Bovine lactoferrin and lactoferricin exert antitumor activities on human colorectal cancer cells (HT-29) by activating various signaling pathways

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Complete List of Authors:	Jiang, Rulan; University of California at Davis Lonnerdal, Bo; University of California at Davis
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6 Rulan Jiang and Bo Lönnerdal

7 Department of Nutrition, University of California, Davis

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11 Address correspondence to:

12 Bo Lönnerdal, PhD

13 Department of Nutrition

14 University of California

15 Davis CA 95616, USA

16 Phone: (530) 752-8347

17 Fax: (530) 752-3564

18 E-mail: blonnerdal@ucdavis.edu

Abstract

Lactoferrin (Lf) is an iron-binding glycoprotein and is present at high concentrations in milk. Bovine lactoferricin (LfcinB) is a peptide fragment generated by pepsin proteolysis of bovine lactoferrin (bLf). LfcinB consists of amino acid residues 17–41 proximal to the N-terminal of bLf and a disulfide bond between residues 19 and 36 forming a cyclic loop. Both bLf and LfcinB have been demonstrated to have antitumor activities. Colorectal cancer is the 2nd most common cause of cancer deaths in developed countries. We hypothesized that bLf and LfcinB exert antitumor activities on colon cancer cells (HT-29) by triggering various signaling pathways. bLf and LfcinB significantly induced apoptosis in colon cancer cells but not in normal human intestinal epithelial cells (HIEC) revealed by the ApoTox-Glo triplex assay. The live/dead cell viability assay showed that both bLf and LfcinB inhibited the viability of HT-29 cells. Transcriptome analysis indicated that bLf, cyclic LfcinB, and linear LfcinB exerted antitumor activities via differentially activating diverse signaling pathways, including p53, apoptosis, and angiopoietin signaling. Immunoblotting results validated that both bLf and LfcinBs increased expression of caspase-8, p53, and p21, critical proteins in tumor suppression. These results provide valuable information regarding bLf and LfcinB for potential clinical applications in colon cancer therapy.

Key words: bovine lactoferrin, bovine lactoferricin, colorectal cancer, antitumor, signaling pathways

Introduction

Lactoferrin (Lf) is an iron-binding glycoprotein found in most exocrine secretions and at high concentrations in some mammalian milks, especially human milk (Lönnerdal 2009). Lf is a pleiotropic protein and is involved in a wide range of bioactivities including antimicrobial and immunomodulatory activities, antitumor properties, and modulation of cell proliferation and differentiation (Lönnerdal and Iyer 1995; Vogel 2012). Bovine lactoferrin (bLf) exhibits several functions similar to those of human Lf (hLf) (Lönnerdal 2009), and it is commercially available (Tomita et al. 2009); therefore it may be used as a nutraceutical to prevent and treat diseases. The unique structure of Lf confers its resistance to digestion both *in vivo* and *in vitro* (Davidson and Lönnerdal 1987; Lönnerdal et al. 2011), and it may therefore play important roles in the intestine. Bovine lactoferricin (LfcinB) is a cationic peptide naturally generated by acid-pepsin hydrolysis of bLf in the stomach (Bellamy et al. 1992). LfcinB consists of amino acid residues 17–41 proximal to the N-terminal of bLf, and an intramolecular cysteine–cysteine disulfide bond is formed between residues 19 and 36 creating a distinctive cyclic form (Gifford et al. 2005). The role of the disulfide bond is not fully characterized as inconsistent results on antimicrobial activities of LfcinBs were found in previous publications (Liu et al. 2011; Vorland et al. 1998). There is a cluster of positively charged residues in the N-terminal lobe of Lf, and it thus exhibits potent antimicrobial activities by interacting with negatively charged regions of the bacterial membrane (Baker and Baker 2009). LfcinB has stronger antimicrobial activities than intact bLf (Bellamy et al. 1992),

and LfcinB exhibits more potent bioactivities than human, murine, and caprine lactoferricins (Vorland et al. 1998). In addition to its antimicrobial activities, LfcinB displays strong antitumor activities on a number of cancer cells (Pan et al. 2013; Riedl et al. 2014), but it does not harm normal cells (Mader et al. 2005).

Colorectal cancer is the leading cause of cancer mortality and malignancy worldwide. Colorectal cancer is also the 2nd most common cause of cancer deaths in the US and other developed countries, despite important advances in detection, surgery, and chemotherapy (Terzic et al. 2010). Current treatments for colorectal cancer includes combination of surgery, radiation therapy, chemotherapy, targeted therapy, and cytotoxic drugs (Chidambaram et al. 2011). All current therapies for colorectal cancer have deleterious side effects, and it is therefore important to find safe alternative therapies for colon cancer.

