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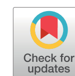
Extract of *Lactobacillus plantarum* strain 06CC2 induces JNK/p38 MAPK pathway-mediated apoptosis through endoplasmic reticulum stress in Caco2 colorectal cancer cells



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ABSTRACT

Colorectal cancer is a multi-factorial disease involving genetic, environmental and lifestyle risk factors. In recent years, many changes in the bacterial composition of the intestinal microflora have been reported in colorectal cancer, suggesting the involvement of the intestinal microflora in the development and progression of colorectal cancer. Along with these reports, research on lactic acid bacteria that have a beneficial effect on the human body for the purpose of improving the intestinal environment and treating intestinal diseases has advanced. Among these studies, biogenics (defined as a component derived from lactic acid bacteria that acts directly on diseases regardless of the state of intestinal microflora) is a recent concept derived from the work on probiotics. Based on this concept, it is important to evaluate the effectiveness of various components derived from lactic acid bacteria in the treatment to diseases from and apply them in prevention and treatment. In this study, we investigated the antitumor effect of an extract obtained from *Lactobacillus plantarum* strain 06CC2 on colorectal cancer cells. In in vitro experiments, the extract derived from *Lactobacillus plantarum* 06CC2 significantly suppressed the proliferation of Caco2 colorectal cancer cells in comparison to control and non-cancer cells. Furthermore, we found that endoplasmic reticulum stress and the JNK/p38 MAPK signaling system are involved in the induction of apoptosis. These findings indicate the direct antitumor effect of the *Lactobacillus plantarum* 06CC2 extract on Caco2 colorectal cancer cells, and that this extract may have potential application as a biogenics.

1. Introduction

Colorectal cancer is a multi-factorial disease involving genetic, environmental and lifestyle risk factors, and is the fourth most common cause of cancer-related death worldwide [1]. In colorectal cancer patients, abnormal changes have been reported in the intestinal microflora in comparison to healthy control group [2,3], suggesting that the perturbation of intestinal microflora is closely correlated with the initiation and progression of colorectal cancer cells. From these reports and the viewpoint of treatment for colorectal cancer, studies of lactic acid bacteria (LAB) with probiotic properties have attracted attention. Probiotics are defined as living microorganisms giving a beneficial effect for host health by improving intestinal microflora balance and are associated with various health benefits, including the conditioning of the intestinal microflora, suppression of excess allergic responses and tumor-suppressive effects [4,5]. Furthermore, it has been reported that the useful biological activity of LAB can be exerted by using not only living bacteria but also the metabolites of bacteria and dead bacteria [6–8]. Taking these reports into account, a new concept, biogenics, which makes use of bioactive substances as foods or medicine, has recently been advocated [9]. Biogenics are food ingredients that have direct beneficial physiological effects without the involvement of the intestinal microflora. Thus, it is hoped that various LAB-derived substances, such as bacterial components, extracellular products and fermented products will be applied to disease and cancer treatment [10–12].

Recent studies have demonstrated that endoplasmic reticulum (ER) stress is involved in cancer, metabolic disorders, inflammatory diseases, and neuro-degenerative diseases [13,14]. ER stress is caused by the accumulation of misfolded or unfolded proteins, called the unfolded protein response (URP), which activates the following three specific stress transducers: protein kinase RNA-like endoplasmic reticulum kinase (PERK); inositol-requiring enzyme 1 (IRE1); and activating transcription factor 6 (ATF6). These factors also induce the phosphorylation

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of the eukaryotic translation initiation factor 2 subunit α (eIF-2α)/ATF4/CHOP signaling pathway and JNK/p38 MAPK signaling pathway, which has multiple downstream targets that stimulate apoptosis and cell death [15]. Understanding these signaling pathways is important for evaluating the apoptosis induced by antitumor activity.

Therefore, the aim of this study is to investigate the antitumor effects and its action mechanism of the extract derived from Lactobacillus plantarum strain 06CC2 (LP06CC2) for colorectal cancer cells. The 06CC2 strain was isolated from traditional Mongolian dairy products and has been reported to have probiotic activity [16–18]. However, there have been no reports on its antitumor effects. In the present study, we demonstrated that an extract derived from the 06CC2 strain suppressed the proliferation of colorectal cancer Caco2 and HT29 cells, while not significantly suppressing the proliferation of non-cancer IEC6 and IEC18 cells. Notably, the growth of Caco2 cells was significantly suppressed and the features of apoptosis such as the activation of caspase and decreasing expression of anti-apoptotic factor Bcl2 were observed. In addition, the increasing expression of CHOP protein, indicating ER stress response, and enhanced phosphorylation of JNK/p38 MAPK were also observed in Caco2 cells. From these results, it is suggested that ER stress and the JNK/p38 MAPK signal transduction system are involved in apoptosis induced by the LP06CC2 extract.

