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 Ca²⁺ 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. (J Cancer Prev 2014;19:118-124)

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.¹³ 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.⁴⁵ 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 Ca²⁺ storage. The Ca²⁺ concentration in the ER lumen reflects a balance between active Ca²⁺ entry through the calcium ATPase and passive Ca²⁺ leakage. Thus, the ER plays a crucial role in Ca²⁺ 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...
correctly folded in the ER; however, folding can be impaired under various physiological and pathological conditions collectively termed ER stress. 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. 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.

Several mechanisms have been proposed for ER stress-induced cell death. 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.

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. 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. In addition, the antitumor compound falcarnidol, a natural polyyne, promotes cancer cell apoptosis by activating ER stress.

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

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. 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 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 CO2. 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.
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-G1 hypodiploid cells

Apoptotic sub-G1 hypodiploid cells were quantitated by flow cytometry. 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 FACS Calibur instrument (Becton Dickinson, Mountain View, CA, USA). Numbers of sub-G1 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 (IC50) was 40 μg/mL, 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).
2. *Dictyopteris undulata* extract induces endoplasmic reticulum stress and expression of endoplasmic reticulum stress-related proteins

Because DUE induces apoptotic cell death, 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. 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.

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. 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. 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. 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-G1 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).

Mitochondrial Ca2+ 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-G1 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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