Effects of celecoxib on cell apoptosis and Fas, FasL and Bcl-2 expression in a BGC-823 human gastric cancer cell line

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Abstract. Fas, which is an apoptotic-related protein, has an important role in cell apoptosis. Fas ligand (FasL) binds to Fas and activates apoptosis signal transduction. We previously demonstrated that the efficiency of celecoxib inhibited the proliferation and apoptosis of HT-29 colon cancer cell line. The BGC823 cell line was used as an experimental model to evaluate the potential role of celecoxib on gastric cancer cell apoptosis. Inhibitory effects of celecoxib on cell viability were determined by MTT assay. Cell apoptosis was evaluated by flow cytometric analysis and laser confocal microscopy. The results of the present study demonstrated that celecoxib inhibited the viability of BGC823 cells in a concentration- and time-dependent manner. Furthermore, the effect of BGC823 cells apoptosis was increased in a concentration-dependent manner. Western blotting was used to determine the protein expression levels of Fas, FasL, and B-cell lymphoma-2 (Bcl-2). During the celecoxib-induced apoptosis of BGC823 cells, celecoxib upregulated Fas expression and downregulated FasL and Bcl-2 expression in a concentration-dependent manner. These results suggest that celecoxib inhibited the growth and induced apoptosis of BGC823 gastric cancer cells by regulating the protein expression of Fas, FasL and Bcl-2.

Introduction

Apoptosis, called also cell suicide or programmed cell death, is a genetically directed process of cell self-destruction (1). Abnormal apoptosis is a component of the pathogenesis of most malignant tumors (1). Fas, a member of the tumor necrosis factor-R (TNF-R) family and a cell surface death receptor, may be an important protein for apoptosis that is induced by ligand binding, such as to Fas ligand (FasL), and leads to subsequent activation of apoptotic signal transduction pathways (2-4). FasL (CD95L or APO-1L), belongs to the family of TNF-related cytokines. Repeated activation of antigen receptors on T cells induces FasL expression and leads to Fas-transduced apoptosis (5). Cytochrome C release is a critical step in the apoptotic cascade, which involves the release of cytochrome C into the cytosol where it binds with apoptotic protease-activating factor (Apaf-1) (6). Also, apoptotic pathways dependent on cytochrome C may be influenced by Bcl-2, which can inhibit the release of cytochrome C and thereby inhibit cell apoptosis (7).

Our previous observations (8) that the cyclooxygenase-2 (COX-2) inhibitor, celecoxib, was able to inhibit viability in the BGC-823 gastric cancer cell line led us to investigate whether celecoxib could induce apoptosis in the same gastric cancer cell line and whether celecoxib modified expression of Fas, FasL, and Bcl-2, which regulate several aspects of apoptosis (9).

Materials and methods

Cell culture and treatment. The human BGC-823 gastric cancer cell line was obtained from Central South University Cancer Institute (Changsha, China). BGC-823 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Beijing TianShiDai Inc., Beijing, China), and 100 U/ml penicillin/streptomycin (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. Celecoxib (Pfizer, Inc., New York, NY, USA) was a generous gift from Professor Guangsheng Zhang at the Second Xiangya Hospital, Central South University (Changsha, China). Celecoxib was dissolved in dimethyl sulfoxide (DMSO) when stored at 30 mmol/l (4°C) and was diluted to a final concentration of 0.4% in culture medium.

Cell viability analysis. Cell viability inhibition was measured using an MTT (Sigma-Aldrich, Merck KGaA, Darmstadt Germany) colorimetric method. Briefly, 2x10⁵/ml BGC-823 gastric cancer cells/well were inoculated into 96-well culture plates, until they reached 80% confluence. Subsequently, various concentrations of celecoxib in DMSO (20, 40, 60, 80, 100 and 120 µM) were added to the cell culture medium. Control groups included a blank control, treated with RPMI-1640 cell culture medium, and a DMSO control, treated
with DMSO. Cells were cultured for 24, 48, and 72 h prior to incubation for 4 h with 0.5% MTT (20 µl per well). An ELISA (wavelength, 570 nm) was performed to obtain optical density (OD) values. Each experiment was repeated three times. Cell viability inhibitory rate was calculated using the following formula: Cell viability inhibitory rate=1−(test group OD value−blank control OD value)/(DMSO control group OD value−blank control OD value).

