Cell-bound exopolysaccharide from probiotic bacteria induces autophagic cell death of tumour cells

Y. Kim1, S. Oh2, H.S. Yun3, S. Oh4 and S.H. Kim3

1 Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
2 Division of Cellular and Developmental Biology, Brain Korea 21 Program for Biomedical Science, Korea University, Seoul, Korea
3 Division of Food Bioscience & Technology, Korea University, Seoul, Korea
4 Department of Animal Science, Chonnam National University, Gwangju, Korea

Keywords
antitumour activity, autophagy, cb-EPS, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Abstract

Aim: Lactic acid bacteria (LAB) are beneficial micro-organisms that have been associated with several probiotic effects in both humans and animals. Here, using proteome analysis, we investigate the antitumour effects of cell-bound exopolysaccharides (cb-EPS) isolated from Lactobacillus acidophilus 606 on colon cancer cells and explore the proteins critical for their antitumour activity.

Methods and Results: cb-EPS inhibited the proliferation of HT-29 colon cancer cells by directly affecting cell morphology and not the cell cycle. Using two-dimensional polyacrylamide gel electrophoresis coupled with matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and immunoblot analysis, we found that cb-EPS dramatically induced Beclin-1 and GRP78, and affected Bcl-2 and Bak regulation.

Conclusions: The results of this study indicate that cb-EPS are antitumourigenic against HT-29 colon cancer cells and that this activity is because of the activation of autophagic cell death promoted directly by the induction of Beclin-1 and GRP78, as well as indirectly through the induction of Bcl-2 and Bak.

Significance and Impact of the Study: These results may contribute to understanding the novel mechanisms by which probiotic bacteria induce tumour cell death via autophagy.

Introduction

Lactobacillus acidophilus is a major species of lactic acid bacteria (LAB), a diverse group of economically important micro-organisms that have been used in various food and agricultural fermentation processes (Kim et al. 2008b). Exopolysaccharides (EPS), one of the primary metabolic products of LAB, have recently received an increasing amount of attention because of their health benefits. We previously found that EPS from probiotic bacteria have health-promoting effects, including antitumour (Choi et al. 2006) activity, cholera toxin neutralization (Kim et al. 2006a), and inhibition of biofilm formation (Kim et al. 2008a). Therefore, there is a high level of interest in understanding the specific mechanisms by which EPS function in relation to their potential use as a novel drug or food adjunct. A number of results from epidemiological and experimental studies have also suggested that the ingestion of specific LAB strains, or of fermented dairy products, might alleviate the risk of certain types of cancers and inhibit tumour growth (Kato et al. 1994). Although it has been postulated that several antitumour components contained in dairy food, including LAB, may induce apoptosis (Di Marzio et al. 2001; Belury 2002), a molecular understanding of the distinct mode of action by which LAB exerts these effects has not been achieved.

In addition to the well-established pathways involved in apoptosis (programmed cell death type I), another interesting programmed cell death pathway, referred to as...
autophagy, has been identified (programmed cell death type II) (Gozuacik and Kimchi 2004). Autophagy is a dynamic process of long-lived protein degradation, which is typically observed during nutrient deprivation (Gozuacik and Kimchi 2004). Recently, interest in autophagy has been renewed among oncologists because different types of cancer cells undergo autophagy after various anticancer therapies. Indeed, various anticancer therapies that induce autophagy have been recently introduced (Kondo and Kondo 2006). Among these, tamoxifen and other antioestrogen agents induce autophagy in breast cancer MCF-7 cells (Bursch et al. 1996). This autophagy is mediated by a cell-permeable short chain analogue of a second mesenger C2-ceramide and increased expression of Beclin-1, an autophagy-related protein (Scarlatti et al. 2004). However, relatively little is known about the mechanisms by which autophagy occurs in type II cell death (Kondo and Kondo 2006).

Here, we investigated the antitumour properties of cell-bound exopolysaccharide (cb-EPS) isolated from Lact. acidophilus 606 by comparing the protein profiles of HT-29 cells in the presence and absence of cb-EPS using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). We provide evidence using comparative proteome analysis and immunoblotting that autophagic processes mediated by Beclin-1 and GRP78 along with Bcl-2 and Bak are involved in inhibiting the proliferations of colon cancer cells. To our knowledge, this report is first to describe specific antitumour activity of food-related materials via the autophagy pathway.

