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Full Length Research Paper

Hydroclathrus clathratus induces apoptosis in HL-60 leukaemia cells via caspase activation, upregulation of pro-apoptotic Bax/Bcl-2 ratio and ROS production

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Hydroclathrus clathratus **is a well-known endemic alga in Korea having antiviral effects. In this study, its ability to induce cytotoxicity and apoptosis in cultured HL-60 human promyelocytic leukaemia cells was investigated. Treatment of the human promyelocytic leukaemia cell line (HL-60), cells with various concentrations of *H. clathratus* ethyl acetate extract (HCE) resulted in growth inhibition and induction of apoptosis in a dose-dependent manner, as determined by the cell viability, chromatin condensation, DNA fragmentation and sub-G1 phase accumulation. The HCE-induced apoptotic cell-death was associated with caspase-3 and caspase-9 activation, and poly ADP-ribose polymerase (PARP) degradation in the HL-60 cells. This increase in the HCE-induced apoptosis was also associated with a reduction in the levels of Bcl-xL, a potent cell-death inhibitor, and an increase in the levels of the Bax protein, which heterodimerises with and thereby inhibits Bcl-2. Finally, the intracellular reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), were found to be elevated after HCE treatment of these cells. In addition, antioxidant *N*-acetyl cysteine (NAC) pretreatment almost completely inhibited the HCE-induced apoptosis, suggesting that ROS are the key mediators of HCE-induced apoptosis. In conclusion, HCE induces apoptosis of human leukaemia cells through caspase activation, upregulation of the pro-apoptotic Bax/Bcl-2 ratio and ROS generation. Therefore, it may have anticancer properties valuable for application in food and drug products.**

Key words: Apoptosis, Bcl-2, *Hydroclathrus clathratus*, reactive oxygen species.

INTRODUCTION

As alternative medicine, herbal therapies are widely used in all regions of the developing world and are rapidly growing in developed countries (Yan et al., 2006). Even with the extensive application of herbal therapies, there is still insufficient scientific data validating their efficacy and safety. Thus, basic research aimed at elucidating the underlying mechanisms of any potential herbal effects is very important for the application of herbal medicine (Li et al., 2009b; Liu et al., 2009). Recently, several researchers have focussed on the potential role of seaweeds as an alternative and complementary medication for cancer treatment. Seaweed extracts are often used together with traditional cancer therapy to improve the survival rate and quality of life, as they are much less expensive than the standard anticancer therapies currently in use (Gamal-Eldeen et al., 2009; Kong et al., 2009; Palozza et al., 2009). However, the available evidence is not adequate to allow their use as therapeutic agents, and there is a need for comprehensive, systematic, multidisciplinary evaluation of these extracts prior to their introduction in modern clinical practice.

Apoptosis, or programmed cell death, plays an essential role in controlling the cell population in many developmental and physiological settings. It is a strictly

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regulated pathway responsible for the ordered removal of unnecessary, old and damaged cells (Thompson et al., 1995; Li et al., 2007). Its morphological characteristics include plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation and formation of apoptotic bodies (Wyllie, 1997). Apoptosis also plays an important role in the development of various diseases such as cancer. Recently, interest has been focussed on the manipulation of apoptotic processes in the treatment and prevention of cancer. Thus, much effort has been directed towards the search for compounds or herbs that influence apoptosis and its mechanism of action (Ward et al., 2008; Sun and Peng, 2009; Tan et al., 2009; Yang et al., 2009).

Hydroclathrus clathratus is an endemic alga in Korea having antiviral effects (Wang et al., 2007; Wang et al., 2008). On the other hand, it is still unclear whether this alga can prevent various cancers. Therefore, in this study, the ability of *H. clathratus* ethyl acetate extract (HCE) to induce apoptosis was investigated by using human leukaemia cell line HL-60. The following methods were used for this purpose: (1) MTT assay was performed for the cytotoxicity test; (2) typical morphological changes, including chromatin condensation and apoptotic bodies, were observed under light microscopy; (3) DNA fragmentation was demonstrated by agarose gel electrophoresis; (4) the cell cycle was analysed by flow cytometry; and caspase activation and ROS generation were observed by the HCE treatment.

