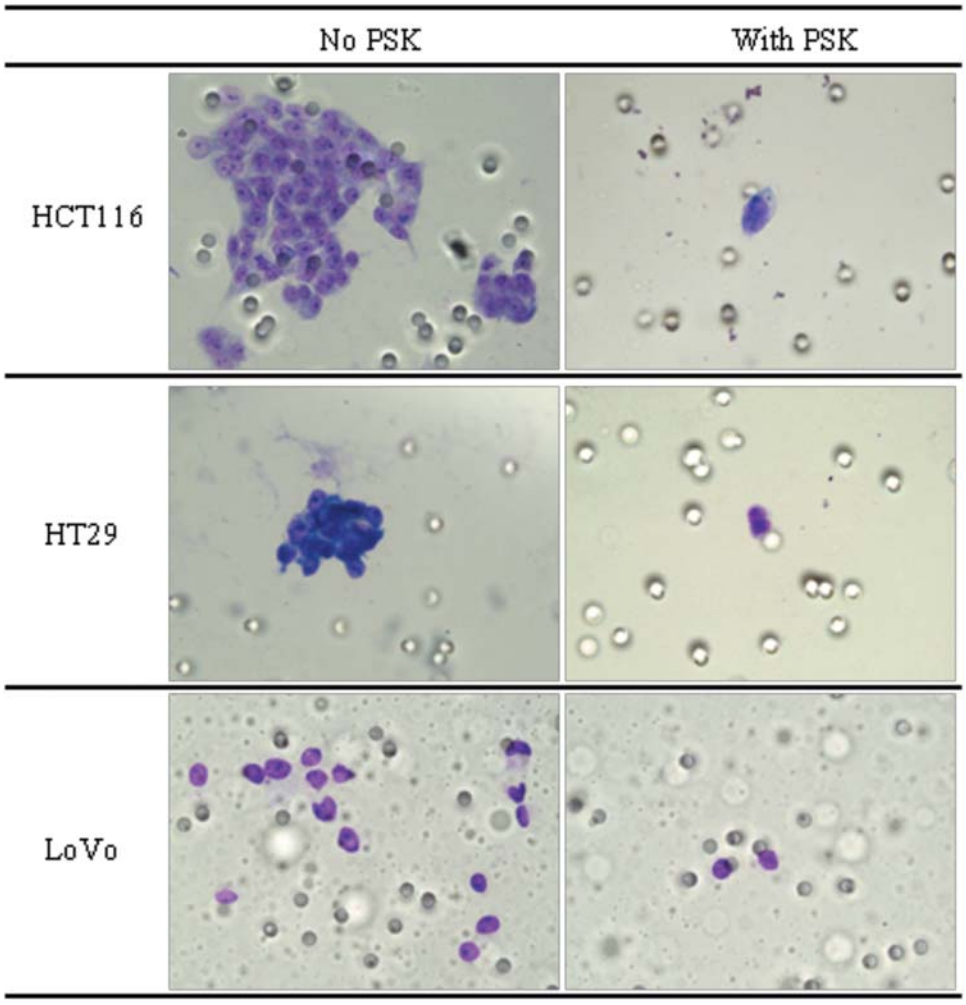

Figure 2. *Colon cancer cell invasion. Cells were treated with PSK (300 μg/ml), and the invasive cells in the lower chamber were stained using Matrigel assay and identified by Microscopy.*

showed *MMP2* and *MMP9* mRNA expression without PSK stimulation, while expression was significantly reduced on PSK stimulation, although the amount of expression was different between the cell lines (quantitative densitometric analysis; 20-80%, $p<0.05$).

Discussion

Colon cancer ranks high in terms of morbidity and death rate in Japan and Western countries (15, 16). A large number of reports have been published on the growth, invasion, and metastasis of colon cancer cells (12, 13, 17-23); however, colorectal cancer is often difficult to completely control in cases with distant metastasis. We examined the effects of PSK, which is obtained from the cultured mycelium of *Coriolus versicolor*. It is known as a biochemical response modifier and used in non-specific immunological therapy (1, 7-11). Several reports are available on the efficacy of PSK; PSK administration improved the survival rate of patients with malignant gastrointestinal tumors such as gastric and colon cancer (2-6).

