Deoxycholic acid inhibits the growth of BGC-823 gastric carcinoma cells via a p53-mediated pathway

HAI-BO YANG*, WEI SONG*, MEI-DIE CHENG, HAI-FANG FAN, XU GU, YING QIAO, XIN LU, RUI-HE YU and LAN-YING CHEN

Department of Biological Science, School of Life Science and Engineering, Henan University of Urban Construction, Pingdingshan, Henan 467044, P.R. China

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Abstract. The aim of the present study was to investigate the effects of deoxycholic acid (DCA) on BGC-823 human gastric carcinoma cells and to explore the possible mechanisms underlying any such effects. Cell proliferation was detected using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, cell morphology was observed by inverted microscopy, and cell cycle progression and the mitochondrial membrane potential were analyzed using flow cytometry. The expression of Bcl-2, Bax, p53, Cyclin D1 and cyclin-dependent kinase (CDK)2 proteins in BGC-823 cells was analyzed with western blotting. The results demonstrated that DCA significantly inhibited cell growth, and that the cell cycle was arrested at the G_1 phase. DCA was also shown to induce BGC-823 cell apoptosis, which was associated with the collapse of the mitochondrial membrane potential. The mitochondria-dependent pathway was activated via an increase in the ratio of Bax:Bcl-2 in BGC-823 cells. In addition, the expression of p53, cyclin D1 and CDK2 was altered following DCA treatment. These results suggest that DCA induces apoptosis in gastric carcinoma cells through activation of an intrinsic mitochondrial-dependent pathway, in which p53 is involved.

Introduction

Gastric carcinoma is a common malignant tumor of the digestive system. The morbidity and mortality of gastric cancer are the highest of any type of cancer in China (1). Surgery, chemotherapy, radiotherapy and immunotherapy are the primary methods of treatment for gastric cancer. However, the overall outcome of these therapies remains unsatisfactory (1,2). With the rapid development of tumor molecular biology and genetic engineering technology, the development of antitumor drugs has become a focus of interest in the treatment of a variety of types of cancer.

Bile acids are synthesized in the liver and secreted with the bile into the duodenum, where they perform essential functions, including the digestion and absorption of dietary lipids, and the regulation of gene expression (3-6). Previous studies have demonstrated a variety of roles for different bile acids in the treatment of cancer, depending on the nature of their chemical structures. For instance, it has been reported that certain components of bile, such as tauroursodeoxycholic acid, ursodeoxycholic acid and deoxycholic acid possess different capacities to induce apoptosis in tumor cell lines (7-10). Deoxycholic acid is a free bile acid derivative, which is composed of metabolized bile acids, and is known to promote bile secretion and to produce anti-inflammatory effects. In recent years, DCA has gained increasing attention as a potential anticarcinogenic agent. DCA has been shown to rapidly induce apoptosis in the HCT116 colon tumor cancer cell line (11,12). As a result of these studies, it was postulated that DCA may inhibit cell proliferation and induce apoptosis, and may thus be amenable to development as an antitumor agent. However, although DCA has been shown to possess anticancer properties, the mechanisms underlying these effects remain unclear. The present study aimed to assess the effects of DCA on the growth of gastric carcinoma cells by determining the ability of this compound to induce apoptosis and to explore the possible mechanisms of action.

Materials and methods

Reagents. Deoxycholic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). DCA was maintained as 100 mM stock solutions in ethanol and were stored at -20˚C. DCA stock solutions were diluted to the final concentration in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY, USA), as required. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), bisbenzimide (Hoechst 33258), acridine orange (AO), ethidium bromide (EB) and propidium iodide (PI), were purchased from Sigma-Aldrich. RPMI-1640 medium and fetal calf serum were purchased from Gibco Life

Correspondence to: Professor Lan-Ying Chen, Department of Biological Science, School of Life Sciences and Engineering, Henan University of Urban Construction, 7A Daxiangshan Road, Pingdingshan, Henan 467044, P.R. China
E-mail: lychen66@163.com

*Contributed equally

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Technologies (Grand Island, NY, USA). Mouse monoclonal antibodies against human p53, Bax, Bcl-2, cyclin D1, cyclin E1 and cyclin-dependent kinase (CDK)2 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals used were of the highest purity grade available.

