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Diethylaminoethyl chitosan induces apoptosis in HeLa cells via activation of caspase-3 and p53 expression

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ABSTRACT

In our previous study, diethylaminoethyl chitosan (DEAEC) was found to inhibit the viability of HeLa cells, a human uterine cervix cancer cell line. To identify the anti-proliferation activity of DEAEC, its mode of action was studied in further detail in HeLa cells. After incubation for 24 h, DEAEC had significantly inhibited HeLa cell growth with an IC_{50} of 23.2 μg/mL and showed strong apoptotic activity. After Hoechst 33258 staining, morphological changes and DNA degradation were observed. Cell cycle analysis and an Annexin V assay by flow cytometry revealed that DEAEC induced apoptosis in the HeLa cells by sub-G1 involving early stage apoptosis. RT-PCR and Western blotting analysis results showed that DEAEC induced apoptosis in the HeLa cells via up-regulation of caspase family enzyme (caspase-3, -8 and -9), p53, and Bax protein expressions, and down-regulation of Bcl-2 protein expression.

1. Introduction

Apoptosis, or programmed cell death, is an active physiological process that occurs during embryonic development and tissue remodeling, and is a natural process for removing unneeded or damaged cells with potentially harmful mutations. However, deregulation of apoptosis can disrupt the delicate balance between cell proliferation and cell death and can lead to diseases such as cancer (Danial & Korsmeyer, 2004). Most cancer cells block apoptosis via anti-apoptotic signaling pathways in order to survive despite undergoing genetic and morphologic transformations. Therefore, drugs promoting apoptosis may be effective against many cancers and should become an important strategy to counteract cancer (Fesik, 2005). Apoptotic cells are characterized by certain morphologic features, including condensation of the cytoplasm and nucleus, cell surface expression of phosphatidylserine, and internucleosomal cleavage of DNA (Park et al., 2008). The important component of apoptosis is the caspase family, which are intracellular cysteine proteases. The caspase family is divided into 2 functional categories including initiator caspase such as caspases-8, -9, and -12, which can activate downstream effector caspases such as caspase-3 (Creagh & Martin, 2001). Caspase-3, in particular, is widely recognized as a key member of the caspase family and its activation is a typical characteristic of apoptosis (Porter & Janicke, 1999). Caspase-3 is an essential player in the DNA fragmentation process and other morphological changes associated with apoptosis (Cohen, 1997). In addition, expressions of apoptotic regulatory proteins (Bcl-2, Bax, and p53) have been recently recognized as an important component of cancer cell apoptosis (Chao & Korsmeyer, 1998; Prives & Manfredi, 1993). Tumor suppressor protein p53 is one of the major regulators of the apoptotic process in response to DNA damage and environmental stress and induces cell cycle arrest and apoptosis (Haupt, Berger, Goldberg, & Haupt, 2003). Bcl-2 family members, such as Bax and Bcl-2, are key regulators of apoptosis and essential for the maintenance of major organ systems, and mutations affecting them are implicated in cancer (Adams & Cory, 1998) p53 participates as a site-specific transactivator of transcription and has been shown to regulate the cell cycle by activating the expression of pro-apoptotic or anti-apoptotic members of the B cell leukemia-2 gene product (Bcl-2) family, with apoptosis being prevented by Bcl-2 and promoted by Bax (Kroemer, 1997).

Chitosan, which is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units, is derived from chitin by deacetylation. Chitosan is the second most abundant polysaccharide in nature and its production is low cost and ecologically appealing. Due to its biocompatibility and less

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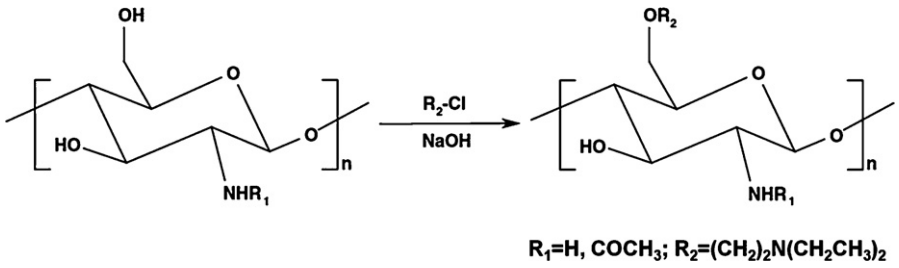

Scheme 1. Synthesis and chemical structure of DEAEC.

toxic nature, it has been developed as a new physiologically bioactive material, because it possesses various biological activities such as antioxidant activity (Park, Je, & Kim, 2004), antitumor activity (Qin, Du, Xiao, Li, & Gao, 2002), antibacterial activity (Jeon, Park, & Kim, 2001), β-secretase inhibitory activity (Byun, Kim, Park, Lin, & Kim, 2005) and acetylcholinesterase inhibitory activity (Yoon, Ngo, & Kim, 2009).