MATERIALS AND METHODS

Preparation of *H. clathratus* extract

H. clathratus was prepared on Jeju Island from May 2006 and then identified by one of the authors (W.J.L.). Previously, sample specimens were deposited at the herbarium of the Jeju Biodiversity Research Institute (JBRI), Korea. The materials for extraction were cleaned, dried at room temperature for two weeks and then ground into a fine powder. The dried alga (100 g) were extracted with 80% ethanol (2 L) at room temperature for 24 h and then evaporated *in vacuo*. The evaporated extract was suspended in water (4 L) and partitioned with ethyl acetate (4 L) at room temperature; this partitioning was repeated thrice.

Cell culture

Human promyelocytic leukaemia cell line (HL-60), human colon carcinoma cell line (HT-29) and A549 (human lung cancer cell line) cells were grown in RPMI-1640 medium; mouse melanoma cell line (B16F10) cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cultures were maintained at 37°C in a 5% CO_2 incubator.

Cell growth inhibition assay

The cytotoxicity of *H. clathratus* against the tumour cells was determined by a colorimetric MTT assay as described previously (Kim et al., 2009). Suspended cells (HL-60 cells) were seeded (5×10^4 cells/ml) together with various concentrations of HCE and incubated for up to 72 h before MTT treatment. Attached cells (HT-29, B16 and A549 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells/ml. Sixteen hours after seeding, the cells were treated with HCE and then incubated for an additional 72 h at 37°C. MTT stock solution (50 µl; 2 mg/ml in PBS) was added to each well for a total reaction volume of 250 µl. After incubating for 4 h, the plate was centrifuged at 2000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was studied by using cell-permeable DNA-specific dye Hoechst 33342. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The HL-60 cells were placed in a 24-well plate at a concentration of 4×10^5 cells/ml. The cells were treated with various concentrations of HCE and further incubated for 24 h. Then, Hoechst 33342 was added to the culture medium at a final concentration of 10 µg/ml, and the plate was incubated for another 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

Determination of DNA fragmentation

The characteristic ladder pattern of DNA breakage was analysed by agarose gel electrophoresis. The HL-60 cells were placed in a 6-well plate at a concentration of 4×10^5 cells/ml. The cells were treated with various concentrations of HCE and further incubated for 24 h. The DNA was isolated by using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and analysed electrophoretically on 1.2% agarose gel containing 0.1 µg/ml ethidium bromide.

Cell cycle analysis

Cell cycle analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The HL-60 cells were placed in a 6-well plate at a concentration of 4.0×10^5 cells/ml and treated with various concentrations of HCE. After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with SGE and incubated in the dark in 1 ml PBS containing 100 µg PI and 100 µg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometre (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis

Flow cytometric analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The HL-60 cells were placed in a 6-well plate at a concentration of 1.0×10^5 cells/ml and treated with various concentrations (6, 13 and 25 µg/ml) of the sample. After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. They were washed twice with PBS and incubated in the dark in 1 ml PBS containing 100 µg PI and 100 µg RNase A for 30 min at 37°C.