A variety of mechanisms are involved in antitumor activities, such as apoptosis promotion, proliferation suppression, angiogenesis inhibition, and facilitation of cell cycle arrest. bLf and LfcinB have been demonstrated to have antitumor activities *in vivo* and *in vitro*. bLf and LfcinB enhanced apoptosis of colon tumor cells in azoxymethane-treated rats by increasing apoptosis-related gene expression (Fujita et al. 2004a, b), and initiated apoptosis in different types of cells, such as T cell leukemia cells (Jurkat), breast cancer cells (MCF-7, MDA-MB-435), colon cancer cells (Colo-35), and melanoma cells (Furlong et al. 2006; Gibbons et al. 2015; Mader et al. 2005) (Riedl et al. 2014). However, bLf- and LfcinB-induced signal transduction and effects on whole genome gene expression in colon cancer cells have not been established.

In the present study, we aimed to identify novel signaling pathways and gene targets, and thus provide a theoretical foundation for possible future clinical applications of bLf and LfcinB in colon cancer therapy. Effects of bLf and LfcinB on apoptosis were assessed, and microarray assays were conducted to determine the signal transduction pathways and gene expression profiling in a colon cancer cell line (HT-29) treated with bLf or LfcinB.

Materials and methods

Cell culture

Human colon adenocarcinoma HT-29 cells (American Type Culture Collection, HTB-37, Rockville, MD) were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO₂ in Minimum Essential Medium (MEM, Life Technologies Inc., Gaithersburg, MD) supplemented with fetal bovine serum (FBS, 10%, MP Biomedicals, Irvine, CA), streptomycin (10 µg/mL), and penicillin (10 units/mL, Sigma, St. Louis, MO). Cells between passages 5-15 were harvested at 90% confluence, and the medium was changed every other day.

Non-transformed human intestinal epithelial crypt-like (HIEC) cells (a gift from Dr. Jean-François Beaulieu, Université de Sherbrooke, Canada) were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO₂ in Opti-MEM (Life Technologies Inc., Gaithersburg, MD) supplemented with fetal bovine serum (FBS, 5%, MP Biomedicals), GlutaMax (1%, Gibco), and epidermal Growth Factor (EGF, 5 ng/mL). Cells between passages 18-24 were harvested at 90% confluence and medium was changed every other day.

Immunoblotting

111 After HT-29 cells ($1.6 \times 10^4/\text{cm}^2$, 6-well plate) were grown overnight, they
 112 were treated with bLf (800 $\mu\text{g}/\text{mL}$), LfcinB-C (400 $\mu\text{g}/\text{mL}$) or LfcinB-L (400 $\mu\text{g}/\text{mL}$)
 113 for 12 h. Cells were then lysed in RIPA buffer (Cell Signaling, Danvers, MA)
 114 supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis,
 115 IN), sonicated briefly, and centrifuged at $5,000 \times g$ for 10 min at 4°C . Protein
 116 concentrations were measured by the Bradford assay. Protein samples (50
 117 $\mu\text{g}/\text{lane}$) were boiled for 5 min in Laemmli sample buffer (Bio-Rad, Hercules, CA)
 118 with β -mercaptoethanol (5%), separated by SDS–PAGE, and then transferred to
 119 a nitrocellulose membrane. The membrane was incubated in blocking buffer (5%
 120 skim milk in PBST: 0.1% Tween-20 in PBS) for 45 min at room temperature,
 121 washed three times with PBST, and subsequently probed with an antibody
 122 specific for human LfR as described previously (Lopez et al. 2008) (rabbit anti-
 123 LfR, 1 $\mu\text{g}/\text{mL}$), rabbit anti-LRP1 (1:50,000, Abcam, Cambridge, MA), rabbit anti-
 124 caspase-3 (1:1000, Cell Signaling, #9662), mouse anti-p53 (1:1000, Cell
 125 Signaling, #2524), rabbit anti-p21 (1:1000, Cell signaling, #2947), or rabbit anti-
 126 caspase-8 (1:1000, Cell Signaling, #9746) in blocking buffer overnight at 4°C .
 127 After three washes with PBST, primary antibodies were detected with
 128 horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:20,000,
 129 Amersham Pharmacia Biotech, Piscataway, NJ) or HRP-conjugated rabbit anti-
 130 mouse IgG (1:20,000, DakoCytomation, Carpinteria, CA) in blocking buffer for 45
 131 min. Bands were visualized with SuperSignal™ West Femto Maximum Sensitivity
 132 Substrate (Thermo Scientific, Rockford, IL) after a final wash (30 min wash with
 133 PBST). To evaluate the equivalence of protein loading, blots were incubated in

Restore Western Blot Stripping buffer (Thermo Scientific) for 15 min at room temperature, blocked for 1 h, and then reprobed with an HRP conjugated β -actin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA).

bLf, cyclic LfcinB (LfcinB-C) and linear LfcinB (LfcinB-L)

Commercial bLf was obtained from Friesland Campina DOMO (Amersfoort, Netherlands), and the iron saturation rate was around 15%. The purity and concentration of lipopolysaccharides (LPS) were ~95% and 0.82 endotoxin units/mg, respectively. Cyclic and linear forms of LfcinB (amino acid sequence: FKCRWQWRMKKLGAPSITCVRRAF) were synthesized by Genemed (Genemed Biotechnologies Inc., San Antonio, TX).

