Cantharidin induces G2/M phase arrest and apoptosis in human colorectal cancer colo 205 cells through inhibition of CDK1 activity and caspase-dependent signaling pathways

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Abstract. Cantharidin (CTD) is a traditional Chinese medicine and an effective component isolated from *blister beetle*, and it has been demonstrated to have anticancer, antibiotic, antivirus activities and immune-regulated functions. It has been reported that CTD induces cell cycle arrest and apoptosis in many cancer cell types. However, there are no reports showing that CTD would induce cell cycle arrest and apoptosis in human colorectal cancer colo 205 cells. In this study, we studied colo 205 cells which were treated with CTD and demonstrated its molecular mechanisms in apoptosis. CTD induced growth inhibition, G2/M phase arrest and apoptosis in colo 205 cells. The IC_{50} is 20.53 μM in CTD-treated colo 205 cells. DAPI/TUNEL double staining and Annexin V assays were used to confirm the apoptotic cell death in colo 205 cells after CTD exposure. CTD caused G2/M arrest, down-regulated CDK1 activity, decreased Cyclin A, Cyclin B, CDK1 and increased CHK1 and p21 protein levels. Colorimetric assays also indicated that CTD triggered activities of casapse-8, -9 and -3 in colo 205 cells. Moreover, CTD increased ROS production and decreased the level of mitochondrial membrane potential ($\Delta\Psi m$) in colo 205 cells. Consequently, CTD-induced growth inhibition was significantly attenuated by *N*-acetylcysteine (NAC, a scavenger). CTD stimulated the protein levels of Fas/CD95, the caspase-3 active form, cytochrome *c* and Bax, but suppressed the protein levels of pro-caspase-8, pro-caspase-9 and Bcl-2, determined by Western blot analysis. Based on our observations, we suggest that CTD is able to induce G2/M phase arrest and apoptosis in colo 205 cells through inhibition of CDK1 activity and caspase-dependent signaling pathways.

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Key words: cantharidin, traditional Chinese medicine, human colorectal cancer colo 205 cells, G2/M arrest, apoptosis

Introduction

Colorectal cancer is a frequent reason of cancer death in the world and in Taiwan based on reports from the Department of Health, R.O.C. (Taiwan) (http://www.doh.gov.tw/EN2006/index_EN.aspx.). Colorectal cancer is a multistep process including progressive disruption of intestinal epithelium growth (1). The treatment of colorectal cancer in clinic consists of surgery, radiation and chemotherapy, but these treatments of colon cancer are limited and the result is not acceptable. Induction of cell cycle arrest and apoptosis in tumor cells may be a considered strategy for colon cancer. The process of cell cycle has been investigated frequently, particularly CDK1/Cyclin B complex play an important role for regulation of G2/M phase (2,3). Much evidence shows that tumor cells can be induced to cell death through cell arrest and apoptosis (4,5).

Apoptosis (programmed cell death type I), is vital in shaping an organism during embryonic development. Apoptosis causes specific morphological modification such as cell membrane blebbing, induced translocation of phosphatidylserine (PS) from inner membrane to the outer membrane, chromatin condensation, caspases activation and DNA fragmentation (6,7). Many studies have demonstrated that death-receptor, mitochondria and endoplasmic reticulum played an important role in apoptosis (8,9). The activation of effector caspases such as caspase-3 and -7 by initiator caspases (caspase-8 and -9) are responsible for the cleavage of cellular substrates degrading the chromosomes into nucleosomal fragments during apoptosis (10,11). Two major pathways are involved in cell apoptosis; the death receptor pathway and the mitochondria-dependent pathway. The death receptor pathway is involved in Fas/CD95 and caspase-8 activation and then activates caspase-3. The mitochondrial signaling pathway is mediated by the mitochondrial depolarization, decrease of mitochondrial membrane potential ($\Delta\Psi m$), increase of reactive

oxygen species (ROS) production, leading to the release of cytochrome *c*, Apaf-1, AIF and procaspase-9, and combination with Apaf-1 and procaspase-9 to form the apoptosome (12). Therefore, many reports have focused on selectively killing of cancer cells through the induction of apoptosis.