2. Materials and methods

2.1. Preparation of extract from L. plantarum strain 06CC2

Powdered heat-killed *L. plantarum* strain 06CC2 was obtained from Minami Nihon Rakuno Kyodo. To obtain the extract from the LP06CC2 strain, the LP06CC2 powder was well suspended in PBS and then incubated with rotation for 1 h. After centrifugation, all insoluble bacterial bodies and debris were removed from the supernatant using a 0.22-μm sterile filter membrane.

2.2. Cells and cell culture

The human colorectal cancer cell lines (Caco2 and HT29) and normal rat small intestine cell lines (IEC18 and IEC6) were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in Dulbecco's modified Eagle Medium (Gibco, Gland Island NY) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Gland Island NY) in a humidified 5% CO_2 atmosphere at 37 °C.

2.3. Cell viability assay

The cytotoxicity of several cells was determined by Cell Count Reagent SF using WST-8 as a chromogenic substrate (Nacalai Tesque, Kyoto, Japan) [19]. The cells were seeded in 12-well plates and cultured with PBS or *L. plantarum* extract for 24, 48 and 72 h. At the end of treatment, the media were replaced with fresh medium and added mixed solution, including WST-8 and 1-Methoxy PMS. After incubation at 37 °C for 1 h, the media supernatants were measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. TUNEL staining

The cells were plated on a collagen-coated cover glass. The cover glass was fixed in 4% paraformaldehyde and washed extensively with PBS. The cover glass was stained using an *In Situ* Cell Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The cells were mounted with anti-fade mounting medium with DAPI, and the TUNEL-positive cells were visualized by fluorescence microscopy (KEYENCE Corporation).

2.5. Annexin V-FITC/PI double-stained assay

Apoptosis was assessed using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan) according to manufacturer's instructions. Briefly, the cells were seeded in 60-mm dishes and cultured with PBS or *L. plantarum* extract. The cells were collected and stained in binding buffer with 5 μl of PI solution and 10 μl of FITC-conjugated annexin V for 15 min in the dark at room temperature. Apoptotic cells were detected with a CytoFLEX flow cytometer (Beckman Coulter, Tokyo, Japan) and the data were analyzed by the FlowJo software program (version 10, FlowJo, Ashland, OR, USA).

2.6. Western blotting and antibodies

The cells were harvested with RIPA buffer containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were incubated with rotation for 20 min at 4 °C and cell debris was removed by centrifugation at 14,000 rpm for 30 min at 4 °C. The supernatant containing the total cellular protein was collected. The protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) based on the Lowry assay method. Equal amounts of the protein samples (40 μg) were loaded onto 4–20% gels (Bio-Rad, Hercules, CA, USA) and electrophoresis was performed. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and the membranes were blocked with super block T20 blocking buffer (Thermo, Rockford, IL). The membranes were then incubated with primary antibodies overnight. After washing, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibody at room temperature. The proteins were visualized with chemiluminescence detection using ECL Western blotting detection reagent (GE Healthcare Life Sciences, Marlborough, MA, USA).

The primary antibodies against βactin (#4970), cleaved-caspase 3 (#9664), cleaved-caspase 9 (#9501), Bcl-2 (#2876), Bim (#2933), PERK1 (#5683), IRE1α (#3294), eIF2α (#9079), p-eIF2α (#5199), ATF4 (#11815), CHOP (#2895), p-MKK4 (#4514), JNK (#9252), p-JNK (#4668), p38 (#9212), p-p38 (#4511), p-ATF2 (#5112) and p-c-Jun (#2361) were purchased from Cell Signaling Technology (Beverly, MA, USA). The HRP-conjugated anti-mouse and -rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.7. Real-time PCR

Total RNA from cells was prepared using TRIzol (Invitrogen). Aliquots of RNA were then applied to synthesize the first strand cDNA using a Prime Script RT Regent Kit (TaKaRa, Dalian, China). To quantify the transcriptional levels of Bim and Bcl-2, a qRT-PCR was performed according to the recommendations of the TB Green Premix EX *Taq*II Kit (TaKaRa, Dalian, China). The specific primer pairs used for amplification were as follows:

Bim (Forward, 5′-CATCATCGCGGTATTCGGTTC-3'; Reverse, 5′-AAGGTTGCTTTGCCA TTGGTC-3′), Bcl-2 (Forward, 5′-AACATCGCC CTGTGGATGAC-3'; Reverse, 5′-AGAGTCTTCAGAGACAGCCAG GAG-3′). For normalization, βactin was used as an endogenous control. The expression changes were quantified using the $2^{-\Delta\Delta CT}$ formula.

