Research Article

Development of chitosan/heparin nanoparticle-encapsulated cytolethal distending toxin for gastric cancer therapy

Aim: The aim of this work was to develop pH-responsive nanoparticles encapsulating CdtB and to demonstrate that these particles represent a potential therapeutic agent for gastric cancer. **Materials & methods:** Chitosan/heparin nanoparticle-encapsulated CdtB was prepared and the delivery efficiency was monitored by confocal laser scanning microscopy. The molecular basis of the nanoparticle-encapsulated CdtB-mediated p53 activation pathway was explored by immunoblot analysis. Antitumoral activities were investigated by analyzing the cell cycle and apoptosis. **Results:** Chitosan/heparin nanoparticle-encapsulated CdtB preferentially inhibited the proliferation of cells derived from gastric cancer, but not in primary gastric epithelial cells. Treatment of cells with nanoparticle-encapsulated CdtB enhanced cell-cycle arrest at G2/M, followed by apoptosis. Moreover, our data showed that the mechanism for nanoparticle-encapsulated CdtB-induced cell death was mediated by ATM-dependent DNA damage checkpoint responses. **Conclusion:** These findings indicate that chitosan/heparin nanoparticle-encapsulated CdtB could represent a new CdtB delivery strategy for the treatment of gastric cancer.

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Cytolethal distending toxin (CDT) is a protein-based bacterial genotoxin composed of three subunits, namely CdtA, CdtB and CdtC, which are encoded by an operon in *Campylobacter jejuni* comprising *cdtA*, *cdtB* and *cdtC* [1,2]. Several studies have demonstrated that cholesterol plays an important role in the binding of CDT to the cell membrane, and also serves as a portal for CdtB delivery into host cells for the induction of cell toxicity [3–5]. Nuclear-translocated CdtB exhibits type I deoxyribonuclease activity, which causes DNA damage, and subsequently leads to cell-cycle arrest in G2/M phase [6]. Given the genotoxic activity of CDT, several studies have investigated the use of CdtB for cancer therapy [7–9]. So far, no studies have developed CDT as a

therapeutic agent for gastric cancer. In part, this may be due to the acidic environment in the stomach, which is not favorable for CDT's stability.

Gastric cancer is the fourth most common cancer, and the second leading cause of cancer-related deaths in the world [10]. Cholesterol-enriched microdomains (also called lipid rafts), which provide platforms for signaling, are thought to be associated with the development of various types of cancer [11]. Recently, a population-based case–control study demonstrated that patients treated with statins, which inhibit 3-hydroxy-3-methyl glutaryl coenzyme A reductase, have a reduced risk for gastric cancer [12]. These results suggest that cholesterol-enriched rafts play a crucial role in gastric cancer

progression. Therefore, we sought to develop a new strategy to target and eradicate cholesterol-enriched gastric cancer cells.

Nanoparticles appear to be potential delivery carriers for various substances, including antigens, antibiotics and cancer drugs [13–15]. Recently, we developed pH-responsive chitosan/heparin nanoparticles that are stable at pH 1.2–2.5 and protect the delivered drug from destruction by gastric acids [13,16]. Heparin is a polyanionic mucopolysaccharide composed of repeating disaccharide units of D-glucosamine and uronic acid linked by 1→4 interglycosidic bonds. It is reported to have the ability to bind to cell receptors and accelerate mucosal regeneration, proliferation and angiogenesis [17]. Chitosan is a biodegradable polysaccharide that has been demonstrated to maintain prolonged interaction between the delivered drug and the cell membrane, facilitating more efficient drug diffusion into the mucosal/epithelium layer [18]. It has also been suggested that chitosan polyplexes can induce endocytic uptake of the polyplexes via nonspecific charged-mediated interactions with proteoglycans present on cell membranes [19,20]. Therefore, these chitosan/heparin nanoparticles could be used to develop an efficient system for the delivery of CdtB for gastric cancer therapy. We recently provided evidence that membrane cholesterol plays an essential role in the binding of *C. jejuni* CDT subunits to membrane rafts, which is critical for its activities in host cells [5]. Considering the acidic environment of the stomach (pH 1–3) and the stability of CDT, we have developed chitosan/heparin nanoparticles encapsulating CdtB and, in this paper, we demonstrate that these particles represent a potential therapeutic agent against gastric cancer.

Materials & methods
Antibodies & reagents
Antibodies against His (His-probe), β-actin, Bcl-2, Bcl-xl, Bax and Bak were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against CHK2, ATM, NBS and phospho-p53 were purchased from Cell Signaling (MA, USA). Anti-phospho-γH2AX antibody was obtained from Millipore (MA, USA). 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (CA, USA).

Purification of CdtB subunit
Recombinant His-tagged CdtB subunit was cloned following standard protocols, as described previously [5]. Briefly, *Escherichia coli* BL21-DE3 cells harboring the *cdtB* expression plasmid were induced at an optical density at 600 nm measure of 0.8 by 0.5 mM of isopropyl β-D-thiogalactopyranoside at 37°C for 3 h. The expressed His-tagged CdtB fusion protein was purified by metal affinity chromatography (Clontech, CA, USA) and assessed by SDS-PAGE and western blot.

Preparation of chitosan/heparin nanoparticle-encapsulated CdtB
The nanoparticles were prepared by a simple ionic gelation method with magnetic stirring at room temperature. The aqueous heparin (1.0 mg/ml, 1 ml, pH 7.4) were added by flush mixing with a pipette tip into aqueous chitosan at various concentrations (0.3, 0.6, 0.9, 1.2 or 1.5 mg/ml, 5 ml, pH 6.0). The nanoparticles were collected by ultracentrifugation at 15,000 × g for 50 min. The supernatant was discarded and the nanoparticles were resuspended in deionized water for further studies. The size distribution and ζ-potential of the particles in deionized water were then determined with a dynamic light-scattering analyzer (Zetasizer™ ZS90, Malvern Instruments Ltd, UK), and the morphology of the prepared nanoparticles was examined using transmission electron microscopy. The nanoparticle suspension was placed onto a 400-mesh copper grid coated with carbon. After 2 min of deposition, the grid was tapped with a filter paper to remove surface water and positively stained with an alkaline bismuth solution [21]. Various concentrations of CdtB (20.0, 10.0 and 5.0 mg/ml, 0.2 ml) were mixed with a heparin solution (2.0 mg/ml, 0.2 ml) and added to a chitosan solution (1.2 mg/ml, 2 ml) under magnetic stirring, as described previously [16]. To determine the loading efficiency, the CdtB-loaded nanoparticles were collected by centrifugation at 15,000 × g, 4°C for 50 min, and the concentration of free CdtB in the supernatant was determined by a protein concentration assay (Bio-Rad, CA, USA). The CdtB loading efficiency of nanoparticles was calculated from the following equation [22]:

$$Loading\ efficiency = \frac{Total\ CdtB\ protein - Free\ CdtB\ protein}{Total\ CdtB\ protein} \times 100$$

■ Cell culture
Human AGS cells (ATCC CRL 1739) were cultured in F12 medium (Invitrogen, CA, USA). MKN45 cells (JCRB0254; RIKEN Cell Bank, Japan) were cultured in DMEM. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, UT, USA) and maintained at 37°C. Primary cultures of human gastric epithelial cells were isolated using enzymatic methods, as previously described by Lai *et al.* [23]. The isolated cells were grown in plastic cell culture dishes in 95% air and 5% CO_2 with F12 medium, which was supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and 0.2% Fungizone® (Sigma-Aldrich, MO, USA). The isolated human gastric epithelial cells

were incubated at 37°C and maintained for further analyses.