Cantharidin (CTD) is extracted from *mylabris* (*blister beetle*, a traditional Chinese medicine) and a type of terpenoid. It has been demonstrated that CTD possesses antitumor, antibiotic, antivirus and regulation of immunity (13,14). Also, CTD has been used as a medical agent over 2,000 years and there are many applications, including abortifacient, dropsy, aphrodisiac and warts (13). In recent studies, CTD was shown to be able to inhibit cell proliferation in many types of cancer cell lines such as pancreatic cancer, hepatoma, bladder carcinoma and breast cancer (15-17). CTD can also reduce the activity of protein phosphatase 2A (PP2A) (15) which is likely to suppress tumor proliferation and also used as a target molecule to detect the suppression of tumor (17). Therefore, the purpose of this study was to examine whether CTD might inhibit proliferation and *in vitro* anticancer mechanisms of apoptosis in human colorectal cancer colo 205 cells.

Materials and methods

Chemicals and reagents. Cantharidin (CTD, Fig. 1), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide), propidium iodide (PI), Tris-HC1, Triton X-100, *N*-acetylcysteine (NAC) and RNase A were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen Corp. (Carlsbad, CA, USA). The primary antibodies were obtained as follows: antibodies for caspase-8, -9 and-3 were purchased from Cell Signaling Technology (Beverly, MA, USA); antibodies for Cyclin A, Cyclin B, CDK1, CHK1, p21, cytochrome c, Bax, Bcl-2, β-actin and horseradish peroxidase (HRP)-linked goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-9, -8 and-3 activity assay kits were obtained from R&D System Inc. (Minneapolis, MN, USA). Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay kit was purchased from Roche Diagnostics Corp. (Indianapolis, IN, USA). Annexin V assays kit and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen.

Cell culture. The human colorectal cancer cell line colo 205 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm^2 tissue culture flasks in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine and grown at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere (9).

Cell morphological examinations. Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 24-well plates and then incubated with 20 μM CTD for 24 h. Cells were directly examined and photographed under a contrast-phase microscope (18).

Assay for cell viability. Colo 205 cells ($2.5x10^4$ cells/well) were seeded onto 96-well plates and then treated with 0, 10, 20 and 40 μM CTD for 24, 48 and 72 h. After CTD incubation, MTT (0.5 μg/ml, 100 μl) was added to each well and then cells were incubated at 37˚C for 4 h. One hundred microliters of 0.04 N HCl/isopropanol was added and the absorbance at OD570 nm was measured for each well. The cell survival ratio was expressed as % of control. All results were performed in three independent experiments (19,20).

Determinations of cell cycle distribution and apoptosis by flow cytometric analysis. Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 12-well plates and then incubated with 20 μM CTD for 12, 24 and 48 h. Cells were then harvested and washed by centrifugation to determine the DNA content. Subsequently, cells were fixed by 70% ethanol at -20˚C overnight and then re-suspended in PBS containing 40 μg/ml PI, 100 μg/ml RNase A and 0.1% Triton X-100 in dark room for 30 min. The cell cycle distribution and apoptotic cells (sub-G1 phase) were determined by flow cytometry (FACSCalibur™ , Becton-Dickinson, Franklin Lakes, NJ, USA). All results were performed in three independent experiments (21,22).

DAPI/TUNEL double staining. Colo 205 cells ($2x10^5$ cells/well) were seeded onto 24-well plates and then exposed to 20 μM CTD for 24 h. After treatment, the cells were harvested and immediately incubated with working strength terminal deoxynucleotidyl transferase (Tdt) enzyme (Roche Diagnostics Corp.) at 37˚C for 1 h. The cells were immersed in stop/wash buffer and gently rinsed with PBS. FITC-labeled anti-digoxigenin conjugate was then applied to cells and incubated at 37˚C for 30 min in the dark. The cells were washed in PBS then stained with DAPI and mounted with DABCO (Sigma-Aldrich Corp.). DAPI and TUNEL positive cells were visualized with a fluorescence microscope (23,24).

CDK1 kinase activity. Colo 205 cells ($1x10^7$ cells) were seeded onto 75 cm^2 tissue culture flask and then treated with 20 μM CTD for 0, 12 and 24 h. Cells were suspended in a buffer containing, in a final volume of 0.2 ml, 50 mM Tris-HC1 (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 50 pg/ml leupeptin, 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol, 0.01% Brij35, 25 mM *b*-glycerophosphate, and 0.5 M NaCl. Cell suspensions were sonicated and centrifuged at 10,000 x g for 30 min. To determine the CDK1 kinase activity condition using MV Peptide and determined by measuring OD492 was as described previously (25).

Annexin V assay and flow cytometry to detect apoptotic cells. Colo 205 cells ($2.5x10^5$ cells/well) were seeded onto 24-well plates and then incubated with 20 μM CTD for 12 h. The harvested cells were then conjugated with Annexin V-FITC (1 μg/ml) incubated for 30 min at room temperature in the dark, and analyzed by flow cytometry (26,27).