2.8. Statistical analysis

The results are presented as the mean ± standard deviation (SD) from three independent experiments. The assay data were analyzed by Student's t-test using the SPSS software program (SPSS Inc., Chicago, IL, USA). P values of < 0.05 were considered to indicate statistical significance.

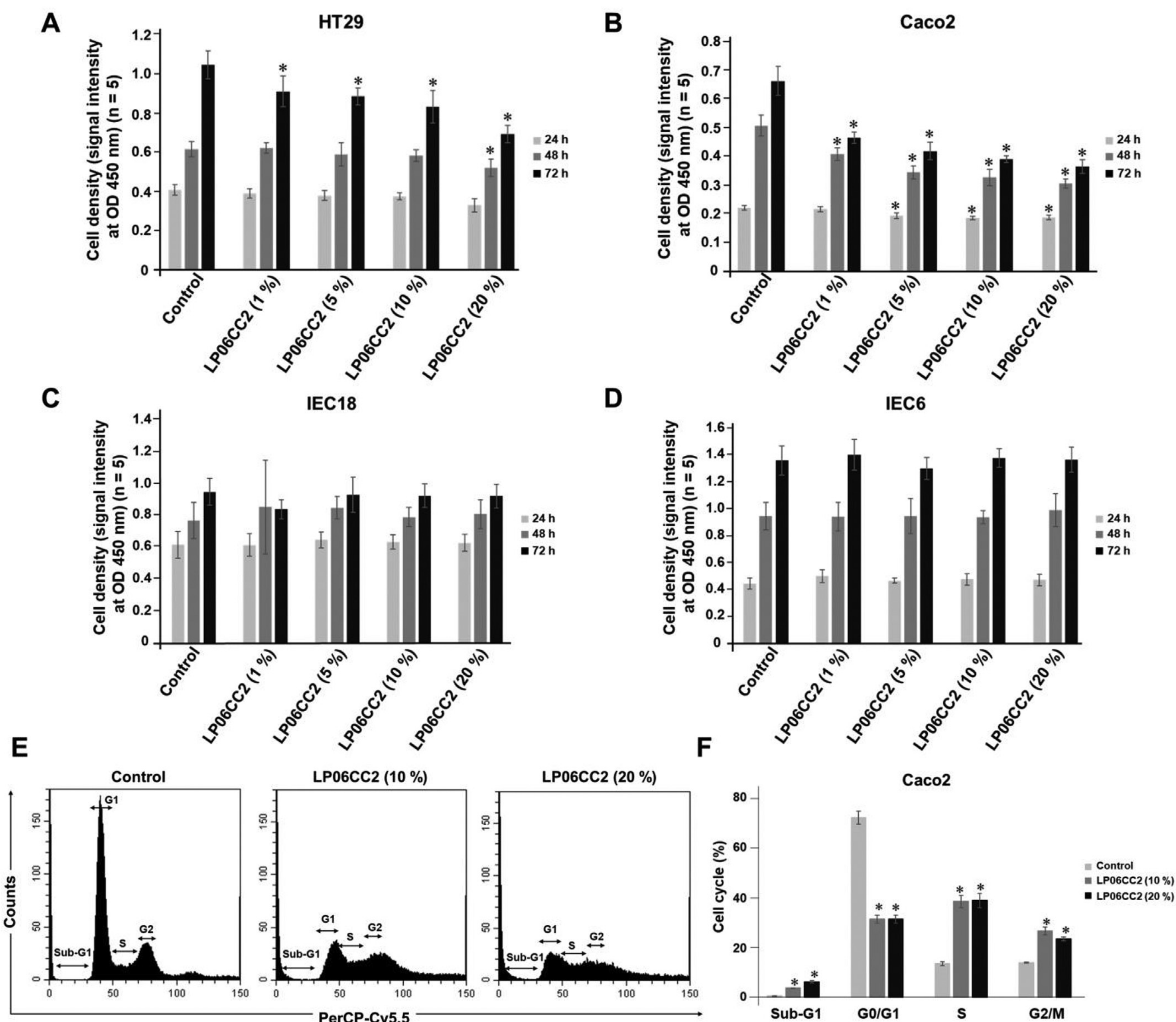

Fig. 1. The effect of the extract derived from the *L. plantarum* 06CC2 strain on the proliferation of colon cancer cells (A, Caco2 cells; B, HT29 cells) and non-cancer cells (C, IEC18 cells; D, IEC6 cells). The LP06CC2 extract was added in range of 1–20% of the total medium volume and cells were incubated for 24, 48 and 72 h. Cells treated with PBS (20%) were used as a control group. Data are presented as the mean ± SD of at least three independent experiments. *, $P < 0.05$, in comparison to the control group.