ApoTox-Glo triplex assay

HT-29 and HIEC cells were seeded onto 96-well plates in MEM with 2% FBS or Opti-MEM with 1% FBS, respectively, and cultured overnight. After HT-29 cells or HIEC were treated with bLf (50, 100, 200, 400, 800, or 1000 μ g/mL) or LfcinBs (50, 100, 200, 400, or 800 μ g/mL) for 4, 12, 24 and 48 h, effects on apoptosis and cytotoxicity were evaluated using the ApoTox-Glo Triplex Assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, after the treatment, the viability/cytotoxicity reagent was added to all wells and incubated for 30 min at room temperature. Caspase-Glo 3/7 was added to the wells, mixed briefly for 30 s, and then incubated for 30 min at room temperature. Fluorescence was measured at 380EX/510EM to assess viability, 485EX/520EM to assess cytotoxicity, and luminescence was measured to assess apoptosis. Each concentration was repeated in 8 wells for 3 independent experiments.

Live/dead cell viability assay

To determine effects of bLf, cyclic LfcinB (LfcinB-C), and linear LfcinB (LfcinB-L) on cell viability, HT-29 cells ($1.6 \times 10^4/\text{cm}^2$, 24 well plate) were treated with bLf (800 $\mu\text{g/mL}$) or LfcinBs (400 $\mu\text{g/mL}$) for 12 h, and then the Live/dead cell viability assay (Molecular Probe) was conducted following the manufacturer's instructions. Briefly, after HT-29 cells were incubated with the Live/Dead solution (100 μL) for 15 min at room temperature, the staining solution was removed. The cells were observed under a fluorescence microscope. Live cells were labeled with green fluorescence, and dead cells were labeled with red fluorescence.

RNA extraction and array hybridization

HT-29 cells ($1.6 \times 10^4/\text{cm}^2$, 6-well plate) were grown over night and then treated with bLf (800 $\mu\text{g/mL}$), LfcinB-C (400 $\mu\text{g/mL}$), or LfcinB-L (400 $\mu\text{g/mL}$) for 12 h. RNA was extracted from the harvested cells using Trizol Reagent (Invitrogen). RNA was then purified by an RNease kit (Qiagen) according to the manufacturer's instructions. Measurement of RNA yield was performed using a NanoDrop 1000A Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was verified by using Bioanalyzer RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA) following the manufacturer's procedure. Total RNA samples were amplified and labeled with biotinylated nucleotides using a kit (Ambion, TotalPrep RNA Amplification Kit). Bioanalyzer analysis was then performed to verify if cRNA was at the expected 1.2 kb average size before applying to bead chips (HumanHT-12 v.4.0, Illumina, San Diego, CA). Bead chips were scanned with the Illumina iScan using standard conditions.

Microarray data analysis

GenomeStudio (Illumina) was used for microarray data analysis, and rank invariant normalization was conducted to normalize the microarray data. Only probe sets with adjusted p values less than 0.05 were selected for subsequent analysis, and a total of 13,248 genes were analyzed. Differentially expressed genes were selected following two criteria: fold-change >1.5 for up-regulation, and fold-change < 0.5 (represents as -2.0) for down-regulation. Samples were clustered with a centroid linkage method on the horizontal axis, and the lengths of the branches were measure by a Euclidean algorithm. Pathway analysis was conducted using IPA (Ingenuity® Systems, Mountain View, CA), and the Core analysis included in IPA was used to interpret data in the context of biological processes, pathways, and networks. The p-value was calculated by IPA using the right-tailed Fisher's exact test for each canonical pathway. P-values less than 0.05 were considered significantly relevant. A heat map of altered genes (fold-changes>3 or <0.4) was generated by using Heatmapper (<http://www.heatmapper.ca/>).

Validation of microarray data by quantitative real-time PCR

RNA (1 µg) was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Gene-specific primers are listed in Table 1. Real-time polymerase chain reaction (RT-PCR) was performed on the cDNA reaction mixture (2 µL) and SYBR Green (Thermo Scientific) using the iCycler real-time PCR system (Bio-Rad). The cycling parameters were 95 °C 15 min and 40 cycles

including 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Linearity of the dissociation curve was analyzed using the iCycler software, and the mean cycle time of the linear part of the curve was designated as Ct. Each sample was analyzed in triplicate and normalized to GAPDH using the following equation: fold change = $2^{(Ct_{\text{Gene}} - Ct_{\text{GAPDH}})}$. Values are shown as the mean fold change $\pm$ standard deviation, relative to the control (set to 1).