Detection of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi m$). Colo 205 cells were plated onto 24-well plates and exposed to 20 μM CTD for various time periods before being harvested, washed twice, and re-suspended in the 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA, Invitrogen) and 3,3'-Dihexyloxacarbocyanine

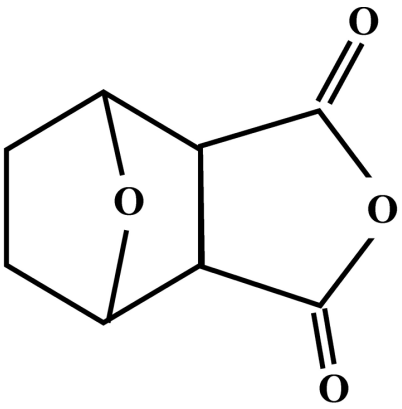

Figure 1. The structure of cantharidin (CTD).

iodide (DiOC6, Invitrogen) to determine the levels of ROS and $\Delta\Psi m$, respectively. Cells were then incubated at 37˚C for 30 min and then measured by flow cytometry as previously described (12,28).

Caspase-3, -8 and -9 activities. Colo 205 cells ($5x10^6$ cells) were seeded in 6-well plates and treated with 20 μM CTD for 24 h. Cells were harvested and lysed in 50 ml lysis buffer which contained 2 mM DTT for 10 min. The supernatant containing protein was incubated with caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA), respectively, in reaction buffer. The samples were incubated in 96-well flat bottom plate at 37˚C for 1 h. The amounts of released pNA were measured at OD405 nm with ELISA reader (19,29).

Western blot analysis. Colo 205 cells ($2.5x10^7$ cells) were seeded in 75-T flask and treated with 20 μM CTD for 0, 6, 12, 18 and 24 h. The cells were harvested and the total proteins were collected. The protein concentration was measured by using a BCA assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Equal amounts of cell lysate were run on 10-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a nitrocellulose membrane by using iBot™ Dry Blotting System (Invitrogen). The blot was soaked in blocking buffer (5% non-fat dry milk/0.05% Tween-20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then incubated with anti-caspase-8, -9, -3, Cyclin A, Cyclin B, CDK1, CHK1, p21, cytochrome c, Bax, Bcl-2 and β-actin antibodies in blocking buffer at 4˚C overnight. Membranes were washed three times with Tris-buffered saline/Tween-20 for 10 min and incubated with secondary horseradish peroxidase (HRP)-conjugated antibody. The blots were developed using a chemiluminescence (ECL) detection kit (Millipore, Billerica, MA, USA) followed by development on Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY, USA). All results were performed in three independent experiments (23,30).

Statistical analysis. Student's t-test was used to analyze the differences between the CTD-treated groups and control sample. All data were expressed as the mean ± SD from at least three independent experiments. ***p0.001 is indicative of significant difference.

Results

CTD decreases cell viability and increases morphological changes in colo 205 cells. We investigated the cell growth inhibition effects and cell viability of CTD in colo 205 cells. Cells were treated with different concentrations (0, 5, 10, 20 and 40 μM) of CTD, and then MTT assay was used to detect cell viability at 0, 24, 48 and 72 h. As shown in Fig. 2A, the cell viability was significantly decreased in a dose- and time-dependent manner in CTD-treated colo 205 cells. The IC_{50} is 20.53 μM in colo 205 cells at 24-h exposure, cell morphological changes were observed, including cell rounding and shrinkage after 24 h-incubation with 20 μM CTD as shown in Fig. 2B.

CTD induces G2/M phase arrest and apoptosis in colo 205 cells. We investigated the CTD-induced cell growth inhibition in colo 205 cells to evaluate whether it may be through the cell cycle arrest and/or apoptotic mechanisms. Cells were treated with 20 μM CTD for 0, 12, 24, and 48 h, and then cell cycle distribution was analyzed by flow cytometry. CTD induced G2/M phase arrest and increased the apoptotic cells in colo 205 cells (Fig. 3A). DAPI/TUNEL double staining was used to confirm the CTD-induced DNA fragmentation and apoptosis in colo 205 cells (Fig. 3B).

CTD reduces CDK1 activity and G2/M phase-associated protein levels in colo 205 cells. CTD-treated colo 205 cells were arrested in G2/M phase and decreased CDK1 activity at 12 and 24 h (Fig. 4A). We also determined the G2/M phase-associated proteins by Western blot analysis as seen in Fig. 4B. CTD caused decrease in protein levels of Cyclin A, Cyclin B and CDK1 and increase in CHK1 and p21 proteins. Our results suggest that CTD is able to decrease the CDK1 activity and induce G2/M phase arrest in colo 205 cells.