3. Results

3.1. The extract derived from L. plantarum strain 06CC2 suppresses the proliferation of human colon cancer cells

First, we examined whether the extract obtained from the heat-killed *L. plantarum* strain 06CC2 (LP06CC2) has the effect of suppressing the proliferation of cancer cells. Colorectal cancer cells (Caco2 and HT29 cells) were incubated with the LP06CC2 extract added in the range of 1–20% of the total medium volume for 24, 48 and 72 h. A cell viability assay indicated that the LP06CC2 extract suppressed the growth of Caco2 and HT29 cells in a dose-dependent manner (Fig. 1A and B). Notably, the growth of Caco2 cells was significantly suppressed in comparison to control cells (Fig. 1A). Next, to assess the toxicity of extract on non-cancer cells, the LP06CC2 extract was incubated with IEC18 and IEC6 cells derived from the normal rat small intestine. Under the same conditions as the experiments using colon cancer cells, the LP06CC2 extract was not observed to have a toxic effect on cell growth in these non-cancer cells (Fig. 1C and D). These results suggest that the LP06CC2 extract suppressed the cell growth of colon cancer cells, while exhibiting less harmful effects in non-cancer cells.

3.2. L. plantarum strain 06CC2 induces mitochondrial-mediated apoptosis in Caco2 cells

To clarify the effects of LP06CC2 on colorectal cancer cells, Caco2 cells were treated with the LP06CC2 extract and apoptotic cells were detected by a TUNEL-staining assay (Fig. 2). The number of TUNEL-positive cells in the LP06CC2 extract-treated cells was higher than that in the control cells (Fig. 2A TUNEL lane and B). The number of DAPI-stained cells among LP06CC2 extract-treated cells was also greatly decreased (Fig. 2A DAPI lane). Similarly, in an Annexin V-FITC/PI double-staining assay, the numbers of early-apoptotic cells (Q3: Annexin-FITC positive) and late-apoptotic cells (Q2: PI and Annexin-FITC double