Even though chitosan has very strong functional properties in many areas, it has some disadvantage for its broad application, such as water-insoluble properties and poor bioavailability. Therefore, in the realm of chitosan research and to overcome its disadvantages, derivatives with water-soluble and functional properties are being developed as pharmaceuticals and nutraceuticals. Several reports exist regarding the antitumor and apoptosis effects of chitosan and its oligomers (Hasegawa, Yagi, Iwakawa, & Hirai, 2001; Suzuki et al., 1986). However, there is currently scanty information regarding the anticancer activity of chitosan derivatives.

In our previous report, six kinds of aminoderivatized chitosans showed strong cytotoxic effects against several cancer cell lines (Je, Cho, & Kim, 2006). In particular, diethylaminoethyl-chitosan (DEAEC) had potent cytotoxic activity as compared to other derivatives. Therefore, in the present study, we investigated DEAEC's mode of action on apoptotic pathways in a human uterine cervix cancer cell line (HeLa) using Hoechst 33258 staining, an Annexin V assay, and immunoblotting analysis for caspase family, p53, Bax, and Bcl protein expressions.

2. Materials and methods

2.1. Materials

Chitosan (average MW 310 kDa, degree of deacetylation, DD, 90%) prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). To prepare the chitosan derivative, 2-(diethylamino) ethylchloride hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media and other materials required for culturing were obtained from Gibco (Grand Island, NY). All other reagents used in this study were reagent grade chemicals.

2.2. Synthesis of DEAEC

DEAEC was synthesized according to our previous study as shown in Scheme 1 (Je et al., 2006). A 15 mL amount of 3.0 M 2-(diethylamino) ethylchloride hydrochloride was added to 0.3 g of chitosan with stirring at 65 °C. Then, 15 mL of 3.0 M NaOH was added to the reaction mixture dropwise and continuously stirred for 18 h. Subsequently, the reaction mixture was neutralized with 3.0 M HCl and dialyzed against water for 2 days. The product was freeze-dried to give the DEAEC. DEAEC was characterized by FT-IR and ^{1}H NMR spectrums. In the FT-IR, two peaks of absorptions, at 2965 cm^{-1} due to C–H stretching and at 1000–1150 cm^{-1} due to C–O–C stretching, were observed. This supports the occurrence of DEAE group substitution instead of C-6 hydroxyl groups. ^{1}H NMR (400 MHz, D_2O) δ: 4.8 (D_2O), 5.3 (1H, H-1), 3.63–4.35 (1H, H-2/6), 3.28 (methylene protons of DEAE group), 1.30 (methyl protons of DEAE group) (20).

2.3. Cell culture and viability assay

HeLa cells, a human cervical cancer cell line, were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, NY, USA) containing 100 μg/mL of penicillin–streptomycin and 10% fetal bovine serum (FBS) and maintained at 37 °C under a humidified atmosphere with 5% CO_2. The medium was changed two or three times each week. The inhibitory effects of DEAEC on HeLa proliferation were measured using the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) assay. The HeLa cells were cultured in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and were treated with different concentrations of DEAEC. After 24 h of incubation, the cells were washed two times with PBS, and 100 μL of MTT solution (1 mg/mL) was added to each well. After 4 h of incubation, 100 μL of DMSO was added to solubilize the formed formazan salt. The optical density was measured at 540 nm using a UV microplate reader (GENios microlate reader, Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan and calculated as a percentage compared to the non-treated group. The data were expressed as means of at least three independent experiments.

2.4. Morphological analysis and Hoechst staining

The HeLa cells were pre-incubated with different concentrations of DEAEC for 24 h. Morphological changes of the HeLa cells were observed under a phase-contrast microscope (DMI6000B, Leica Microsystems, Wetzlar, Germany). Apoptotic cells were identified by Hoechst 33258 staining. The HK-2 cells were seeded on 6-well plates, followed by incubation with DEAEC at various concentrations. The cells were fixed with 5% paraformaldehyde in PBS for 10 min. After staining with Hoechst 33258 for another 5 min in the dark, the cells were observed under a fluorescence microscope.

2.5. Annexin V-FITC and propidium iodide staining apoptosis tests

The HeLa cells were cultured in 6-well plates at a density of 5×10^5 and DEAEC was added at different concentrations. The cells were then incubated for 24 h. The subsequent procedures were carried out according to the manufacturer's manual provided with the Annexin V-FITC kit (BD Sciences). Briefly, the cells were washed 3 times with PBS and suspended in binding buffer at a concentration of 1×10^5 cells/mL followed by additions of FITC Annexin V and propidium iodide and left for 15 min in the dark. The samples were then analyzed by a flow cytometer (FACS Calibur, BD Sciences, Heidelberg, Germany) within 1 h.