CTD induces phosphatidylserine (PS) exposure and caspase-8, -9, -3 activities in colo 205 cells. To confirm the CTD-induced early apoptotic characteristics in colo 205 cells, cells were treated with 20 μM CTD for 12 h and the apoptotic cells were determined by Annexin V analysis. Treatment of colo 205 cells with CTD induced the exposure of phosphatidylserine (PS) from inner side of the plasma membrane to the outer layer of the cell membrane by Annexin V (positive cells of CTD-treated colo 205 cells: 44.22±2.68%; Fig. 5A). To investigate the mechanism of CTD-induced apoptosis, we investigated caspase-8, -9 and -3 activities in colo 205 cells. CTD caused an increase of caspase-8, -9 and-3 activities in 24-h treatment (Fig. 5B). Our results suggest that caspase activation may be involved in CTD-induced apoptotic cell death in colo 205 cells.

CTD-triggered apoptosis is accompanied by ROS production and loss of mitochondrial membrane potential ($\Delta\Psi m$). We examined the effects of CTD on the ROS production and loss of $\Delta\Psi m$ by using the specific dye DCFH-DA and DiOC6, respectively. Our results shown in Fig. 6 indicate a remarkable increase of ROS production (Fig. 6A), and decrease the $\Delta\Psi m$ was observed after 3, 6 and 12 h in CTD-treated colo 205 cells (Fig. 6B). Cells were pretreated with a ROS scavenger

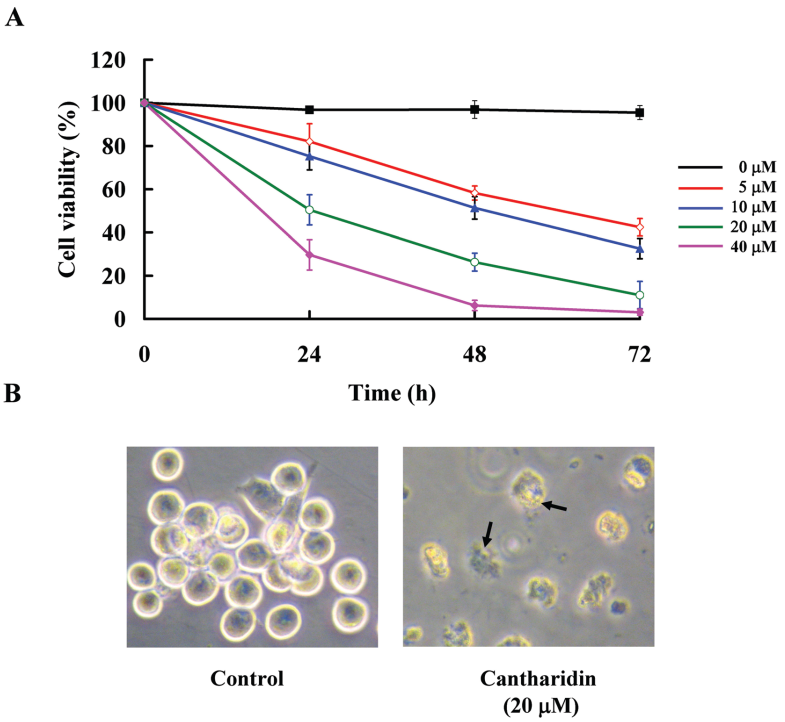

Figure 2. Effects of CTD on cell viability and morphological changes of colo 205 cells. Cells were treated with 0, 5, 10, 20 and 40 μM CTD for 0, 24, 48 and 72 h and then harvested for determination of cell viability by using MTT assay (A). Cells were exposed to 20 μM CTD for 24 h, and then were examined and photographed for morphological changes by a phase-contrast microscope (B). Data represent the mean ± SD of three experiments.