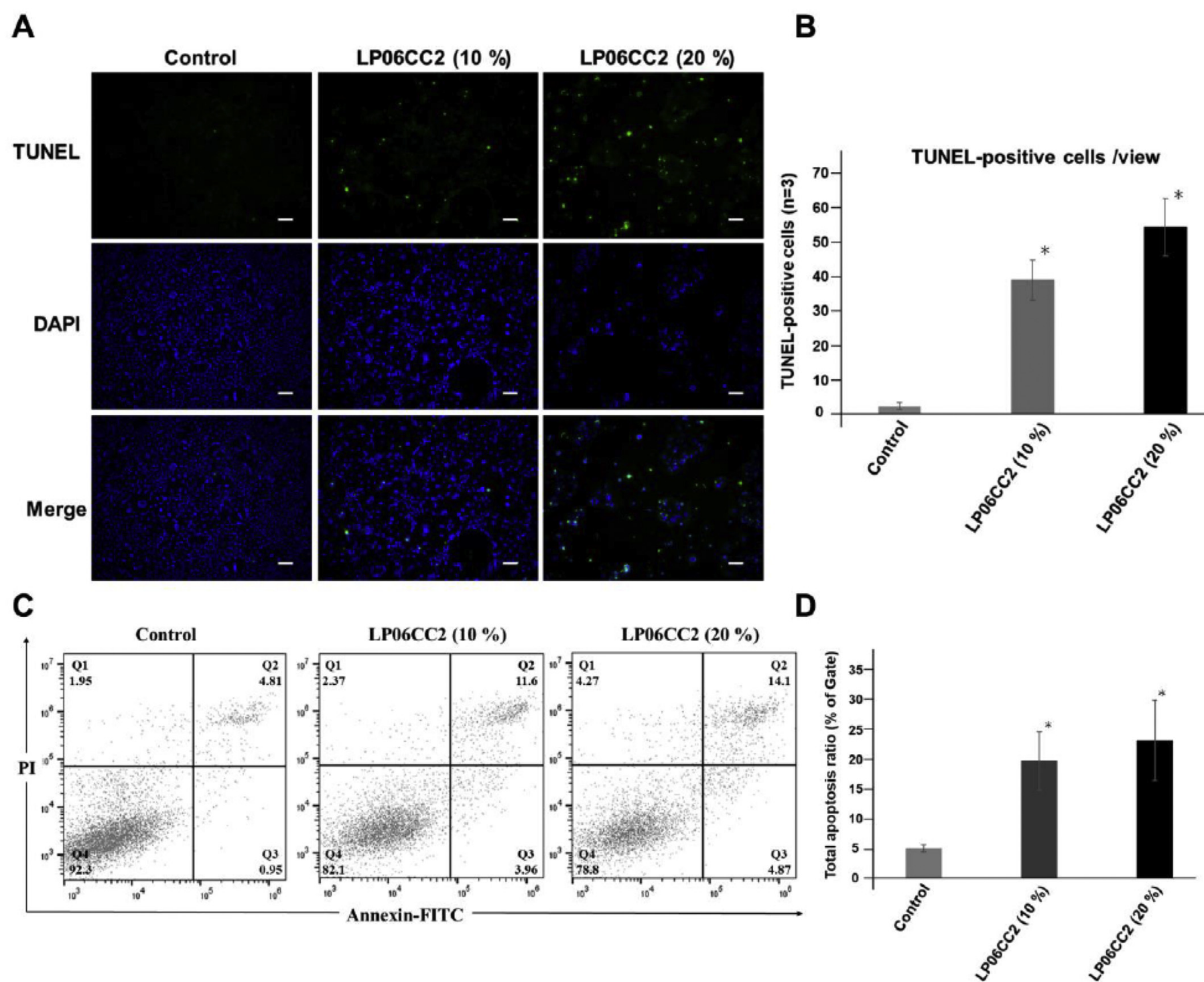

Fig. 2. The LP06CC2 extract induced apoptosis in Caco2 colon cancer cells. (A) Apoptosis of Caco2 cells was increased by the LP06CC2 extract. DAPI staining for nuclear visualization (blue) and TUNEL staining (green) were performed, and cells were observed by fluorescence microscopy. Bars, 100 μm. (B) The quantification of TUNEL-positive cells. The number of cells with TUNEL-positive nuclei was counted as the number of apoptotic cells. (C) Flow cytometry was performed with Annexin V-FITC/PI staining. The upper left quadrant (Q1) shows necrotic cells and cellular debris. The upper right quadrant (Q2) shows late apoptotic cells. The lower right quadrant (Q3) shows early apoptotic cells. The lower left quadrant (Q4) shows normal cells. (D) The total apoptotic cell rate was calculated as the rate of both early and late apoptotic cells. Data are presented at the mean ± SD of at least three independent experiments. *, $P < 0.05$, in comparison to the control group.

positive) were increased in a dose-dependent manner in comparison to the control cells (Fig. 2C and D). We also examined the expression of apoptotic-marker proteins (Fig. 3). A Western blotting analysis revealed that the expression of cleaved-caspase 9 and 3 was markedly increased in the LP06CC2 extract-treated cells (Fig. 3A). In order to evaluate the markers of mitochondria-mediated apoptosis, the expression levels of Bcl-2 and Bim (Bcl-2 family proteins) were examined. The expression of Bim_L and Bim_S (isoforms of Bim protein) were increased in extract-treated cells (Fig. 3A). In accordance with the expression of these proteins, the Bim mRNA level was also increased in the cells (Fig. 3B a). In contrast, the Bcl-2 protein and mRNA expression levels were decreased in LP06CC2 extract-treated cells (Fig. 3A and Bb). These data indicated that the extract derived from the LP06CC2 strain exhibited its tumor-suppressive effect through the induction of apoptosis in colon cancer cells.

3.3. The endoplasmic reticulum stress response and the activation of JNK/p38 MAPK signaling are induced in Caco2 cells by L. plantarum strain 06CC2

Endoplasmic reticulum (ER) is essential for most cellular activities and survival [20]. In order to investigate the role of ER stress in the colon cancer cell apoptosis induced by the LP06CC2 extract, we further analyzed marker proteins involved in ER stress using Western blotting. While the protein expression levels of the PERK1 and eIF2α proteins were not altered in comparison to the control, the IRE1α protein level was tended to slightly increase in cells treated with LP06CC2 extract (Fig. 4A). In addition, the phosphorylated eIF2α (p-eIF2α), ATF4 and CHOP protein levels were significantly increased in Caco2 cells after treatment with LP06CC2 extract (Fig. 4A). The results suggest that the LP06CC2 extract activated the ER stress signaling pathway. Next, we examined the activation of the JNK/p38 signaling pathway. The JNK/p38 signaling pathway is involved in many aspects of the control of cellular proliferation and apoptosis and is reported to be associated with the activation of the ER stress response [15]. In Caco2 cells treated