2.6. RT-PCR

The HeLa cells were treated with different concentrations of sample for 24 h. The cells were harvested and washed 2 times with PBS. The total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA). Chloroform was added to the cell lysate and centrifuged at 13,000 × *g* for 12 min and the supernatant was collected. The same volume of isopropanol was added and the RNA pellet was collected following centrifugation. After washing with 70% ethanol, the extracted RNA was dissolved in diethylpyrocarbonate-treated RNase free water and incubated for 10 min at 60 °C. RNA concentrations were quantified by measuring optical density at 260 nm using a microplate reader (Tecan Austria GmbH, Groedig, Austria). The 1 μg of RNA obtained from the cells was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) at 42 °C for 45 min to produce the cDNAs. The RT-generated cDNAs were used as a template to amplify capase family, p53, Bax, Bcl-2, and GAPDH genes in a PCR mixture containing dNTPs and taq DNA polymerase (Promega, Madison, WI, USA). PCR was performed using a Whatman thermocycler (Biometra, Kent, UK) with selective upstream and downstream primers for GAPDH (forward 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse 5′-GACAAGCTTCCCGTTCTCAG-3′), p53 (forward 5′- GCGCACAGAGGAAGAGAATC-3′ and reverse 5′- CTCTCGGA-ACATCTCGAAGC-3′), Bax (forward 5′-TGCCAGCAAACTGGTGCTCA-3′ and reverse 5′-GCACTCCCGCCACAAAGATG-3′), Bcl-2 (forward 5′-CGCATCAGGAAGGCTAGAGT-3′ and reverse 5′-AGCTTCCAGAC-ATTCGGAGA-3′), caspase-3 (forward 5′- CCCAGGCCGTGAGGA-GTTAGC-3′ and reverse 5′-CAGCATCACTGTAACTTGCTAATC-3′), caspase-8 (forward 5′-CACTAGAAAGGAGGAGATGGAAAG-3′ and reverse 5′-CTATCCTGTTCTCTTGGAGAGTCC-3′) and caspase-9 (forward 5′-GCTCTTCCTTTGTTCATCTCC-3′ and reverse 5′-CATCTG-GCTCGGGGTTACTGC-3′). The amplified DNA was analyzed by 2% agarose gel electrophoresis and visualized by ethiduim bromide staining. G3DPH was used as an internal control for sample loading and mRNA integrity.

2.7. Western blotting

1×10^6 HeLa cells were cultured and treated with different concentrations of sample for 24 h. The HeLa cells were lysed in RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, at 4 °C for 20 min. The total protein was extracted and separated by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane from the gel. The blots were blocked for 90 min at 25 °C using TBS-T buffer containing 0.1% Tween-20 and 3% BSA. After washing the membrane with TBS-T buffer twice, the blots were incubated for 1 h with specific primary antibodies, such as mouse anti-human β-actin, mouse anti-human p53, rabbit anti-human Bax, mouse anti-human Bcl-2, rabbit anti-human caspase-3, -8 and -9 antibodies (1:1000 dilution; Santa Cruz Biotechnology) at 25 °C. After washing the blots with TBS-T buffer, the blots were incubated for 1 h with secondary antibodies at 25 °C. The blots were visualized with enhanced chemiluminescence detection and imaged using an LAS-3000 system (Fujifilm Life Science, Tokyo, Japan).

2.8. Statistical analysis

All results are expressed as the mean ± S.E.M. ($n = 3$). Differences between the means of each group were assessed by one-way ANOVA with Duncan's multiple range tests ($p < 0.05$). The statistical software, SPSS v10.01 (SPSS, Chicago, IL), was used for the analysis.

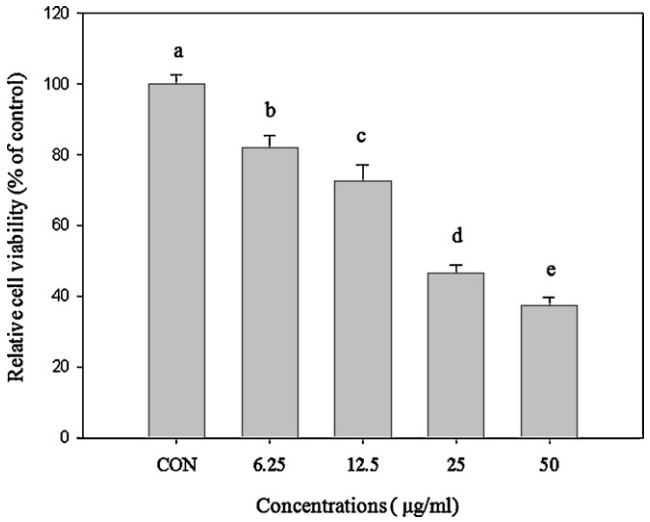

Fig. 1. Inhibitory effects of DEAEC on HeLa cell proliferation by MTT assay. The HeLa cells were treated with various concentrations of DEAEC for 24 h. Values represent means ± SE ($n = 3$). (a–e) Different letters indicate significant differences ($p < 0.05$) within each group by Duncan's multiple range test.

3. Results and discussion

3.1. Inhibition of cell proliferation in DEAEC-treated HeLa cells

In order to evaluate the inhibitory effects of DEAEC on HeLa cell proliferation, we first investigated its effects on HeLa cell viability. The HeLa cells were treated with different concentrations of DEAEC for 24 h, followed by the MTT assay. The DEAEC showed strong anti-proliferative activity in a dose-dependent manner ($p < 0.05$), by presenting relative HeLa cell viabilities of 82%, 73%, 46%, and 37% at concentrations of 6.25, 12.5, 25, and 50 μg/mL, respectively, compared to the control group (Fig. 1). DEAEC appeared to be a potent inhibitor of HeLa cell viability with an IC_{50} of 23.2 μg/mL.