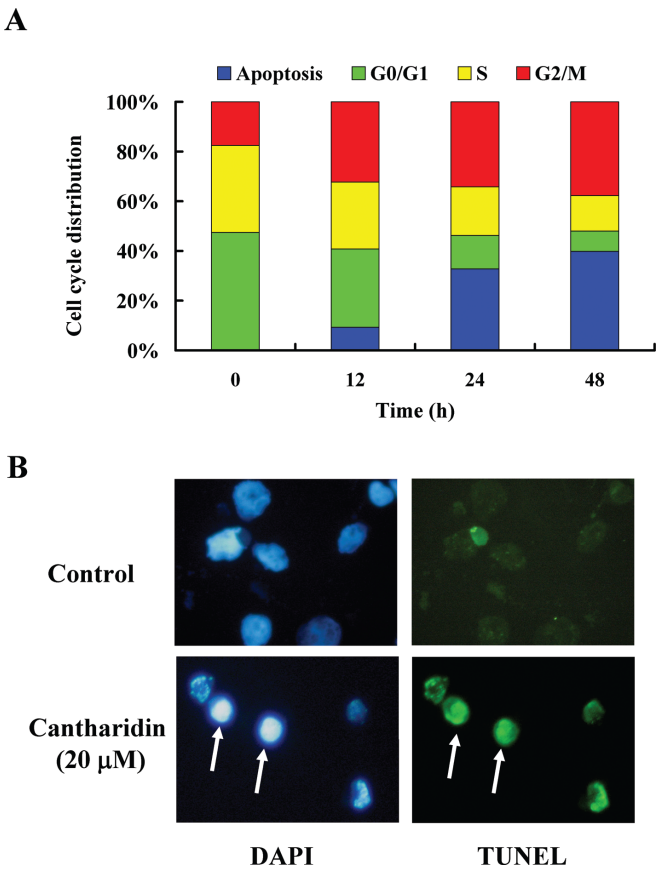

Figure 3. Effects of CTD on cell cycle distribution and apoptosis of colo 205 cells. Cells were treated with 20 μM CTD for 0, 12, 24 and 48 h and then cell cycle distribution was detected by flow cytometry (A). Apoptotic cells after exposure to 20 μM CTD for 24 h were detected by DAPI/TUNEL double staining, and then examined and photographed under a fluorescent microscopy (x400) as described in Materials and methods (B).

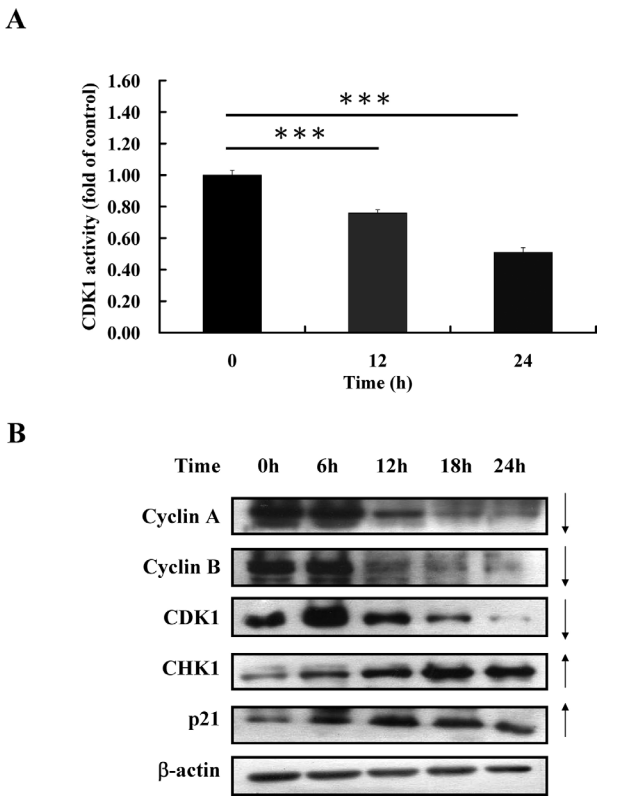

Figure 4. Effects of CTD on CDK1 activity and G2/M-associated protein levels of colo 205 cells. Cells were treated with 20 μM CTD for 0, 12 and 24 h and CDK1 kinase activity was measured in cellular extracts for the ability to phosphorylate the MV Peptide, a CDK1 kinase specific substrate according to the manual of Medical & Biological Laboratory's CDK1 kinase assay kit (A). Colo 205 cells were exposed to 20 μM CTD for 0, 6, 12, 18 and 24 h, and then the total proteins were detected by Western blotting. Respectively, primary antibodies for Cyclin A, Cyclin B, CDK1, CHK1 and p21 were examined (B). $^{***}p<0.001$.