Analysis of cell viability

The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan (MTT) assay was used to measure the effects of nanoparticle-encapsulated CdtB (nanoparticle–CdtB) on gastric epithelial cell viability. Cells (5×10^3) were cultured for 24 h, and were then treated with or without various concentrations of nanoparticle–CdtB for 24 h. Cell viability was measured by the ability of viable cells to reduce MTT (Sigma-Aldrich) to formazan.

In vitro cellular uptake & confocal laser scanning microscopy visualization

To observe the cellular distribution of nanoparticle–CdtB, the fluorescent cyanine 3 (Cy3)–chitosan/heparin-encapsulated fluorescein isothiocyanate (FITC)-labeled CdtB (FITC–CdtB) nanoparticles were prepared according to the procedure described in the 'Preparation of chitosan/heparin nanoparticle–CdtB' section. The FITC–CdtB protein and *N*-hydroxy-succinimide (NHS)-functionalized Cy3–chitosan were prepared for confocal laser scanning microscopy (CLSM) analysis. Synthesis of the FITC–CdtB was based on the reaction between the isothiocyanate group of FITC and the amino groups of CdtB. Briefly, 1.0 mg of FITC in 1 ml of dehydrated methanol was added to 2 ml of 5.0 mg/ml CdtB protein in phosphate-buffered saline (PBS) with continuous stirring for 3 h in the dark at 4°C. To remove the unconjugated FITC, FITC–CdtB was dialyzed in the dark against 1000 ml of PBS, which was replaced on a daily basis until no fluorescence was detected in the supernatant. The resultant FITC–CdtB was lyophilized in a freeze dryer. Additionally, the synthesis of Cy3–chitosan was based on the reaction between the free amines on the chitosan and NHS on Cy3–NHS. A solution of 0.5 ml of Cy3 in dimethyl sulfoxide (1 mg/ml) was prepared and added gradually to 10 ml of soluble chitosan (10 mg/ml, pH 6.0, 1.0% acetic acid) with continuous stirring for 12 h in the dark. To remove the unconjugated Cy3, Cy3–chitosan was dialyzed in the dark against 5000 ml of 0.5% acetic acid,
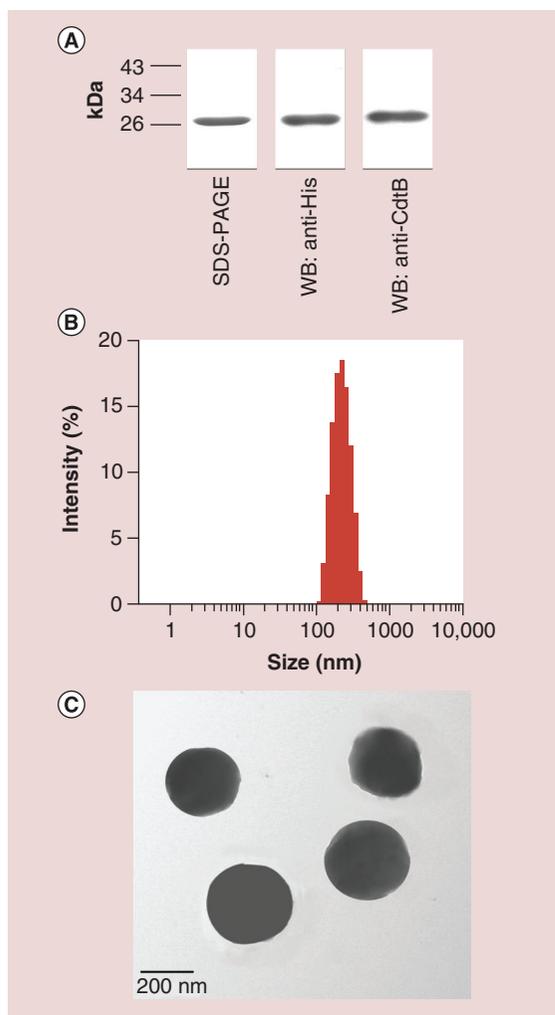
Figure 1. Purification of recombinant CdtB and characterization of chitosan/heparin nanoparticle-encapsulated CdtB. (A) Recombinant CdtB protein was subjected to SDS-PAGE, or WB analysis with a monoclonal antibody specific to the His-tag or antiserum against CdtB. Molecular weight markers (kDa) are shown on the left. **(B)** Particle size distributions of the prepared chitosan/heparin nanoparticle-encapsulated CdtB. **(C)** Transmission electron micrograph of the chitosan/heparin nanoparticle-encapsulated CdtB.
WB: Western blot.

which was replaced on a daily basis until no fluorescence was detected in the supernatant. The resultant Cy3–chitosan was lyophilized in a freeze dryer.

Table 1. Particle sizes and z-potentials of the prepared chitosan/heparin nanoparticle-encapsulated CdtB in deionized water (n = 5).				
Protein:heparin (mg/ml)	Chitosan (mg/ml)	Mean particle size (nm)†	ζ-potential (mV)†	Polydispersity index†
10.0:1.0	1.2	983.5 ± 163.9	20.8 ± 3.1	0.98 ± 0.12
5.0:1.0	1.2	434.3 ± 8.3	29.9 ± 1.7	0.67 ± 0.14
2.5:1.0	1.2	312.4 ± 15.7	32.3 ± 0.7	0.31 ± 0.08
†Data are presented as mean ± standard deviation.				

To track the internalization of the fluorescent Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticles, the cells were seeded onto glass coverslips at a density 2×10^5 cells/cm^2 and incubated for 2 days. The test samples containing FITC–CdtB solution or Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticles with 200 nM of CdtB were then added to the cells for specific times. After incubation, the test samples were aspirated. The cells were then washed three times with PBS before they were fixed in 3.7% paraformaldehyde. The cells were washed again three times with PBS and permeabilized with 0.2% Triton™ X-100 (Sigma-Aldrich) for 15 min at 37°C. The washes were repeated and the cells were stained with DAPI, which specifically bind to the nuclei. The stained cells were examined with excitation at 340, 488 and 543 nm under a CLSM. The images were superimposed with the LCS Lite™ software (version 2.0; Leica, Germany).

Flow cytometry analysis of the cell cycle

AGS cells (2×10^5) were cultured at 37°C for 24 h, followed by treatment with medium (mock), nanoparticles, CDT holotoxin, nanoparticle–CdtB (each 200 nM) and ICRF-193 (2 µg/ml) for 2 h. The treatments were removed then replaced with culture medium and incubated for a further 2–24 h. The treated cells

were harvested and fixed with ice-cold 70% ethanol for 1 h, and stained with 20 µg/ml propidium iodide containing 1 mg/ml RNase (Sigma-Aldrich) for 1 h. The stained cells were analyzed with a FACSCalibur™ flow cytometer (Becton-Dickinson, CA, USA). The data were collected using 20,000 cells from each sample, and analyzed using CellQuest™ software WinMDI (Verity Software House, ME, USA). All samples were examined in triplicate from at three independent experiments.

Observation of cell morphology

AGS cells were cultured at 37°C for 24 h in six-well plates containing F12 medium supplemented with 10% FBS. After two washes with PBS, cells were incubated with Hank's buffered salt solution (containing 50 mM glucose, pH 6.5) containing nanoparticle–CdtB at 37°C for 2 h. The culture supernatant was carefully removed and replaced with fresh completed medium for 24–72 h. The cell morphology was observed by phase-contrast microscopy (Carl Zeiss, Germany). The images were captured and analyzed by the AxioVision™ software (Carl Zeiss).