3.2. Effects of DEAEC on morphological changes and Hoechst 33258 stained fluorescence images of HeLa cells

To confirm that DEAEC may induce apoptotic death in HeLa cells, morphological changes after 24 h of treatment of different concentrations of DEAEC were observed by a microscope under phase contrast mode and fluorescence mode following Hoechst 33258 staining. Under the phase contrast mode, cell shape and morphological changes were clearly observed. As shown in Fig. 2A, the control group showed regular polygonal shapes with few round cells. Cells treated with 6.25 μg/mL for 24 h showed slight morphological changes with only a small number of cells showing elongations. When the HeLa cells were incubated in the presence of 25 μg/mL of DEAEC, the cells showed significant morphological chages including loss of adhesion and filamentous protrusions. In the presence of 50 μg/mL of DEAEC, the HeLa cells showed obvious cell shrinkages, small vesicles, and membrane asymmetry, indicating the possibility of apoptosis occurrence. Furthermore, in order to determine whether the anti-proliferative effects of DEAEC on HeLa cells were due to apoptotic cell death, the cells were stained with Hoechst 33258 dye and observed under a fluorescence microscope (Fig. 2B). Viable cells (control group) with intact DNA and no morphological changes were negative to Hoechst 33258 or just slightly activated in the fluorescence microscope image. However, after incubation with DEAEC for 24 h, apoptotic cells showed morphological changes and green colored nuclei with fragmented DNA. Furthermore, Hoechst 33258 positive cells and their intensities increased in a DEAEC dose-dependent manner. This indicates that

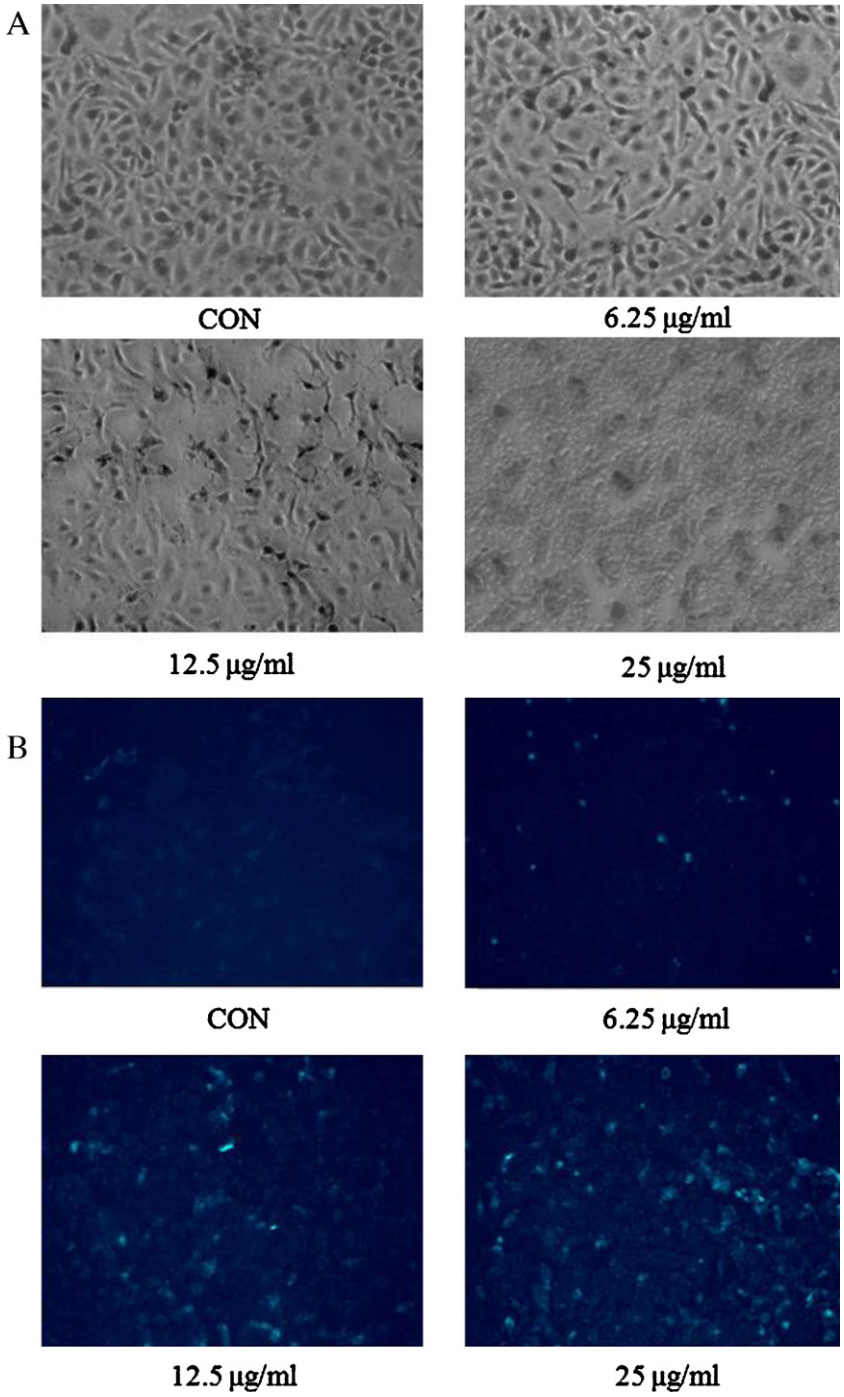

Fig. 2. Morphological changes of DEAEC-induced apoptosis in HeLa cells. (A) Phase contrast microscope images of HeLa cells. DEAEC was treated at different concentrations in HeLa cells for 24 h (B) Fluorescence microscope images of HeLa cells stained with Hoechst 33258. The cells were detected by fluorescence light microscopy at 360 nm/470 nm excitation/emission.