Flow cytometric analysis was performed with a FACSCalibur flow

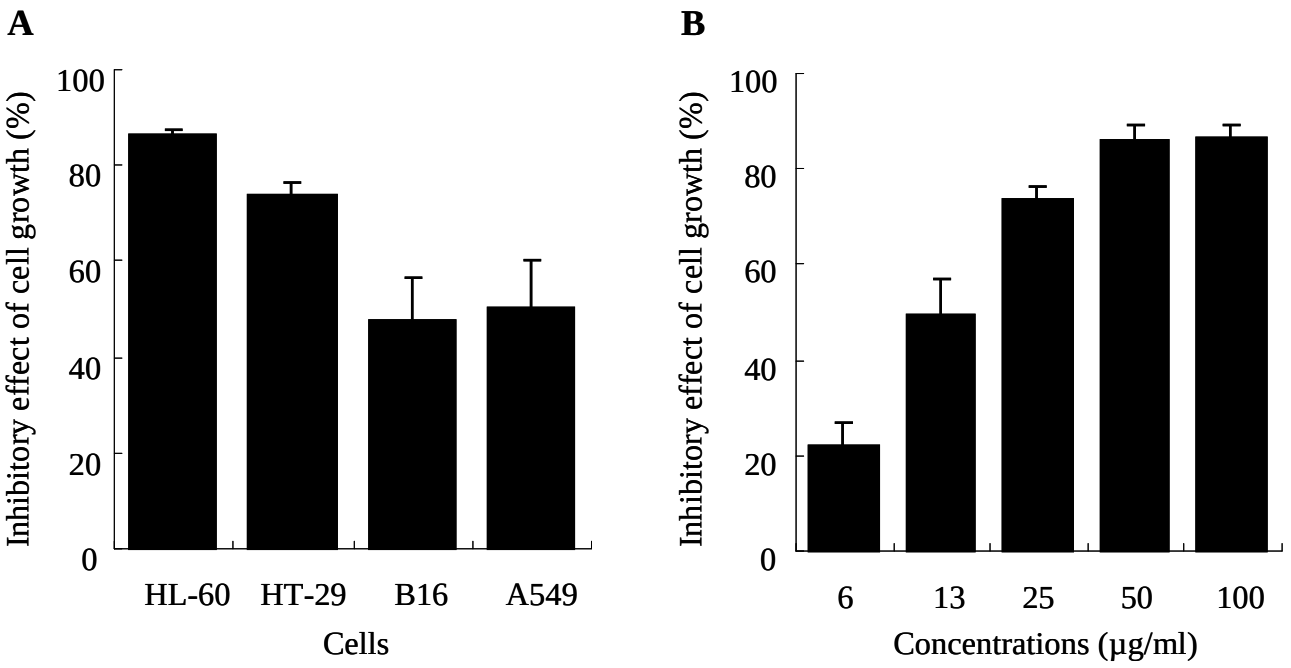

Figure 1. Inhibitory effect of HCE against the growth of the tumour cells. (A) HL-60, HT-29, B16 and A549 cells were incubated with HCE (100 µg/ml) for 72 h. (B) HL-60 cells were incubated with various concentrations of HCE for 72 h, and the cell viability was examined by MTT assay.

cytometre (Becton Dickinson, San Jose, CA, USA). The effect on the cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the programs CellQuest and Mod-Fit.

Measurement of ROS

The accumulation of intracellular H_2O_2 and $O_2^{\bullet-}$ was detected with the probes 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) and hydroethidine (HE), respectively, as described previously. In brief, the HL-60 cells were placed in a 6-well plate at a concentration of 2×10^5 cells/ml and treated with various concentrations (6, 13 and 25 µg/mL) of HCE. After 2 h, the cells were labelled with 5 µM HE or 10 µM $DCFH_2$-DA for 30 min at 37°C. The labelled cells were collected, and the fluorescence was analysed using a flow cytometre.

Western blot analysis

Cells (2×10^5 cells/ml) were treated with various concentrations (6, 13 and 25 µg/ml) of the sample for 24 h and harvested. The cell lysates were prepared with lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/L EDTA) and washed by centrifugation. The protein concentrations were determined by using a BCA protein assay kit (Pierce Chemical Company, Rockford, IL, USA). The lysates containing 30 µg protein were subjected to electrophoresis on 10 or 12% sodium dodecyl sulphate–polyacrylamide gel, and the gel was transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with primary antibodies against Bax, Bcl-xL, cleaved caspase-3, caspase-9, poly ADP-ribose polymerase (PARP) and β-actin in TTBS (25 mmol/L Tris-HCl [pH 7.4], 137 mmol/L NaCl, 0.1% Tween 20) containing 0.5% non-fat dry milk at 1 h. The membranes were then washed with TTBS and incubated with secondary antibodies. The signals were developed by using an ECL Western blotting detection kit (Amersham Pharmacia Biotech., Piscataway, NJ, USA) and exposed to X-ray films (Fujifilm Corporation, Tokyo, Japan).

Statistical analysis

All data were obtained in triplicate and are represented as means ± standard error (SE). Significant differences between treatments were determined by the Student's *t* test in a one-way analysis of variance (ANOVA).

RESULTS

Cytotoxic effects of HCE on human cancer cell lines

By using MTT assay, the cytotoxicity of HCE on the various cells is shown in Figure 1A. Different cells had different sensitivity to the inhibitory effect of HCE. The cytotoxicity values were 73.7 ± 2.6, 50.9 ± 9.8, 47.6 ± 8.9 and 86.6 ± 0.9% at 100 µg/ml concentrations of HT-29, A549, B16F10 and HL60 cells, respectively. At these concentrations, the extract had significantly less effect on human keratinocyte HaCaT cells (data not shown). To determine the cytotoxic effects of HCE on HL-60 cells, the cells were exposed to various concentrations of HCE (6 to 100 µg/ml) for 24 h. Cells treated with 0.1% DMSO were used as controls. As shown in Figure 1B, the

cytotoxicity of HCE on the HL-60 cells showed dose-dependent changes. The 50% inhibitory concentration (IC_{50}) of HCE calculated from the graph was 14.0 µg/ml.