Cell lines and cell culture. The BGC-823 gastric carcinoma cell line, which was provided by the China Center for Type Culture Collection (Shanghai, China), were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml kanamycin (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C in a 5% CO_2 in air atmosphere. Following seeding for 24 h, cells were treated with culture medium containing various concentrations of DCA (0.2, 0.4, 0.6 or 0.8 mM).

MTT cytotoxicity assay. The cytotoxicity of DCA to the BGC823 cell line was evaluated by an MTT assay (13). Briefly, cells were arrayed in a 96-well plate at a density of $1x10^5$/ml. Following overnight growth, cells were treated with DCA at various concentrations for 24, 48 or 72 h. Following treatment with DCA, 20 μl MTT (5 g/l) was added to each well and cells were continually cultured for another 4 h at 37˚C. The medium was then removed and 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to each well. The color intensity was measured with an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 490 nm.

Giemsa staining. BGC-823 cells from the control group (treated with RPMI-1640, including 0.5% DMSO) and the group treated with 0.4 mM DCA for 36 h were seeded onto cover slips and grown for 24 h. Cells were washed with phosphate-buffered saline (PBS) three times, stained with Giemsa staining solution (Sangon Biotech Co., Ltd, Shanghai, China) for 10 min and observed under a light microscope.

Hoechst 33258 and AO/EB staining. Cells that had been treated with 0.4 mM DCA for 36 h were harvested and fixed with a mixture of glacial acetic acid and methanol (1:3, v/v; Sangon Biotech Co., Ltd) for 5 min and then washed twice with PBS. Cells were resuspended in Hoechst 33258 solution (5 μg/ml) and incubated at room temperature for 10 min. Following three washes with PBS, the cells were thoroughly dried naturally at room temperature and observed under a fluorescence microscope (Eclipse Te2000-E; Nikon Corp., Tokyo, Japan). For AO/EB staining, the cells were harvested and washed twice with PBS. Cells were then incubated with 100 μl PBS plus 4 μl AO/EB solution (100 μg/ml AO and 100 μg/ml EB in PBS) for 3 min at room temperature in darkness and immediately observed under a fluorescence microscope.

Flow cytometry. Following treatment with DCA at different concentrations (0.2, 0.4, 0.6 and 0.8 mM) for 36 h, the BGC-823 cells were harvested, washed twice with PBS and fixed with 70% ethanol at 4˚C overnight. Cells were then centrifuged at 800 x g for 5 min, resuspended in 100 μg/ml RNase A (Sigma-Aldrich) at 37˚C for 30 min and stained with 50 μg/ml propidium iodide at 4˚C for 30 min in darkness. Cells were analyzed by flow cytometry (Guava easyCyte 8; EMD Millipore, Billerica, MA, USA) at 488 nm, and the data were analyzed with CellFit software (Guava InCyte; EMD Millipore).

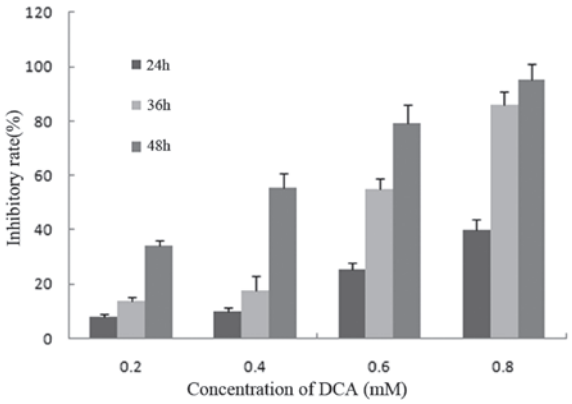

Figure 1. Growth inhibitory effects of bile extract on BGC-823 cells. Exponentially growing BGC-823 cells were treated with varying concentrations of deoxycholic acid for different time periods. The cell growth inhibition was analyzed by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.

