Antineoplastic effect of a novel chemopreventive agent, neokestose, on the Caco-2 cell line via inhibition of expression of nuclear factor-κB and cyclooxygenase-2

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Abstract. Neokestose is a ^{6}G-fructooligosaccharide (FOS) and an important prebiotic. When FOS are ingested by patients with colorectal cancer, they may come into contact with cancer cells prior to being fermented by bifidobacteria in the colon. In the present study, the effects of neokestose on cell proliferation, cell cycle and apoptosis of the colorectal cancer cell line Caco-2 were investigated to evaluate its anti-cancer effect. An MTT assay showed that neokestose-treated Caco-2 cells exhibited a significant and dose-dependent loss of viability. Flow cytometric analysis indicated that the sub-G1 population of Caco-2 cells was significantly increased following treatment with neokestose, and the percentage of Caco-2 cells in the stage of late apoptosis was also significantly increased in a dose-dependent manner. Western blot analysis showed that the overexpression of nuclear factor-κB, a central molecule responsible for the transition from inflammation to cancer, and cyclooxygenase-2, an important enzyme in colorectal tumorigenesis, in colorectal carcinoma cells was inhibited by neokestose. Accordingly, the present study provided *in vitro* evidence that neokestose may be used as a dietary chemopreventive agent, whose application is more rational than that of COX-2 inhibitors or aspirin for preventing colorectal cancer.

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Introduction

Sporadic colorectal cancer accounts for 75% of total cases of colorectal cancer, and is the third most common cancer in the world as well as the second leading cause of cancer mortality (1). Screening tests are able to reduce the mortality rate of colorectal cancer; however, a large proportion of individuals do not undergo colorectal cancer screening due to its inconvenience and high cost (2-5). The increasing frequency of colorectal cancer worldwide makes the prevention of this disease an urgent problem to be solved.

Previous studies suggested that fructooligosaccharides (FOS) can inhibit the process of colon cancer development, mainly by its prebiotic effect and the short-chain fatty acids (SCFA) produced during the fermentation of FOS in the colon (6-9). FOS are important prebiotics, since FOS cannot be digested in the small intestine of humans, but can be fermented in the colon to form short-chain fatty acids (SCFA) and lactic acid (10). FOS have recently attracted attention due to their beneficial physiological effects. These include relief of constipation, improved mineral absorption, as well as reduced blood levels of total cholesterol, triglycerides and phospholipids (8,11,12).

FOS are classified into three categories: ^{1}F-, ^{6}F- and ^{6}G-FOS (13-15). Among these FOS, only ^{1}F-FOS is commercially available. However, ^{6}G-FOS, also called neo-FOS, has fructosyl units bound at the β (2→6) position in the glucose moiety of sucrose. Hence, neo-FOS has superior bifidogenecity as well as better heat and chemical stability than ^{1}F-FOS (16,17). Neo-FOS comprises neokestose and neo-nystose (18); the structure of neokestose, together with the structures of 1-kestose (a ^{1}F-FOS) and 6-kestose (a ^{6}F-FOS), is shown in Fig. 1.

When FOS are ingested by patients with colorectal cancer, they may come into contact with cancer cells prior to being fermented by bifidobacteria in the colon. Among various FOS, neokestose was selected as the candidate in the present study, as it is the prebiotic with the highest potency, as mentioned above. The effects of neokestose on the colon cancer cell line Caco-2 were investigated, and the potential applications of neokestose for the dietary chemoprevention of colorectal cancer were also evaluated. The present study aimed to determine the biological

anti-cancer activity of neokestose towards the colon cancer cell line Caco-2, since neokestose is the most potential prebiotic among the fructooligosaccharides. For this, neokestose was produced using yeast fermentation of sucrose, isolated using high-performance liquid chromatography (HPLC), and its anti-cancerous effect against Caco-2 cells was assessed using MTT and flow cytometric assays as well as western blot analysis. The results indicated that neokestose may be an effective dietary chemopreventive against colorectal cancer.

