Polyphosphate Derived from *Lactobacillus brevis* Inhibits Colon Cancer Progression Through Induction of Cell Apoptosis.

(*Lactobacillus brevis*由来ポリリン酸によるアポトーシス誘導を介した大腸癌抑制作用に関する研究)
Polyphosphate Derived from *Lactobacillus brevis* Inhibits Colon Cancer Progression Through Induction of Cell Apoptosis

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**Abstract.** Although probiotics are known to have antitumor activity, few bacteria-derived antitumor molecules have been identified. The present study explored an antitumor molecule derived from *Lactobacillus brevis* SBL8803 (L. brevis 8803) and the mechanisms that underlie its effects. Cell viability and apoptosis were assessed by a sulforhodamine B assay and terminal deoxynucleotidyl transferase dUTP staining, respectively. Phosphorylated extracellular signal-regulated kinase (ERK) and cleaved poly ADP-ribose polymerase (PARP) expression were detected by western blotting. The conditioned medium of *L. brevis* 8803 inhibited SW620 cells viability and the effect was reduced by the degradation of polyphosphate (poly P) in the conditioned medium. A xenograft model showed that poly P inhibited the growth of SW620 cells. Poly P induced the apoptosis of SW620 cells through activation of the ERK pathway. In contrast, in primary cultured cells derived from normal mouse, poly P did not affect cell viability. Probiotic-derived poly P is expected to be an antitumor drug with fewer adverse effects than conventional drugs.

Colon cancer is one of the most frequent causes of cancer-related death in Western and Eastern countries. Most cases of colon cancer are detected at an advanced phase, at which time they are treated with surgery as well as antitumor agents. Recently developed drugs, including molecular-target drugs, have helped to improve the survival rate of patients with colon cancer (1-3). However, such antitumor treatments are frequently stopped due to their adverse effects, which include dermatological problems, mucosal disorders and interstitial pneumonia, particularly in elderly patients. The development of new drugs, that have fewer adverse effects, is needed.

Probiotics, which are defined as microorganisms that confer beneficial effects on the health of the host, are thought to have antitumor effects (4-7). However, the mechanisms underlying the antitumor effects are, for the most part, unclear. Some bioactive molecules which are derived from probiotics, including parasporin-2Aa1 (from *Bacillus thuringiensis* strain A1547) (8), and ε-poly-L-lysine (ε-PL) (from marine *Bacillus subtilis* SDNS) (9), have been identified as antitumor molecules. Because these types of probiotics have been used as functional foods, they are thought to have low toxicity, suggesting the potential use of these probiotic-derived molecules as antitumor drugs with fewer adverse effects than the current generation of antitumor agents.

*Lactobacillus brevis* SBL8803 (L. brevis 8803) is a type of plant bacterium that has been identified in fermented malt. We previously showed that the bacterium maintains intestinal homeostasis under conditions of oxidative stress (10), which damages epithelial DNA, suggesting that the bacterium has an inhibitory effect on tumor cells (11). The present study investigated the antitumor effect of conditioned medium of *L. brevis* 8803 and identified an antitumor molecule, polyphosphate (poly P), which is derived from the bacterium and the mechanisms that underlie its antitumor effects.

**Materials and Methods**

**Cell culture.** Human colon carcinoma SW620 cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin at 37°C in 5% CO2. Before subculturing, the cells were digested in 0.25% trypsin containing 0.02% EDTA.
Primary cultures were made from the small intestinal epithelial cells of mice using a previously reported method (12).

**Bacterial strains and the preparation of conditioned medium.** The L. brevis 8803 that was used in the present study was supplied by Sapporo Breweries Ltd. (Tokyo, Japan). L. brevis 8803 were cultured overnight in de Man–Rogosa–Sharpe (MRS) broth (Difco Laboratories, Detroit, MN, USA) at 37°C. Bacteria were pelleted for 5 min at 1,000 × g. The bacteria were then resuspended in 10 ml of DMEM and cultured overnight at 37°C. To separate the conditioned medium and the bacterial pellets, the media were centrifuged for 5 min at 1,000 × g. The conditioned medium sample was sterilized using a 0.22 μm filter and stored at −80°C until use.