Analysis of mitochondrial transmembrane potential ($\Delta\Psi m$). Following treatment with various concentrations of DCA (0.2, 0.3, 0.4 and 0.5 mM) for 48 h, the BGC-823 cells were incubated with Rh123 (1 mg/ml in DMSO; Sigma-Aldrich) at 37˚C for 30 min and washed three times with PBS. Cells were then harvested and analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Western blotting analysis. Western blotting analysis was performed as previously described (13). Briefly, cell lysates were prepared, separated with 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). Non-specific reactivity was blocked by incubating the membranes for 1 h in 5% nonfat milk at room temperature. The membranes were incubated with primary antibody overnight at 4˚C. After three washes for 10 min with phosphate-buffered saline Tween-20 (PBST), the membranes were incubated at 37 °C for 1 h with the appropriate secondary antibody (1:5,000; Sigma-Aldrich) and washed three times with PBST. Reactive proteins were detected with an enhanced chemiluminescence detection system (Pierce Biotechnology Inc., Rockford, IL, USA). β-actin was used as an internal control.

Statistical analysis. Data were analyzed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean of separate experiments ($n \geq 3$). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of DCA on the proliferation of BGC-823 cells. With DCA concentrations of 0.2, 0.4, 0.6 and 0.8 mM, the proliferation of BGC-823 cells was significantly inhibited in a dose- and time-dependent manner. Following treatment with various concentrations of DCA the percentage of inhibition