most of the cells underwent cell death occurring primarily through apoptosis by the treatment of DEAEC. In a previous study, among the aminoderivatized chitosans, DEAEC showed the strongest cytotoxic activity, especially against HeLa cells. However, the influence of DEAEC on signaling molecules directly involved in apoptosis has not been fully elucidated in the human uterine cervix cancer cell line, HeLa. As shown in Fig. 2A and B, its application caused specific features of the apoptotic process with dose-dependent morphological changes and DNA fragmentation. DNA fragmentation is a key feature of apoptosis and is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments. Thus, the evaluation of internucleosomal DNA fragmentation has been widely accepted as one of the best-characterized biochemical markers for apoptosis (Yang et al., 2010). Therefore, these results suggest that the effects of DEAEC on HeLa cells were primarily apoptosis rather than necrosis.

3.3. Induction of apoptosis by DEAEC in HeLa cells

To investigate the effects of DEAEC on cell cycle regulation, HeLa cells were treated with various concentrations of DEAEC for 24 hand

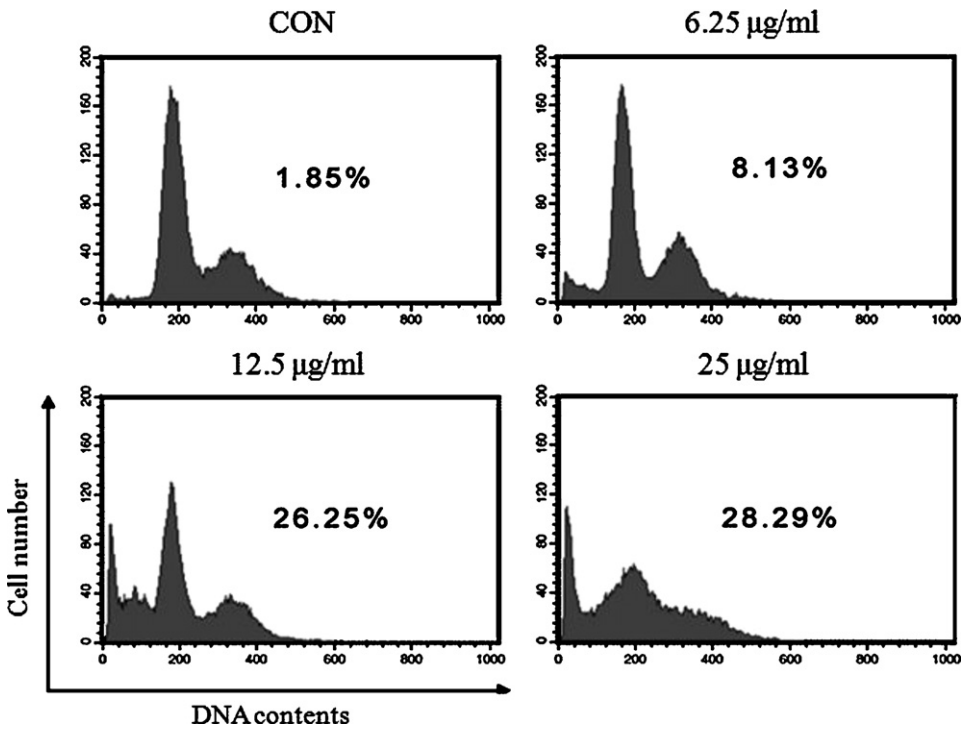

Fig. 3. Effects of DEAEC on cell cycle distribution in HeLa cells using flow cytometry. The cells were treated with various concentrations of DEAEC for 24 h and stained with PI. The percentage of the cell population in sub-G1 was calculated by flow cytometry software.

DNA content was analyzed by flow cytometry (FACS Calibur, BD Sciences, Heidelberg, Germany). As shown in Fig. 3, the treatment of DEAEC for 24 h resulted in a decrease of the G1 population followed by an increase in the sub-G1 population of HeLa cells. DEAEC induced increases of the sub-G1 population by 8.13%, 26.25%, and 28.29% at the concentrations of 6.25, 12.5, and 25 μg/mL, respectively. Futhermore, to assess whether DEAEC induced apoptosis or necrosis, Hela cells were treated with DEAEC for 12 h follwed by Annexin V-PI staining assay. Phosphatidylserine-specific fluorescein isothiocyanate (FITC)-Annexin V staining assay offers the possibility of detecting early stage apoptosis before the loss of cell membrane integrity, and permits kinetic measurements of apoptotic cell death in relation to the cell cycle (Vermes, Haanen, Steffens-Nakken, & Reutellingsperger, 1995). Propidium iodide