Cell apoptosis analysis. Cells grown to 80% confluence were treated with celecoxib (20, 40, 60, 80, 100 and 120 µM). Cells were incubated for 48 h and cell suspensions were subsequently centrifuged (350 x g; 4°C; 5 min). Following treatment, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended with binding buffer to regulate cell concentration to 10⁶/ml. Subsequently, the cell suspension was stained with Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD; Beijing Korad Biotech Co., Ltd.) according to the manufacturer's protocol, prior to incubation for 15 min at room temperature in the dark. A FACSVersus flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze the population of Annexin V-positive and 7-AAD-negative early apoptotic cells. Confocal laser fluorescence microscopy was used to observe fluorescence signals from early apoptotic cells. Western blot analysis. Since cells were almost inhibited by 120 µM celecoxib (8), BGC-823 gastric cancer cells were only treated with 0, 20, 40, 60, 80 and 100 µM celecoxib for 48 h, and used to measure Fas, FasL, and Bcl-2 protein expression levels via western blot analysis. Rabbit anti-human GAPDH (catalogue no. BA2913), Fas (catalogue no. PB0214), FasL (catalogue no. BA0049) and Bcl-2 (catalogue no. BA0412) monoclonal antibodies and goat anti-rabbit antibodies (catalogue no. BA1055) labeled with horseradish peroxidase were purchased from Boster Biological Technology, Ltd., (Wuhan, China). Proteins were extracted from the BGC-823 gastric cancer cell line, and protein quantification was performed using a bicinchoninic acid protein assay (Nanjing KeyGen Biotech Co., Ltd.) with bovine serum albumin as a standard. Proteins (30 µg/sample) in SDS-loading buffer were heated to 100°C for 5 min, separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked overnight in 5% nonfat dry milk in TBST buffer at 4°C. Blots were then incubated at room temperature for 2 h with rabbit anti-human Fas, FasL, and Bcl-2 monoclonal antibodies (1:300) in TBST buffer with 5% nonfat dry milk. Following washing in TBST buffer four times for 7 min, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:800) in TBST buffer with 5% nonfat dry milk. Finally, membranes were washed three times and developed with enhanced chemiluminescence reagent (Boster Biological Technology, Ltd.). Immunoreactive bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data were processed by SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA) statistical software. Data were represented as means ± standard deviation and compared using one-way analysis of variance and Tukey’s multiple comparison test among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Celecoxib inhibits the viability of BGC-823 gastric cancer cells. MTT analysis showed that celecoxib inhibited cell viability of BGC-823 gastric cancer cells in a time- and concentration-dependent manner (P<0.001 and P<0.05, respectively). Inhibition ratio of cell viability peaked at 98% at 120 µM after 72 h (Fig. 1).

Celecoxib induces apoptosis in BGC-823 gastric cancer cells. Flow cytometric analysis revealed the effect of celecoxib on BGC-823 gastric cancer cell apoptosis. Intact cells were used as a blank control group. Remaining cells were treated with 0 (DMSO control), 20, 40, 60, 80, 100 and 120 µM celecoxib concentration for 48 h. In the 0, 20, 40, 60, 80, 100 and 120 µM celecoxib concentration groups, the respective percentages of early apoptotic cells were 17.43±0.82, 17.37±0.94, 21.77±1.27, 29.13±3.1, 41.47±5.20, 75.60±3.76, and 88.37±3.27%. These results suggested that celecoxib may induce BGC-823 gastric cancer cell apoptosis in a concentration-dependent manner (Fig. 2).

Apoptotic cells were also assessed by fluorescence-activated cell sorter analysis of Annexin V-PE/7-AAD staining. Nuclei were counterstained with DAPI. The percentage of early apoptotic cells and dead cells increased with the concentration of celecoxib (Fig. 3).

Celecoxib regulates the expression of Fas, FasL and Bcl-2 protein in BGC-823 gastric cancer cells. Cells were treated with 0, 20, 40, 60, 80, and 100 µM celecoxib for 48 h. Western blot analysis verified that, with increased celecoxib concentration, FasL and Bcl-2 protein expression levels decreased, as demonstrated by thinner protein bands; whereas Fas protein expression increased, as demonstrated by thicker bands, in a concentration-dependent manner (Fig. 4). OD values for semi-quantitative determination of the relative protein content are depicted in Table I.
Discussion

Cell proliferation and apoptosis are two critical cellular behaviors and their mechanisms are often the core of diseases or disorders. Uncontrolled cell proliferation or apoptosis can give rise to tumors as cancer research has shown that malignant cell proliferation involves abnormal apoptosis (10). Therefore, selective apoptosis of tumor cells may be a viable cancer treatment strategy.

Apoptosis, which is a type of programmed cell death, is a spontaneous biological process that follows a predetermined pathway. Cell volume shrinks, chromatin condenses, and the nucleus collapses. As the cell dies, unused nutrient macromolecules are transferred to neighboring cells. Apoptosis offers a unique perspective for tumor studies in which two apoptotic pathways exist (11,12). One pathway is mediated by cell death receptors (tumor necrosis factor receptor or Fas) and caspase-8 activation by Fas-associated death domain protein (FADD). The second pathway of apoptosis is induced by developmental signals or growth factor withdrawal.
which triggers cytochrome C release from mitochondria. Subsequently, apoptosis activated factor-1 (apoptotic protease activating factor-1) polymerization and caspase-9 activation occur in a process controlled by a gene that regulates the B cell lymphoma/leukemia 2 (Bcl-2) protein family. Fas has an important role in cell apoptosis. Its ligand, FasL, binds and activates apoptosis signal transduction (2,3).

