The anticancer effect of Bioconverted Danggui Liuhuang Decoction EtOH extracts in human colorectal cancer cell lines

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Abstract Objective: The objective of our study was to investigate anti-cancer effects of Danggui Liuhuang Decoction extract bioconverted by protease liquid coenzyme of Aspergillus kawachii (DLD-BE), compared to a non-bioconverted DLD extract (DLD-E) and determine the underlying mechanisms. Methods: DLD-E and DLD-BE were evaluated for their ability to modulate these signaling pathways and suppress the proliferation of human colorectal cancer (CRC) cells, HCT-116, LoVo, and HT-29. The anti-cancer effects of DLD-E and DLD-BE were measured by using proliferation and migration assays, cell cycle analysis, Western blots, and real-time PCR. Results: In this study, treatment with DLD-E and DLD-BE at concentrations of 25-100 μg/mL inhibited proliferation and migration in human CRC cells. DLD-BE induced apoptotic cell death and decreased COX-2 expression in HT-29 cells. The mechanisms of action included modulation of the AKT and extracellular-signal-regulated kinase signaling cascades along with inhibition of COX-2 expression. The results demonstrate novel anti-cancer mechanisms of DLD-BE against the growth of human CRC cells. Thus, we propose that DLD-BE can be developed as a more potent supplement to inhibit colorectal tumor growth and intestinal inflammation than DLD-E.

Keywords Bioconverted extract of Danggui Liuhuang Decoction · Colorectal cancer · Cyclooxygenase-2 · Decoction extract · Extracellular-signal-regulated kinase · Liuhuang · Protein kinase B

Introduction Colorectal cancer (CRC) is one of the most common malignancies in humans, with a high incidence and rising mortality rates worldwide [1,2]. Along with loss or mutation in tumor suppressor genes, regulation of cell survival, cell proliferation, inflammation and angiogenesis also play important role in CRC development and progression [3,4].

Cyclooxygenase (COX)-2, a key rate-limiting enzyme in the conversion of arachidonic acid to prostanoids, is undetectable or expressed at low levels in most normal tissue, but it is known to be overexpressed in inflammatory bowel disease (IBD) and most colorectal adenomas and adenocarcinomas [5-7].

COX-2 expression is regulated by intracellular signal transduction including mitogen-activated protein (MAP) kinase [8], and it is also associated with AKT signaling in colon cancer cells [9,10]. Inhibition of COX-2 expression with selective COX-2 inhibitors has been reported to effectively prevent proliferation, angiogenesis and induction of apoptosis in human colon cancer cells [11,12]. However, many of these anticancer drugs such as selective COX-2 inhibitors and nonsteroidal anti-inflammatory drugs have disadvantages either by having side effects or resistance of cancer to these chemotherapeutic drugs [13-15]. Thus, there has been a noticeable shift towards alternative therapeutic strategies using naturally available sources, recently. Many natural dietary phytochemicals present in fruits, vegetables and herb tea have been shown to be protective against cancer in vitro and in vivo.

Danggui Liuhuang Decoction (當歸六黃湯, DLD), a traditional Chinese medicine, known as Dangkwiyughwangtang (local name in korea), has been used as a treatment for fever, night sweats, red face, distress, dry mouth, and constipation. DLD consists of seven medicinal plants, i.e., Astragali Radix, Rehmanniae Radix preparata, Rehmanniae Radix, Angelicae Sinensis Radix, Scutellariae Radix, Coptidis Rhizoma, and Phellodendri Cortex [16,17].

Previous studies have reported that immunomodulatory effects of DLD in mice and anti-inflammatory effects in murine macrophage...
cell line. It has also been investigated that DLD can reverse premature ovarian failure. However, the effects of DLD on anti-tumor or anti-inflammation in human colorectal cancer cells and the mechanisms have not yet been investigated [18,19].