Materials and Methods

Bacteria and cell culture
Cell-bound EPS (cb-EPS) from Lact. acidophilus 606 [isolated from human faeces; deposited in Korea Culture Center of Microorganisms (KCCM) as KCCM-10663P; http://www.kccm.or.kr/] was prepared using the same methods described previously (Choi et al. 2006; Kim et al. 2006a). Prior to use in experiments, the strain was subcultured at least three times in de Mann, Rogosa and Sharpe broth (MRS; Difco Laboratories, Detroit, MI, USA) at 37°C for 18 h. The HT-29 colon cancer cell line was routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY, USA). The cells were cultured at 37°C in 5% CO₂ in a humidified atmosphere.

Cell proliferation assay
The antitumour activity of cb-EPS was determined as previously described (Choi et al. 2006). Briefly, HT-29 cells were seeded in a 96-well plate (1·0 × 10⁴ cells per well). After 24 h, each well was washed three times with prewarmed phosphate-buffered saline (PBS; 37°C) to remove nonattached cells. Ten milligrams per millilitre cb-EPS was then added to the cancer cells for 96 h. No significant differences were observed in the pH of the medium during the incubation. Thereafter, 50 µl of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2·0 g l⁻¹ stock) was added to each well. After 4 h, the culture medium in each well was replaced with dimethyl sulfoxide, and the plate was gently agitated for 20 min. Absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of viable cells was calculated as follows: cell proliferation (%) = (OD of experimental group/OD of control group) × 100. PBS was used as a negative control. Additionally, lactate dehydrogenase (LDH) cytotoxicity was quantified with a commercially available kit (LDH Cytotoxicity Detection kit; TaKaRa, Tokyo, Japan) according to the manufacturer’s instructions. The absorbance was measured at 600 nm using a plate reader (Molecular Devices).

Cell staining and cell cycle analysis
HT-29 cells incubated in the presence or absence of 100 µg ml⁻¹ cb-EPS were washed twice with PBS and then fixed in ice-cold methanol for 10 min at −20°C. Fixed cells were washed with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) solution for 10 min at room temperature. The cells were then quickly washed with PBS and the DAPI stained nuclei were visualized using a fluorescence microscope (Olympus IX71, Tokyo, Japan). Flow cytometric analysis (FACS) was used to follow the kinetics of cell cycle progression of G0/G1-phase synchronized cells after release from quiescence. For FACS analysis, HT-29 cells treated with 100 µg ml⁻¹ cb-EPS were harvested and fixed overnight in cold 70% ethanol. Cells were resuspended in 300 µg ml⁻¹ RNase A (Qiagen, Hiden, Germany) in PBS containing 5 µg ml⁻¹ propidium iodide (PI; Sigma Chemical Co., St Louis, MI, USA) at 37°C for 30 min. One-colour flow cytometric analysis was performed using a fluorescence-activated cell sorter with ModFit LT (Verity Software, Topsham, ME, USA) and CellQUEST software (Becton Dickinson, Franklin Lakes, NJ, USA).

2D-PAGE and MALDI-TOF/MS
2D-PAGE procedures, including isofocusing, SDS-PAGE, and staining, were adapted from a previously described...
Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of Beclin-1 and GRP78

Total RNA was extracted from HT-29 cells incubated in the presence or absence of 100 mg ml⁻¹ cb-EPS for 96 h using TRIZOL™ (Invitrogen, Carlsbad, CA, USA) and was treated with DNase I (Promega) for 30 min at 37°C. The quantity of purified RNA was determined by measuring the absorbance at 260 and 280 nm using an Ultrospec™ 2100 pro UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). The RNA integrity was determined by running it on a denaturing 1.5% agarose gel. One microgram of total RNA was reverse transcribed in a 20 µl reaction mixture with an AMV First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). The following primers were used to amplify the corresponding transcripts of interest: Beclin-1 (Scarlatti et al. 2004) forward primer, 5′-CCAGGATGG-TGTCTCTGGCA-3′; reverse primer, 5′-CTGCGTCTGG-GGATACGCA-3′; and GRP78 (Szczesna-Skorupa et al. 2004) forward primer, 5′-ATCACGCCGTCCTATGTCGC-3′; reverse primer, 5′-TCTCCCCCTCCCTCTTATCC-3′. Amplification products were visualized using a Kodak DC290 zoom digital camera and Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA).

Western blot analysis

To further explore whether cb-EPS influences apoptosis-related signalling factors, the levels of Bcl-2, Bak, Bax, poly (ADP ribose) polymerase (PARP), apoptosis inducing factor (AIF), and caspase-3 in HT-29 cells were examined by immunoblot analysis after exposure to cb-EPS for 96 h. Primary antibodies against Bcl-2, Bak, and Bax (BD-Pharmingen, San Diego, CA, USA); AIF, PARP, and caspase-3 (Biomol, Plymouth Meeting, PA, USA); and Horseradish Peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockward, IL, USA) were used for protein detection. Immunoreactive bands were visualized using the Super Signal West Pico Chemiluminescent Substrate kit (Pierce). Protein levels at each time point were normalized against α-tubulin as a protein loading control using Kodak 1D image analysis software (Eastman Kodak Company).

