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ORIGINAL ARTICLE

Dictyopteris undulata Extract Induces Apoptosis via Induction of Endoplasmic Reticulum Stress in Human Colon Cancer Cells

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Background: Induction of endoplasmic reticulum (ER) stress-mediated apoptosis in cancer cells represents an alternative approach for cancer therapy. The objective of this study was to elucidate whether ethanol extract of the marine brown alga *Dictyopteris undulata* can induce apoptosis, via ER stress, in human colon adenocarcinoma cells.
Methods: Anti-proliferative activity was evaluated by the colony forming assay. ER stress response was evaluated using flow cytometry and confocal imaging after Rhod2 and ER tracker staining. The expression of ER stress-related proteins was assessed by Western blotting.
Results: *D. undulata* extract (DUE) inhibited colony forming ability in SW480 cells. Furthermore, DUE induced characteristic signs of ER stress: mitochondrial Ca^{2+} overloading, ER staining, expression of ER stress-related proteins, phosphorylation of RNA-dependent protein kinase-like ER kinase and inositol requiring enzyme 1, cleavage of activating transcription factor 6, and induction of the pro-apoptotic factors, CCAAT/enhancer-binding protein-homologous protein (CHOP) and caspase-12. Moreover, down-regulation of CHOP by siCHOP RNA attenuated DUE-induced apoptosis.
Conclusions: The ER stress response plays an important role in DUE-induced apoptosis in human colon cancer cells.
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Key Words: Marine brown alga, Colon adenocarcinoma cells, Apoptosis, Endoplasmic reticulum stress

INTRODUCTION

Colon cancer is the fourth most common cancer in men and the third most common in women worldwide and has become an important problem in Asian countries.[1-3] Current treatments for colon cancer generally involve surgical resection combined with radiation therapy and chemotherapy using one or more cytotoxic drugs. This therapy is only moderately successful for late-stage cancers and is often limited by severe side effects and dose-limiting toxicity.[4,5] Accordingly, identification of new, less toxic adjuvants for chemotherapy that can selectively kill cancer cells or enhance the effects of existing chemotherapeutic agents could lead to improved treatments for late-stage colorectal cancers. Such adjuvants might be derived from natural compounds.

Endoplasmic reticulum (ER) is the major site of intracellular Ca^{2+} storage. The Ca^{2+} concentration in the ER lumen reflects a balance between active Ca^{2+} entry through the calcium ATPase and passive Ca^{2+} leakage. Thus, the ER plays a crucial role in Ca^{2+} homeostasis and cell physiopathology.[6] The ER is also a principal site for protein production: following synthesis, folding, and modification in the ER, proteins are subsequently delivered to other secretory organelles. Under normal conditions, proteins are

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correctly folded in the ER; however, folding can be impaired under various physiological and pathological conditions collectively termed ER stress.[7,8] Accumulation of unfolded or misfolded proteins in the lumen of the ER induces the unfolded protein response. During this cellular stress response, specific signaling pathways are induced to cope with the accumulation of aberrant proteins.

Cells exhibit various adaptive responses to relieve ER stress. The unfolded protein response, an adaptive response, induces the expression of ER-resident chaperones, such as glucose-regulated protein (GRP) 78 and GRP94.[9] In addition, RNA-dependent protein kinase-like ER-resident kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) serve as proximal sensors that regulate components that up-regulate the capacity of the ER to fold newly synthesized proteins and degrade misfolded/unfolded proteins.[10-13]

Several mechanisms have been proposed for ER stress-induced cell death.[14,15] Modulators of ER stress-induced apoptosis include caspase-12, C/EBP homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153), and members of the Bcl-2 family. CHOP is a key pro-apoptotic transcription factor that is induced during ER stress.[15,16]