Statistical analysis

Data represent means $\pm$ standard deviations from 3 independent experiments. Comparisons between treatments and the control were conducted using one-way ANOVA (Prism Graph Pad, Berkeley, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Effects of bLf, LfcinB-C, and LfcinB-L on apoptosis, cytotoxicity, and viability of HT-29 and HIEC cells

We have previously shown that the small intestine LfR (intelectin 1) mediates functions of Lf by partly regulating internalization of Lf by intestinal epithelial cells (Jiang et al. 2011). LRP1 has also been documented as a functional LfR (Grey et al. 2004; Suzuki et al. 2005). We therefore examined expression of LfR and LRP1 in HT-29 cells by immunoblotting. As shown in Fig.1, HT-29 cells expressed LRP1 and the two isoforms of LfR seen in HIEC (Jiang and Lönnerdal 2014). Another bLf receptor, transferrin receptor, is also expressed in HT-29 cells (Fan et al. 2012). LfcinB may not bind to any of the three LfRs expressed by HT-29 cells, however, owing to the abundant Arg

residues, LfcinB may be spontaneously internalized by these cells as a penetratin (Vogel 2012). To evaluate effects of bLf, LfcinB-C, and LfcinB-L on apoptosis and cytotoxicity of colon cancer cells, HT-29 cells were treated with varying concentrations of bLf (50, 100, 200, 400, 800 or 1000 $\mu\text{g/mL}$), or LfcinBs (50, 100, 200, 400, or 800 $\mu\text{g/mL}$) for 4, 12, 24, or 48 h. Both bLf and LfcinBs significantly stimulated apoptosis of HT-29 cells (Fig. 2 A-C), and displayed cytotoxic activity on HT-29 cells (Fig. 2 D-F). The effects of bLf and LfcinBs on apoptosis of HT-29 cells were also confirmed by immunoblotting, showing that bLf or LfcinB treatments increased cleaved caspase-3 (Fig. 3). Based on the results, the maximum effects were observed at 12 h of the treatment, and the optimal concentrations for bLf and LfcinBs were 800 $\mu\text{g/mL}$ and 400 $\mu\text{g/mL}$, respectively. Figure 2 shows that 800 $\mu\text{g/mL}$ bLf was more effective at inducing apoptosis than 1000 $\mu\text{g/mL}$ bLf, and that 400 $\mu\text{g/mL}$ LfcinBs was more effective at inducing apoptosis than 800 $\mu\text{g/mL}$ LfcinBs. 1000 $\mu\text{g/mL}$ bLf and 800 $\mu\text{g/mL}$ LfcinBs might cause cell death, as shown by their higher toxicities (Fig. 2B). To evaluate effects on apoptosis, a caspase-3/7 substrate was used to examine activities of caspase3/7 in the ApoTox-Glo™ Triplex Assay. If cells die, the activity of caspase3/7 might not be detected. In addition, cells might die in a non-programmed way. As a consequence, low activities of caspase3/7 (low apoptosis activities) were seen in cells treated with 1000 $\mu\text{g/mL}$ bLf or with 800 $\mu\text{g/mL}$ LfcinBs. Live/dead cell viability was subsequently assessed. The results showed that both bLf and LfcinBs considerably reduced the viability of colon cancer cells after 12 h of treatment (Fig. 4). In order to evaluate effects of bLf and LfcinBs on

normal intestinal epithelial cells, HIEC cells were treated with the same concentrations of bLf and LfcinBs as HT-29 cells for 4, 12, 24, or 48 h. No significant effects of bLf and LfcinBs on apoptosis of HIEC were found, so only one time point and one concentration of treatments are shown. Cell cytotoxic effects were seen for treatments at some concentrations and time points (Fig. 5 B-E).

bLf and bLfcinBs diversely regulated gene expression

HT-29 cells were treated with bLf (800 µg/mL), LfcinB-C (400 µg/mL), or LfcinB-L (400 µg/mL) for 12 h, and then RNA was extracted for microarray assays. Transcription of genes was significantly modified by bLf and LfcinBs (Fig. 6), and the numbers of genes regulated by bLf, LfcinB-C, and LfcinB-L are 461, 595, and 901, respectively. Among the regulated genes, 349 genes were similarly altered by LfcinB-C and LfcinB-L, whereas 798 genes were differentially affected, suggesting that the disulfide-bond loop plays a role in regulation of gene transcription. Totally, only 98 genes were similarly modified by bLf, LfcinB-C, and LfcinB-L.