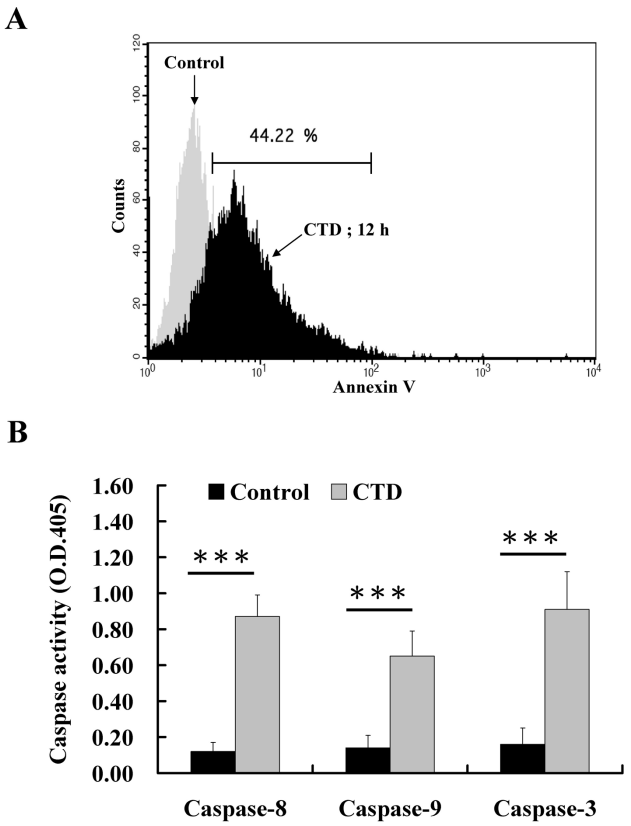

Figure 5. Effects of CTD on phosphatidylserine (PS) exposure and caspase-8, -9, -3 activities of colo 205 cells. Cells were treated with 20 μM CTD for 0 and 12 h and then the phosphatidylserine (PS) translocation was detemined by flow cytometry (A). Colo 205 cells were incubated with 20 μM CTD for 24 h. The total cell extracts were incubated with caspase-3, -9, and -8 specific substrates respectively (Ac-DEVD-pNA, Ac-LEHD-pNA and Ac-IETD-pNA, respectively). The release of pNA was measured at 405 nm by spectrophotometry (B). ***p<0.001.

(NAC) to block CTD-induced growth inhibition and cell death by MTT assay (Fig. 6C). Our results suggest that CTD-promoted ROS production and CTD-induced growth inhibition was significantly attenuated by NAC and loss of $\Delta\Psi m$. Therefore, CTD-induced apoptotic cell death in colo 205 cells is mediated through ROS and mitochondria-dependent pathways.

CTD affectes the levels of apoptosis-associated proteins in colo 205 cells. We investigated the protein levels of Fas/CD95, caspase-8, -3, cytochrome *c*, caspase-9, Bax and Bcl-2 proteins by using Western blot analysis. As shown in Fig. 7A, CTD increased the levels of Fas/CD95, cleavage of caspase-3 and decreased the levels of pro-caspase-8. CTD also promoted the levels of cytochrome *c*, Bax and attenuated the levels of pro-caspase-9 and Bcl-2 (Fig. 7B). Our results suggest that CTD-induced cell death in colo 205 cells is through the death receptor and mitochondria-mediated apoptotic pathways.

Discussion

In this study, we investigated the anti-colorectal cancer activity of CTD on colo 205 cells *in vitro* and focused on cell cycle arrest, cell apoptosis and its apoptotic signaling pathways. Our results indicated CTD induced growth inhibition and cell death in colo 205 cells in a dose- and time-dependent manner (Fig. 2A). Colo 205 cells treated with CTD resulted in G2/M phase arrest in cell cycle distribution (Fig. 3A). After exposure to CTD in colo 205 cells, the CDK1 kinase activity was reduced, leading to broken G2/M progression. Our results also showed that the protein levels of CDK1, Cyclin A and Cyclin B were decreased after treatment with CTD in colo 205 cells. Moreover, the level of p21 was up-regulated. It was also reported that the G2/M checkpoint plays an important