Recent work has shown that some natural compounds induce ER-stress mediated apoptosis in numerous cancer cell lines. For example, curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells via activation of CHOP and GRP78, as well as other pathways dependent on caspase cascades and the mitochondria.[17] In addition, in HeLa human cervical cancer cells, the natural triterpenoid compound celastrol induces ER stress, activates CHOP, increases transcription of ER stress target genes such as Bim, induces Bax translocation into mitochondria, and ultimately activates mitochondrial apoptosis.[18] In addition, the antitumor compound falcarindiol, a natural polyyne, promotes cancer cell apoptosis by activating ER stress.[19]

Marine algae are rich in dietary fiber, minerals, lipids, proteins, omega-3 fatty acids, essential amino acids, polysaccharides, and vitamins.[20-24] Studies on the bioactivities of marine algae have revealed numerous health-promoting effects, including antioxidant, anti-inflammatory, anti-microbial, and anti-cancer effects.[25-30]

The brown alga *Dictyopteris undulata* belongs to the *Dictyotaceae* family, commonly found in east Asian countries such as Japan, China, and Korea. Cyclozonarone, a sesquiterpene-substituted benzoquinone derivative from *D. undulata*, exerts anti-cancer effects by inducing dissipation of the mitochondrial membrane potential and activation of caspase-3 in the human prostate cancer cell lines PC-3, DU-145 and the human colon cancer cell line HT-29.[31,32]

Recently, we have shown that *D. undulata* ethanol extract (DUE) induced apoptosis by reducing Bcl2 levels, upregulating Bax, and disrupting the mitochondrial membrane potential, leading to activation of caspase-3 and caspase-9 in human colon cancer cells.[33] However, little is known about the involvement of ER stress in this process. To address this issue, we investigated the induction of ER stress and apoptosis by DUE in human colon cancer cells.

MATERIALS AND METHODS

1. Preparation of *Dictyopteris undulata* extract

D. undulata was collected on Jeju Island, Korea. The voucher specimen number was deposited in the herbarium of Jeju Biodiversity Research Institute (Jeju, Korea). *D. undulata* was washed various times with tap water to remove salt and extracted with 80% ethanol at room temperature for 24 hours, and the resultant extract was subjected to vacuum evaporation and then was freeze-dried. The dry DUE was then freshly dissolved in dimethyl sulfoxide, yielding a final concentration that did not exceed 0.01%.

2. Reagents

Rhod2-AM and ER-tracker Blue-White DPX probe were purchased from Molecular Probes (Eugene, OR, USA). Primary antibodies against phospho PERK, phospho IRE1, and ATF6 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and antibodies against CHOP and caspase-12 were purchased from Cell Signaling Technology (Beverly, MA, USA).

3. Cell culture

The human colon cancer cell line SW480 was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained at 37°C in an incubator without CO_2. Culture medium was RPMI 1640 containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL), and penicillin (100 U/mL).

4. Colony formation analysis

SW480 cells were seeded at a density of 500 cells per 60 mm dish in triplicate and cultured for 10 days with either PBS (as a negative control) or 40 μg/mL DUE. During colony growth, the culture medium was replaced every 3 days. Colonies with more than 50 cells were counted. The cells were counted under microscopic observation using a Diff-Quick staining kit (Sysmex,

Kobe, Japan).

5. Endoplasmic reticulum staining

Cells were seeded at a concentration of 1×10^5 cells/well, and 16 hours after plating, they were treated with DUE at 40 μg/mL and incubated for 48 hours. Cells were harvested, washed, and re-suspended in phosphate buffer saline (PBS) containing the ER-tracker Blue-White DPX probe. After 30 minutes of incubation at 37°C, cells were washed, suspended in PBS, and analyzed by flow cytometry. For confocal imaging analysis, cells were seeded in chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of 1×10^5 cells/mL; 16 hours after plating, they were treated with DUE at 40 μg/mL and incubated for 48 hours. Then, ER-tracker was added to the cells, and the samples were incubated for 30 minutes under the same growth conditions. The loading solution was removed, and the cells were washed with PBS before addition of fresh medium without the stain. Microscopic images were collected using the LSM 5 PASCAL software (Carl Zeiss, Jena, Germany).