**Synthesis and degradation of poly P by polyphosphate kinase (PPK).** To synthesize poly P, 1 ml of reaction mixture containing 50 mM Tris-HCl (pH 7.4), 40 mM ammonium sulfate, 4 mM MgCl₂, 40 mM creatine phosphate, 20 ng/ml creatine kinase, 1 mM ATP (pH 7.2), and 1 U of PPK from Propionibacterium shermanii was incubated for 3 h at 37°C. To separate the enzymatically synthesized poly P from the reaction mixture, 100 ml of 50 mM CaCl₂ was added to the mixture and the poly P was centrifuged at 5,000 × g for 10 min. The precipitates were dissolved by adding 50 mM ethylene-diaminetetraacetic acid (EDTA) solution. The low molecular weight components, such as Ca²⁺ ions and EDTA, were then removed from the solution by dialysis using a tube equipped with a 3-kDa MWCO membrane.

To degrade poly P, L. brevis 8803 conditioned medium was incubated overnight at 37°C in the final reaction mixture [50 mmol/l (NH₄)₂SO₄, 4 mmol/l MgCl₂, 10 μmol/l ADP, 40 mmol/l HEPES-KOH (pH 7.5), and 0.1 U/μl PPK].

**Salmonella B (SRB) cell viability assay.** We used an SRB assay (13) to assess cell viability. SW620 cells were seeded into 96-well plates (10,000 cells/well) and incubated overnight. The cells were then incubated with 100 μl of fresh serum-free medium containing treatment agents such as conditioned medium of L. brevis 8803 and PPK. After 24, 48, and 72 hours, the cells were added to 100 μl of 20% TCA, were washed, and stained with 100 μl of 0.05% (w/v) SRB. Unbound dye was removed by washing the cells three times with 0.5% acetic acid. The plates were then air dried. Bound SRB was solubilized with 200 μl of 10 mM Tris base solution (pH 10.5) and the absorbance was read at 510 nm.

**In vivo BALB/cnu/nu mouse SW620 xenograft model.** Animal Care and Use Committee of Asahikawa Medical University approved our study (approval number: 13104, 13175 and 13197). Female nude mice (BALB/cnu/nu) (6-8 weeks of age) were obtained from Sankyo Laboratory Service (Tokyo, Japan). All of the animals were housed in a barrier facility and acclimated to 12-h light-dark cycles. Water and food were autoclaved and provided to all of the animals. Two hours after treatment with mice with anti-asialo ganglio-N-tetraosylceramide (GMI) antibody, SW620 human colon cancer cells in RPMI-1640 medium with matrigel were subcutaneously injected into the flanks of mice (2.5x10⁶ cells/tumor). After 5 days, treatment was initiated. One hundred microliters of poly P or ATP (25 μg/ml) was injected directly into each of the mouse tumors every day and the tumor size was measured each day using a Vernier caliper. The tumor volume was estimated according to the following formula:

\[ \text{Tumor volume (mm}^3\text{)} = L \times W^2/2 \]

where L is the length and W is the width. The final measurement was taken 9 days after tumor cell inoculation. At the end of the experiment (9 days after cell inoculation), the animals were sacrificed.