Therefore, we aimed to evaluate the effects of non-bioconverted DLD extract (DLD-E) and bioconverted DLD extract (DLD-BE) on carcinogenesis of human cancer cells and determine the possible mechanisms of action. In the present study, we evaluated the effects of DLD-E and DLD-BE on cell survival, cell proliferation, migration and cell colony formation in human colon cancer cell lines, and confirmed whether DLD-BE inhibits the expression of proteins that induces apoptosis and inhibit survival of HCT-116 or HT-29 cells compared to DLD-E. In addition to, we confirmed that the effect of DLD-BE depends on AKT signaling that inhibit cell proliferation and COX-2 expression in HT-29.

Materials and Methods
Preparation of Bioconverted Danggui Lihuahng Decoction
Seven medicinal herbs, the components of Danggui Lihuahng Decoction (DLD) extracts were purchased from Oriental Medicine Market in Korea (Daegu) and was refluxed twice with 3.5 L of 70% MeOH (a sample/MeOH ratio of 1:7 (w/w)). The extract was filtered through filter paper and concentrated using a Rotary evaporator (EYELA, Tokyo, Japan). The resulting extract was bioconverted using the enzyme isolated from the soybean paste fungi, *Aspergillus kawachii* (*A. kawachii*), according to a previous study with some modifications [20]. Briefly, extracts were resuspended in 200 mL of distilled water, and a 100 mL aliquot of the suspension was treated with 100 mL of active or inactive crude enzyme extract. The mixture was incubated with shaking at 100 rpm at 30 °C. Inactive crude enzyme prepared by autoclaving the mixture at 121 °C for 15 min served as the control.

Preparation of *A. kawachii* Enzyme
The *A. kawachii* enzyme was prepared as previously described [20]. Briefly, *A. kawachii* grown in potato dextrose agar medium (Difco Laboratories, Detroit, MI, USA) was inoculated into sterilized wheat bran and incubated at 30 °C for 3 days. The resulting mixture was suspended in sodium phosphate buffer (pH 7.0) and incubated for 18 h at 4 °C. The reaction mixture was then centrifuged, and the supernatant was used for fermentation of DLD extracts. The supernatant provided 0.276 U/mL (1 U is defined as the enzyme activity needed to produce 1 mmol of ρ-nitrobenzene from ρ-nitrophenyl-β-D-glucopyranoside per min) β-glucosidase activity [20].

Materials and Cell Culture
Human colon cancer cell line (HCT-116, HT-29) and rat normal intestinal cell line (IEC-6) were obtained from the Korean Cell Line Bank (Seoul, Korea). RPMI 1640 medium, fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from HyClone (Logan, UT, USA). Antibodies specific for COX-2, TNF-α, IL-1β and P-AKT were purchased from Cell Signaling (Beverly, MA, USA). Secondary antibodies (goat anti-rabbit and anti-mouse) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell viability was assessed using the CellTiter 96 Aqueous One Solution Assay kit (Promega, Madison, WI, USA) and primers for real time PCR were purchased from MBiotech Inc. (Hanam, Korea).

Measurement of Cell Viability and Proliferation Assay
Cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C with humidified air containing 5% CO₂, and plated at a density of 1×10⁴ cells/mL in 96-well plates for measurement of cell viability. The optical densities were measured at 490 nm using a microplate reader (Tecan System, San Jose, CA, USA) by MTS assay method. As a second method for determining the cytotoxicity of DLD-E or DLD-BE on cells we used the crystal violet assay. Upon solubilization, Cells (5×10⁴) were seeded in 6-well plates. They were incubated for 48 h with DLD-E or DLD-BE. After washing with phosphate-buffered saline (PBS) the cells were incubated and were slightly shaked at RT with staining solution (0.5% crystal violet, 20% methanol) which stains DNA. The plate was washed with dH₂O and dried completely. Image were acquired using phase contrast microscope (Nikon, Tokyo, Japan, magnification×100). The uptake crystal violet was solubilized by 30% acetic acid and the amount of dye was quantified by measuring the absorbance at 550nm in a microplate reader.

Measurement of Migration Assay
Cells were also seeded in a 6-well plate, allowed to reach 90% confluence, and then they were scratched using a 1000 mL pipet tip. After washing with PBS, serum-free medium containing DLD-E or DLD-BE were added to the cells, followed by 48 h incubation. The scratches were monitored, and images were acquired using a phase contrast microscope (Nikon, Tokyo, Japan).