Quantification of apoptosis by flow cytometry

Quantification of apoptotic cells was analyzed by flow cytometry, as described previously with slight modification [24]. In brief, cells were treated with or without nanoparticle–CdtB and cultured for the indicated times. Cells were collected and incubated with pre-chilled ethanol at 4°C for 30 min. After treatment, cells were washed twice with PBS and resuspended in staining buffer containing 100 µg/ml propidium iodide in the presence of an equal volume of DNase-free RNase (200 µg/ml). Cells were immediately analyzed with the FACSCalibur and the CellQuest programs. The apoptotic cells were determined by measuring the DNA content of the cells below the sub-G1 peak. Alternatively, the nanoparticle–CdtB-treated cells were permeabilized with 0.1% Triton X-100 and stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Roche Diagnostics, Germany) reaction mixture at 37°C for 30 min. The reaction was blocked in stop/wash buffer for 10 min. The TUNEL cells were determined using the FACSCalibur and the CellQuest programs.

Western blot analysis

AGS cells (5×10^5) were seeded onto six-well plates at 37°C for 24 h, followed by treatment with medium (mock), CdtB, nanoparticles and nanoparticle–CdtB (each 200 nM) for 2 h. The nanoparticles were removed then replaced with culture medium and incubated for the indicated time. Nanoparticle–CdtB-treated cells were washed three times with PBS and then boiled in

Figure 2. Nanoparticle-encapsulated CdtB-induced cell death of cultured gastric cancer cells. Gastric cancer cell lines (AGS and MKN-45 cells) and normal human gastric epithelial cells (5×10^3) were cultured at 37°C for 24 h followed by incubation with different concentrations (0–1000 nM) of nanoparticle–CdtB for 24 h. The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assay was used to analyze cell viability. Results are presented as the mean ± standard deviation values from three independent experiments. p-values were < 0.01 for both AGS and MKN-45 cells when compared with the vehicle control. No statistically significant difference was observed between normal gastric epithelial cells and control medium.
Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.

Figure 3. Delivery of nanoparticle-encapsulated CdtB into cell nuclei. AGS cells (2 × 10⁵) were cultured for 24 h, followed by treatment with FITC–CdtB alone or Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticles (200 nM each) for the indicated times. Cells were fixed and stained with 4′,6-diamidino-2-phenylindole (blue) to visualize the cell nuclei. Samples were analyzed by confocal microscopy. After the treatment of cells with nanoparticles for 120 min, the colocalization of CdtB (green) with chitosan (red) in the cytoplasm appears as yellow in the overlay (white arrows). Cells were treated with nanoparticles for 120 min, and then the nanoparticles were removed, and the incubation was continued for an additional 240, 360 and 1320 min. The colocalization of CdtB (green) with chitosan (red) in the cell nuclei appears as yellow in the overlay (black arrows).
Cy3: Cyanine 3; FITC: Fluorescein isothiocyanate.

SDS-PAGE sample buffer for 5 min. The samples were then resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with antiserum against the CdtB subunit [5], antibodies against Bax, Bak and Bcl-2 (Santa Cruz Biotechnology) or antibodies against CHK2, ATM, NBS and phospho-p53 (Cell Signaling) at room temperature for 1 h. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies. The proteins of interest were visualized using the ECL™ Western Blotting Detection Reagents (GE Healthcare, NJ, USA) and detected using x-ray film (Kodak, NY, USA).

In vivo tumor growth study

Five-week-old male BALB/c nude mice (BALB/cAnN. Cg-*Foxn1ⁿᵘ*/CrlNarl) were obtained from the National Laboratory Animal Center (Taiwan). The mice were
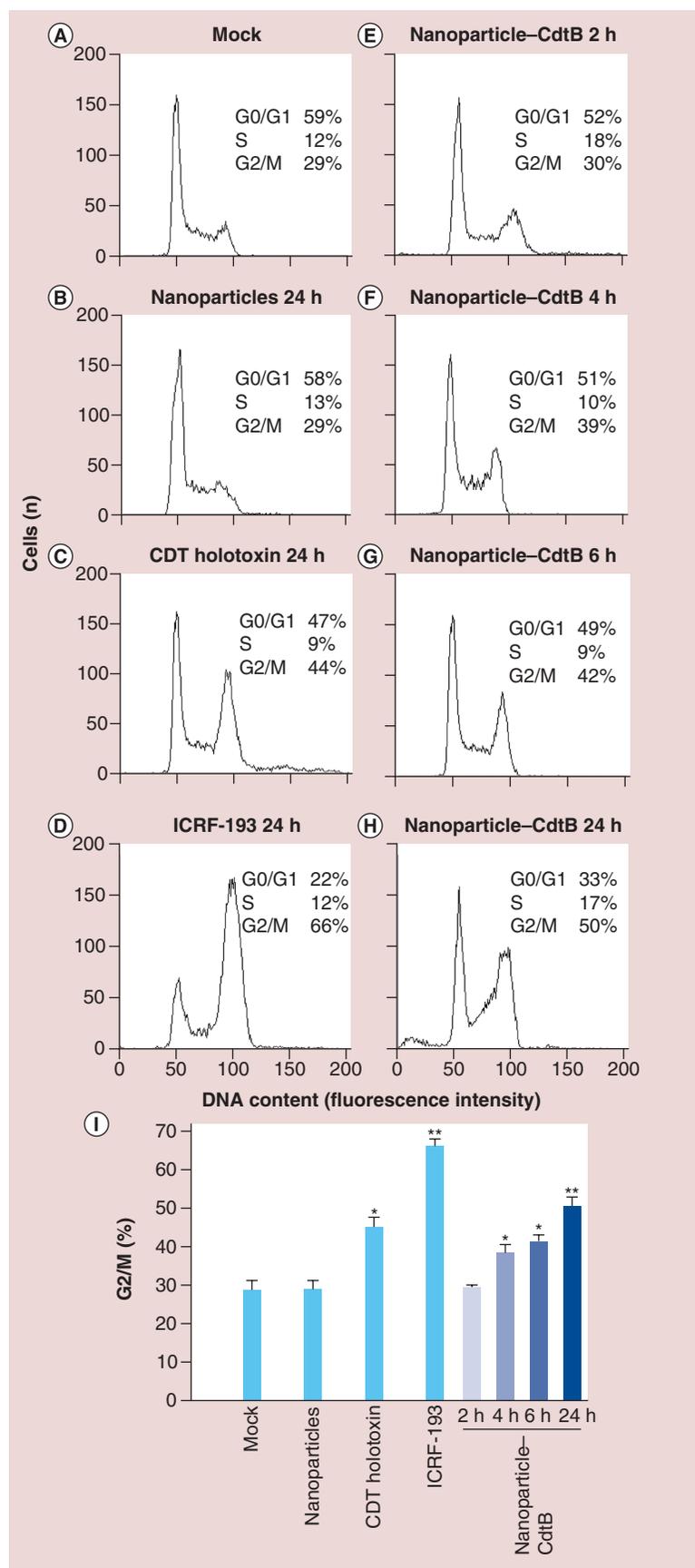
Figure 4. Nanoparticle-encapsulated CdtB-induced cell-cycle arrest of gastric cancer cells. AGS cells (2×10^5) were cultured at 37°C for 24 h, followed by exposure to **(A)** medium alone, **(B)** nanoparticles alone, **(C)** CDT or **(D)** ICRF-193 (2 μg/ml) for 24 h, or **(E–H)** nanoparticle–CdtB (200 nM each) for the indicated time. The cell-cycle distribution was based on the DNA content, which was analyzed by flow cytometry. The percentage of cells in the G0/G1, S and G2/M phases of the cell cycle are indicated at the right of each histogram. **(I)** The percentages of cells in the G2/M phase were calculated and plotted as intensity histograms. Results represent three independent experiments and are expressed as the mean ± standard deviation values. *$p < 0.05$ and **$p < 0.01$ were considered to indicate statistical significance.
CDT: Cytolethal distending toxin; Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.