Hierarchical clustering gene expression analysis was carried out. The control and treatment groups are clustered on the horizontal axis (Fig. 7). The lengths of the branches in the dendrogram represent the degrees of correlation between the control and treatment groups. bLf displayed higher correlation with LfcinB-C than LfcinB-L. LfcinB-L exhibited the most potent effects on transcription of HT-29 cells compared with bLf and LfcinB-C. The heat map shows genes were markedly and variously modified by bLf and LfcinBs. Genes

involved in apoptosis, cell cycle control, and immune functions were altered by bLf and LfcinBs (Table 2). bLf and LfcinBs variously regulated transcription of genes involved in the p53 signaling pathway, such as PMAIP-1, TP5313, and SFN. PMAIP-1 is a pro-apoptotic member of the Bcl-2 protein family, TP5313 is similar to oxidoreductase involved in cellular responses to oxidative stresses and irradiation, and SFN is a p53 mediated inhibitor of cell cycle progression. These three genes are induced by the tumor suppressor p53, and are believed to be involved in p53-mediated cell death. CAPN2 is a cysteine proteinase involved in apoptosis and cell survival (Demarchi and Schneider 2007). Transcription of anti-oxidation related genes was increased, including GCLC, GCLM, MAFF, and SQSTM1. GCLC and GCLM are enzymes in glutathione synthesis, MAFF is a transcriptional factor regulating cytoprotective genes (Katsuoka et al. 2005), and SQSTM1 is an autophagy receptor involved in apoptosis and immune response. Three NF κ B genes including NFKBIA, NFKBIB, and NFKBIE were upregulated. These three genes play roles in dendritic cell maturation (Palucka and Banchereau 2012).

Validation of microarray results by RT-PCR

The microarray results were verified by RT-PCR using primers listed in Table 1. Five genes significantly modified by bLf or LfcinBs were chosen, and RT-PCR was performed. As shown in Fig. 8, microarray results were verified by real-time PCR. Fig. 8A shows RT-PCR results and correlation between microarray and RT-PCR is shown in Fig. 8B. Results of RT-PCR were

significantly correlated to the findings from microarray ($R^2 > 0.9$ for three treatments), confirming the reliability of the microarray results.

bLf and LfcinBs exert their antitumor activities via activating various signaling pathways

Activated signaling pathways involved in antitumor activities are shown in Fig. 9. bLf and LfcinBs triggered a series of signaling pathways in colon cancer cells, such as p53, cell cycle control, apoptosis, and dendritic cell maturation signaling pathways. bLf mainly activated p53 and cycle control pathways including ATM, CHK and BRC1 signaling to hinder growth of cancer cells, whereas LfcinBs initiated more diverse pathways, such as immune related signaling pathways and the angiopoietin signaling pathway. Three critical proteins involved in apoptosis and p53 pathways, caspase 8, p53, and p21 were evaluated by immunoblotting. As shown in Fig.10, expression of caspase 8, p53, and p21 was markedly upregulated by bLf and LfcinBs, implying that bLf and LfcinBs trigger these two pathways to exert their antitumor activities. Decreased expression or loss of p21 was found in colorectal cancer specimens (Ogino et al. 2006). In line with this, low expression of p21 is seen in HT-29 cells (Fig. 10).

Discussion

bLf and LfcinBs suppressed the growth of colon cancer cells. bLf and LfcinB have been shown to exhibit antitumor activities on different types of cancers, including gastric cancer, lung cancer, head and neck squamous cell carcinoma, and leukemia (Pan et al. 2013; Tung et al. 2013; Wolf et al. 2007) (Mader et al. 2005). The positive charges from the N-terminal of Lf may play essential roles in

destabilizing cancer cell membranes by interacting with negatively charged structures on cancer cells (Hoskin and Ramamoorthy 2008), and/or binding to DNA as a transcriptional factor (He and Furmanski 1995) to activate transcription of anti-tumor genes, which might be two major antitumor mechanisms of Lf.

bLf and LfcinBs displayed pro-apoptotic activities. The activities of caspase 3/7 were induced by bLf and LfcinBs as indicated by the results from the ApoTox-Glo™ Triplex Assay (Fig. 2), and activities and expression of caspase 3 as shown by immunoblotting (Fig. 3). Moreover, the activities of caspase 8 were also significantly increased by bLf and LfcinBs. These results suggest that promotion of apoptosis is one of the mechanisms by which bLf and LfcinBs exert their antitumor effects in colon cancer, which is in agreement with previous findings (Pan et al. 2013). Caspases are aspartate specific cysteine proteases that play critical roles in the initiation and execution of the extrinsic/death receptor and intrinsic/mitochondria-dependent pathways, two main pathways that mediate apoptosis (Ashkenazi and Dixit 1998; Earnshaw et al. 1999; Green and Reed 1998). Apoptosis is a distinct form of cell death that plays important roles in carcinogenesis, embryonic development, immune system maturation, and cytotoxic effector function (Fleisher 1997). Cancer cells can escape apoptosis, and therefore apoptosis promotion is one of mechanisms to fight cancer (Hanahan and Weinberg 2000). LfcinB was reported to induce apoptosis of B-lymphoma cells a caspase independent pathway (Furlong et al. 2010), implying that LfcinB differently exerts pro-apoptotic activities in different types of cancers.