DNA fragmentation of the HL-60 cells

Apoptosis is characterised by a series of morphological changes such as chromatin condensation, cell shrinkage, membrane blebbing, packing of organelles, formation of apoptotic bodies and inter-nucleosomal DNA fragmentation. The cleavage of the DNA double strand, which can be visualised as a ladder pattern on agarose gel, is a late event and a hallmark of apoptosis. In order to determine whether the cytotoxic activity of HCE was due to apoptosis, we used the DNA fragmentation assay to confirm the induction of apoptosis. The DNA ladder pattern is shown in Figure 2A. Consistent with the MTT and apoptosis assays, the DNA fragmentation assay showed that HCE induced HL-60 cell death dose-dependently. The amount of fragmented DNA was obviously increased with the concentrations of 6, 13 and 25 µg/ml. No chromosomal DNA cleavage was observed in the untreated control cells.

Induction of apoptosis by HCE in the HL-60 cells

In order to determine whether the anti-proliferative effect of HCE was due to apoptosis, the HL-60 cells were treated with HCE for 48 h, and nuclear 4'-6-Diamidino-2-phenylindole (DAPI) staining was performed. As shown in Figure 2B, nuclei with condensed chromatin and apoptotic bodies, the typical characteristics of apoptosis, were observed in the HL-60 cells incubated with HCE. The number of apoptotic cells increased as the concentration of HCE increased.

Cell cycle analysis

The cell cycle of the HL-60 cells was analysed by using flow cytometry after exposure to 6, 12.5 or 25 µg/ml HCE for 48 h. Figure 2C shows that the treatment with 6 to 25 µg/ml HCE resulted in a significant increase in the sub-G1 population in the HL-60 cells (6 µg/ml, 10.1%; 12.5 µg/ml, 35.1%; 25 µg/ml, 49.6%), which indicate apoptotic cells, compared with the controls (7.9%). In the non-apoptotic population, the portion of cells in the G1 phase increased at lower HCE concentrations (control, 37.9%; 6 µg/ml, 37.2%; 12.5 µg/ml, 24.2%) with a decrease in the S phase (control, 34.0%; 6 µg/ml, 34.4%; 12.5 µg/ml, 26.3%) and G2/M phase (control, 20.7%; 6 µg/ml, 19.0%; 12.5 µg/ml, 15.0%). The portion of cells in the G1 phase slightly decreased (17.8%) at the higher concentration (25 µg/ml) compared with that at the lower concentrations. This decrease in cell number was associated with a concomitant increase in the number of cells in the sub-G1 phase. These results suggested that HCE induced cell cycle arrest in the G1 phase and apoptosis in the HL-60 cells in a dose-dependent manner.

Effects of HCE on the up-/downregulation of apoptosis-related proteins

The activation of a family of caspase is necessary for the execution of apoptosis. Procaspase-8 and procaspase-9 as initiators are activated by self-processing and cleave downstream procaspase-3 to an active dimeric form of caspase-3 as the executioner of apoptosis. To verify whether HCE induces the activation of these caspases, the HL-60 cells were exposed to HCE and the processing levels of procaspase-3 and procaspase-9 were examined by Western blotting. After exposure to HCE (6 and 13 µg/ml) for 48 h, HCE induced the activation of procaspase-9 (43 kDa) into caspase-9 (35 kDa) and the cleavage of procaspase-3 (32 kDa; Figure 3). The cleavage of poly (ADP-ribose) polymerase (PARP), a substrate for caspase-3, is also a characteristic of apoptosis. Active caspase-3 cleaves 116 kDa PARP proteins into 85 kDa fragments. Figure 3 shows that PARP was cleaved into an 85 kDa fragment after treatment with HCE. These results strongly indicated that apoptosis induced by HCE in the HL-60 cells occurred via the mitochondria-dependent signal pathway.