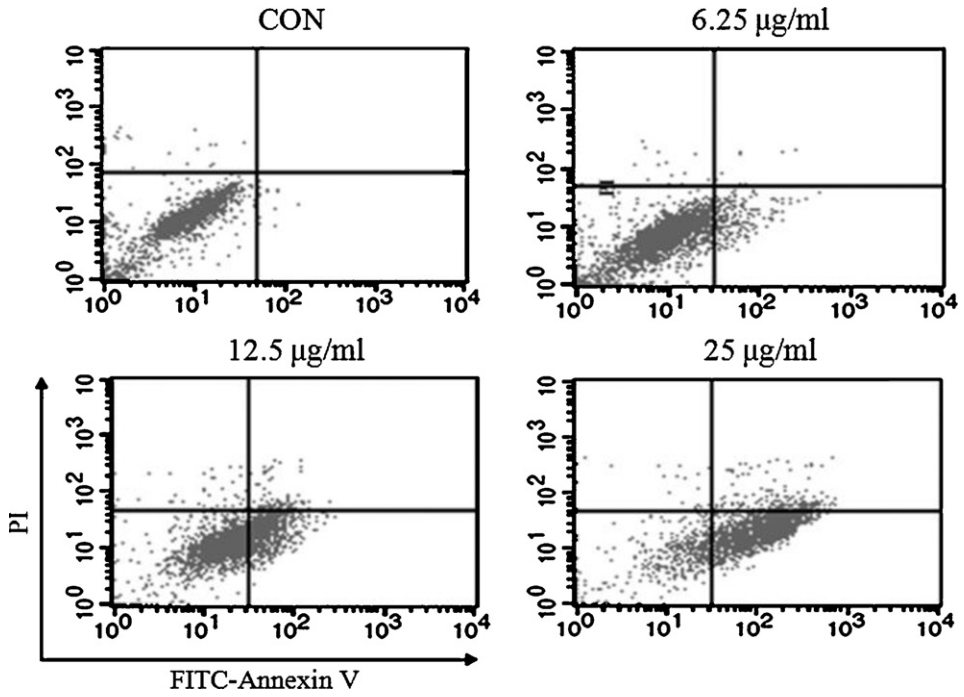

Fig. 4. Assessment of apoptosis induced by DEAEC in HeLa cells using an FITC-Annexin V assay. The cells were treated with various concentrations of DEAEC for 24 h and stained with FITC-Annexin V and PI followed by flow cytometric analysis. (The lower left quadrant: Annexin V− and PI−, viable cells; The lower right quadrant: Annexin V+ and PI−, apoptic cells; The upper right quadrant: Annexin V+ and PI+, dead cells).

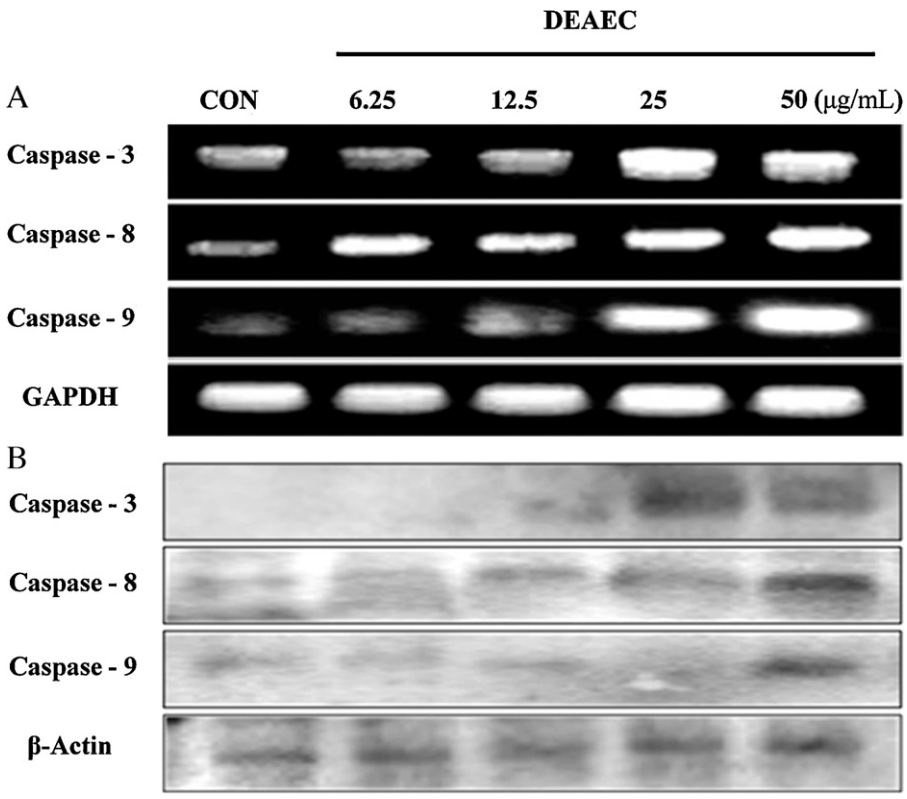

Fig. 5. Effects of DEAEC on the mRNA (A) and protein (B) expression levels of caspase-3, -8, and -9 in HeLa cell using RT-PCR and Western blotting analysis. The cells were treated with various concentrations of DEAEC for 24 h. GAPDH and β-actin were used as an internal control.