Materials and methods

Preparation of neokestose. As previously reported, neo-FOS was produced by the culture of *Xanthophyllomyces dendrorhous* (BCRC 22367; Bioresource Collection and Research Center, Hsinchu, Taiwan), using 250 g/l sucrose as the substrate (15). Neokestose was purified by HPLC on a semipreparative YMC-pack ODS-AQ column (20x250 mm; YMC Co., Ltd., Kyoto, Japan) with a Waters 410 differential refractive index detector (Waters Corporation, Milford, MA, USA). Water at a flow rate of 8 ml/min was used as the mobile phase. A 200-μl sample of neo-FOS (containing 109 g/l neokestose) was injected for the purification of neokestose. The respective peak fractions were collected and concentrated by rotary evaporation.

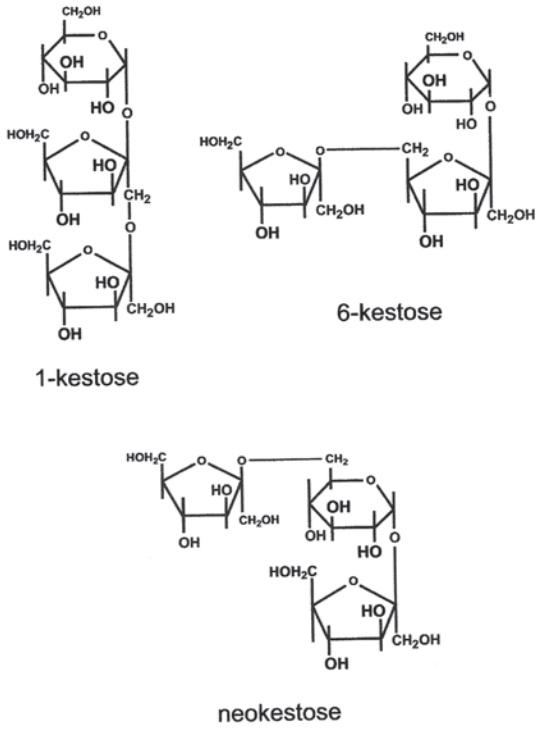

Figure 1. Chemical structures of 1-kestose, 6-kestose and neokestose.

Cell culture. The Caco-2 cell line (BCRC 60182) was obtained from the Bioresource Collection and Research Center. Cells were routinely maintained and subcultured in 10 cm^2-dishes at 37˚C in a humidified CO_2 incubator (95% air and 5% CO_2). The medium for cell maintenance consisted of 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and 100 g/ml streptomycin in Dulbecco's modified Eagle's medium (DMEM) solution, which are all from HyClone Laboratories, Inc. (Logan, UT, USA). When cells were 80% confluent, they were subcultured using 0.25% trypsin and 0.02% EDTA in D-Hanks' balanced salt solution (HyClone Laboratories, Inc.). The medium was replaced every 48 h.

MTT assay. MTT (Sigma, St Louis, MO) is a tetrazolium salt that can be cleaved by mitochondrial dehydrogenase in living cells. The effects of neokestose (0, 0.5, 1.0 or 2.5 mg/ml) on cell growth were tested using the MTT assay. Caco-2 cells were cultured in 96-well plates at a density of $1.0x10^4$ cells/well for two days, then the medium was discarded, 20 μl MTT solution [0.5 mg/ml in phosphate-buffered saline (PBS)] was added to all wells, and the cells were incubated for 2 h. At the end of the incubation, 100 μl dimethylsulfoxide was added to each well to lyse the cells, and the plates were transferred to a microplate reader (Synergy™ HT; BioTek, Winooski, VT, USA). Absorbance was recorded at 570 nm. All experiments were performed at least four times with four wells of each concentration.