6. Western blot analysis

Cells were harvested, washed twice with PBS, lysed on ice for 30 minutes in 100 μL of lysis buffer (120 mM NaCl, 40 mM Tris [pH 8], and 0.1% NP 40), and centrifuged at 13,000 rpm for 15 minutes. Supernatants were collected from the lysates, and the protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 minutes and electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. The resolved proteins were then transferred onto nitrocellulose membranes, which were subsequently incubated with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK), followed by exposure of the membranes to X-ray film.

7. Measurement of mitochondrial Ca^{2+}

Rhod2 AM was used to determine the mitochondrial Ca^{2+} level.[34] Rhod2 AM has a net positive charge, which facilitates its sequestration into mitochondria via membrane potential-driven uptake. The use of Rhod2 AM improves the selectivity of mitochondrial loading, because the dye exhibits Ca^{2+}-dependent fluorescence only after it is oxidized, a process that occurs preferentially within mitochondria. Cells were seeded at a concentration of 1×10^5 cells/well; 16 hours after plating, they were treated with DUE at 40 μg/mL and incubated for an additional 48 hours. Cells were harvested, washed, and re-suspended in PBS containing Rhod2 AM. After 15 minutes of incubation at 37°C, cells were washed, suspended in PBS, and analyzed by flow cytometry. For image analysis, cells were loaded with Rhod2 AM and incubated for 30 minutes at 37°C. The stained cells were then washed and mounted on microscope slides in mounting medium (DAKO, Carpinteria, CA, USA). Microscopic images were obtained using a confocal laser scanning microscope and LSM 5 PASCAL software (Carl Zeiss).

8. Transient transfection of small interfering RNA

Cells were seeded in 24-well plates at a density of 1.5×10^5 cells/mL, and allowed to reach approximately 50% confluence on the day of transfection. The small interfering RNA (siRNA) constructs used were mismatched siControl (Santa Cruz Biotechnology) and siCHOP (Bioneer Corporation, Daejeon, Korea). Cells were transfected with 10-50 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 hours, cells were treated with DUE for 48 hours and examined by Western blot

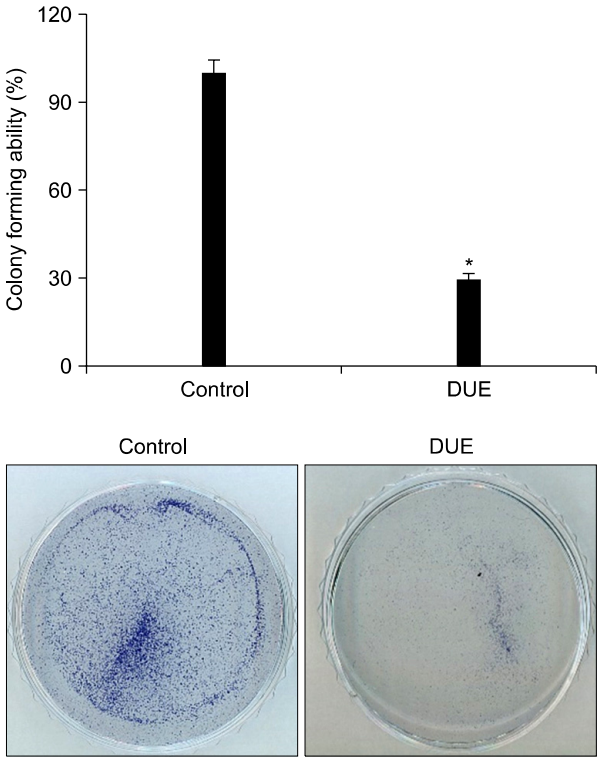

Figure 1. *Dictyopteris undulata* extract (DUE) inhibits growth of human colon cancer cells. For colony forming assays, cells were cultured in the absence or presence of DUE (40 μg/mL) for 10 days. Colonies with more than 50 cells were counted. *Significantly different from control cells ($P<0.05$).

analysis, DNA fragmentation assay, and PI staining.