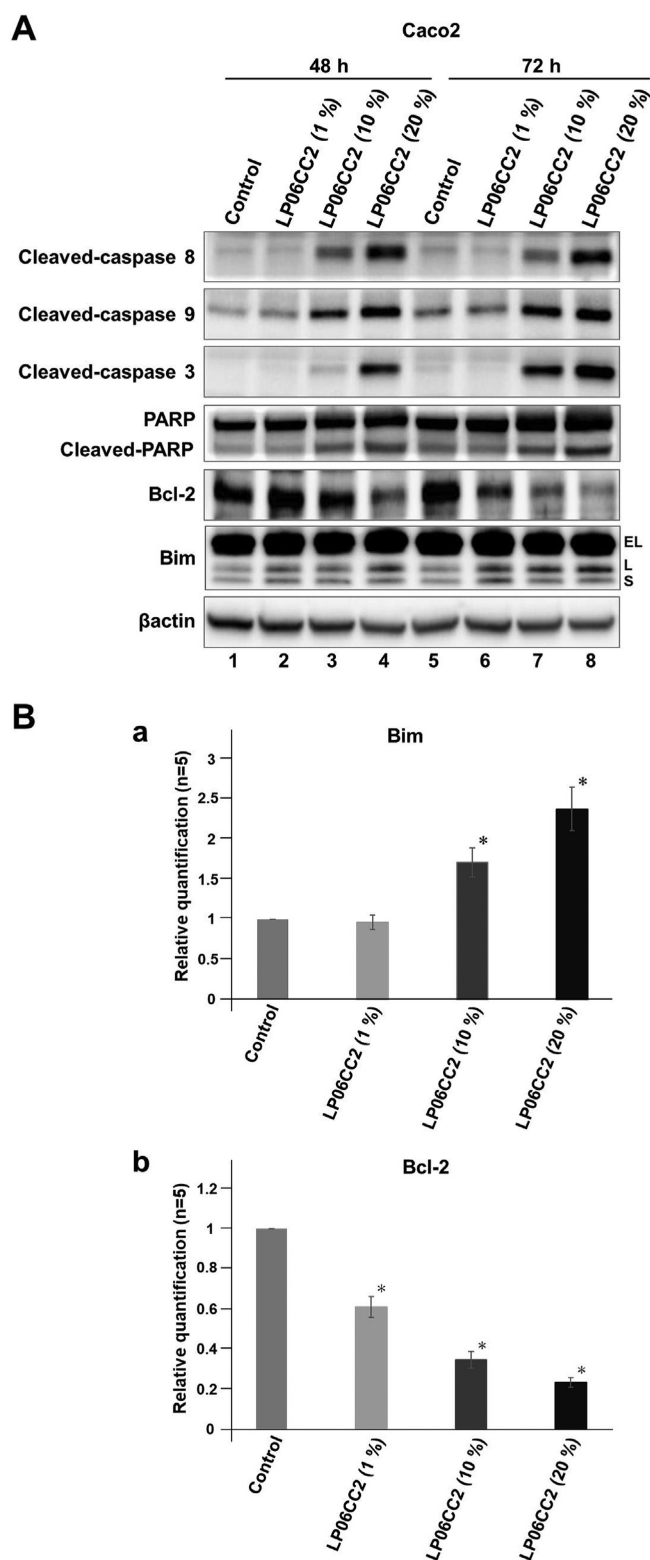

Fig. 3. The activation of the caspase cascade and mitochondria dysfunction induced by the LP06CC2 extract. (A) Cell lysates were subjected to Western blotting with anti-cleaved caspase 3, anti-cleaved caspase 9, anti-Bcl-2, anti-Bim, and βactin antibodies. βactin was used as the loading control. (B) The Bim and Bcl-2 mRNA expression levels were assessed by a quantitative RT-PCR. Data are presented as the mean ± SD of at least three independent experiments. *, $P < 0.05$, in comparison to control.

with LP06CC2 extract, the levels of phosphorylated JNK (p-JNK) and p38 (p-p38), indicating the activation of JNK/p38 signal transduction, were significantly increased in a dose-dependent manner (Fig. 4B). Similarly, the levels of phosphorylated ATF2 (p-ATF2) and c-Jun (p-c-Jun), which are transcription factors located downstream of the JNK/p38 MAPK signaling cascade, were increased in these cells in comparison to control cells (Fig. 4B). These results suggested that the extract of *L. plantarum* strain 06CC2 induced apoptosis through the ER stress response and the JNK/p38 MAPK signaling pathway.