Effect of HCE on Bcl-xL and Bax proteins

As shown in Figure 3, incubation of the HL-60 cells with HCE dramatically reduced the level of Bcl-xL, a potent cell-death inhibitor, and increased the level of the Bax protein in a dose-dependent manner. These results indicated that HCE induced dysregulation of Bcl-xL and Bax in the HL-60 cells.

Increase in ROS generation by HCE treatment

As many anticancer drugs and DNA damage-causing agents activate the apoptotic pathway through ROS generation, the possibility that ROS elevation is a key step in HCE-induced apoptosis was assessed. ROS elevation was tested by using two dyes: $DCFH_2$-DA for the detection of cytosolic H_2O_2 and HE for the detection of cytosolic $O_2^{\bullet-}$ in live cells. As shown in Figure 4, intracellular H_2O_2 was elevated by about twofold in the HL-60 cells after treatment with HCE for 2 h, compared with the untreated or DMSO control cells. The increased H_2O_2 generation in the HCE-treated cells was comparable to or even higher than that in positive control cells directly treated with 200 µM H_2O_2. HCE treatment also led to the elevation of $O_2^{\bullet-}$ generation in the HL-60 cells, indicating

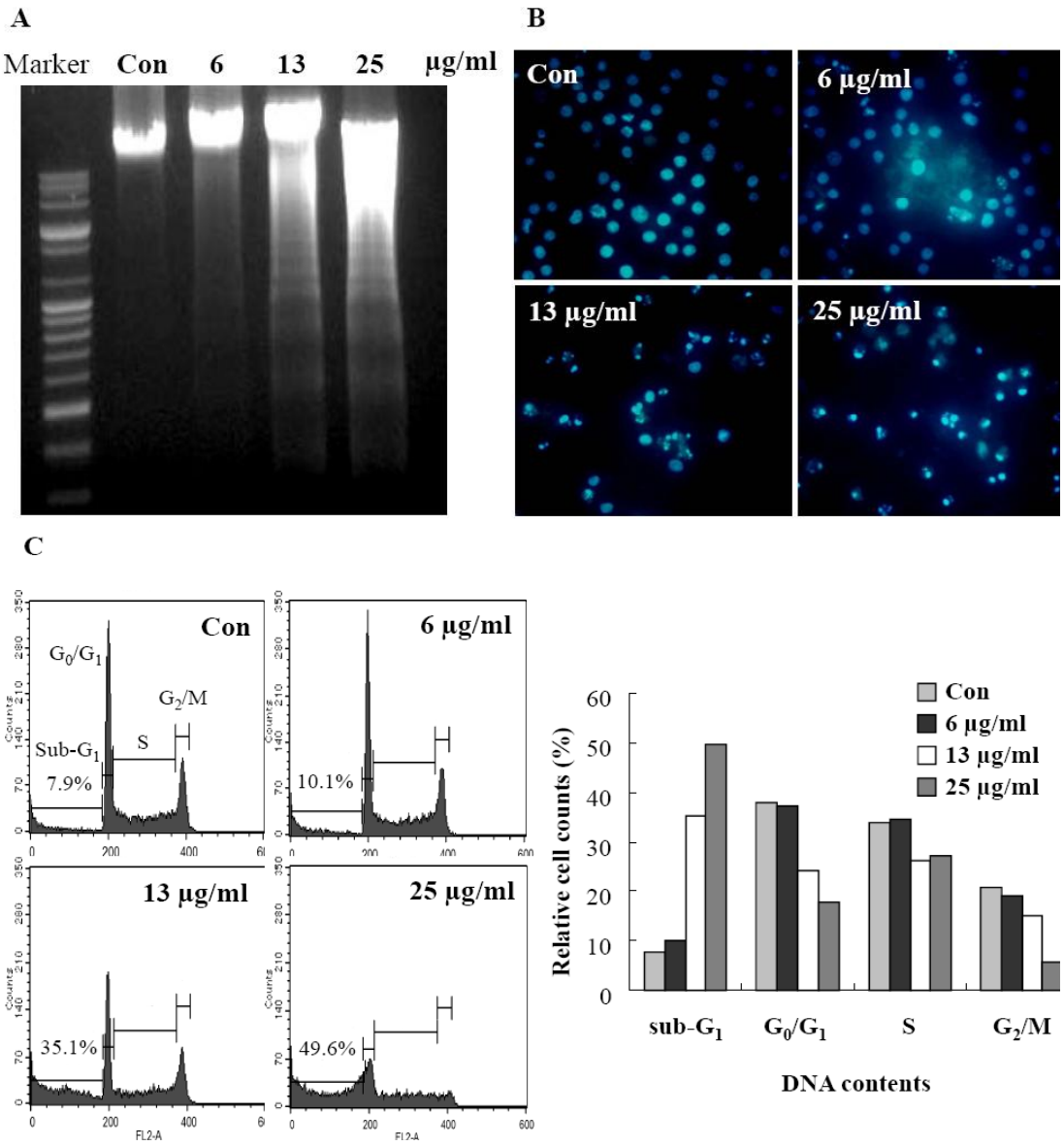