9. DNA fragmentation

A hallmark of apoptosis is DNA cleavage, which can be measured by ELISA quantitation of histone-complexed DNA fragments. Cells were treated with DUE for 48 hours. DNA fragmentation was assessed using a cellular DNA fragmentation ELISA kit (Roche Diagnostics, Mannheim, Germany).

10. Detection of sub-G_1 hypodiploid cells

Apoptotic sub-G_1 hypodiploid cells were quantitated by flow cytometry.[35] siRNA-transfected cells were treated with DUE for 48 hours, harvested, washed twice with PBS, and fixed in 70% ethanol for 30 minutes at 4°C. Subsequently, the cells were incubated in 10 μg/mL PI and 50 μg/mL RNase A in the dark for 30 minutes at 37°C. Flow-cytometric analysis was performed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA, USA). Numbers of sub-G_1 hypodiploid cells were assessed based on histograms generated by the CellQuest and ModFit software.

11. Statistical analysis

Values are expressed as means ± standard error. Results were analyzed using analysis of variance and Tukey's test to determine pairwise differences. $P<0.05$ was considered significant.

RESULTS

1. *Dictyopteris undulata* extract inhibits growth of human colon cancer cells

Recently, we reported that DUE inhibited SW480 cell growth in a dose-dependent manner, as determined by the MTT assay. The concentration of DUE that yielded 50% growth inhibition (IC_{50}) was 40 μg/mL,[33] which we selected as the dose for all experiments in this study. Cell viability was assessed by colony-formation assays, which revealed that the viability of cells treated with 40 μg/mL DUE was lower than that of control cells (Fig. 1).