bLf and LfcinB-L activated the p53 signaling pathway. The p53 protein is activated in a specific manner by a post-translational modification which results in cell cycle arrest, cell senescence, cellular apoptosis, and necrotic/necroptotic cell death via mitochondria (Dashzeveg and Yoshida 2015; Jin and Levine 2001). Both bLf and LfcinBs significantly up-regulated expression of p53 and p21. p21 is an inhibitor of cyclin E-cdk2 and a major player in p53-mediated G1 arrest (Dashzeveg and Yoshida 2015). p21 has been shown to play a pro-apoptotic role and an anti-apoptotic role against p53-mediated apoptosis, depending on the biological context (Abbas and Dutta 2009). p21 has been associated with cancer progress (Gartel 2006) in breast carcinoma MDA-MB-231 cells (Damiens et al. 1999) and head and neck cancer cells (Xiao et al. 2004). In our study, the increased p21 might occur via a p53 dependent pathway and play a pro-apoptotic role.

bLf and LfcinBs exert antitumor activities by variously modulating signaling pathways. In addition to the p53 pathway, bLf also up-regulated three signaling pathways mainly leading to cell cycle arrest in response to DNA damage, which are the BRC1, CHK, and ATM pathways. LfcinB-C and LfcinB-L considerably up-regulated the NRF2-mediated oxidative stress response, interferon signaling, and hindered angiopoietin signaling. Elevated levels of reactive oxygen species (ROS) in cancer cells can cause DNA mutations and induce pro-oncogenic signaling, and thereby contribute to the carcinogenesis process (Gorrini et al. 2013). The NRF2-mediated oxidative stress response protects against the development of cancer by converting highly reactive ROS to less reactive products (Cheung et al.

2014). Cancer cells are less responsive to immune modulation (Schreiber et al. 2011), and elevated IFN signaling by LfcinBs may activate endogenous antitumor cytotoxic T cells to act against cancer (Shalapour and Karin 2015). Angiogenesis is required for tumor growth and metastasis (Sun 2012). Both LfcinB-C and LfcinB-L inhibited angiopoietin signaling, suggesting that LfcinBs suppress metastasis by hindering angiogenesis. Besides the above common pathways, LfcinB-C increased dendritic cell maturation and apoptosis signaling, and LfcinB-L promoted G2/M DNA checkpoint regulation, indicating that the loop structure plays a role in specific and unique functions. The generation of protective antitumor immunity relies on dendritic cell maturation (Palucka and Banchereau 2012). Due to mutations in tumour suppressors and oncogenes, cancer cells exhibit uncontrolled cell proliferation. LfcinL may cause cell cycle arrest of HT-29 cells by stimulating G2/M DNA checkpoint regulation.

bLf and LfcinBs specifically target cancer cells and show no apoptosis effects on normal human intestinal epithelial cells. Although bLf and LfcinBs exhibited cytotoxic activities on HIEC cells at high dosages (400 µg/mL and 800 µg/mL), the effects on HIEC are weaker than those on HT-29 cells. Current therapies for colon cancer are usually accompanied by severe side effects. Safe and effective alternative therapies are increasingly needed. Non-specificity is one of the reasons for the severe side effects. According to our results, bLf selectively exerts its antitumor effects on cancer cells but not normal cells. Compared to non-malignant cells, cancer cells have a larger quantity of anionic molecules in cell membrane composition, increased membrane fluidity, as well as more

microvilli (Camilio et al. 2014). Due to the highly positively charged regions in bLf and LfcinBs, both bLf and LfcinBs are capable to distinguish between cancer cells and normal cells, and thus only target cancer cells to exert their antitumor activities.

In summary, bLf and LfcinB exert their potent antitumor activities by activating various signaling pathways including apoptosis, p53 signaling, and cell cycle arrest signaling. These mechanisms contribute to antitumor activities of LfcinBs, but also include the antioxidant response, inhibition of angiogenesis, and immunity signaling. Both bLf and LfcinBs specifically target cancer cells and these three protein/peptides are safe for normal cells. Our results need to be examined and confirmed in animal experiments and clinical trials. However, our findings provide valuable information regarding the therapeutic potential for bLf in colorectal cancer.

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Draft

Fig. 1.

Fig. 1. HT-29 cells express LfR and LRP1. Total protein of HT-29 cells was extracted, subjected to immunoblotting, and probed with LfR or LRP1 antibodies.

254x190mm (96 x 96 DPI)

Fig. 2.