Figure 2. Induction of apoptosis by HCE in the HL-60 cells. The HL-60 cells were incubated with various concentrations of HCE for 24 h. (A) DNA was isolated and subjected to 1.2% agarose gel electrophoresis for staining with ethidium bromide. (B) The cells were stained with Hoechst 33342, and the stained nuclei were observed under a fluorescent microscope. (C) To quantify the degree of apoptosis induced by HCE, the cells were evaluated for their sub-G1 DNA content, representing the fractions undergoing apoptotic DNA degradation, by flow cytometry.

that HCE treatment indeed enhanced the intracellular ROS generation in human leukaemia cells.

HCE-induced apoptosis mediated by ROS generation in human leukaemia cells

As HCE elevated ROS, antioxidant *N*-acetyl cysteine (NAC) was used to gain an insight into whether HCE induced apoptosis through ROS generation. As shown in Figure 5, NAC pretreatment almost completely inhibited HCE-induced apoptosis in the HL-60 cells. These data indicated that elevated ROS are indeed upstream molecules or key signalling molecules for HCE-induced apoptosis in HL-60 cells.

DISCUSSION AND CONCLUSIONS

In this study, we have demonstrated HCE-induced cell

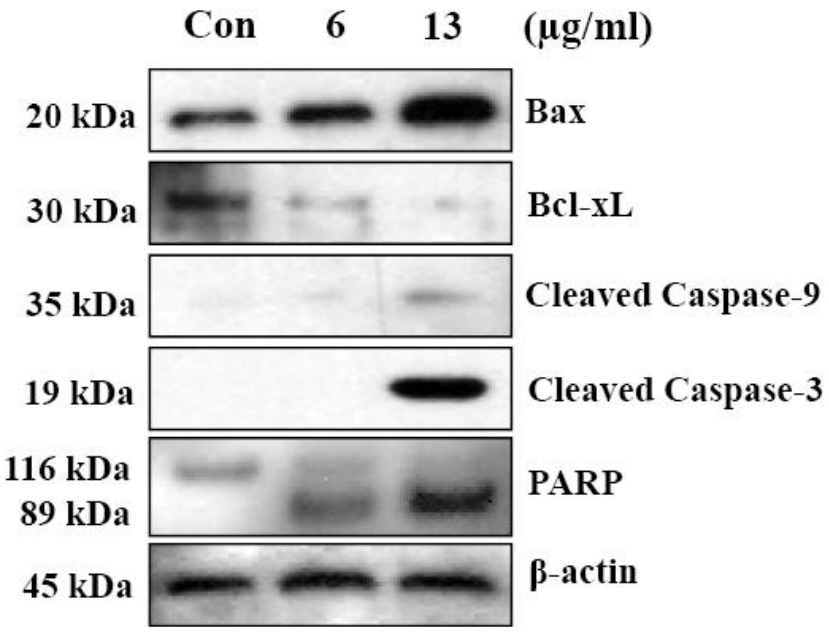

Figure 3. Expression levels of apoptosis-related proteins by HCE treatment of the HL-60 cells. The HL-60 cells were incubated with various concentrations of HCE for 24 h. The cell lysates (30 μg) were immunoblotted with the indicated antibodies (Bax, Bcl-xL, capsase-3, capsase-9, PARP and β-actin) for Western blotting.