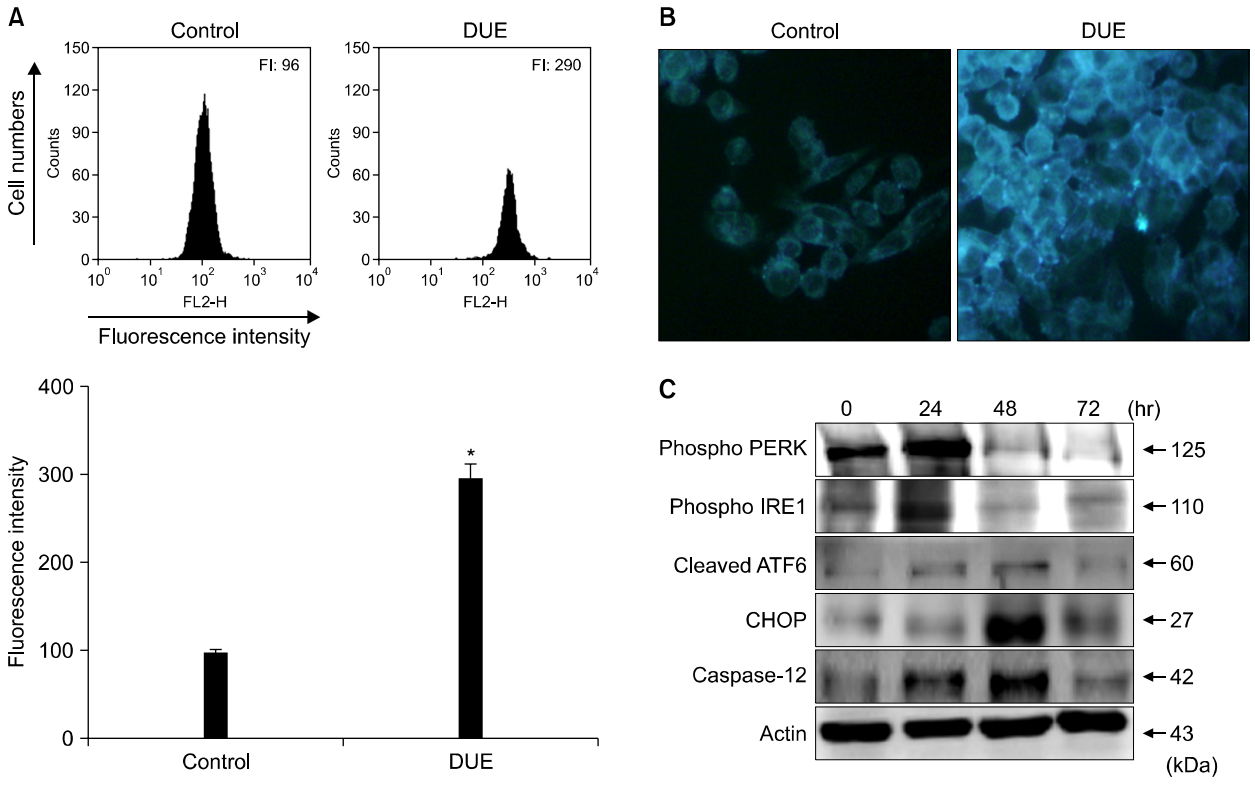

Figure 2. *Dictyopteris undulata* extract (DUE) increases ER-stress staining and expression of ER stress-related proteins. Cells were treated with DUE for 48 hours, and then stained with the ER-tracker Blue-White DPX dye. Fluorescence intensity was measured by flow cytometry (A) and confocal microscopy (B). Representative confocal microscopic images show the increase in blue fluorescence intensity of ER-tracker in DUE-treated cells relative to control cells. *Significantly different from untreated cells ($P<0.05$). (C) Cell lysates were subjected to electrophoresis, and phosphorylated protein kinase-like ER kinase (PERK), phosphorylated inositol requiring enzyme 1 (IRE1), cleaved activating transcription factor 6 (ATF6), CCAAT/enhancer-binding protein-homologous protein (CHOP), and caspase-12 were detected using the appropriate specific antibodies.

2. *Dictyopteris undulata* extract induces endoplasmic reticulum stress and expression of endoplasmic reticulum stress-related proteins

Because DUE induces apoptotic cell death,[33] we investigated whether ER stress is involved in DUE-induced apoptosis. Treatment with DUE for 48 hours significantly increased ER stress, as indicated by elevation of fluorescence intensity in ER-tracker-stained cells (Fig. 2A and 2B). During ER stress, activated PERK (phospho PERK) phosphorylates IRE1, leading to the attenuation of translational initiation and protein synthesis.[11-13] As shown in Figure 2C, DUE treatment for 24 hours induced the expression of both phospho PERK and phospho IRE1; subsequently, the levels of both phospho proteins returned to basal values. DUE treatment also induced other hallmarks of the ER stress responses: cleavage of ATF6, up-regulation of CHOP, and activation of caspase-12.[14,15]