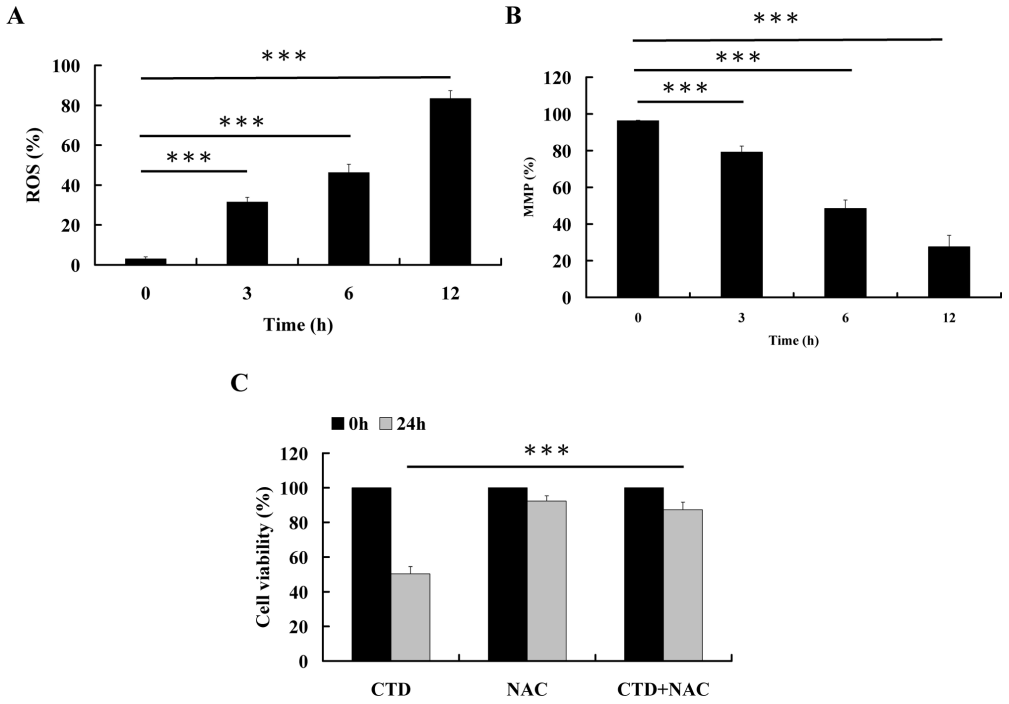

Figure 6. Effects of CTD on ROS production and loss of mitochondrial membrane potential ($\Delta\Psi m$) of colo 205 cells. Cells were treated with 20 μM CTD for 0, 3, 6 and 12 h and then the changes of ROS production (A) and loss of $\Delta\Psi m$ (B) was determined by DCFH-DA and DiOC6 specific dyes, respectively, by flow cytometry. Cells were pretreated with N-acetylcysteine (NAC), a ROS scavenger, for 1 h and then treated with 20 μM CTD for 24 h. Cells were harvested for determination of cell viability by using MTT assay (C). ***p<0.001.

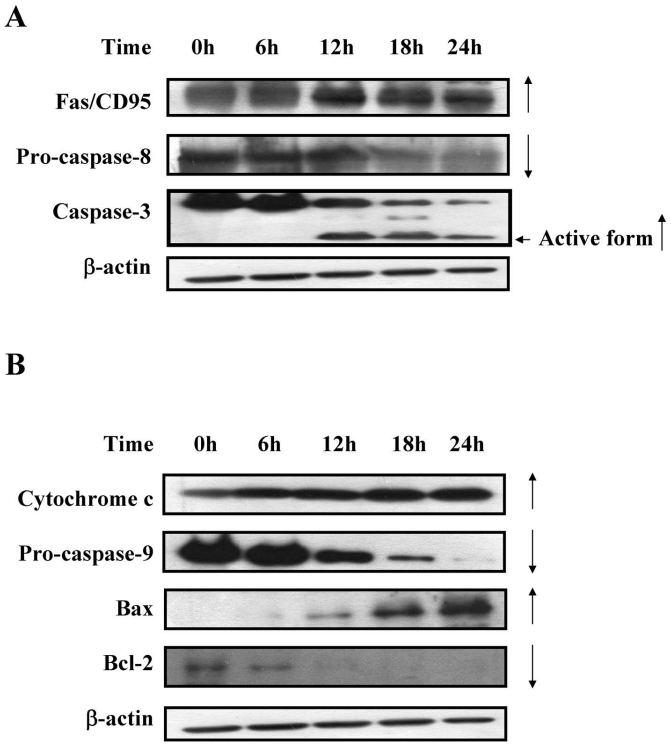

Figure 7. Effects of CTD on apoptosis-related protein levels of colo 205 cells. Western blot analysis was used for protein levels of Fas/CD95, caspase-8, caspase-3 (A) and cytochrome *c*, caspase-9, Bax, Bcl-2 (B) in CTD-treated colo 205 cells.

role for DNA damage-induced apoptosis. The CDK1/Cyclin B complex is the major regulator leading the G2 to M phase progression (2,9). Recently, it was reported that p21, a potent cyclin-dependent kinase inhibitor (CKI), is a key regulator, and it can inhibit the CDK1 activity and strengthen the G2/M arrest of cell cycle distribution (15,31). It was also reported that CTD is a potent inhibitor of PP2A, which may block the antigen-presenting cell (APC) activity (32). Cdh1 can inhibit CDK1 activity and it is a co-activator of APC (33). Our results suggest that colo 205 cells, after treatment with CTD, might be able to induce APC through inhibition of PP2A and down-regulation of CDK1.