staining can be visible in late apoptotic or dead cells because the membranes of damaged and dead cells are permeable to PI. Viable cells are negative to Annexin V and PI. However, early apoptotic cells are Annexin V positive and PI negative, and late apoptotic or already dead cells are positive to both Annexin V and PI. Fig. 4 shows the contour diagrams of Annexin V and PI stained HeLa cells using flow cytometry after 12 h of incubation with different concentrations of DEAEC. As you can see in Fig. 4, in the lower right quadrants (Annexin V + and PI −) the cell population increases dose dependently, and at the concentration of 25 μg/mL there is a slight increase of the cell population in the upper right quadrant (Annexin V+ and PI+). Dying cells undergoing the final stages of apoptosis translocate phosphatidylserine to the cell surface (Li, Sarkisian, Mehal, Rakic, & Flavell, 2003). Phosphatidylserine is normally located on the cytosolic surface of the plasma membrane, but is redistributed to the extracellular surface by a scramblase during apoptosis (Wang et al., 2003). These results represent that DEAEC actually induced cell death via apoptosis rather than a necrotic pathway involving early stage apoptosis.

3.4. Effects of DEAEC on expression of caspase family enzymes in HeLa cells

Caspases are expressed in almost all cell types as inactive proenzymes (Enari, Talanian, Wrong, & Nagata, 1996). Much evidence suggests that activation of caspases triggers the apoptotic process in various cell types (Grimm, Bauer, Baeuerle, & Schulze-Osthoff, 1996; Kwon, Park, Ryu, & Park, 2002). Activated caspase-3 then cleaves some specific substrates, such as poly (ADP-ribosyl) polymerase (PARP) and D4-GDI proteins, which have important roles in the occurrence of typical biochemical and morphological changes in apoptotic cells (Krieser & Eastman, 1999). To elucidate the possible mechanisms of apoptosis in HeLa cells, we investigated the effects of DEAEC with respect to mRNA expression by RT-PCR. Different concentrations of DEAEC were added to HeLa cells for 24 h. As shown in Fig. 5A, the expression levels of caspase-3, 8, and 9 increased notably at the concentration of 12.5 μg/mL compared to the untreated group, and continuously increased dose-dependently. Furthermore, we investigated the effects of DEAEC on caspase family protein expression by Western blotting analysis, in which similar findings were observed to those of RT-PCR (Fig. 5B). DEAEC treatment promoted caspase-3, -8, and -9 protein expressions dose-dependently in the HeLa cells. Taken together, these results suggest that the mechanism of DEAEC-induced apoptosis in HeLa cells involves the activation of caspase family expression.

3.5. Effects of DEAEC on expressions of p53, Bcl-2, and Bax in HeLa cells

To determine whether p53 was involved in modulating apoptosis induced by DEAEC, we investigated the effects of DEAEC on mRNA and protein expressions of p53, Bax, and Bcl-2 in HeLa cells. Different concentrations of DEAEC were added to HeLa cells for 24 h, and RT-PCR and Western-blotting were carried out. In the RT-PCR results, DEAEC treatment promoted the mRNA expression of p53, and down-regulated Bcl-2 and up-regulated Bax expressions in a dose-dependent manner (Fig. 6A). According to the Western blotting assay results, protein expression levels of p53, Bax, and Bcl-2 complied with the RT-PCR results (Fig. 6B). It is well known that tumor suppressor p53 protein can be induced and activated in DNA damage-mediated cell death (Thomas, Giesler, & White, 2000) p53 can control the regulation of Bcl-2 family proteins, which are the most important regulators for characterizing apoptosis by leading to the disruption of mitochondrial membranes. Apoptotic pathways can be divided into two major group, such as intrinsic or mitochondrial and extrinsic or death receptor-related (Li, Nie, Yu, & Xie, 2009). Bcl-2 family members have important roles in regulating