treated in accordance with the Animal Care and Use Guidelines for China Medical University, under a protocol approved by the Institutional Animal Care Use Committee. The mice were permitted to acclimatize to local conditions for 1 week before being injected with the cancer cells. Each mouse was subcutaneously injected with MKN-45 cells (1×10^7 cells) mixed in a 50% matrix gel (BD Biosciences, MA, USA) in the right flank. The tumor volume reached up to 70 mm³ after the mice were inoculated with MKN-45 cells. The animals were randomly divided into five groups (four mice each): treatment with vehicle (PBS); CdtB alone (2.5 mg/kg); nanoparticles alone (2.5 mg/kg); CDT holotoxin (2.5 mg/kg); or nanoparticle–CdtB (2.5 mg/kg) once in 3 days. The tumors were measured with calipers at 3-day intervals, and the volume of each tumor (mm³) was calculated in accordance with the following formula: $0.5236 \times$ length $\times$ width $\times$ height [25]. After 16 days, the weight of the tumor was measured upon its surgical removal from the sacrificed mice in each group.

Statistical analysis

The Student's *t*-test was used to calculate the statistical significance of experimental differences between two groups. The analysis of variance test was made by means of one-way analysis of variance. The relationship of compared groups was performed using the Duncan multiple-comparison test. Statistical analyses were carried out using the SPSS program (version 11.0; SPSS Inc., IL, USA).

Results

Preparation & characterization of nanoparticle–CdtB

Recombinant CdtB fusion protein was purified and assessed by SDS-PAGE and western blot analysis using anti-His monoclonal antibodies or polyclonal anti-CdtB antibodies (Figure 1A). To prepare chitosan/heparin

nanoparticle–CdtB, various concentrations of chitosan were mixed with a heparin solution. As shown in Supplementary Table 1 (see online at www.futuremedicine.com/doi/suppl/10.2217/nnm.13.54), mixing chitosan and heparin with distinct concentrations of chitosan (0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml; 10.0 ml) and heparin (1.0 mg/ml; 2.0 ml) resulted in the formation of complexes on the nanometer scale, with the exception of the chitosan concentration of 0.3 mg/ml. The mean particle sizes of nanoparticles ranges from 200 to 300 nm, with positive ζ-potentials, depending on the relative concentrations of chitosan and heparin used, and the amount of positively charged chitosan significantly exceeded that of negatively charged heparin. In our selected particulate system, the chitosan concentration was 1.2 mg/ml, and we produced particles of sizes (mean ± standard deviation) of 273.1 ± 10.6 nm with a significant ζ-potential of 37.4 ± 1.9 mV (Supplementary Table 1). Therefore, this specific composition was used to prepare the rest of the nanoparticle–CdtB in this study.

As shown in Table 1, the chitosan (1.2 mg/ml; 2.0 ml) and CdtB:heparin in distinct ratios (10.0:1.0, 5.0:1.0 and 2.5:1.0 by mg/ml; 0.4 ml) gave a mean size range of 310–980 nm, with different positive ζ-potentials, depending on the relative concentrations of recombinant CdtB used. Furthermroe, the CdtB loading efficiencies were 76.9 ± 4.6% (for the CdtB:heparin composition of 10.0:1.0 mg/ml, n = 5), 69.9 ± 1.6% (5.0:1.0 mg/ml) and 52.1 ± 7.5% (2.5:1.0 mg/ml). The high loading efficiency of chitosan/heparin nanoparticle–CdtB may be attributed to the high electrostatic interaction between the negative charge on CdtB (ζ-potential of -12.3 ± 2.7 mV) and the positive charge on chitosan (ζ-potential of 33.7 ± 4.5 mV). This technique is promising as the nanoparticles can be prepared under deionized water at room temperature in order to protect proteins from degradation in this system. In addition, the polydispersity index of nanoparticles (CdtB:heparin ratio of 2.5:1.0 mg/ml) measured by dynamic light scattering revealed a narrower distribution (polydispersity index: 0.31 ± 0.08) when compared with other formulation (Table 1). Transmission electron microscopy examination showed that the morphology of the prepared chitosan/heparin nanoparticle–CdtB remained spherical and smooth-shaped (Figure 1C). Therefore, the CdtB-encapsulated nanoparticles prepared with this chitosan concentration (1.2 mg/ml; 2.0 ml) and CdtB:heparin ratio (2.5:1.0 mg/ml; 0.4 ml) were employed for the remaining studies.

Delivery of chitosan/heparin nanoparticle–CdtB into gastric cancer cells

To examine the ability of nanoparticle–CdtB to induce cell death in human gastric cancer cells, we first analyzed the effect of chitosan/heparin nanoparticle–CdtB on the cell viability in human gastric epithelial cells by using the MTT assay. Treatment of human gastric cancer cells (AGS and MKN-45 cells) with nanoparticle–CdtB decreased total cell number in a concentration-dependent manner (Figure 2). However, nanoparticle–CdtB did not have any influence on the viability of normal primary gastric epithelial cells (Figure 2). Additionally, our data from the MTT test showed that the viability of AGS cells decreased dramatically with the increase in the concentration of nanoparticle–CdtB up to 200 nM. We therefore chose 200 nM for the following study.