Fig. 2. Effects of bLf, LfcinB-C, and LfcinB-L on apoptosis (A, B & C) and cytotoxicity (D, E & F) of HT-29 cells. HT-29 cells were seeded onto 96-well plates (1.6 x 10⁴/cm²), and cultured overnight. After HT-29 cells were treated with bLf or LfcinBs (50, 100, 200, 400, 800 or 1000 µg/mL) for 4, 12, 24 or 48 h, effects on apoptosis and cytotoxicity were evaluated using the ApoTox-Glo Triplex Assay (Promega) according to the manufacturer's instructions. Results represent means ± SDs from 3 independent experiments. *p<0.001, **p<0.01, #p<0.05

254x190mm (96 x 96 DPI)

Fig. 3

Fig. 3. Effects of bLf, LfcinB-C, and LfcinB-L on activities of caspase-3. HT-29 cells ($1.6 \times 10^4/\text{cm}^2$, in 6-well plates) were cultured overnight, and then treated with bLf (800 $\mu\text{g}/\text{mL}$) or LfcinBs (400 $\mu\text{g}/\text{mL}$) for 12 h. Cell lysates were subjected to immunoblotting and probed with a caspase-3 antibody.

254x190mm (96 x 96 DPI)

Fig. 4.

Fig. 4. Effects of bLf and LfcinBs on viability of HT-29 cells. To determine effects of bLf and LfcinBs on cell viability, HT-29 cells were treated with bLf (800 µg/mL) or LfcinBs (400 µg/mL) for 12 h. Effects of bLf and LfcinBs on cell viability were evaluated using a live/dead cell viability assay according the manufacturer’s instructions. The cells were observed under a fluorescence microscope. Live cells were labeled with green fluorescence and dead cells were labeled with red fluorescence (Scale bar, 20 µm).

254x190mm (96 x 96 DPI)

Fig. 5.

Fig. 5. Effects of bLf, LfcinB-C, and LfcinB-L on apoptosis and cytotoxicity of HIEC cells. HIEC cells were seeded onto 96-well plates ($1.6 \times 10^4/\text{cm}^2$) and cultured overnight. After HIEC were treated with bLf or LfcinBs (50, 100, 200, 400, 800 or 1000 $\mu\text{g}/\text{mL}$) for 4, 12, 24 or 48 h, effects on apoptosis and cytotoxicity were evaluated using the ApoTox-Glo Triplex Assay (Promega) according to the manufacturer's instructions. Results represent means $\pm$ SDs from 3 independent experiments.

254x190mm (96 x 96 DPI)

Fig. 6.

Fig. 6. Venn diagram of genes significantly regulated by bLf, LfcinB-C, and LfcinB-L. Differentially expressed genes were selected following three criteria: fold change >1.5 for up-regulation, fold-change < 0.5 for down-regulation, and detection probability greater than 0.95 in all samples.

254x190mm (96 x 96 DPI)

Fig. 7. Hierarchical clustering gene expression analysis and a heat map of HT-29 cells upon bLf or LfcinBs treatments. Probe sets for genes significantly regulated (adjusted p value <0.05 by significance analysis of microarrays) were selected, clustered A heat map was generated using altered genes (fold-changes>3 or <0.4 (B). Each row represents one probe. Gene expression level is depicted by the intensity of the red (low intensity) and green (high intensity) boxes.

254x190mm (96 x 96 DPI)

Fig. 8.

Fig. 8. Real-time PCR verification of microarray results. (A) RT-PCR. (B) Correlations of results from microarrays and RT-PCR. Values are shown as mean fold-changes $\pm$ SDs, relative to the control (set to 1).

254x190mm (96 x 96 DPI)

Fig. 9.

Fig. 9. Significant signaling pathways associated with antitumor activities of bLf, LfcinB-C, and LfcinB-L. The angiopoietin signaling pathway was down-regulated by LfcinB-C and LfcinB-L and the other pathways were up-regulated by bLf, LfcinB-C, or LfcinB-L. $P<0.05$ was used as a threshold.

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Fig. 10.

Fig. 10. Effects of bLf, LfcinB-C, and LfcinB-L on expression of caspase 8, p53, and p21. After HT-29 cells were treated with bLf (800 μ g/mL) or LfcinBs (400 μ g/mL) for 12 h, total proteins of HT-29 cells was extracted and subjected to immunoblotting, and probed with caspase-8, p53, or p21 antibodies.