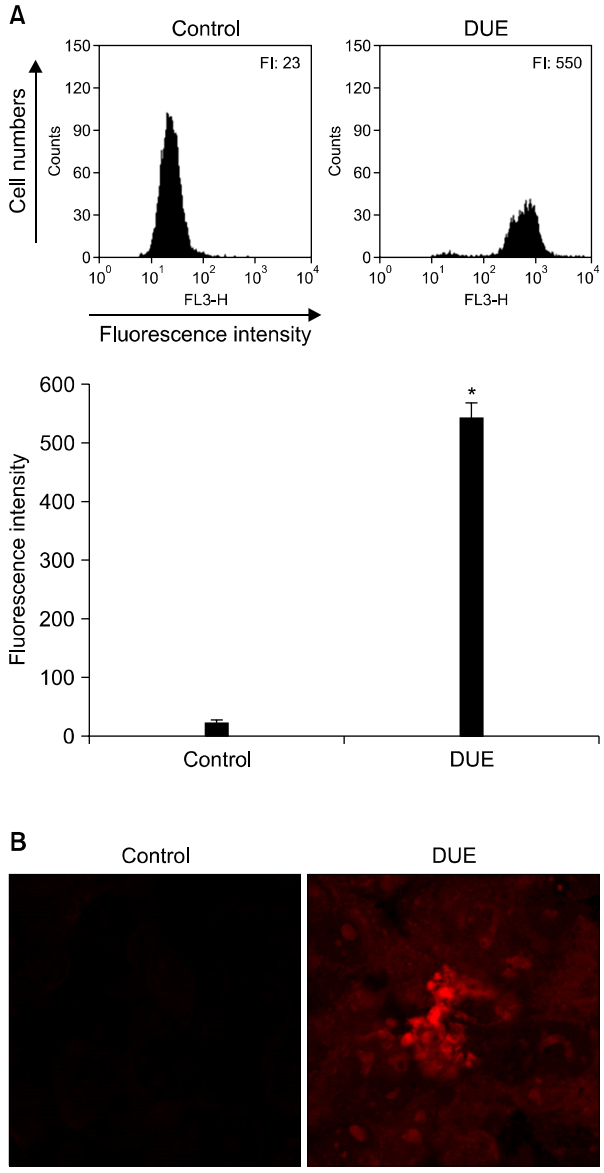

Figure 3. *Dictyopteris undulata* extract (DUE) causes mitochondrial Ca^{2+} overloading. Cells were treated with 40 μg/mL of DUE for 48 hours, harvested, and treated with the fluorescent probe Rhod2 AM. Mitochondrial Ca^{2+} levels were measured by flow cytometry (A) and confocal microscopy (B). FI indicates the fluorescence intensity of Rhod2 AM. *Significantly different from untreated cells ($P<0.05$). Representative confocal microscopic images show the increase in red fluorescence intensity of Rhod2 AM, produced by mitochondrial Ca^{2+} overloading, in DUE-treated cells relative to control cells.

3. *Dictyopteris undulata* extract induces mitochondrial Ca^{2+} overloading

Depletion of ER calcium stores induces ER stress, which leads to an increase in cytosolic and mitochondrial Ca^{2+} levels.[36,37] Therefore, we examined the effect of DUE on mobilization of Ca^{2+}. DUE treatment for 48 hours resulted in significant increases in mitochondrial Ca^{2+} levels, as determined by Rhod2 AM staining (Fig. 3).

4. Suppression of CCAAT/enhancer-binding protein-homologous protein expression attenuates *Dictyopteris undulata* extract-induced apoptosis

CHOP plays a pro-apoptotic role during ER stress.[15] To determine whether CHOP contributes to induction of apoptosis by ER stress in our system, we transfected cells with siRNA targeting CHOP (siCHOP) or a control siRNA (siControl). Cells transfected with siCHOP exhibited a significant decrease in DUE-induced DNA fragmentation, one of the characteristic findings of apoptotic cells (Fig. 4A), compared with siControl-transfected cells. Similarly, CHOP knockdown significantly reduced the sub-G_1 cell population induced by DUE treatment (Fig. 4B). These observations indicate that suppression of siRNA-mediated CHOP expression attenuated the apoptotic cell death induced by DUE. Collectively, these results suggest that up-regulation of CHOP during ER stress induced by DUE contributes to apoptosis.

DISCUSSION

The results of this study demonstrate the involvement of ER stress in DUE-induced apoptosis in human colon cancer cells. ER stress arises due to accumulation of unfolded or misfolded proteins within the ER or depletion of Ca^{2+} stores.[7,8] Our results show that DUE induced both ER stress and elevation of mito-