Cell cycle and apoptosis analyses
HT-29 cells at 5×10⁵ cell/well were grown in a 6-well plate with different concentrations (50 and 100 μg/mL) of DLD-E or DLD-BE. Cells were harvested, washed with cold PBS, trypsinized, and centrifuged. Cells were suspended in 50 μL cold PBS, 450 μL of cold ethanol was added, and incubated at 4 °C for 1 h. After centrifugation at 1000 rpm for 5 min, the pellet was washed with cold PBS, resuspended in 500 μL PBS, and then incubated with 5 μL RNase (20 μg/mL final concentration) at 37 °C for 30 min. The cells were chilled on ice for 10 min and incubated with propidium iodide at a final concentration of 50 μg/mL for 1 h in the dark. Cell cycle distribution was determined by flow cytometry.
and DNA histograms for cell cycle analysis were determined using the FlowJo software (Tree Star, San Jose, CA, USA).

**RNA extraction and real time (RT)-PCR**
Total RNA was isolated from HT-29 cells using TRI Solution™ according to the manufacturer’s instructions (BSK Bioscience, Gyeongbuk, Korea). cDNA was synthesized from 1 μg of total RNA using OligodT15 and Goscript™ Reverse transcription system kit (Promega, Madison, WI, USA). For RT-PCR, the primers were purchased from M-Biotech Inc. (Daejeon, Korea).

The RT-PCR reaction was carried out on the StepOne Plus™ (Applied Biosystems, Foster City, CA, USA) using HotStart® SYBR® Green qPCR Master Mix (USB, Cleveland, OH, USA). Each PCR cycle consisted of the 3 following steps: 95°C for 2 min, 95°C for 5 s, 60°C for 30 s. The results of RT-PCR were presented as pro-inflammatory cytokine gene (COX-2, TNF-α, IL-1β, IL-6) induction fold, and these were calculated using β-actin, which was amplified under the same conditions, as an internal control.

**Western blot analysis**
Cells (1×10⁶ cells/well) were treated with various concentrations of DLD-E or DLD-BE and incubated for 24 h. For the total protein extract, cells were washed once with 10 mM PBS (pH 7.4) containing 150 mM NaCl and then lysed with PROPREP™ Protein Extraction Solution (iNtRON Biotechnology, Daejeon, Korea) according to the manufacturer’s protocol. Thirty micrograms of protein were applied to 10% SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes in 20% methanol/25 mM Tris/192 mM glycine. Next, the membranes were blocked with 5% non-fat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20) and then probed with various first antibodies. After 1 h of incubation followed by three washes, the membranes were incubated for 1 h with a secondary HRP-conjugated antibody. The protein bands were then visualized using an ECL system. The densities of the bands were measured with the ImageQuant LAS 4000 luminescent image analyzer and ImageQuant TL software system (GE Healthcare, Little Chalfont, UK). The GAPDH or total MAP kinase-normalized relative band intensity was presented.

**HPLC analysis**
The chromatographic system was composed of Agilent Technologies 1260 Infinity LC system coupled to SPD-M20A diode array detector. Detection and quantification were performed using Chemstation software. Each sample was analyzed on a Kinetex C18 column of 150 mm×4.6 mm, 2.6 μm size (Phenomenex, Milford, CA, USA). The mobile phase consisted of water (A) and acetonitrile (B), which were applied in the gradient elution as follows: 20% Solvent B at 0 min, 20% B at 2 min, 80% B at 46 min. The flow rate of the mobile phase was 1.0 mL/min, and the detection wavelength was 210 nm. Calycosin and Wogonin were commercially available from Sigma and ChromaDex (Irvine, CA, USA).

**Statistical analysis**
The statistical analyses were performed using SPSS for Windows, version 18 (Chicago, IL, USA). Treatment effects were analyzed by one-way ANOVA, offered by Tukey’s multiple range tests.

*p* <0.05, **p** <0.01 and ***p*** <0.001 was considered statistically significant.