254x190mm (96 x 96 DPI)

Table 1 Sequences of the primers for Real-time PCR

Gene		Primer sequence	Accession number
CASP6	Forward	TCACACCTGTAATCCCAGCA	NM_032992.2
	Reverse	GTCAGGCTGGTCTCGAACTC	
CASP8	Forward	AGAGTCTGTGCCCAAATCAAC	NM_033358.2
	Reverse	GCTGCTTCTCTCTTTGCTGAA	
CDKN1A	Forward	CGATGGAACCTTCGACTTTGTCA	NM_078467.1
	Reverse	GCACAAGGGTACAAGACAGTG	
SOD2	Forward	GGAAGCCATCAAACGTGACTT	NM_001024466
	Reverse	CCCGTTCCTTATTGAAACCAAGC	
TNFRSF21	Forward	GTAGCATCCAGGAAGGGACA	NM_014452.3
	Reverse	TTTGGGAGGGTCTTGTTTAC	
GAPDH	Forward	CAACGAATTTGGCTACAGCA	NM_002046.2
	Reverse	GGTGGTCCAGGGGTCTTACT	

Table 2 Selected genes altered by bLf and LfcinBs

Accession number	Gene symbol	Description	Fold changes		
			bLf	LfcinB-C	LfcinB-L
p53 signaling					
NM_021127.1	PMAIP 1	Phorbol-12-myristate-13-acetate-induced protein 1	1.77	2.82	3.51
NM_006216.2	SERPINE 2	Serpin peptidase inhibitor, clade E, member 2	1.62	1.61	2.51
NM_147184.1	TP5313	Tumor protein p53 inducible protein 3	1.53	1.25	1.45
NM_078467.1	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.58	2.51	3.93
NM_002639.2	SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin)	1.43	1.43	1.99
NM_006142.3	SFN	Stratifin	1.09	1.38	2.32
NM_003842.3	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	1.14	1.43	1.59
Cell cycle control					
NM_005590.3	MRE11A	MRE11 meiotic recombination 11 homolog A	1.63	1.37	1.14
NM_018313.2	PBRM1	Polybromo 1	1.83	1.26	1.06
NM_139266.1	STAT1	Signal transducer and activator of transcription 1	-1.90	1.26	-1.59
NM_004156.2	PPP2CB	Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	1.48	1.24	1.20

NM_001790.3	CDC25C	Cell division cycle 25 homolog C (S. pombe)	0.83	-1.44	-2.0
NM_006142.3	SFN	Stratifin	2.39	1.47	1.15
NM_003390.2	WEE1	WEE1 homolog (S. pombe)	1.15	1.24	1.62
NM_005983.2	SKP2	S-phase kinase-associated protein 2 (p45)	0.81	-1.75	-1.88
Apoptosis					
NM_032992.2	CASP6	Caspase 6, apoptosis-related cysteine peptidase	1.47	1.32	1.02
NM_033358.2	CASP8	Caspase 8, apoptosis-related cysteine peptidase	1.53	1.57	1.45
NM_001748.3	CAPN2	Calpain 2, (m/II) large subunit	1.71	2.29	3.07
Anti-oxidation					
NM_182810.1	ATF4	Activating transcription factor 4	1.52	1.73	1.72
NM_001757.2	CBR1	Carbonyl reductase 1	1.49	1.59	1.70
NM_012328.1	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.19	1.59	1.66
NM_001498.2	GCLC	Glutamate-cysteine ligase, catalytic subunit	1.1	1.79	2.88
NM_002061.2	GCLM	Glutamate-cysteine ligase, modifier subunit	1.11	1.60	2.76
NM_012323.2	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	1.10	1.82	2.72
XM_944206.1	MAP2K3	Mitogen-activated protein kinase kinase 3	1.26	1.62	1.71
NM_00102446	SOD2	Superoxide dismutase 2,	1.39	3.57	3.29

6.1		mitochondrial			
NM_003900.3	SQSTM1	Sequestosome 1	1.05	1.86	2.27
NM_003330.2	TXNRD1	Thioredoxin reductase 1	1.35	1.73	2.54
Dendritic cell maturation					
NM_001675.2	ATF4	Activating transcription factor 4	1.49	1.68	1.66
NM_001779.1	CD58	CD58 molecule	1.27	1.33	1.64
NM_004233.2	CD83	CD83 molecule	1.29	1.51	1.88
NM_030582.2	COL18A1	Collagen, type XVIII, alpha 1	1.11	1.32	1.51
NM_004001.3	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	1.72	1.86	1.79
NM_003088.2	FSCN1	Fascin homolog 1, actin- bundling protein	0.90	1.20	1.66
NM_002175.1	IFNA21	Interferon, alpha 21	1.12	0.97	1.48
NM_173842.1	IL1RN	Interleukin 1 receptor antagonist	1.20	1.92	2.20
NM_000230.1	LEP	Leptin	1.28	1.36	1.60
NM_020529.1	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.08	2.62	2.90
NM_002503.3	NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	1.22	1.83	2.37
NM_004556.2	NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	1.24	2.00	1.89
NM_004620.2	TRAF6	TNF receptor-associated factor 6	1.37	1.51	1.78

Fold changes are indicated for each gene significantly up or downregulated ($p < 0.05$, fold change >1.5 or <0.5) in bLf, LfcinB-C and LfcinB-L groups compared to the control. Positive data indicate upregulation and negative data indicate downregulation.

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