Cell cycle analysis. To analyze the cell cycle distribution, cells were washed twice with PBS, collected by centrifugation at 725 x g for 5 min at 4˚C, and fixed in 70% (v/v) ethanol at 4˚C for 30 min. After fixation, cells were resuspended in PBS and stained with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) solution (containing 48 μg/ml propidium iodide and 48 μg/ml RNase A; Invitrogen Life Technologies, Carlsbad, CA, USA) for 20 min. The DNA content of the cells was examined by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Apoptosis analysis. Apoptosis was measured by using a fluorsecein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and collected by centrifugation at 725 x g for 5 min at 25˚C. Cells were resuspended in 100 μl of binding buffer and labeled with 5 μl FITC Annexin V and 5 μl PI solution for 15 min in the dark. After labeling, cells were resuspended in 400 μl binding buffer and detected by flow cytometry (FACSCalibur).

Western blot analysis. Caco-2 cells were lysed with Cell Extraction Buffer (M-PER® Mammalian Protein Extraction Reagent, Thermo Fisher Scientific, Waltham, MA, USA). The bicinchoninic acid protein assay kit (cat. no. 23225; Thermo Fisher Scientific) was used to measure the protein concentration. The cell extracts were subjected to 10% SDS-PAGE. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 3% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% Tween 20 (T-TBS) for 1 h, nitrocellulose membranes were incubated with the following primary antibodies (all diluted 1:1,000): Rabbit monoclonal anti-COX-2 (cat. no. 12282; Cell Signaling Technology, Danvers, MA, USA) mouse monoclonal anti-NF-κB (cat. no. MA5-16160; Thermo Fisher Scientific) and mouse monoclonal anti-actin (cat. no. MA1-744; Thermo Fisher Scientific), for 8 h at 4˚C and then washed in T-TBS.

The secondary antibody, horseradish peroxidase-conjugated rabbit anti-mouse IgG(H+L) (cat. no. 315-035-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, MA, USA) or mouse anti-rabbit IgG(H) (cat. no. GTX628140-01; GeneTex, Inc., Irvine, CA, USA), was incubated with the membranes at a dilution of 1:2,000 in T-TBS. After washing, the antibody complexes were detected by chemiluminescence using ECL reagents (Clarity™ Western ECL Substrate; Bio-Rad Laboratories, Inc.) and ImageQuant LAS 4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Statistical analysis. The results of multiple experiments are expressed as the mean ± standard error. Differences between the treatment and control groups were analyzed using Student's *t*-test. A $P<0.05$ was considered to indicate a statistically significant difference, which was denoted by asterisks in the figures.

Results

Preparation of neokestose. Neokestose was isolated from neo-FOS, which is produced from sucrose by the culture of *Xanthophyllomyces dendrorhous* BCRC 22367, which has ^{6}G-fructofuranosidase (^{6}G-FFase) activity (15). Fig. 2 shows the HPLC chromatogram of the neo-FOS mixture. Peak separation for the neo-FOS components was achieved. In Fig. 2, peak 6 indicates the neokestose fraction, which was the major component of the neo-FOS mixture. Approximately 20 mg pure neokestose was obtained from every HPLC run. The neo-FOS product consisted of 77.0% neokestose, 10.4% neo-nystose, 2.8% 1-kestose, 4.5% monosaccharides (glucose and fructose) and 5.3% sucrose on a dry weight basis. This product is adequate for large-scale preparation of neokestose.

Effect of neokestose on cell viability. The antiproliferative effect of neokestose on Caco-2 cells was measured by an MTT assay. Caco-2 cells were treated with different concentrations (0-2.5 mg/ml) of neokestose for 48 h, and the obtained results are shown in Fig. 3. In Fig. 3, the cell viability of the Caco-2 cells was decreased from 100% to ~59% as the concentration of neokestose was increased from 0 to 2.5 mg/ml. The results showed that Caco-2 cells treated with neokestose exhibited a significant and dose-dependent loss of viability.

Effect of neokestose on the sub-G1 population and tumor cell apoptosis. Caco-2 cells were treated with neokestose for 48 h, and the cell cycle distribution and apoptosis were measured by flow cytometric analysis. Neokestose treatment significantly increased the sub-G1 phase population (Fig. 4). Similarly, neokestose treatment also dramatically increased the percentage of the late apoptotic cells, according to the PI-Annexin V staining assay (Fig. 5).