We decided to study the mechanism of action of PSK, which is mostly unclear. Invasion and metastasis of cancer cells are known to be important for prognosis in patients with cancer. According to previous reports, as vascular invasion becomes more advanced, the recurrence rate of colon cancer increases and prognosis becomes worse (18). Therefore, invasiveness of cancer cells is considered a significant factor for malignancy. We examined the effects of PSK on the invasive ability of cancer cells, and found it reduced invasion. Such effects may improve the survival rate of patients with cancer. We then continued by investigating

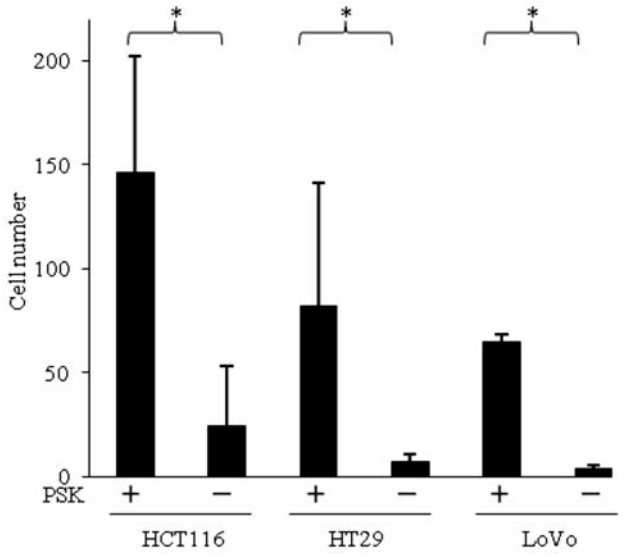

Figure 3. *PSK stimulation reduced colon cancer cell invasion. The number of invading cells was quantitated over 48 h using Matrigel assay. The number of colon cancer cells significantly decreased when stimulated by PSK. Results are presented as the mean±SD.*

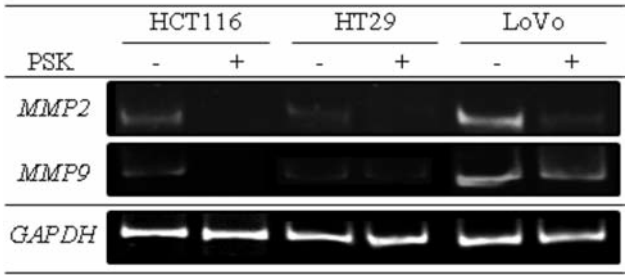

Figure 4. *Matrix metalloproteinase (MMP)-2 and -9 mRNA expression in polysaccharide K (PSK)-stimulated colon cancer cells. The expression of MMP-2 and -9 mRNA expression in colon cancer cells treated with PSK was determined by RT-PCR. MMP2 and MMP9 mRNA expression was significantly reduced (quantitative densitometric analysis; 20-80%, p<0.05) in PSK-stimulated colon cancer cell lines compared to to non-stimulated colon cell lines.*

what genes were affected by PSK by using the MMP enzyme family as a target due to their important role during the degradation of the extracellular matrix when cancer cells move from the primary lesion to interstitium and enter the vasculature (24-26). Among the MMP family, MMP2 and MMP9 are particularly known for their involvement in the invasion of colon cancer cells (27-30). We found that the expression of *MMP2* and *MMP9* genes was reduced by PSK.

This experiment has, to our knowledge for the first time, demonstrated that exposure of colon cancer cells *in vitro* to PSK inhibited their invasion most likely through its action on MMP2 and MMP9 (Figure 5).

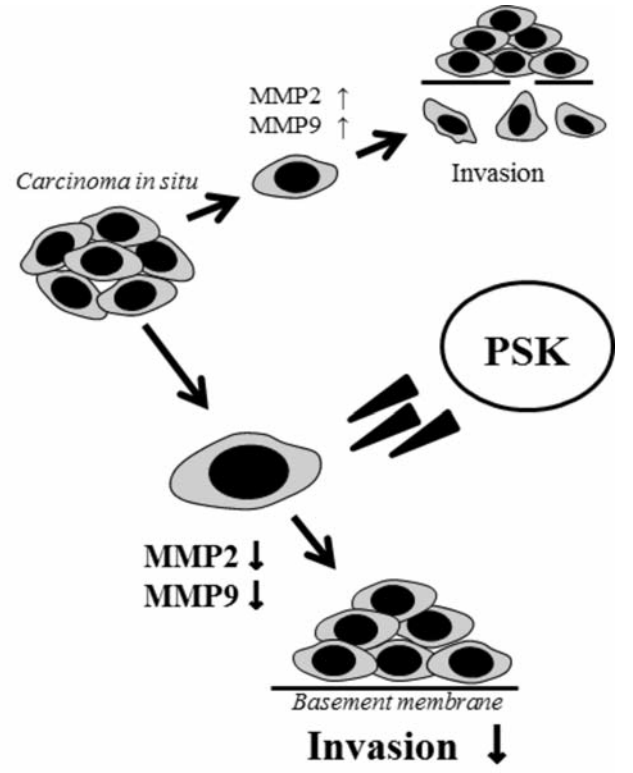

Figure 5. *New actions of polysaccharide K (PSK). Treatment with PSK reduced the invasive ability of colon cancer cells, possibly through reduction of MMP2 and MMP9 mRNA levels.*

Conflicts of Interest

The Authors do not have any financial interest in any company making any products discussed in the article. The Authors report no conflicts of interest.

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