**Western blotting.** After washing SW620 or primary culture cells with phosphate-buffered saline (PBS), proteins were extracted from the samples using a Mammalian Cell Extraction Kit (BioVision, Mountain View, CA) and analyzed by western blotting. Ten to twenty micrograms of each sample was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and immediately transferred to a nitrocellulose membrane using a transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine with 20% (vol/vol) methanol). Nitrocellulose membranes were incubated in PBS with 0.1% (vol/vol) Tween 20 (T-PBS) containing 1% (wt/vol) bovine serum albumin (Wako, Osaka, Japan) for 1 h at room temperature to block nonspecific binding. The blots were incubated overnight at 4°C with the following primary antibodies: anti-cleaved poly ADP-ribose polymerase (PARP), anti-phospho-p44/42 [extracellular signal-regulated kinase (ERK) 1/2], anti-phospho-p44/42 (ERK1/2) (Cell Signaling Technology, Danvers, MA, USA), anti-actin (BD Biosciences, San Jose, CA, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Novus Biologicals, Littleton, CO, USA). The blots were washed three times for 15 min each with T-PBS at room temperature, incubated for 60 min in species-appropriate horseradish peroxidase-conjugated secondary antibodies (R&D Systems, Minneapolis, MN, USA) in T-PBS, washed three times in T-PBS, and developed using Super-Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining assay.** SW620 cells were seeded in 8-well chamber slides and were treated with poly P. After washing with PBS, the SW620 cells were exposed to 4% paraformaldehyde for 1 hour at room temperature. A tetramethylrhodamine red kit (Roche, Palo Alto, CA, USA) was used to perform TUNEL staining. The slide was then analyzed under a fluorescence microscope. The TUNEL-positive cells and total cells in three areas of each well were counted under a high-power view (×200).

**Phospho-mitogen-activated protein kinase (MAPK) protein array.** Total cellular protein was obtained using the reagents and protocol provided with a Human Phospho-MAPK Array kit (R&D Systems). The arrays were incubated with 200 μg of protein and the phospho-MAPK levels were detected by chemiluminescence according to the manufacturer’s instructions.

**Statistical analysis.** Student’s two-tailed t-test was used for the statistical analyses. The p-values are given in each figure. A p-value greater than 0.05 was considered to be statistically not significant (N.S). The mean ± standard error (SE) are given as error bars for the columns of the figures.

**Results**

**Conditioned medium of L. brevis 8803 inhibits SW620 cell viability.** Firstly, in order to determine the inhibitory effect of L. brevis 8803-secreted molecules, SW620 cells were incubated with DMEM and the conditioned medium of the bacteria was filtered using a 0.22-μm filter. An SRB assay
showed that the cell viability decreased in the group that was incubated with the conditioned medium of \textit{L. brevis} 8803 in comparison to the cells that were only incubated with DMEM at 24, 48 and 72 h after incubation (Figure 1). \textit{L. brevis} 8803-derived poly P mediates the antitumor effect of the bacterium. We previously suggested that \textit{L. brevis} 8803-derived poly P maintained intestinal homeostasis under oxidative stress (14), which accelerates tumor growth (11). Poly P has also been reported to inhibit the growth of myeloma cells (15) and the pulmonary metastasis of B16Bl6 cells \textit{in vivo} (16). These reports suggest the possibility that poly P mediates the antitumor effect of \textit{L. brevis} 8803.

To determine whether poly P mediates the antitumor effect of the bacteria, PPK was used to degrade poly P in the conditioned medium of the bacteria. An SRB assay showed that conditioned medium of \textit{L. brevis} 8803 inhibited the viability of SW620 cells and that the effect was suppressed by treatment with PPK, suggesting that poly P mediated the antitumor effect of the bacteria (Figure 2).

\textbf{Poly P inhibits the growth of SW620 cells in vitro and in vivo.} Poly P was chemically synthesized to investigate its effects. The tumor-inhibitory effects of several concentrations of chemically synthesized poly P were investigated. The application of more than 25 and 50 μg/ml of poly P was found to significantly reduce the viability of SW620 cells at 48 h. We selected the concentration of 25 μg/ml (Figure 3A). Poly P at 25 μg/ml inhibited the cell viability of SW620 cells at 24, 48 and 72 h after incubation (p<0.01) (Figure 3B). A xenograft model showed that direct injection of poly P into the tumors of SW620 cells on the backs of mice significantly inhibited the enlargement of the tumor in comparison to the injection of adenosine triphosphate (ATP) (Figure 3C).