**Results and Discussion**

**DLD-BE significantly inhibited cell proliferation and migration and induced apoptosis in human CRC cells**
Apoptosis plays a pivotal role in controlling cell proliferation and migration, hence is significant to the prevention of cancer pathogenesis [21]. The Bcl-2 family proteins, including the anti-apoptotic Bcl-2 members and pro-apoptotic Bax members, are the most characteristic regulators of mitochondria-mediated apoptosis.

Bcl-2 conserves the integrity of the outer mitochondrial membrane and thereby, prevents the release of pro-apoptotic members from the mitochondria. The caspases play a critical role during apoptosis, particularly caspase-3. The activation of caspase-3 is necessary for efficient apoptosis. Activated caspase-3 induces degradation of proteins, such as PARP, which are involved in the morphological features of apoptosis. When PARP is cleaved and inactivated by caspase-3, the cleaved PARP level is increased, indicating that the cell is undergoing apoptosis [22,23].

In the current study, the HT-29 colorectal cancer cells were treated with DLD extracts for 24 h before and after bioconversion and the effects on cell proliferation and migration were compared. The results showed that DLD-E and DLD-BE inhibited the proliferation of cancer cells in a dose-dependent manner compared to the control group that was not treated with DLD-E or DLD-BE and that DLD-BE was more effective than DLD-E. In addition, DLD-BE treatment induced a sub G1 phase suggestive of apoptotic cell death by stopping the progression from the G0 phase to the G1 phase of the cell cycle. DLD-E and DLD-BE treatment also had no effect on a normal intestine cell line.

Since intracellular signals are led to apoptosis by DLD-BE, it decreased the expression of Bcl-2 protein, which inhibited apoptosis and increased Bax protein expression compared to the control group. The expression of these molecules appears to increase the permeability of the mitochondrial membrane and increase the release of cytochrome c. The expression of cleaved caspase-3 and cleaved PARP protein was also increased by the activation of caspase-3. The results confirmed that DLD-BE is involved in the inhibition of cell proliferation by inducing apoptotic signals in HT-29 cells.
Fig. 1 Effect of non-bioconverted DLD extract (DLD-E) and bioconverted DLD extract (DLD-BE) on cell colony formation in colon cancer cells, compared to a normal intestinal cell line. Cells were stained with crystal violet, and then images were acquired using phase contrast microscope (A). Quantification of stained cell colonies was measured in 550 nm (B). Values are expressed as the means ± standard deviation of three separate experiments. **p < 0.05, ***p < 0.005 vs. control. Cell migration was determined by the wound healing assay and the wounds were photographed at 0 and 48h after creating the wound using an optical microscope (C). Original magnification (40X)
DLD-E and DLD-BE suppressed the expression of COX-2, TNF-α, and IL-1β in HT-29 cells

COX-2 is rarely expressed in normal tissues, but is overexpressed in various cancers, including colorectal cancer, and has been reported to reduce the survival rate of colorectal cancer patients. It has been recognized as an important target for the prevention...
and treatment of colorectal cancer [4,7]. It is also associated with the overall mechanisms of cancer growth, such as tumorigenesis and development, inhibition of apoptosis, angiogenesis and metastasis, and the inflammatory response of pro-inflammatory cytokines, such as TNF-α and IL-1β, that contribute to colorectal cancer [5-7].

In this study, COX-2 protein was constitutively expressed in HT29 cells, although not expressed in IEC-6 cells. We showed that DLD-E and DLD-BE suppressed the over-expressed COX-2 protein and mRNA levels in HT-29 cells while reducing the protein and mRNA expression levels of TNF-α and IL-1β. It was confirmed that DLD-BE was more effective than DLD-E.