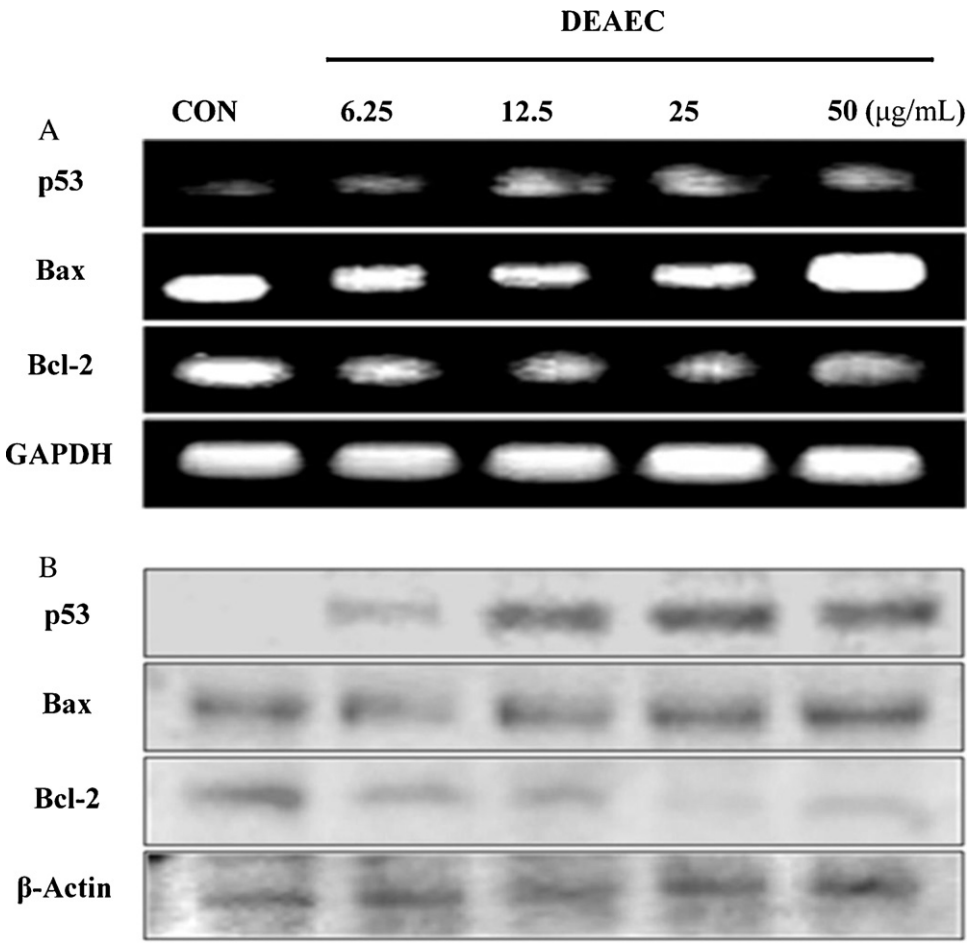

Fig. 6. Effects of DEAEC on the mRNA (A) and protein (B) expression levels of p53, Bax, and Bcl-2 in HeLa cell using RT-PCR and Western blotting analysis. The cells were treated with various concentrations of DEAEC for 24 h. GAPDH and β-actin were used as an internal control.

mitochondrial integrity and mitochondria-initiated caspase activation (Cheng et al., 2001). The mitochondrial pathway is thought to be important in both events, and is mediated by Bcl-2 family proteins: the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax (Antonsson & Martinou, 2000). Over-expression of Bcl-2 has been reported to protect tumor cells from apoptosis, whereas increased Bax expression promotes apoptosis (Hockenbery, Nunez, Milliman, Schreiber, & Korsmeyer, 1990). The anti-apoptotic function of Bcl-2 is thought to be primarily derived from Bcl-2 presented in the mitochondria (Li et al., 2009). Bax mostly exists as a soluble monomer in the cytosol. However, upon apoptotic stimulation, Bax translocates to the mitochondria where it forms oligomers that are inserted into the outer mitochondrial membrane, and mitochondrial Bcl-2 decreases (Antonsson, Montessuit, Sanchez, & Martinou, 2001). Collectively, these results suggest that DEAEC induced apoptosis by activating expressions of p53 and Bax and inhibiting Bcl-2.

Our previous report suggests that the cytotoxic activity of DEAEC seems to depend on the hydrophobicity of its diethylaminoethyl groups, which can interact with tumor cell surfaces, and its activity may be caused by increased cationic charge due to free amino groups with electrostatic interactions between chitosan and negatively charged functional residues on the tumor cell surface such as mucosal membranes (Je et al., 2006). Several papers have also reported the importance of cationic characteristics related to cytotoxic effects in tumor cell lines. Chitosan nanoparticles and copper-loaded chitosan nanoparticles exhibited cytotoxic activities against various tumor cell lines. Copper-loaded chitosan nanoparticles with high surface zeta potentials showed excellent cytotoxic activity compared to chitosan nanoparticles (Qi, Xu, Jiang, Li, & Wang, 2005). As a kind of cationic polymer, the surface charge of chitosan derivatives is a major factor affecting its cytotoxic activity due to electrostatic interactions between the positively charged amino groups of chitosan and the negatively charged groups of tumor cells (Lee, Lim, & Kim, 2002). In summary, increasing hydrophobic moieties and cationic characteristics are important factors for the development of anticancer drugs using chitosan, and positively charged DEAEC with high hydrophobicity may have an important role in inducing apoptosis by interactions with cancer cell surfaces.

4. Conclusion

In the present study, we demostrated that DEAEC exerted inhibitory activity against HeLa cell proliferation by causing apoptosis with morphological changes and arresting cells in the sub-G1 phase of the cell cycle. Moreover, we also found that DEAEC induced apoptotic cell death in HeLa cells in association with activating expressions of caspase family enzymes and p53 followed by Bax/Bcl-2 regulation. This study is the first to provide clear evidence that DEAEC induces apoptosis in HeLa cell and suggests that DEAEC has strong potential as a therapeutic agent for preventing cancers, including human uterine cervix cancer.

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