\textbf{Poly P induces apoptosis of SW620 cells.} To investigate the apoptotic status of cells, a TUNEL staining assay was performed. The numbers of TUNEL-positive cells were significantly increased in SW620 cells that were treated with poly P in comparison to the control (Figure 4A). Western blotting was performed using antibody against cleaved PARP. The western blotting showed that the expression of cleaved PARP at 24 h was increased by treatment with poly P (25 μg/ml) in comparison to untreated cells, suggesting that poly P induces apoptosis of SW620 cells (Figure 4B).

\textbf{Poly P does not induce apoptosis nor inhibits the viability of the primary cultured normal mouse intestinal cells.} In primary cultured cells, poly P had a small inhibitory effect at 24 and 48 h, while cell viability had recovered at 72 h (Figure 5A). TUNEL staining showed that poly P did not
increase the induction of apoptosis of the primary cultured cells (Figure 5B). Poly P treatment did not change the expression of cleaved PARP in primary cultured cells (Figure 5C). These data suggest that poly P inhibited the growth of SW620 cells but not of cells derived from normal epithelium.

Activation of ERK signaling mediates the effect of poly P. To clarify the mechanisms underlying the induction of apoptosis, the activation of the MAPK pathway was investigated using a protein array. The phosphorylation of ERK was increased within 30 min by poly P treatment, while changes in the phosphorylation of other kinases, including p38 MAPK and c-JUN N-terminal kinase (JNK) were not significant (Figure 6A). Western blotting showed that the phosphorylation of ERK was significantly increased by poly P treatment (Figure 6B). Cleaved PARP was increased by poly P treatment and the increase of cleaved PARP was

Figure 3. Polyphosphate (poly P) inhibited the growth of SW620 cells. SW620 cells were incubated with 0, 6.25, 12.5, 25 and 50 μg/ml of poly P for 48 h. The applicable concentration of poly P for achieving the inhibition of SW620 cells was thought to be 25 μg/ml (A). A sulforhodamine B assay showed that poly P (25 μg/ml) inhibited the viability of SW620 cells at 24, 48 and 72 h after incubation (n=15) (B). A xenograft model showed that the direct injection of poly P into the tumors of SW620 cells on the backs of mice significantly inhibited the enlargement of the tumor in comparison to the injection of ATP (Control group) (n=5) (C). *p<0.05, **p<0.01.
suppressed by MEK inhibitor, U0126 within 24 h (Figure 6C). An SRB assay demonstrated that the inhibitory effect of poly P on the viability of SW620 cells was suppressed by U0126 treatment at 48 and 72 hours (Figure 6D). These data indicate that the effect of poly P is mediated by the activation of the ERK pathway.

**Discussion**

The present study demonstrates that conditioned medium of probiotic *L. brevis* 8803 inhibits the progression of colon cancer cells and that the inhibitory effect of the bacterium was suppressed by the degradation of poly P in the

Figure 4. Polyphosphate (poly P) induced apoptosis of SW620 cells. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining assay showed a significant increase in the number of TUNEL-positive SW620 cells among the SW620 cells that were treated with poly P in comparison to the control (A). Western blotting showed that at 24 h, the expression of cleaved poly ADP-ribose polymerase (PARP) was increased by treatment with poly P (25 μg/ml) in comparison to untreated cells (n=9) (B). **p<0.01.
conditioned medium, indicating that the tumor-inhibitory effect of this bacterium was mediated by the bacteria-derived poly P. Poly P reduced the viability of colon cancer cells in vitro and reduced tumor enlargement in an in vivo xenograft model. Our TUNEL staining and western blotting assays with antibody against cleaved PARP clearly showed that poly P induces the apoptosis of colon cancer cells. It is noteworthy that viability of the primary cultured cells of the mouse small intestine was not affected by poly P treatment, illustrating that poly P is less toxic to the cells of the normal intestine. Poly P therefore shows promise as a potential antitumor drug that is likely to be associated with fewer adverse effects in the normal epithelium.