Statistical analysis

All experiments were carried out at least in triplicate. The effects of each treatment were analysed by ANOVA, followed by general linear model procedures using the SAS software package (ver. 9.1; SAS Inc., Cary, NC, USA). The level of significance was defined at \( P < 0.05 \).

Results

cb-EPS inhibit HT-29 colon cell proliferation through inducing morphologic changes

Recently, we reported that heat-killed probiotic bacteria and their components possess certain antitumour properties against various cancer cells, whereas no growth inhibition was observed for noncancer cells (hEF cells) (Choi et al. 2006). As expected, cb-EPS dramatically and consistently inhibited the proliferation of HT-29 cells in a dose- and temporal-dependent manner (Fig. 1a,b). In addition, dramatic differences in cell morphology were observed after exposure to cb-EPS, whereas significant nuclear condensation was not observed by fluorescent microscopy (Fig. 1c). Also, no significant alterations in the cell cycle, including in G0/G1, and S phases, were observed by FACS analysis (data not shown). Therefore, it appears that cb-EPS may inhibit colon cancer cell proliferation by directly affecting cell morphology and not the cell cycle. In these initial experiments, high levels of cb-EPS were
used to examine its antitumour activity (up to 100 mg ml\(^{-1}\)), but cb-EPS did not solubilize well at these concentrations (data not shown). Given these results, we subsequently used 10 mg ml\(^{-1}\) as an optimal concentration in further studies.

The effects of cb-EPS on autophagy-related proteins

To determine the effect of cb-EPS treatment on protein levels in HT-29 cells, a comparative proteome experiment was performed 96 h after cb-EPS exposure (10 \(\mu\)g ml\(^{-1}\)) using a strip covering a pH range from 3.0 to 10.0. Among the approximately 1000 visible spots, a total of 42 spots were detected with a 3-fold increase or decrease compared to the control (31 spots were increased, while eleven were decreased) (Table S1 and Fig. 2). Consistent with the observed changes in cell morphology, the level of cell matrix- and morphology-related proteins, including plectin-1 (spot 23) and microtubule-actin cross-linking factor 1 (spot 21), was strongly altered by cb-EPS. Also, the expression of heat shock cognate 71 kDa protein (HSP7C, spot 22) was significantly induced.

Among the identified proteins, Beclin-1 (spot 10) and GRP78 (spot 16/17) were dramatically induced in the presence of 100 mg ml\(^{-1}\) cb-EPS. Moreover, we found a consistent induction of Beclin-1 (2.1-fold) and GRP78 (2.2-fold) transcription in the presence of cb-EPS by RT-PCR using specific primer sets (data not shown). These data suggest that the antitumour effects of cb-EPS may be caused by activation of the autophagy pathway via the interaction of Beclin-1 and GRP78, and not through the induction of apoptosis.

The influence of cb-EPS on Bcl-2 and Bak

To further test whether cb-EPS influences apoptosis-related signalling factors, the levels of Bcl-2, Bak, Bax, PARP, AIF, and caspase-3 were examined after exposure of HT-29 cells to cb-EPS for 96 h (Fig. 3): Bcl-2 was reduced by 2.2-fold, whereas Bak was slightly induced by 1.5-fold after exposure. These results may suggest that cb-EPS-mediated regulation of Bcl-2 and Bak could contribute to autophagic death, either directly or indirectly.

Discussion

Several anticancer components obtained from probiotic bacteria have been proposed as candidates for potential prevention of a number of cancers (Di Marzio et al. 2001; Belury 2002). However, the mechanisms by which these components affect cancer cells are not clearly understood at the genetic and proteomic levels. Here, based on proteomic analysis, we found that several proteins involved in
autophagic cell death, including Beclin-1 and GRP78, are significantly regulated by cb-EPS. Beclin-1 is a mammalian autophagy protein, while GRP78 (also referred to as BiP) is an endoplasmic reticulum (ER) molecular chaperone. Importantly, both proteins are significantly involved in autophagy-related cell death pathways coupled to ER stress (Li et al. 2008). Beclin-1 is expressed at decreased levels in human breast carcinoma cells (Liang et al. 1999), and functions to induce the expression of several autophagy-related factors and degrade long-lived proteins under amino-acid starvation conditions (Gozuacik and Kimchi 2004). In addition, autophagy was significantly