Fig. 4 clearly indicates that the sub-G1 phase population increased from 8, 22, 28 and 45% when the Caco-2 cells were treated by an increasing concentrations of neokestose from 0 (control), 0.5, 1.0 and 2.5 mg/ml. In the flow cytometric cell cycle distribution curve, the sub-G1 area represents a population of cells with a reduced DNA content, which is a marker for apoptosis. Therefore, the significantly increased sub-G1 phase population indicates increasing apoptosis in the Caco-2 cells.

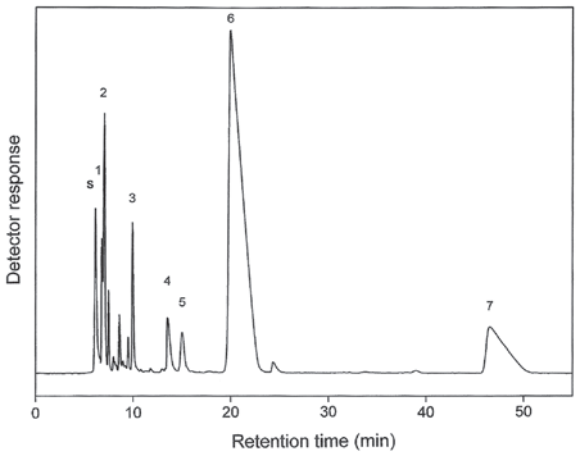

Figure 2. High-performance liquid chromatogram of neo-fructooligosaccharides on a semipreparative ODS-AQ column (Peaks: S, salts; 1, fructose and glucose; 2, glycerol; 3, sucrose; 4, ethanol; 5, 1-kestose; 6, neokestose; 7, neonystose).

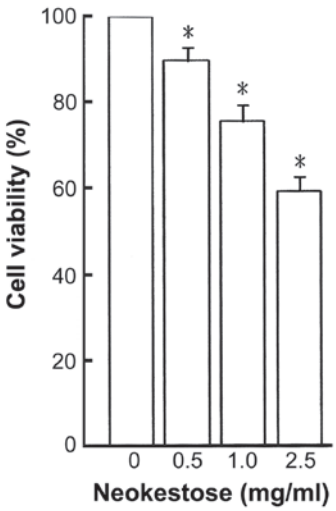

Figure 3. Anti-proliferative effect of neokestose on Caco-2 cells. Caco-2 cells were treated with 0.5, 1.0 or 2.5 mg/ml of neokestose for 48 h, and Caco-2 cells without neokestose treatment served as a control. The cell viability of Caco-2 cells was then determined using the MTT assay. Values are expressed as the mean ± standard error of four independent experiments (*$P<0.05$ vs. control).

In addition, as mentioned above, apoptosis was further verified by annexin V-FITC/PI double staining using a flow cytometry. Therefore, neokestose-treated Caco-2 cells were stained with PI and annexin V-FITC to determine the apoptotic rate of the cells. Cells were distiguished as viable (annexin V-FITC, PI double negative), early apoptotic (annexin V-FITC positive, PI negative) and late apoptotic (annexin V-FITC, PI double positive) cells. In Fig. 6, it can be clearly seen that when the Caco-2 cells were treated with neokestose at concentrations of 0 (control), 0.5, 1.0 and 2.5 mg/ml, the percentage of early apoptotic cells was 8.3, 10.9, 9.0 and 8.7%, respectively, along with percentages of late apoptotic cells of 15.3, 25.3, 27.2 and 31.2%, respectively. This indicated that neokestose efficiently induced apoptosis of Caco-2 cells.

From the results, it was evident that neokestose affected cell cycle progression and apoptosis in colorectal cancer cells.