To observe the localization of CdtB protein, nanoparticles, or nanoparticle–CdtB internalized by AGS cells after various durations of incubation, fluorescent nanoparticles (Cy3–chitosan/heparin-encapsulated FITC–CdtB) and fluorescent protein (FITC–CdtB) were used and the subcellular distributions of Cy3–chitosan (red), FITC–CdtB (green) and nuclei (blue, stained with DAPI) were observed under CLSM. As shown in Figure 3 (second row), after 60 min of incubation with Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticles, fluorescent signals were observed in the intercellular spaces. With incubation time increased to 120 min, the fluorescent signals were significantly observed in the intercellular spaces and cell cytoplasm, as indicated by superimposed red/green spots (i.e., yellow spots, white arrows; Figure 3, third row). Furthermore, when the nanoparticles were incubated with AGS cells for 120 min, and then removed and replaced with culture medium for an additional 240 min, the incubated Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticles, as indicated by superimposed red (Cy3–chitosan)/green (FITC–CdtB) spots (yellow spots, black arrows; Figure 3), in the superimposed images appear in the cell cytoplasm and cell nuclei. In this case, fluorescent signals (shown as green spots [FITC–CdtB]; Figure 3) in the superimposed images appeared in the perinuclear space and cell nuclei, indicating that the CdtB could be released from nanoparticles intracellularly (Figure 3, fourth row). When nanoparticles were incubated with the cells for 120 min and the culture medium was removed and replaced for an additional 360 min, we noted a significant amount of FITC–CdtB in the cell nuclei (Figure 3, fifth row, white arrowheads). Noticeably, the white spots in the superimposed images (superimposed red [Cy3–chitosan]/green [FITC–CdtB]/blue [nuclei]] started to disappear in the perinuclear space and cell nuclei, indicating that the nanoparticles in the cellular spaces were no longer intact. Upon extending the incubation time to 1320 min, the fluorescent signal of Cy3–chitosan/heparin-encapsulated FITC–CdtB nanoparticle expression decreased (Figure 3, sixth row).
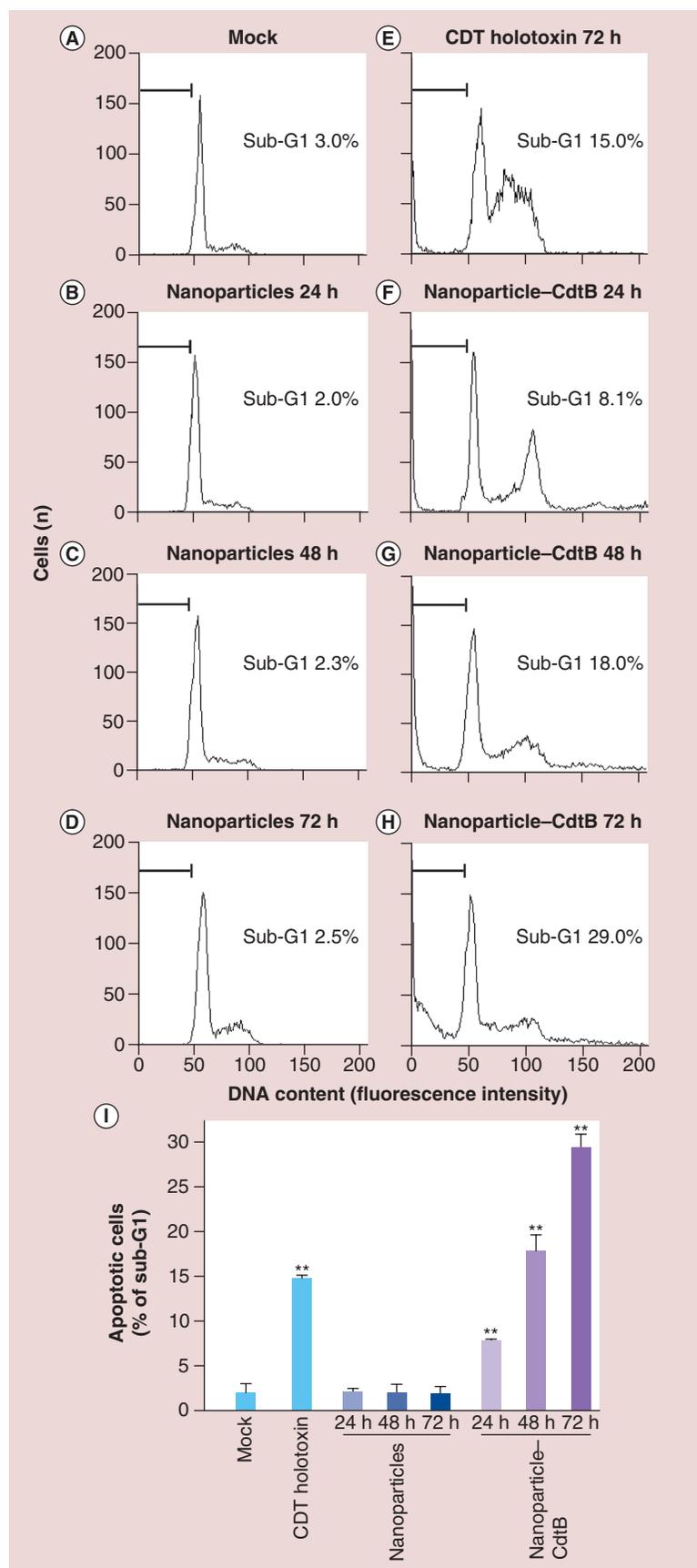
Figure 5. Nanoparticle-encapsulated CdtB-induced cell death of gastric cancer cells. AGS cells (2 × 10⁵) were incubated at 37°C for 24 h, followed by exposure to **(A)** medium alone, **(B–D)** nanoparticles alone, **(E)** CDT holotoxin or **(F–H)** nanoparticle–CdtB (200 nM each) for the indicated times. The cell-cycle distribution was based on DNA content, which was analyzed by flow cytometry. The percentages of cells in the sub-G1 phase of the cell cycle are indicated at the right of each histogram. **(I)** The percentages of cells in the sub-G1 phase were calculated and plotted as intensity histograms. Results represent three independent experiments and are expressed as the mean ± standard deviation values. The horizontal crossbar lines in **(A–H)** represented the cells that were in sub-G1 phase. **$p < 0.01$ was considered to indicate statistical significance.
CDT: Cytolethal distending toxin; Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.

However, in the cells incubated with FITC–CdtB alone, the fluorescent signals observed in the intercellular spaces were not as obvious as those observed with the fluorescent nanoparticle–CdtB (Figure 3, first row).

To further clarify whether CdtB protein was delivered into AGS cells by the nanoparticles, we examined the protein expression levels of CdtB by western blot. As shown in Supplementary Figure 1A, the levels of the CdtB released were found to be elevated with the incubation time in AGS cells. The efficiency of CdtB release into cells was higher than for CDT holotoxin (Supplementary Figure 1B). However, no internalized CdtB was shown by western blot analysis when cells were treated with CdtB alone (Supplementary Figure 1C). Taken together, these results indicate that chitosan/heparin nanoparticle–CdtB provides the most efficient system for delivery of CdtB into nuclei and for the induction of gastric cancer cell death.

Cell-cycle arrest induced by nanoparticle–CdtB in gastric cancer cells

We investigated whether nanoparticle–CdtB has the potential to induce cell-cycle arrest in gastric cancer cells. As shown in Figure 4A & B, exposure of AGS cells to the nanoparticles did not alter their cell-cycle distribution compared with the control cells. After treatment of cells with CDT holotoxin for 24 h, 44% of cells were accumulated in G2/M (Figure 4C). When cells were treated with ICRF-193 (2 μg/ml), which is a DNA topoisomerase II inhibitor, more than 66% of cells were arrested at G2/M (Figure 4D). We employed ICRF-193 as a positive control for typical cell-cycle arrest. With increasing nanoparticle–CdtB incubation times from 2 to 24 h, the proportion of cells arrested in G2/M was elevated in a time-dependent manner (Figure 4E–H).

We then evaluated the cytolethal activity of prolonged nanoparticle–CdtB treatment on AGS cells

by observation of the cell morphology. As shown in Supplementary Figure 2 (second row), when AGS cells were incubated with CDT holotoxin for 72 h, a significantly higher level of cell detachment was observed and the morphology changed to cause shrinkage in CDT holotoxin-treated cells. Consistently, more cell detachment and morphology change was observed in cells exposed to nanoparticle–CdtB for 72 h than in mock-treated cells (Supplementary Figure 2, fourth row). By contrast, no cell detachment and morphology change was observed in the mock-treated cells or the cells treated with nanoparticles alone. These results

indicated that nanoparticle–CdtB has better biological activity than CDT holotoxin.