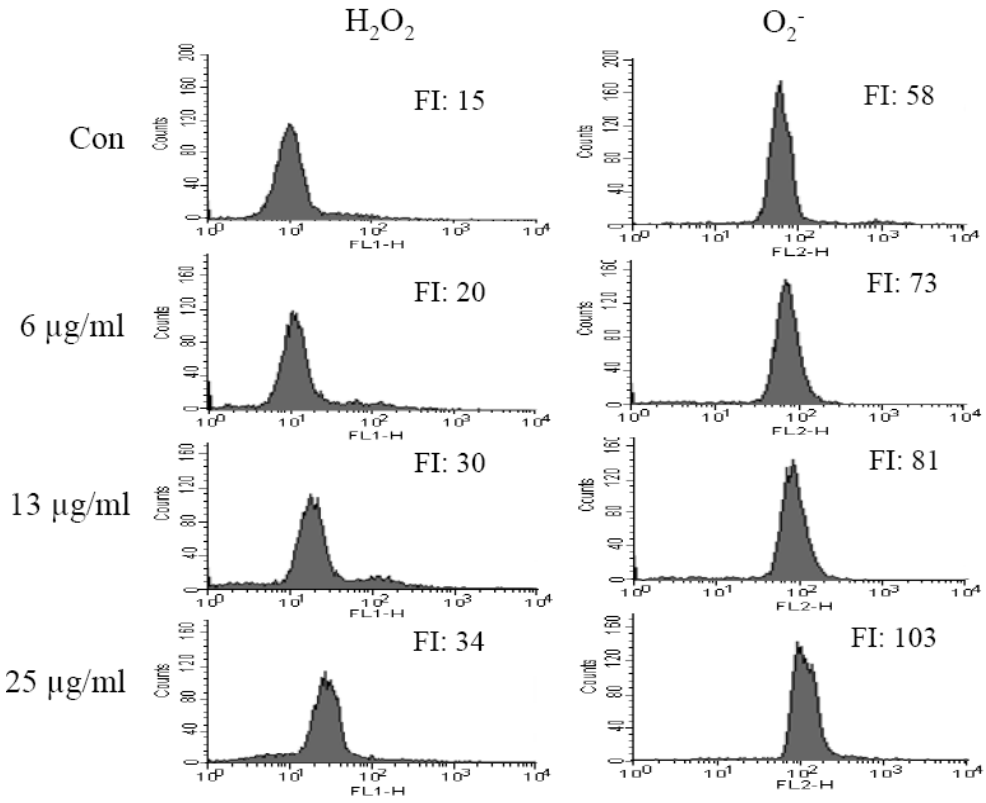

Figure 4. HCE induced ROS production in the HL-60 cells. The cells were incubated with various concentrations of HCE for 2 h, followed by the addition of 5 μM DCFH$_2$-DA (for H_2O_2) or 2 μM HE (for O_2^-) for 30 min at 37°C and subsequent FACS analysis for intracellular accumulation of ROS. FI means fluorescence intensity.

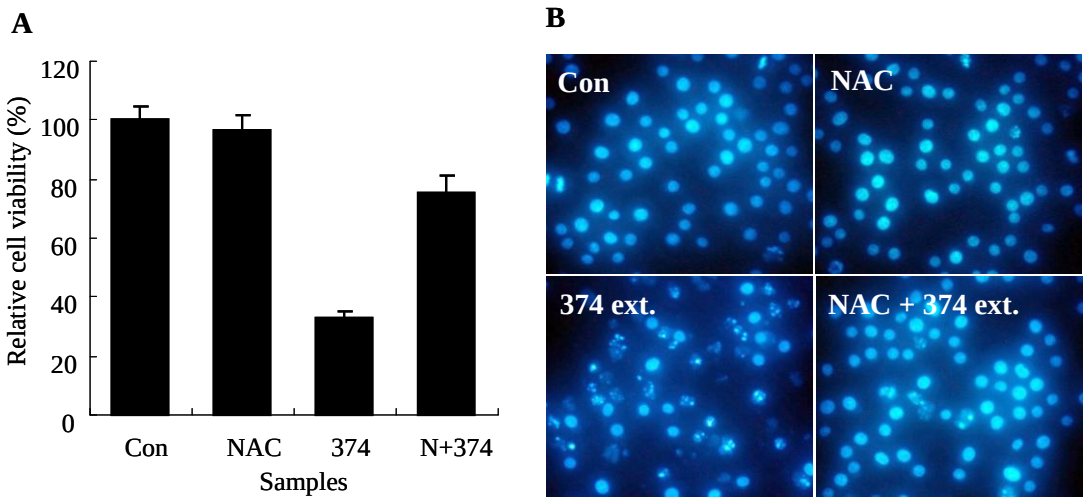

Figure 5. Inhibition of HCE-induced apoptosis by the quenching of ROS generation in the HL-60 cells. The cells were treated with NAC (2 mM) for 1 h before a challenge with HCE (13 µg/ml) for 72 h. (A) The cell viability was examined by MTT assay. (B) The cells were stained with Hoechst 33342, and the stained nuclei were observed under a fluorescence microscope.