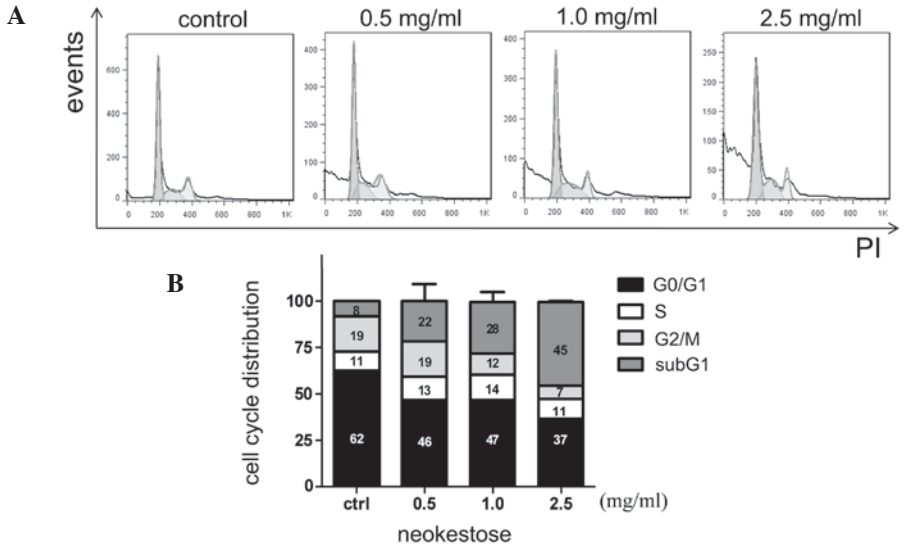

Figure 4. Effect of neokestose on cell cycle distribution of Caco-2 cells. Cells were treated with 0.5, 1.0 or 2.5 mg/ml neokestose, and untreated cells served as a control. (A) Caco-2 cells were stained with PI and their DNA content was analyzed by flow cytometric analysis. (B) Quantified values obtained by integration of the cell cycle distribution curves are expressed as the mean ± standard error of four independent experiments. PI, propidium iodide.

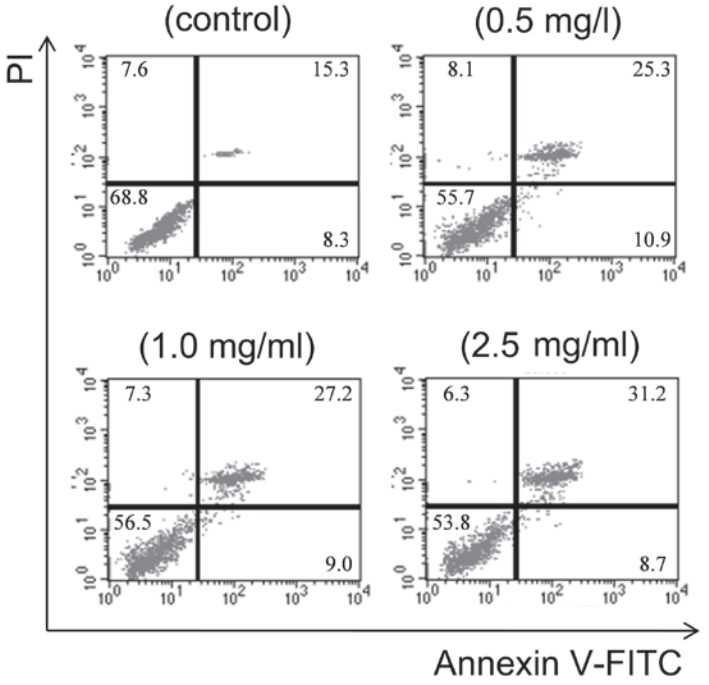

Figure 5. Effect of neokestose on cell apoptosis of Caco-2 cells. Caco-2 cells were treated with 0.5, 1.0 and 2.5 mg/ml neokestose for 48 h, and Caco-2 cells without neokestose treatment served as a control. Apoptosis of Caco-2 cells was then determined by a PI and annexin V-FITC double-staining assay and quantified by flow cytometric analysis. Annexin V-FITC $^{-}$/PI^{-}, annexin V-FITC $^{+}$/PI^{-} and annexin V-FITC $^{+}$/PI^{+} cells were considered to be viable, early-apoptotic, and late-apoptotic cells, respectively. PI, propidium iodide; FITC, fluorescein isothiocyanate.