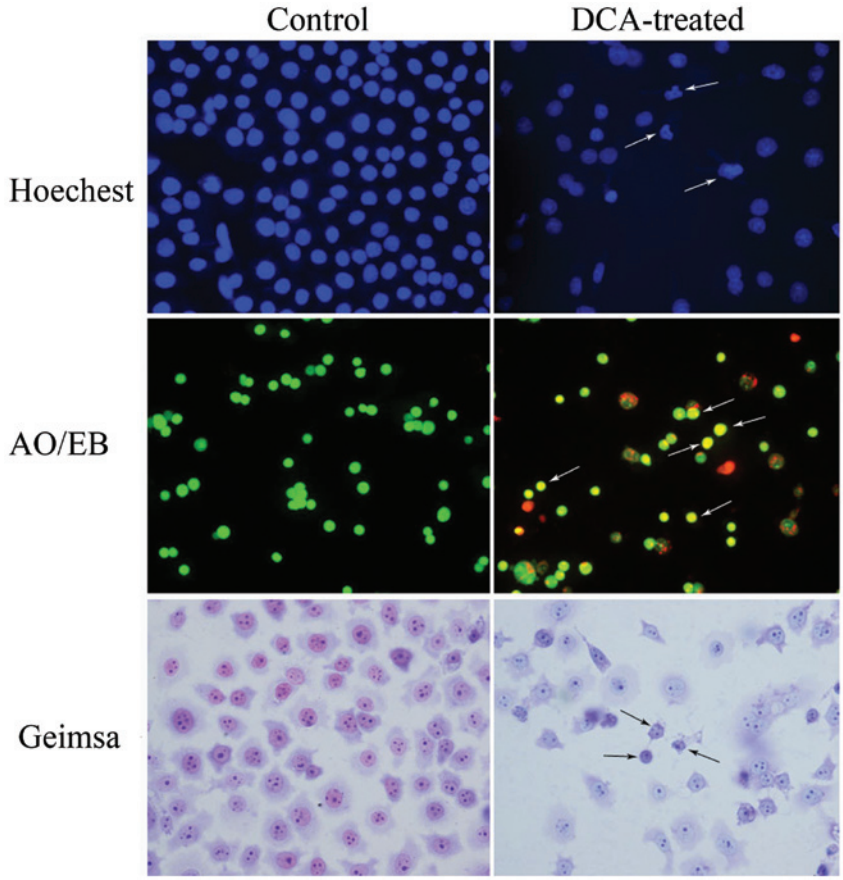

Figure 2. Morphological changes in BGC-823 cells following exposure to 0.35 mM DCA for 36 h. Hoechst 33258 staining demonstrated morphological changes using a fluorescence microscope (magnification, x400). AO/EB staining demonstrated morphological changes when viewed under a fluorescence microscope (magnification, x200). Giemsa staining demonstrated morphological changes when viewed under an optical inverted phase-contrast microscope (magnification, x400). Arrows indicate cells undergoing apoptosis. DCA, deoxycholic acid; AO, acridine orange; EB, ethidium bromide.

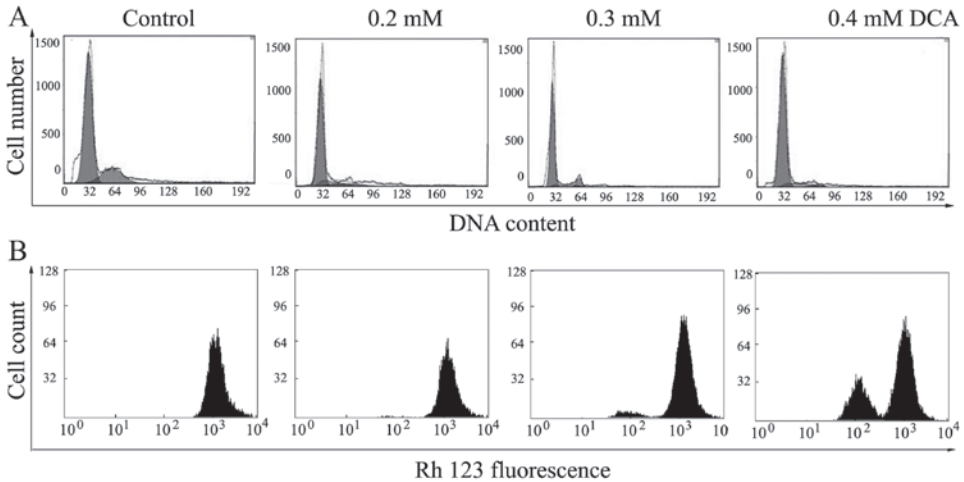

Figure 3. Effect of DCA on BGC-823 cell cycle distribution. (A) Cell cycle analysis using flow cytometry with PI staining showing DNA histograms of BGC-823 cells. (B) Effect of DCA on the ΔΨm of BGC-823 cells. The increase in Rh123 hypofluorescence indicated the decrease in ΔΨm. DCA, deoxycholic acid; PI, propidium iodide; ΔΨm, motochondrial transmembrane potential.

at 24 h were 7.89, 10.12, 25.56 and 40.12%, and at 48 h were 34.09, 55.22, 79.21 and 95.02%, with doses of 0.2, 04.4, 0.6 and 0.8 mM DCA, respectively (Fig. 1).

Morphological changes of BGC-823 cells following DCA treatment. Hoechst 33258 staining showed that the nuclei of cells in the control group exhibited a uniform dispersion of low-intensity fluorescence and had an integral structure. However, following treatment with 0.3 mM DCA, the nuclei exhibited pyknosis or a granular distribution of fluorescence. In these cells, the intensity of fluorescence was uneven and the nuclei appeared deformed and horseshoe-shaped, in addition to