Activation of caspase is one of the major mechanisms that promotes cell apoptosis responding to death receptor signal (extrinsic or death-receptor pathway) and mitochondrial stress (intrinsic or mitochondrial pathway) (34). In this study, we first demonstrated that CTD-reduced cell growth and cell death in colo 205 cells is through induction of cell apoptosis. We provided strong evidence that CTD induced apoptosis through the death receptor and mitochondrial apoptotic pathways in colo 205 cells. We showed in the present study that CTD: i) decreased the percentage of viable cells by MTT assay (Fig. 2A); ii) triggered apoptotic morphological changes (Fig. 2B); iii) induced DNA condensation and DNA fragmentation by DAPI/TUNAL staining (Fig. 3B); iv) stimulated translocation of phosphatidylserine (PS) from inner membrane to the outer membrane by Annexin V analysis (Fig. 5A); v) increased the protein levels of Fas/CD95, cleavage caspase-3, cytochrome *c*, Bax (Fig. 7); vi) promoted caspase-8, -9 and -3 activities (Fig. 5B).

Previous studies have demonstrated that Bcl-2 and Bax locate in the mitochondrial outer-membrane and the Bcl-2/Bax ratio regulates the release of mitochondrial cytochrome *c* to cytosol (24,35). Our results showed that CTD promoted the expression of Bax (a pro-apoptotic protein) and suppressed the levels of Bcl-2 (an anti-apoptotic protein) by Western blotting (Fig. 7B). Furthermore, CTD increased the level of cytochrome *c* and decreased the level of pro-caspase-9 (Fig. 7B) in colo 205 cells. This is in agreement with another report that CTD treatment led to dramatically decreased expression of Bcl-2 in human bladder carcinoma cells (36,37). It was reported that CTD-induced growth inhibition was not mitigated in pancreatic cancer cells with NAC (15). In our study, pretreatment with NAC protected apoptosis against CTD-induced production of ROS (Fig. 6A) and growth inhibition in colo 205 cells. This is in agreement with a previous report that the cytotoxic effect of cantharidin was believed to be related to oxidative stress (38).

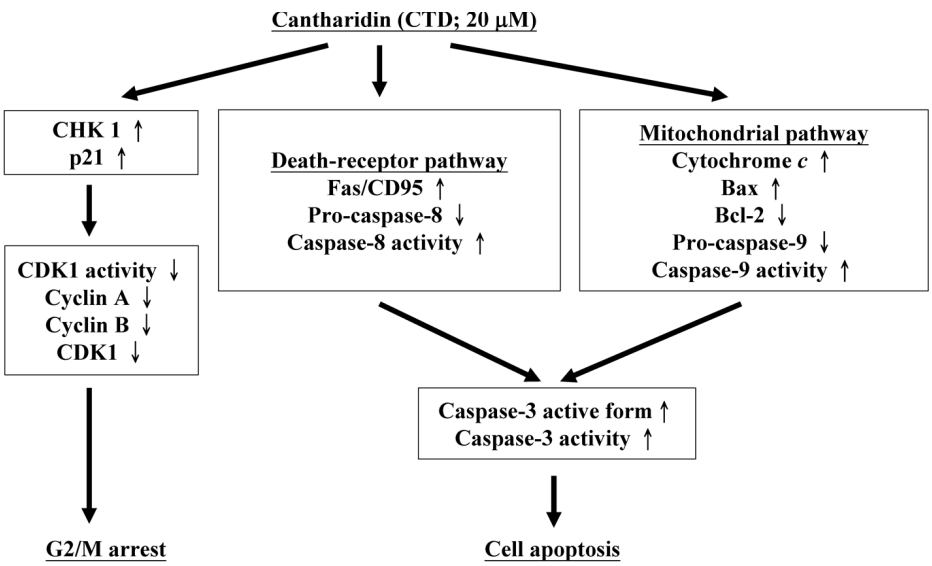

Figure 8. The proposed model of CTD-mediated G2/M arrest and apoptosis in human colorectal cancer colo 205 cells.

In conclusion, based on these observations, we have obtained convincing evidence that CTD efficiently inhibits the growth of a human colorectal cancer cell line (colo 205) through inhibiting CDK1 activity and caspase-dependent signal pathway as shown in Fig. 8. Further investigation on *in vivo* colorectal cancer models is necessary to clarify the biological activity of CTD.

Acknowledgments

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