death via an apoptotic pathway in HL-60 cells. After the HL-60 cells were treated with HCE for 12 h, the microscopic observations demonstrated apoptotic characteristics. The preponderance of apoptotic nuclei was most likely blocked at the G1 and G2 phases, which is in agreement with the DNA fluorescence flow cytometric profiles. We also noted DNA fragment ladder formation, a characteristic gel electrophoretic band pattern is associated with apoptosis. The DNA ladder, the biochemical hallmark of apoptosis, results from the degradation of DNA by endogenous DNase, which cuts the inter-nucleosomal regions into double-stranded DNA fragments of 180 to 200 bp. All of these changes result from the proteolytic cleavage of various intracellular polypeptides, mostly caused by a family of cysteine-dependent proteases called caspases (Zhang et al., 2003). Caspase-3 is a major downstream effector of apoptosis, and the caspase-3-mediated proteolytic cleavage of PARP and other substrates is a critical step leading to DNA fragmentation and chromatin condensation. All these major apoptotic events were detected in the HCE-treated cells in this study. Therefore, the results confirm that apoptosis in the HL-60 cells was induced by HCE treatment and caspase-3 mediated PARP cleavage in the apoptotic process.

Bcl-2 family proteins play a critical role in the induction of apoptosis, particularly in the interaction between pro- and anti-apoptotic proteins of the Bcl-2 family, which integrate the diverse death and survival signals to control the fate of a cell. Many studies have shown that anti-apoptotic Bcl-2 family proteins such as Bcl-xL form heterodimers with Bax, which might neutralise its pro-apoptotic effects and lead to a decrease or cessation of apoptosis (Dai and Grant, 2007; Kang and Reynolds, 2009; Lomonosova and Chinnadurai, 2009). In this study, HCE treatment resulted in a significant increase in pro-apoptotic Bax protein and decrease in the levels of anti-apoptotic Bcl-xL protein, thus shifting the Bax/Bcl-xL ratio in favour of apoptosis.

Seaweed-derived phycocyanin and phlorotannin induce their cytotoxicity on tumour cells by ROS-mediated mechanisms (Kang et al., 2006; Li et al., 2009). It was therefore interesting to observe the induction of ROS in the HL-60 leukaemia cell line after treatment with HCE. The results showed that HCE increased intracellular ROS in the HL-60 cells. The ROS then participate in a set of responses that lead to apoptosis through the generation of more intracellular ROS, driving the cell to oxidative stress-induced cell death. Among the ROS generated by HCE, H_2O_2 and $O_2^{\bullet-}$ are well-known mediators of apoptosis in cancer cells. HCE may exert its cytotoxic effect on tumour cell lines through its major component, generating large amounts of intracellular H_2O_2 and $O_2^{\bullet-}$ in tumour cells (Balsano and Alisi, 2009; Trachootham et al., 2009; Valdivia et al., 2009). Growth inhibition and ROS generation induced by HCE in the HL-60 cells indicate that ROS production probably causes apoptotic cell-death via the mitochondrial pathway.

In conclusion, HCE exhibits an anti-proliferative effect by inducing the loss of cell viability, morphological change, inter-nucleosomal DNA fragmentation, sub-G1 phase accumulation, caspase-3 and caspase-9 activation, PARP degradation and dysregulation of Bcl-xL and Bax in HL-60 cells. In addition, HCE induces ROS (at least H_2O_2 and $O_2^{\bullet-}$) elevation, which is the upstream signalling molecule, or key step, for HCE-induced apoptosis, because NAC effectively blocked apoptosis in the HL-60 cells studied. Our data confirm the potential of HCE as an

agent with chemotherapeutic and cytostatic activity for human leukaemia. Further investigations will focus on the *in vivo* assessment of the biological activity of HCE and on the chemical identification of the major active components responsible for anti-tumor activity in the efficacious seaweed extracts.

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