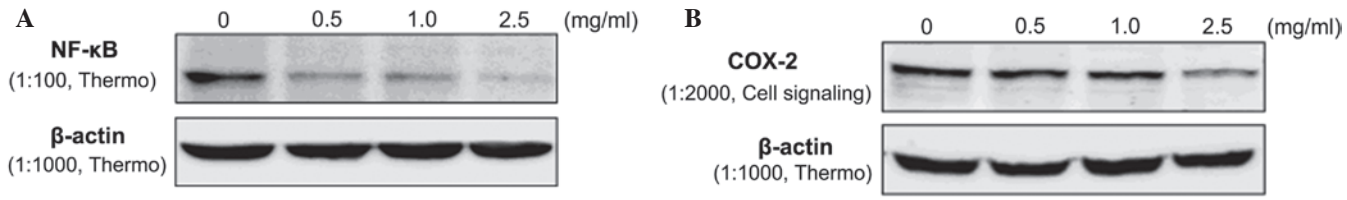

Figure 6. Effect of neokestose on the protein levels of (A) NF-κB and (B) COX-2 in Caco-2 cells. Caco-2 cells were treated with neokestose at 0.5, 1.0 or 2.5 mg/ml for 48 h, and Caco-2 cells without neokestose treatment served as a control. NF-κB and COX-2 proteins were extracted and analyzed by western blotting. Protein expressions were normalized to the β-actin control. NF-κB, nuclear factor kappa B; COX, cyclooxygenase.

Effect of neokestose on expression of NF-κB and COX-2. To evaluate potential molecular mechanisms causing apoptosis, the expression of NF-κB and COX-2 was assessed by western blot analysis (19,20). Caco-2 cells were treated with neokestose at concentrations of 0 (control), 0.5, 1.0 or 2.5 mg/ml for 48 h. Protein was then extracted and analyzed by western blotting. Fig. 6 shows that NF-κB and COX-2 were overexpressed in the Caco-2 cell line; however, following treatment with neokestose, the expression of NF-κB and COX-2 protein was significantly reduced in a dose-dependent manner.

Discussion

In the present study, neokestose produced by the conversion of sucrose by *Xanthophyllomyces dendrorhous* BCRC 22367 induced apoptosis in the colorectal cancer cell line Caco-2. Flow cytometric analysis showed that neokestose increased the population of Caco-2 cells in sub-G1 phase, and increase the percentage of annexin V-FITC-positive cells. These results indicated that neokestose induced apoptosis of Caco-2 cells in a dose-dependent manner, which is consistent with the results of the cell viability test.

NF-κB and COX-2 are commonly used markers of cell apoptosis. Therefore, the protein expression of NF-κB and COX-2 in Caco-2 cells treated with various concentrations of neokestose was evaluated. The results showed that the expression of NF-κB and COX-2 was decreased in Caco-2 cells treated with neokestose. NF-κB is a central molecule responsible for the transition from inflammation to cancer (19), and COX-2 has an important role in colorectal tumorigenesis (4,19). Accordingly, the results of the present study demonstrated that neokestose has a potent bifidogenic effect directed against colorectal cancer.

In conclusion, the results of the present study demonstrated that neokestose significantly induces apoptotic cell death in a dose-dependent manner via inhibition of NF-κB and COX-2 expression in colorectal carcinoma cells. Accordingly, neokestose may be used as a chemopreventive agent for colorectal cancer. Ideally, an anti-cancer agent or chemopreventive should inhibit the growth of cancer cells without affecting normal cell growth. To the best of our knowledge, the present study was the first to provide *in vitro* evidence that neokestose may serve as a potential dietary chemopreventive agent for colorectal cancer.

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