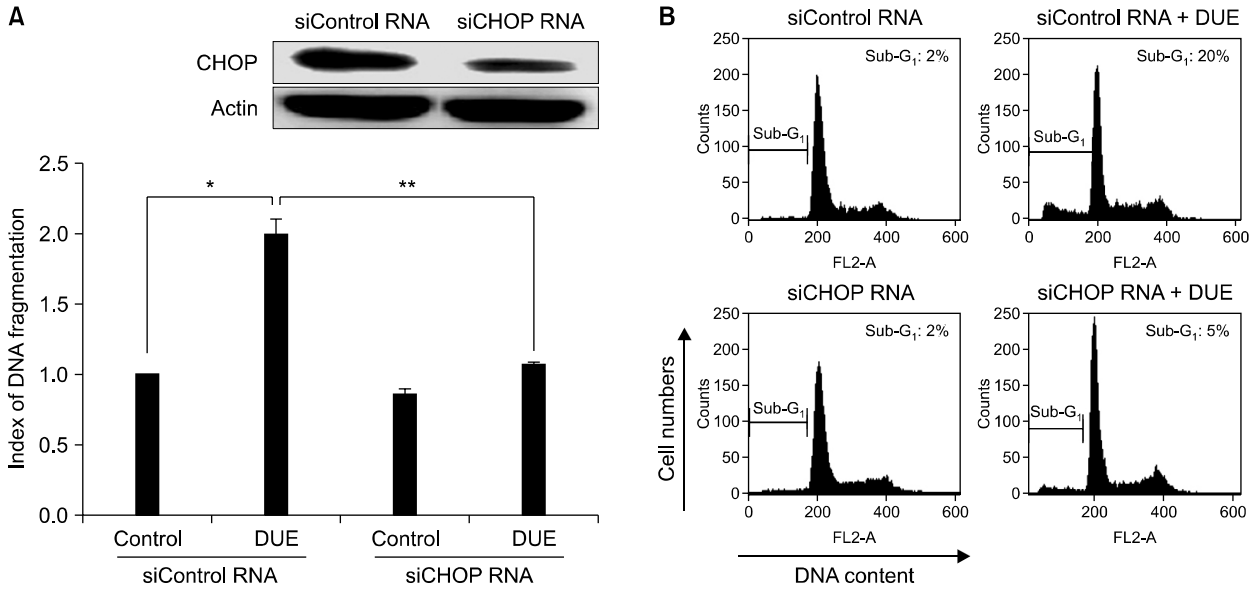

Figure 4. Down-regulation of CCAAT/enhancer-binding protein-homologous protein (CHOP) attenuates *Dictyopteris undulata* extract (DUE)-induced apoptosis. Cells were transfected with siCHOP or siControl for 24 hours, and then treated with DUE for 48 hours. (A) DNA fragmentation was quantitated using an ELISA kit and (B) apoptotic sub-G_1 DNA content was detected by flow cytometry after PI staining. *Significantly different from siControl-transfected cells ($P<0.05$); **significantly different from DUE-treated siControl-transfected cells ($P<0.05$).

chondrial Ca^{2+} levels. ER stress constitutes a physiological as well as pathophysiological stress stimulus; when this stress is overwhelming, it can lead to apoptotic death of the damaged cell. DUE also induced apoptotic body formation, DNA fragmentation, an increase in the population of apoptotic sub-G_1 phase cells, and mitochondrial membrane depolarization.[33] In addition, DUE significantly decreased levels of the anti-apoptotic protein Bcl2, increased levels of the pro-apoptotic protein Bax, and activated caspase-3 and caspase-9.[33] Furthermore, induction of ER stress-related proteins may have been involved in DUE-induced apoptosis: DUE treatment induced phosphorylation of PERK and IRE1, up-regulation of CHOP and caspase-12, and cleavage of ATF6. Among ER-associated apoptotic molecules, CHOP and caspase-12 are major pro-apoptotic factors that are closely associated with ER stress.[38] The CHOP protein is a member of the CCAAT/enhancer-binding protein family, and functions as an inhibitor of gene transcription.[39] Expression of CHOP is mainly regulated at the transcriptional level through the PERK/eIF2α/ATF6 pathway.[39] CHOP knockout mice exhibit reduced apoptosis in response to ER stress.[40] Therefore, CHOP is a component of the ER stress-mediated apoptosis pathway. In this study, suppression of CHOP using siRNA attenuated DUE-induced apoptosis.

In summary, we showed that DUE induced ER stress-mediated apoptosis in SW480 human colon cancer cells. This is the first report to support the involvement of DUE-induced ER stress in apoptosis of colon cancer cells.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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