Similarly to the present study, a small number of studies have identified molecules that are responsible for the antitumor effects of probiotics. El-Sersy et al. proposed the antitumor effect of ε-PL, that is produced by the marine Bacillus subtilis SDNS (9). However, they did not investigate the effects of the antitumor molecule on normal cells. Brasseur et al. identified parasporin-2Aa1 from Bacillus thuringiensis strain A1547 as an antitumor molecule. They proposed that bacillus-derived parasporin-2Aa1 induced apoptosis of hepG2, MCF-7 and PC-3 cells. They investigated the effects of the antitumor molecule in normal cells in several cell lines, including the IOSE-144, HIEEC, HIESC and MCF-10A cell lines, and showed that growth was not inhibited in any of these cell lines, but they did not investigate the effects of parasporin-2Aa1 in primary cultured cells (8). The present study also showed an inhibitory effect of L. brevis 8803-derived poly P on colon cancer cells, and confirmed poly P does not affect the growth of primary cultured cells derived from the normal intestine, strongly suggesting that poly P is less toxic to normal cells.

The present study investigated the signaling pathways which mediate the antitumor effects of poly P and determined that the activation of the ERK pathway was associated with the antitumor effect. Brasseur et al. proposed that bacillus-derived parasporin-2Aa1 induced the apoptosis of cancer cells through the down-regulation of the protein kinase B pathway and the up-regulation of the ERK pathway (8). These results suggest that the ERK pathway is a possible target of such bacteria-derived antitumor molecules. ERK serves as the output of the rat sarcoma virus (RAS)–rapidly accelerated fibrosarcoma (RAF)–MEK–ERK signal transduction pathway (17). Phosphorylated ERK activates many downstream proteins, thereby regulating a large variety of cellular processes, including cell proliferation, cell differentiation and apoptosis (18, 19). It has been reported that ERK activation induces the entry of cells into the S-phase and accelerates cellular growth (20). Conversely, sustained ERK activation also inhibits cell growth and induces neuronal differentiation of PC12 pheochromocytoma cells (21). Aoki et al. investigated the controversial effects of ERK signaling and proposed that a low-frequency activation of ERK signaling inhibits the progression of cells, while high frequency activation of ERK signaling enhances cell growth (22). The present study revealed that poly P activated ERK signaling at
Figure 6. The activation of extracellular signal-regulated kinase (ERK) signaling mediates the effect of polyphosphate (poly P). The activation of the mitogen-activated protein kinase (MAPK) pathways was investigated using a protein array. The phosphorylation of ERK at 30 min was increased by poly P treatment, while changes in the phosphorylation of other kinase, including p38 MAPK and c-JUN N-terminal kinase (JNK) were not significant (A). Western blotting showed that the phosphorylation of ERK at 15, 30 and 60 min was significantly increased by poly P treatment (n=6) (B). The expression of cleaved PARP was increased by poly P treatment and the increase was suppressed at 24 hours by U0126 (a MAPK/ERK kinase inhibitor) treatment (n=9) (C). A sulforhodamine B assay demonstrated that the inhibitory effect of poly P on the viability of SW620 cells was suppressed by U0126 (10 μM) treatment at 48 and 72 h (D) (n=15). *p<0.05, **p<0.01.
15, 30 and 60 min, suggesting that poly P induces sustained long-term activation of ERK signaling, which is thought to reduce the frequency of the signal, thereby inhibiting the progression of cancer cells.

In summary, the present study demonstrated that probiotic-derived poly P induces apoptosis and inhibits the progression of colon cancer cells through the activation of ERK signaling. Poly P did not affect the viability of primary cultured cells derived from the normal small intestine. Based on these findings, poly P is thought to be a promising antitumor drug that will be associated with fewer adverse effects than conventional drugs. Further analysis of the activity that occurs downstream of ERK signaling, which is activated by poly P, may clarify the detailed mechanisms by which the apoptosis of colon cancer cells is induced.

References


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