4. Discussion

The present study revealed that the extract of *L. plantarum* strain 06CC2, which included no insoluble bacterial body or debris components, inhibited the cell growth of colon cancer cells, suggesting that the extract had a tumor-suppressive effect. Notably, the growth of Caco2 colorectal cancer cells was significantly suppressed by the LP06CC2 extract (Fig. 1A). In previous studies, Caco2 cells were reported to show resistance to drugs used in the clinical setting, such as 5FU, due to the inactivation of the caspase cascade induced by mutation of the p53 gene [21,22]. In contrast, the LP06CC2 extract was shown to induce the activation of caspase and apoptosis in Caco2 cells in our experiment (Figs. 2 and 3). Given these previous reports, our results suggest that the components included in the LP06CC2 extract may induce cell death independently of p53 activation. The present study also demonstrated that the LP06CC2 extract did not affect the cell growth of IEC18 and IEC6 cells derived from the normal rat small intestine, suggesting that the extract had a lesser effect on non-cancerous cells (Fig. 1C and D). From these results, it is possible that extracted components derived from LP06CC2 can be applied to the development of anti-cancer drugs and cancer therapy. However, in this study, we did not clarify the substances with antitumor effects that were contained within the LP06CC2 extract. In order to utilize the LP06CC2 strain in the study of anticancer drugs it will be important to identify a substance that has an antitumor effect. Additionally, these data were obtained with the direct treatment of cancer cells. The stability and delivery of substances within the LP06CC2 extract *in vivo* remain problems in relation to its use in the clinical setting. Thus, it is necessary to determine a suitable method of delivery and to clarify the metabolism of the components of LP06CC2 *in vivo* in order to be used in the development of anticancer drugs and effective cancer therapeutics.

Investigating the mechanism underlying the apoptosis of cancer cells and the associated signaling pathway is important for evaluating the antitumor effects on cancer cells. In this study, we showed that the induction of apoptosis by the LP06CC2 extract was mediated by Bcl-2 family proteins, which was activated by endoplasmic reticulum stress and the JNK/p38 MAK kinase pathway (Figs. 3 and 4). The function of the Bcl-2 family proteins in mitochondria have come to be regarded as playing a central role in the triggering of apoptosis [23,24]. The Bcl-2 family proteins such as Bcl-2 and Bcl_{XL} contribute to controlling the permeability of the outer membrane of mitochondria and work to suppress apoptosis, while other factors, such as Bax, Bak, Bad and Bim promote apoptosis [25]. Among these apoptosis promoting factors, Bim has three isoforms—Bim_{EL}, Bim_{L} and Bim_{S}—among which the expression levels of Bim_{L} and Bim_{S} are increased with the promotion of apoptosis [26]. The present study showed that the Bim protein expression and the mRNA level in Caco2 cells were elevated after treatment with LP06CC2 extract (Fig. 3). At the same time, the protein and mRNA levels Bcl-2, an anti-apoptotic factor, were decreased (Fig. 3). Furthermore, the regulation of Bim and Bcl2 activity in the apoptosis process is mediated by the JNK/p38 signaling pathway through the ER stress response. PERK1, one of the receptors that recognizes endoplasmic reticulum stress, phosphorylates eIF2α and increases the expression levels of transcription factors ATF4 and CHOP [27]. The transmembrane protein IRE1α is activated by the accumulation of unfolded proteins in the ER and stimulates JNK/p38 MAPK, promoting