Nanoparticle–CdtB-induced apoptosis in gastric cancer cells

We then examined the ability of nanoparticle–CdtB to induce apoptosis in AGS cells using propidium iodide staining. The results showed that treatment of cells with nanoparticle–CdtB for 24–72 h caused an increase in the proportion of cells in the sub-G1 phase (Figure 5F–H). However, cell viability was not affected by the nanoparticles until 72 h
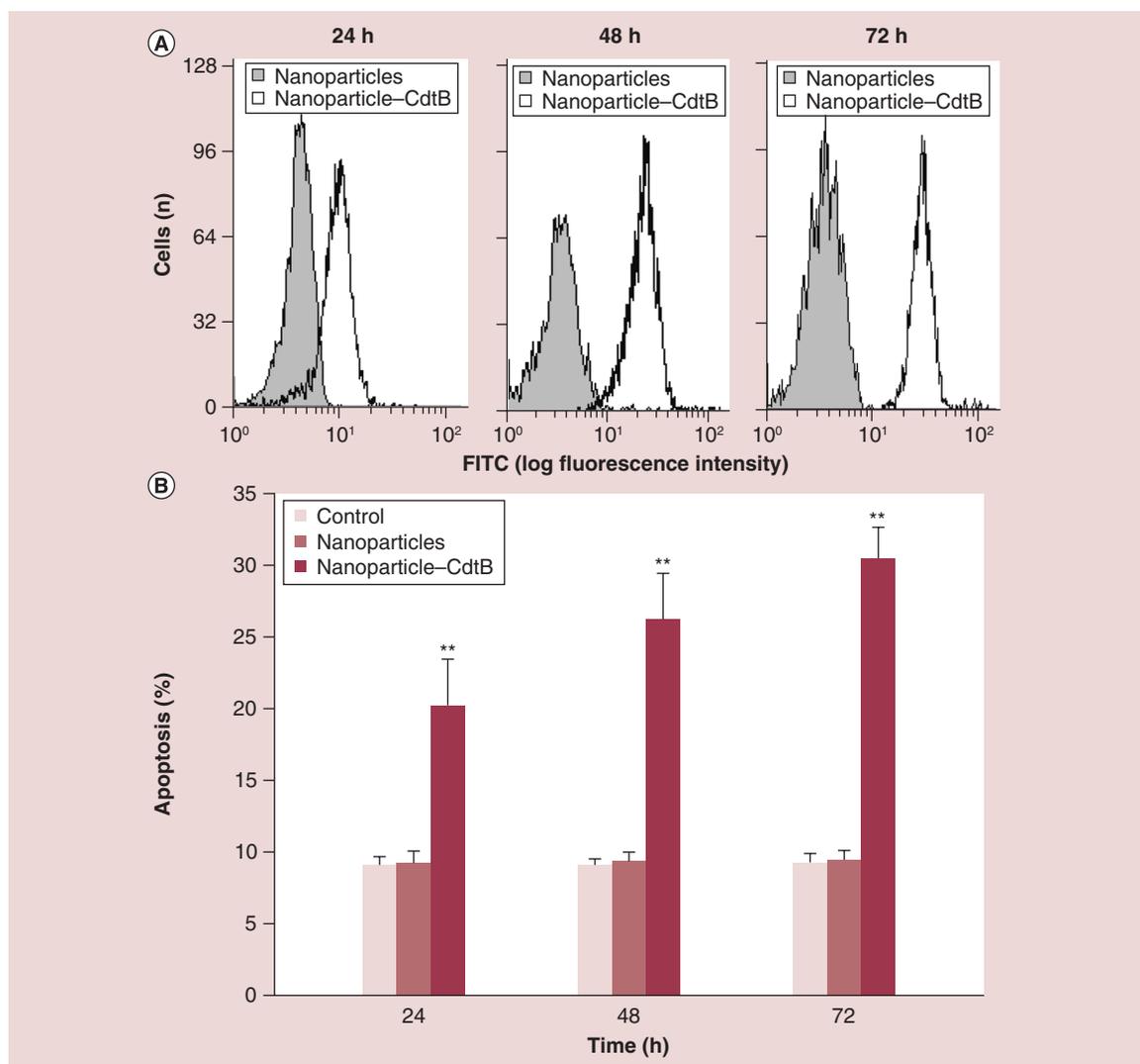
Figure 6. Nanoparticle-encapsulated CdtB increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive staining of gastric cancer cells. AGS cells (5×10^5) were cultured at 37°C for 24 h. Cells were treated with medium alone, nanoparticles alone (200 nM) and nanoparticle–CdtB (200 nM) for 24–72 h. Transferase-mediated deoxyuridine triphosphate nick end labeling fluorescence was analyzed by flow cytometry. **(A)** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells were assessed by flow cytometric analysis. **(B)** The percentages of TUNEL-positive cells were calculated and plotted as intensity histograms. Results represent the mean ± standard deviation values for three independent experiments. **p < 0.01 was considered to indicate statistical significance.
FITC: Fluorescein isothiocyanate; Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.
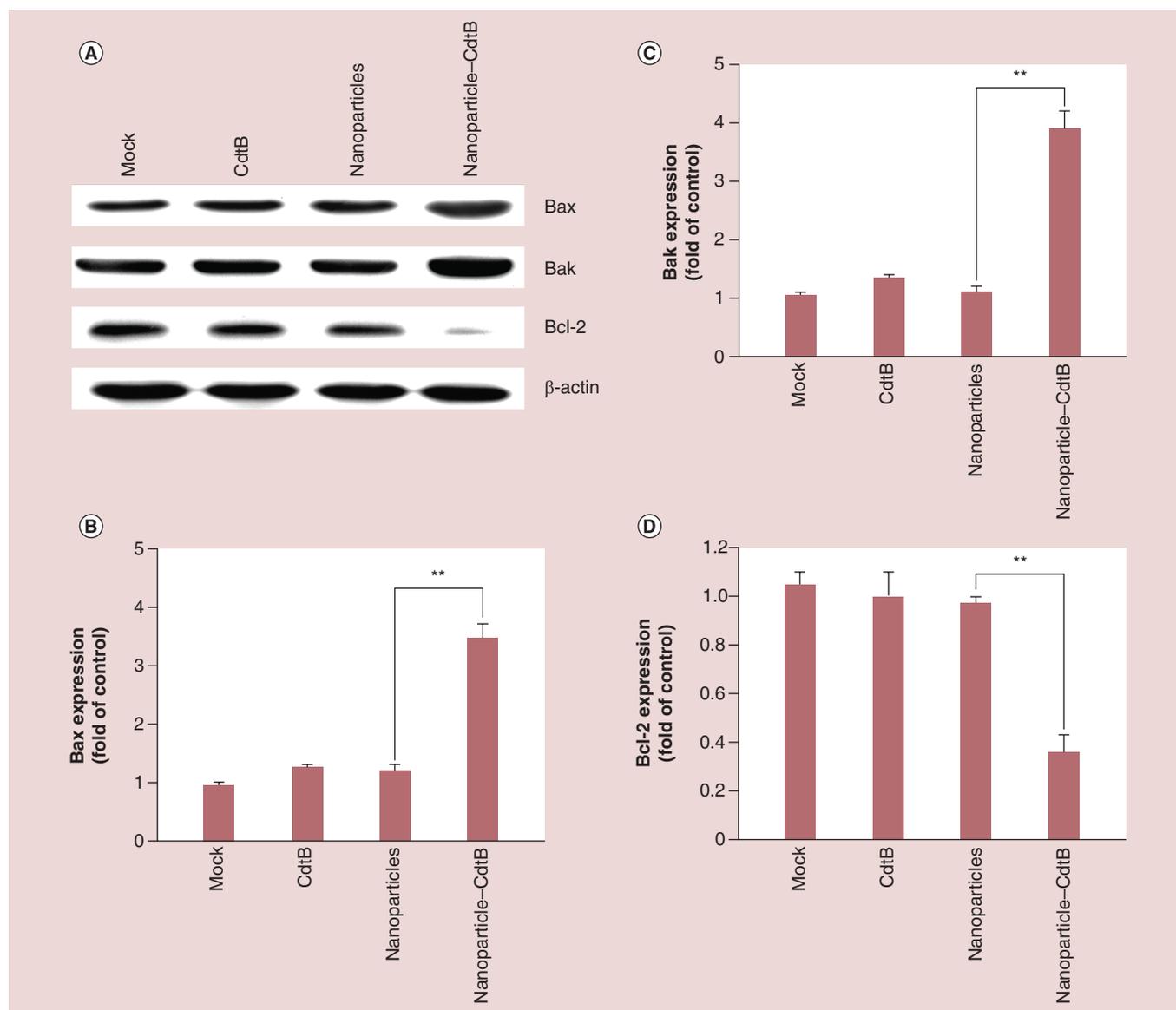
Figure 7. Bcl-2 pathway proteins are involved in nanoparticle-encapsulated CdtB-induced apoptosis of gastric cancer cells. AGS cells (5×10^5) were cultured at 37°C for 24 h. Cells were incubated with 200 nM of nanoparticle–CdtB for 24 h, and **(A)** the expression levels of Bax, Bak, Bcl-2 and β-actin were examined by western blot analysis, while **(B–D)** densitometry was used to quantify the expression of these proteins relative to the control group. Results are expressed as the mean ± standard deviation values for three independent experiments.
**p < 0.01 compared with the control group.
Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.