**Figure 2** 2D-PAGE images of protein extracts from HT-29 cells exposed to control treatment (a) and 10 mg ml^{-1} cb-EPS (b) for 96 h. Crude protein extracts (500 µg) were separated on pH 3.0–10.0 IPG strips, followed by 12.5% SDS-PAGE. The proteins analysed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry are indicated by spot number in (b), and the results shown in Table S1. 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; cb-EPS, cell-bound exopolysaccharides.
reduced in vitro in Beclin-1-deficient embryonic stem cells and in vivo in Beclin-1 mutant (Beclin-1−/−) mice (Yue et al. 1999; Qu et al. 2003). Recently, several reports have also linked Beclin-1 to the antitumour activity of various compounds (Ertmer et al. 2007; Li et al. 2009; Sun et al. 2010). Therefore, we propose that cb-EPS may directly control the expression of Beclin-1 to stimulate autophagy-mediated cell death.

In addition to Beclin-1, the expression of GRP78 was also strongly induced by cb-EPS. GRP78 is not only involved in many cellular processes, including translocating newly synthesized polypeptides across the ER membrane and facilitating the folding and assembly of newly synthesized proteins, but is also a key regulator of ER stress transducers (Li et al. 2008). Recently, it was shown that autophagy is activated upon ER stress as a defensive mechanism for cell survival (Ding et al. 2007). Interestingly, Beclin-1 is required for ER stress-induced autophagy, and GRP78 regulates ER stress-induced autophagy in human cells (Li et al. 2008). In addition, depletion of GRP78 via RNA interference leads to significant suppression of autophagosome formation initiated by nutrient deprivation or ER stress (Li et al. 2008). Therefore, it is possible that cb-EPS treatment may induce ER stress and GRP78 expression, which then promote autophagy-associated cancer cell death by a cascade influenced by the expression of Beclin-1. In addition, stress-related proteins have been shown to be involved in refolding damaged proteins and importing proteins into mitochondria, processes important for cell survival (Lee et al. 2008). Hence, together our data indicate that cb-EPS stimulates a number of stress-induced proteins and affects proteins important in maintaining normal cellular morphology.

In addition to Beclin-1 and GRP78, other proteins have been implicated in autophagy, including the apoptosis-related protein Bcl-2 (Shimizu et al. 2004). Studies have shown that Bcl-2 may function as an antiautophagic protein as over-expression of Bcl-2 in apoptosis-deficient cells could potentiate autophagy. Additionally, Beclin-1/Bcl-2 interactions can influence both apoptosis and autophagy-associated death (Pattingre et al. 2005). Finally, Bak has also been shown to act as a repressor of autophagy (Moretti et al. 2007). We found that cb-EPS induced Beclin-1 (Fig. 2 and Table S1) and Bak (Fig. 3), whereas it repressed Bcl-2 (Fig. 3). Interestingly, the balance between pro-apoptotic (e.g. Bak) and antiapoptotic (e.g. Bcl-2) proteins may act as a regulator of autophagy (Moretti et al. 2007). Moreover, the protective affects of autophagy and Bcl-2 activity have been linked in cell cytotoxicity assays. *Theobroma cacao* L. phenolic extracts, which are widely distributed in plant-derived foods, inhibited drug-triggered liver cytotoxicity by inducing autophagy coupled with the regulation of Bcl-2 (Arlorio et al. 2009). Consistent with these reports, our results indicate that the balance or ratio between Beclin-1, Bcl-2, and Bak may play an important role in the antitumour activity of cb-EPS via the autophagy pathway. Further studies will be required to fully understand the specific relationship between Bcl-2, Bak and autophagy-specific factors in these processes.

To our knowledge, this is the first report that cb-EPS produced by probiotic bacteria commonly used in the food industry can induce antitumour activity via the autophagy pathway. From these data, we developed a working model (Fig. 4), which links cb-EPS to the regulation of (i) Beclin-1 (autophagy protein), (ii) GRP78 (indirectly regulates autophagy via ER stress), and (iii)
interaction with apoptosis-related factors such as Bcl-2 and Bak. These data support the potential use of dietary bacteria to prevent colon cancer development, clearly a matter of great interest. Given that other studies have not yet reported antitumour affects of foods through the autophagy pathway, these results further our understanding of the distinct antitumour pathways induced by probiotic bacteria. Such studies may aid in the development of these bacteria for use in the food industry as adjuncts for cancer therapy and prevention. Studies aimed at evaluating the kinetics of tumour development reduction and verifying cb-EPS-mediated inhibition or prevention of colon cancer in vivo using animal models are ongoing.

Acknowledgements

This study was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (R01-2008-000-20593-0). Y. Kim was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-F00025).

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Table S1. List of differentially expressed proteins for the HT-29 in the presence of 10⁻⁰ mg ml⁻¹ cb-EPS for 96 h.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.