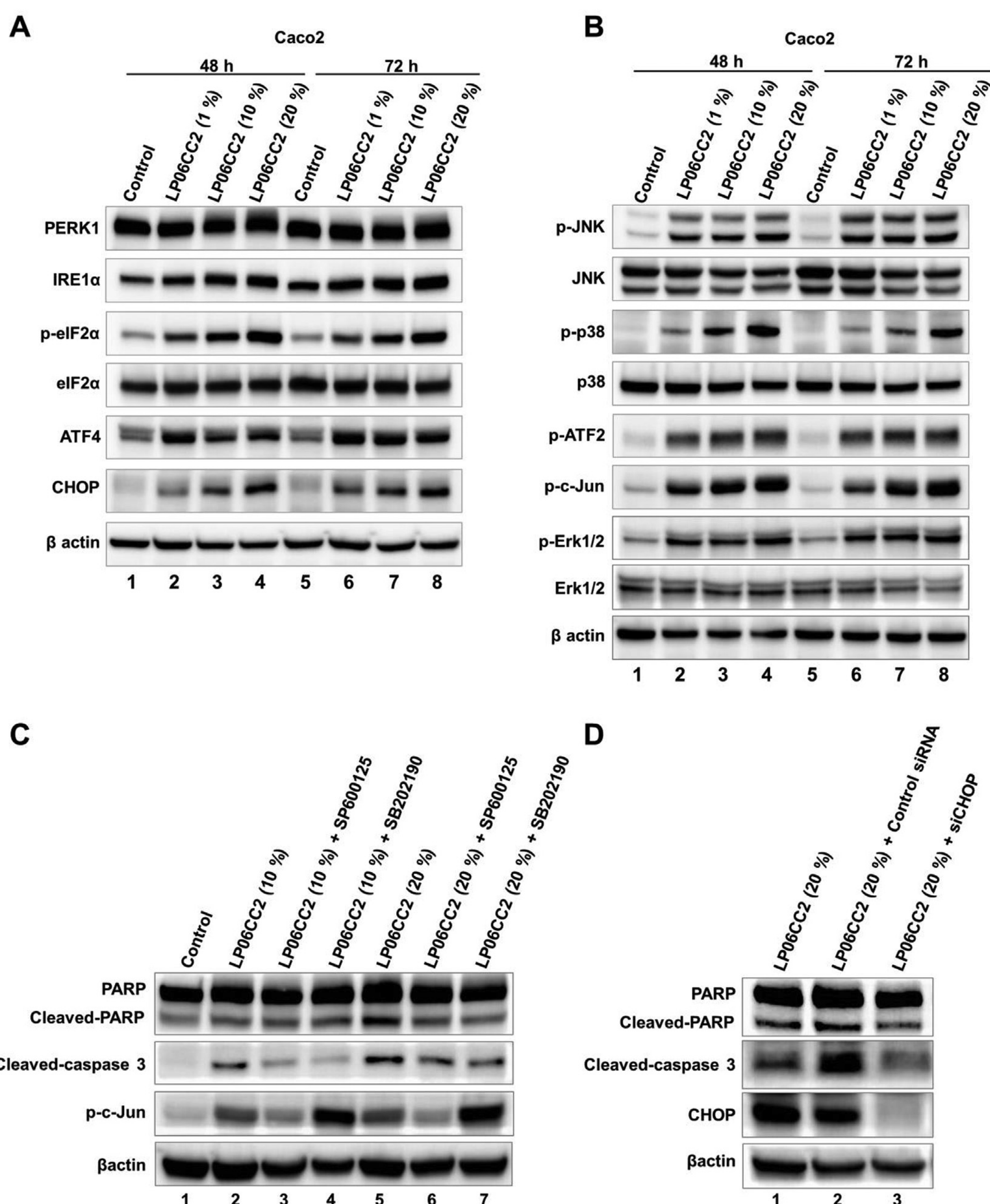

Fig. 4. The LP06CC2 extract induced endoplasmic reticulum stress and JNK/p38 MAPK pathway activation in Caco2 cells. (A) The protein expression of ER stress markers (PERK1, IRE1α, eIF2α, p-eIF2α, ATF4 and CHOP) was detected by Western blotting. (B) The expression of JNK/p38 MAPK-associated proteins (p-MKK4, p-JNK, JNK, p38, p-p38, p-ATF2 and p-c-Jun) was detected by Western blotting. βactin was used as the loading control.

apoptosis [28]. Among the apoptosis inducing substrates of JNK are Bcl2 and Bim, which are inhibited and activated, respectively, by JNK phosphorylation [29,30]. p38 MAPK phosphorylates and activates the transcription factor CHOP (an ER stress marker) and causes gene expression changes that favor apoptosis, including increasing the expression of Bim, while decreasing the expression of Bcl-2 [31]. Our Western blotting data clearly showed the activation of ER stress and JNK/p38 MAPK after the addition of the LP06CC2 extract (Fig. 4). The activation of apoptosis mediated by endoplasmic reticulum stress that was observed in this study is a p53 independent apoptotic mechanism and it is therefore considered that apoptosis could also be induced in Caco2 cells with p53 gene mutations.

In summary, we demonstrated that the extract of the *L. plantarum* strain 06CC2 had anti-tumor activity against Caco2 colon cancer cells *in vitro*. Since the *in vitro* experiments in this study did not involve the intestinal microflora of the living body, the LP06CC2 extract may have biogenic properties with a direct anti-cancer effect in colorectal cancer cells. We therefore expect future research on the application of *L. plantarum* strain 06CC2 in cancer prevention and treatment and in the development of anti-cancer drugs.

Declaration of competing interest

The authors declare no conflicts of interest in association with the present study.

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