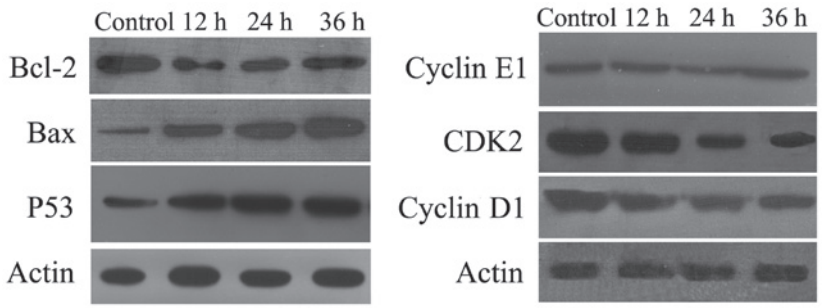

Figure 4. Expression of p53, cyclin E1, CDK2 cyclin D1, Bcl-2 and Bax in BGC-823 cells treated with 0.3 mM deoxycholic acid for different periods of time.

other typical characteristics of apoptosis , including condensed chromatin, gradual disintegration of the nuclear membrane, and pyknotic (shrunken and dark) nuclei (Fig. 2). The results of the AO/EB staining demonstrated that the control BGC-823 cells appeared uniformly green, and contained nuclei with apparently normal structures. However, the DCA-treated cells appeared orange or red, the nuclei exhibited pyknosis and there was cataclastic scattering (non-uniform fluorescence distribution) in the cytoplasm.

Following Giemsa staining, BGC-823 cells exhibited various morphological forms, such as epithelioid, round, and irregular. When treated with a concentration of 0.3 mM DCA, BGC-823 cells were observed to undergo a significant morphological change and appeared shrunken, with a small nucleus and chromatin agglutination. Apoptotic cells were observed singly or in groups.

Flow cytometric analysis. Cell cycle progression in the BGC-823 cells was analyzed using flow cytometry. The results showed that the cell cycle distribution of BGC-823 cells changed markedly following treatment with various concentrations of DCA (0.2, 0.3, 0.4 and 0.5 mM). The proportion of cells in G_0/G_1 phase in the control group was 60.12, which increased to 88.35% following treatment with 0.3 mM DCA, while the proportion of cells at G_2 phase decreased significantly from 25.16 to 4.98%. These results indicate that the cell cycle was arrested in G_0/G_1 phase following treatment with DCA.

Analysis of ΔΨm. In order to explore whether mitochondrial damage is involved in DCA-induced apoptosis, the increase of Rh123 hyperfluorescence that occurs with a decrease in membrane potential, was used to investigate the changes in ΔΨm following treatment with DCA. As shown in Fig. 3B, a significant dose-dependent increase in the mean fluorescence intensity of the cells, associated with collapse of the ΔΨm, were observed in the BGC-823 cells following 48 h of treatment with DCA.

Western blot analysis. To investigate the mechanisms underlying the induction of apoptosis in BGC-823 cells by DCA, the levels of expression of apoptosis-related proteins, including p53, Bax, Bcl-2, CKD2, Cyclin D1 and Cyclin E1 were measured. The tumor-suppressor protein, p53, regulates apoptosis through the transcriptional activation of its target genes, and acts as a key facilitator of cross-talk between numerous pathways. Following treatment with DCA, the level of the p53 protein was significantly increased (Fig. 4A). As the proteins of the Bcl-2 family are known to regulate the mitochondrial pathway by controlling the permeability of the outer mitochondrial membrane during apoptosis, the effect of DCA on the expression of Bcl-2 and Bax was investigated. As shown in Fig. 4A, following exposure to DCA for 48 h, Bcl-2 expression was diminished while Bax expression was elevated. Therefore, the Bcl-2:Bax ratio was markedly decreased in DCA-treated BGC-823 cells. Furthermore, the levels of cyclin D1 and CDK2 decreased in DCA-treated BGC-823 cells in a dose-dependent manner (Fig. 4B). These results suggest that p53 is involved in the induction of apoptosis by DCA.