of exposure (Figure 5B–D). We further investigated whether nanoparticle–CdtB could induce cell death through apoptosis. Flow cytometric analysis of TUNEL was used for detecting apoptotic cells. As compared with vehicle control and nanoparticle-treated cells, a high proportion of TUNEL was detected in cells treated with nanoparticle–CdtB, and this effect increased in a time-dependent manner (Figure 6). Taken together, these results suggest that chitosan/heparin nanoparticle–CdtB-induced cell death occurs through apoptosis.

Bcl-2 family proteins are involved in nanoparticle–CdtB-induced apoptosis in gastric cancer cells

To confirm whether nanoparticle–CdtB-induced AGS cell apoptosis was triggered through the mitochondrial apoptotic pathway, we examined changes in Bcl-2 family proteins. Treatment of cells with nanoparticle–CdtB induced increased levels of Bax and Bak proteins when compared with cells treated with CdtB or nanoparticles individually (Figure 7). In addition, nanoparticle–CdtB reduced the expression of Bcl-2, a

protein with known antiapoptotic activity (Figure 7). These results demonstrate that mitochondrial dysfunction is involved in the apoptotic mechanism induced by the treatment with chitosan/heparin nanoparticle–CdtB.

An ATM-dependent pathway mediates nanoparticle–CdtB-induced apoptosis

Bacterial CDT harbors DNase activity that can cause dsDNA breakage (DSB) and subsequent activation of the ATM-dependent pathway [26]. To ascertain whether nanoparticle–CdtB-induced gastric cancer cell apoptosis was mediated through an ATM-dependent mechanism, we further analyzed the expression levels of ATM-related signaling proteins. In cells treated with nanoparticle–CdtB, the expression levels of phospho-NBS1 and phopho-ATM were significantly higher than in the cells treated with the vehicle control (Figure 8). Elevation of ATM activation in the nanoparticle–CdtB-treated cells was associated with increased phosphorylation of γH2AX, CHK2 and p53. Taken together, these data demonstrate that chitosan/heparin nanoparticle–CdtB-induced cell apoptosis is mediated via the ATM-dependent checkpoint, which is triggered by DNA breakage.

Antitumor effect of nanoparticle–CdtB *in vivo*

We then evaluated the antitumor effect of nanoparticle–CdtB in a set of mouse xenograft experiments. Mice with human gastric cancer xenografts were established. These mice were or were not directly injected with nanoparticle–CdtB into tumors every 3 days for a total of 16 days. Our results showed that the growth of the tumor was significantly lower in mice treated with the nanoparticle–CdtB complex than in the vehicle control group (p < 0.05; Figure 9). We noticed that mice that received the CDT holotoxin showed an intermediate level of inhibition of tumor growth when compared with mice treated with nanoparticle–CdtB. However, no significant difference was observed among the CdtB alone, nanoparticle alone and vehicle control groups. Taken together, our results demonstrate that chitosan/heparin nanoparticle–CdtB effectively inhibited the growth of tumor cells *in vivo*.

Discussion

Gastric cancer is one of the most prevalent malignancies in the world, with approximately 1 million cases diagnosed annually [27]. Despite ongoing research, the mortality rate for gastric cancer remains high, with a 5-year survival rate of only 20% [28]. Although surgery is the preferred treatment for early gastric cancers with no submucosal invasion or angiolymphatic invasion, surgical treatment is much less effective for advanced

gastric cancers [28]. Recently, targeted therapies using small molecules or antibodies that specifically interfere with oncogenic signaling have been recognized as potent therapeutics. We previously reported that a bacterial membrane-associated protein, *C. jejuni* CDT,
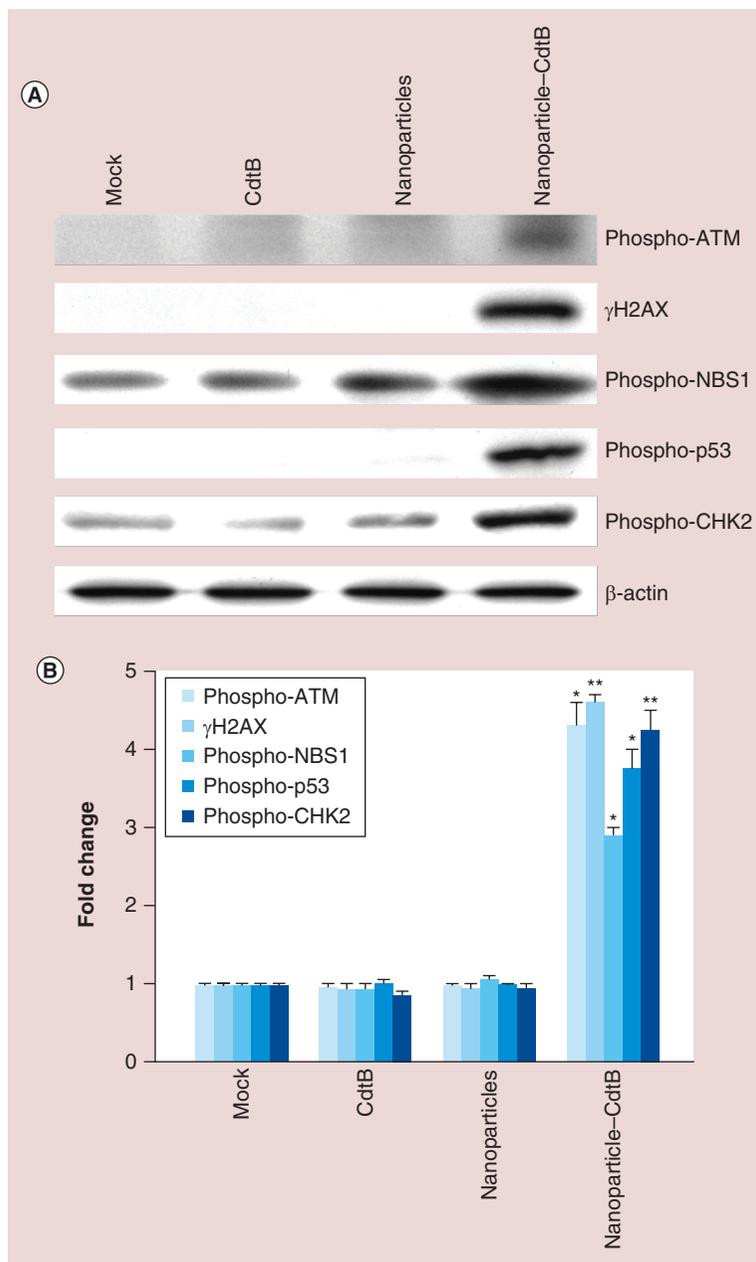
Figure 8. ATM-dependent responses are involved in nanoparticle-encapsulated CdtB-mediated cell apoptosis in gastric cancer cells. AGS cells (5 × 10⁵) were cultured at 37°C for 24 h, followed by incubation with 200 nM of nanoparticle–CdtB for 4 h, and **(A)** the expression level of each phosphoprotein was assessed by western blot analysis. **(B)** The densitometry analysis was carried out to quantify the protein expression levels relative to the control group. Results are expressed as the mean ± standard deviation values for three independent experiments. *p < 0.05; **p < 0.01 compared with the control group. Nanoparticle–CdtB: Nanoparticle-encapsulated CdtB.

induced by nanoparticle–CdtB in gastric cancer cells involve cell-cycle arrest and apoptosis mediated by an ATM-dependent pathway.