**Fig. 4** Effect of non-bioconverted DLD extract (DLD-E) and bioconverted DLD extract (DLD-BE) on the phosphorylation of AKT and ERK in HT-29 cells, and change of COX-2 expression by inhibition of AKT pathway (A). Treatment with LY294002 (an AKT inhibitor) decreased cell proliferation of HT-29 cells, while a combined treatment with LY294002 and DYT-BE resulted in even further inhibits proliferation. Celecoxib, an effective nonsteroidal anti-inflammatory drug was used to compare the activity of DYT-BE (B). *p <0.5, **p <0.05, ***p <0.005 vs. control

**COX-2 regulation and apoptosis induction by DLD-BE are mediated by the AKT pathway**

Many studies have suggested that mutations in phosphatidylinositol 3-kinase (PI3-K)/Akt and mitogen-activated protein kinase (MAPK)/ERK molecules are commonly observed in various types of cancer. Suppression of the PI3-K/Akt and MAPK/ERK signaling pathways leads to the blockade of cell proliferation, demonstrating the importance of these signaling cascades in the control of both cell cycle progression and cell growth during cancer development [24,25]. Therefore, the Akt and MAPK/ERK mechanisms play dominant roles in determining the fate of tumor growth. Studies have reported that COX-2 is derived from
Prostaglandins, particularly PGE2, and these signaling systems are associated with EGFR-PI3K-Akt, Ras-MAPK, PPAR, VEGF, Bcl-2, chemokines, canonical Wnt signaling systems, and the activation of these receptors [26-28].

Phytochemicals, such as selenium, curcumin, and EGCG, have been reported to inhibit the activation of Akt signaling, thereby inhibiting cancer cell proliferation, inducing apoptosis, and inhibiting tumor growth in vitro [29]. Therefore, regulation of the Akt pathways can play an important role in suppressing the abnormal proliferation of cancer cells.

In this study, we found that phosphorylation of AKT and ERK in HT-29 cells was inhibited by treatment with DLD-BE for 24 h. In particular, the results showed that DLD-BE responded more specifically than DLD-E to inhibit the phosphorylation of AKT in the HT-29 cells. Cell proliferation was measured by treatment with LY294002, an AKT inhibitor or celecoxib, a COX-2 inhibitor, alone or in combination with DLD-BE for 24 h. The results confirmed that cell proliferation was more strongly inhibited by combined treatment with LY294002 (40 μM) and DLD-BE (50 μg/mL) or celecoxib (25 μM) and DLD-BE (50 μg/mL), compared to treatment with each agent alone.

Our findings suggest the potential of DLD-BE as a combination treatment or adjuvant anticancer agent for colorectal cancer that could have synergistic effects with existing anticancer drugs.

Bioconversion resulted in a significant change in compounds in the DLD extract

Bioconversion process is considered to be the key factor controlling the quality of herbal extracts and enhances the therapeutic effects of herbal medicine by changing the secondary metabolite composition and altering the mechanisms that are responsible for biological activity [30-32]. The production of a chemical compound that has pharmacological activity through bioconversion processes has great industrial interest due to its large scale production feasibility and environmental value as green chemistry.

In the present study, DLD-E and DLD-BE were analyzed by HPLC to evaluate changes occurring from the bioconversion of DLD. The HPLC analysis showed that components not seen in DLD-E were detected in DLD-BE. These components were identified as calycosin (compound 1) and wogonin (compound 2). Calycosin derived from the dry root extract of Radix Astragali is known to exhibit a variety of biological effects that easily undergo extensive phase II metabolism [33]. It is reported to stimulate the apoptosis of CRC cells and inhibit their invasion by acting as an SIRT1 activator, which induces the activation of AMPK-induced inhibition of the Akt/mTOR signaling pathway [34]. Wogonin is a plant monoflavonoid that has been reported to inhibit cell growth and/or induce apoptosis in various tumors and significantly induces apoptosis via the regulation of Bcl-2 family proteins and activation of caspases in HT-29 cells [35]. These results suggest that bioconversion resulted in a significant change in compounds in the DLD extract.

In conclusion, we compared the anti-cancer efficacy and possible anti-cancer mechanisms of DLD-E and DLD-BE in HT-29 human colorectal tumor cells. DLD-BE inhibited cell proliferation...
and migration and induced apoptosis by activating apoptosis-inducing molecules in HT-29 cells. The mechanism involved inhibition of the AKT/ERK signaling pathways and the suppression of key markers of colon cancer development, such as COX-2, TNF-α, and IL-1β. Thus, we propose that the bioconverted extract of DLD using Aspergillus kawachii can be developed as a potent supplement to inhibit colorectal tumor growth and intestinal tumor growth and inflammatory inflammation.

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