Discussion

Over recent years, it has been shown that DCA induces apoptosis in a number of types of human malignancies (14,15). The results from the present study indicated that DCA significantly inhibits the proliferation of BGC-823 cells in a dose-dependent manner and induces apoptosis via activation of the mitochondrial pathway. p53 was also shown to be involved in the DCA-mediated apoptosis of BGC-823 cells.

Apoptosis was initially described according to its morphological characteristics, and morphology remains a relevant experimental method to demonstrate the occurrence of this process. Morphological features characteristic of apoptosis were observed in BGC-823 cells following exposure to DCA, including cell shrinkage, chromatin agglutination, marginalization, nuclear fragmentation and apoptotic body formation. An important feature of apoptosis is the permeabilization of mitochondria. Mitochondria are known to be an important part of apoptotic signaling. DCA treatment led to a concentration-dependent reduction in mitochondrial membrane potential. This drop in ΔΨm produces changes in mitochondrial biogenesis and activity, decreasing the numbers of mitochondria and influencing the stability of mitochondrial function.

There are several checkpoints during the cell cycle. The most important points are the G_1/S phase transition and the G_2/M phase transition. Healthy cells replicate during S phase and reduce in number during G_1 phase. By contrast, tumor cells are able to bypass the checkpoint of the G_1/S and G_2/M phase transition points, allowing cells to proliferate without restraint. Therefore, the genes controlling the G_1/S or G_2/M phase transitions are potential targets for the development of antitumor drugs (16). To determine whether the toxicity of

DCA in BGC-823 cells is due to the induction of cell cycle arrest, the cell cycle phase distribution of the treated cells was examined by flow cytometry. The results showed that BGC-823 cells were arrested at the G_1 phase when treated with DCA. The ratio of cells in the G_1 phase was positively correlated with the duration and concentration of DCA administered, which indicates that the inhibition of gastric cancer cell growth by DCA may be due to G_1 phase arrest. Furthermore, following treatment of BGC-823 cells with DCA, the expression of Cyclin D1 and CDK2 proteins was downregulated.

The mechanism underlying DCA-induced apoptosis in BGC-823 cells is unclear. The intrinsic apoptosis pathway is a mitochondrial-mediated cell death process, which is regulated by the Bcl-2 family of proteins that comprises proapoptotic proteins, such as Bax, Bak, Bad and Bid, and antiapoptotic proteins, such as Bcl-2 and Bcl-XL. Activation of the intrinsic pathway is often associated with a change in the level of Bax:Bcl-2 expression or the ratio of these proteins (17). In the present study, the expression of Bax was shown to be upregulated, while the expression of Bcl-2 was downregulated. The ratio of Bax:Bcl-2 was increased. This rise in the Bax:Bcl-2 ratio suggested a commitment of BGC-823 cells to apoptosis via activation of the mitochondria-dependent pathway.

A study demonstrated that almost 50% of human tumors carry p53 mutations, and that additional mechanisms are able to disrupt wild-type p53 function (18). Although a number of studies have shown that apoptosis may be induced in a p53-independent manner (19-26), other studies have reported that p53 is actively required for apoptosis to occur (24,27,28). The action of p53 has been shown to operate predominantly through induction of apoptosis in cells via the intrinsic pathway (29,30). Therefore, it is important to further investigate this process and to identify whether the activation of p53 is involved in the mechanisms underlying the induction of apoptosis in BGC-823 cells. The results from the current study demonstrated that p53 was active during DCA-induced apoptosis, and suggested that P53 may have a central role in the apoptosis of BGC-823 cells.

In conclusion, the present study demonstrated that DCA significantly inhibited proliferation and arrested cell cycle progression at the G_0/G_1 phase in BGC-823 cells. The results indicated that DCA-induced apoptosis of BGC-823 cells occurs via activation of the mitochondrial pathway, in which p53 is involved. An improved understanding of the way in which DCA regulates cell death may provide insight into potential anticancer mechanisms, and aid in the selection of novel natural compounds for screening and ultimately the development of cancer treatments.

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