Several reports have demonstrated that CdtB exhibits type I deoxyribonuclease activity, which is responsible for CDT-induced DNA damage [6,29]. Although bacteria that cause persistent infections associated with chronic inflammation pose a higher risk for promoting carcinogenesis [30], very little is known about the ability of CDT to induce tumor formation. Only the CDT secreted by *Helicobacter hepaticus* is known to be associated with dysplasic lesions in hepatocytes [31]. However, neither *C. jejuni* nor its CDT have known associations with malignancies in the GI tract following long-term surveillance [32]. In addition, previous studies have used CDT delivery in the treatment of human gingival squamous carcinoma and oral cancer stem cells [7–9]. These studies support our intent to apply recombinant CDT isolated from *C. jejuni* on cancer therapy.

In the present study, we employed chitosan/heparin nanoparticles as a delivery system. Chitosan is a polycationic, nontoxic, mucoadhesive polymer, which has been proven safe for use *in vivo* [33]. It has been widely used to mediate intracellular uptake of nucleic acids [34]. Moreover, the polycation polyplexes were found to enhance their internalization by cells mediating the interaction of nonspecific charges with membrane proteoglycans, and the highly monodispersed nanoparticles were reported to be passively accumulated and retained at the tissue sites [35–37]. Additionally, we recently developed pH-responsive chitosan/heparin nanoparticles and demonstrated that they are stable in the acidic environment of the stomach, where they can protect the delivered drug from destruction by gastric acids [13,16]. To determine whether nanoparticle–CdtB

interacts with lipid rafts, and this association enhances nuclear translocation of CdtB [5]. CdtB exhibits type I deoxyribonuclease activity, and this activity may lead to cell-cycle arrest and apoptosis [6]. The present study shows that chitosan/heparin nanoparticle–CdtB can induce apoptosis in gastric cancer cells. Our study reveals that nanoparticle–CdtB can deliver CdtB directly into the nuclei, which is similar to holotoxin. The molecular mechanisms of cell death

could attach to and penetrate into gastric cancer cells, we tracked the subcellular localization of our prepared nanoparticles (Figure 3 & Supplementary Figure 1). We found that the amount of nanoparticle–CdtB taken up by cells significantly prolonged its biological effect. The nanoparticle–CdtB system, which was composed of positively charged chitosan and negatively charged heparin, was prepared using an ionic gelation method. Chitosan is a weak base and the amino group on chitosan has a pKa value of approximately 6.5 [16]. When AGS cells are treated with chitosan/heparin nanoparticle–CdtB for 120 min and cells are washed with culture medium (pH 7.4), the nanoparticles become unstable and break apart. This is because, at pH 7.4, chitosan is deprotonated, resulting in the collapse of the nanoparticles and release of the CdtB protein. The delivered CdtB appeared in the nuclei at the highest levels when the cells were treated with nanoparticle–CdtB for 4 h (Figure 3). These results confirmed the notion that recombinant CdtB can be directly delivered into the nuclei by chitosan/heparin nanoparticles. Furthermore, the efficiency of delivery was better than that in the cells treated with CDT holotoxin (Supplementary Figure 1).

Nuclear-translocated CdtB subsequently enhanced the activation of ATM and phosphorylation of its downstream molecules, including CHK2, γH2AX and p53 (Figure 8). Our data show that substantial DSBs occurred following treatment of cells with nanoparticle–CdtB for 24 h, which could lead to apoptosis via a mitochondria-dependent pathway (Figure 7). These findings are consistent with those of a previous study that showed that CDT toxicity activates the ATM-dependent DNA damage checkpoint response, thus leading to cell death [38]. It has been shown that loss of a tumor-suppressor gene, *PTEN*, was found to be significantly associated with gastric malignancy and poor gastric carcinoma prognosis [39,40]. Similarly, decreased PTEN expression was observed to correlate with chemoresistance of gastric cancer cells [41]. Our data show that nanoparticle–CdtB-induced cell death is more likely to occur in gastric cancer cell lines (i.e., AGS and MKN-45) than in primary gastric epithelial cells (Figure 2). These results might be explained by the previous findings that cells with defects in PTEN were more sensitive to CDT [42], which underlies the specificity of CdtB nanoparticles for killing gastric cancer cells (with lower PTEN expression), but not normal cells. Nevertheless, it is also interesting to determine whether nanoparticles can specifically target gastric cancer cells *in vivo*.

Conclusion

We have constructed a chitosan/heparin nanoparticle vehicle system for delivering CdtB into gastric cancer cells. Our data indicate that this particle has the same activity of CDT holotoxin plus better potentcy. The mechanism of nanoparticle–CdtB-induced cell death of gastric cancer cells is to induce DSBs and G2/M cell-cycle arrest, followed by apoptosis. Our findings provide a new strategy for applying chitosan/heparin nanoparticle–CdtB as a chemotherapeutic agent for gastric cancer.

Future perspective

Cell toxicity induced by CDT requires the delivery of CdtB into cell nuclei, where CdtB exhibits type I deoxyribonuclease activity and is thought to induce apoptosis. In the present study, pH-responsive chitosan/heparin nanoparticles were developed in order to deliver CdtB as a therapeutic agent for gastric cancer. The molecular mechanism of nanoparticle–CdtB-induced gastric cancer cell death is to arrest the cell cycle and enhance apoptosis. Future work is warranted in order to develop nanoparticle–CdtB that specifically targets gastric cancer cells and to validate its antitumor activity *in vivo*, particularly for application in clinical therapy.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- The genotoxin cytolethal distending toxin is composed of three subunits: CdtA, CdtB and CdtC. We have utilized the stability of chitosan/heparin nanoparticles in acidic environments in order to develop an efficient system for delivering CdtB into cells for the treatment of gastric cancer.

Materials & methods

- Chitosan/heparin nanoparticle-encapsulated CdtB was prepared for inhibition of gastric cancer. The antitumor effects of nanoparticle-encapsulated CdtB were evaluated in cell-based and *in vivo* xenograft experiments. The molecular mechanisms underlying nanoparticle-encapsulated CdtB-induced cell death were investigated.

Results

- Nanoparticle-encapsulated CdtB induced gastric cancer cell death in a concentration-dependent manner, but this is not seen in normal primary gastric epithelial cells.
- Treatment of gastric cancer cells with nanoparticle-encapsulated CdtB enhanced cell-cycle arrest at G2/M, followed by apoptosis, as determined by flow cytometry analysis.
- The molecular mechanism for nanoparticle-encapsulated CdtB-induced gastric cancer cell death is mediated by the p53 activation pathway and ATM-dependent checkpoint, followed by triggering DNA breakage.
- Our results demonstrate that nanoparticle-encapsulated CdtB effectively inhibited growth of tumor cells *in vivo*.

Conclusion

- Our study suggests that chitosan/heparin nanoparticle-encapsulated CdtB, which exerts genotoxicity and enhances antitumor activity, represents a novel strategy for the treatment of gastric cancer.

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