Previous studies have suggested that non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, tolfenamic acid and aspirin, are able to inhibit the occurrence and development of chemically-induced colon tumors, inhibit tumor cell growth and proliferation and increase tumor cell apoptosis (13-16). Previous studies suggest that the antitumor effect of NSAIDs are associated with cyclooxygenase (COX), specifically COX-2 (13), and this COX-2 inhibition may be the mechanism underlying the anti-tumor effect (17). Pang *et al* (18) reported that celecoxib induces apoptosis in gastric cancer cells that do not express COX-2. Zhu *et al* (19,20) reported that traditional chemotherapy drugs combined with rofecoxib or with celecoxib reduce tumor chemoresistance, improve the overall curative effect, and may sensitize tumor cells to chemotherapy. The mechanism for this may be the induction of cancer cell apoptosis. Selective COX-2 inhibitors, such as nimesulide, NS-398 and celecoxib, have been reported to induce apoptosis in human colon cancer cells and liver cancer cells (21-23). Li *et al* (24) reported that nimesulide induced apoptosis in gastric cancer cells. These studies suggest that COX-2 inhibitors may induce tumor cell apoptosis, via the inhibition of COX-2. To investigate this hypothesis, we treated the BGC-823 gastric cancer cell line with celecoxib and used molecular biological techniques to measure gastric cancer cell apoptosis and the expression of apoptosis-related proteins.

Cell apoptosis, reduced cell volume, alterations in light scattering, membrane permeabilization can all be used to detect apoptotic cells. Cells suspended with fluorescein staining can be subjected to flow cytometry to detect apoptosis in living and fixed dead cells. By flow cytometry, Annexin V-PE can be detected in the PE signal channel and 7-AAD can be detected in the red fluorescent channel. The analysis can be obtained by using two parameter scatter plots (25). The present findings demonstrated that apoptosis in the BGC-823 cell line was concentration-dependent when cells were treated with 20-120 µM celecoxib. Confocal laser fluorescence microscopy also indicated orange staining indicative of early apoptosis by conjugated staining.

**Figure 4.** Celecoxib regulated the expression of apoptosis-related proteins in BGC-823 gastric cancer cells. Cells were treated with 0, 20, 40, 60, 80 and 100 µM celecoxib for 48 h and subjected to (A) western blot analysis and (B) subsequent quantification. Data are expressed as the mean ± standard deviation from three independent experiments. *P*<0.05 vs 0 µM.

Table I. Fas, FasL and Bcl-2 protein expression in a BGC-823 gastric cancer cell line treated with various concentrations of celecoxib for 48 h (n=3; mean ± standard deviation).

| Concentration (µM) | Fas/GAPDH | FasL/GAPDH | Bcl-2/GAPDH |
|-------------------|-----------|------------|-------------|
| 0                 | 0.1113±0.0037 | 0.8070±0.0268 | 0.5448±0.0049 |
| 20                | 0.1017±0.0065 | 0.7311±0.0780* | 0.5213±0.0326 |
| 40                | 0.1975±0.0100* | 0.6254±0.0501* | 0.4322±0.0306* |
| 60                | 0.3297±0.0053* | 0.5465±0.0180* | 0.3901±0.0264* |
| 80                | 0.4595±0.0045* | 0.4378±0.0081* | 0.2659±0.0121* |
| 100               | 0.5204±0.0148* | 0.3760±0.0034* | 0.2228±0.0071* |

*P*<0.05 vs 0 µM. FasL, Fas ligand; Bcl-2, B-cell lymphoma-2.
FasL is predominantly expressed in cytotoxic T lymphocytes, NK cells and some immune tissue (29). FasL expressed in these cells binds to Fas-positive cells to initiate the death signal and apoptosis. Fas expression in benign tumors is similar to normal tissues; whereas Fas expression in malignant tumors is downregulated or lost, and in metastatic tumors is decreased (30). Malignant tumors lacking Fas escape the scavenging effect of the Fas:FasL system in vivo (30). Tumor cells with high FasL expression, such as immune tissue, protect themselves from active killer immune cells. Expression of the Fas antigen in immune cells can trigger apoptotic mechanisms by expression of Fasl in tumor cells (31-33).

Human BGC-823 gastric cancer cells treated with celecoxib for 48 h were found to have less FasL protein expression. The Fas and FasL apoptotic system may mediate apoptosis in this cancer cell line, increasing Fas expression and the activation of FADD (34), which activates the caspase-8 cascade and cancer cell apoptosis. Celecoxib treatment of gastric cancer cells with low FasL expression can reduce immunologically active cells induced by apoptosis, facilitating immune cells’ ability to kill tumor cells (29). Apoptotic pathways are dependent on cytochrome c, and data show that mitochondria are important in apoptosis. The cell apoptosis Bel-2 family is primarily located in the mitochondrial membrane. Cytochrome c is released into the cytosol after mitochondria permeability transition pores (MPT) open to promote apoptosis. The Bel-2 gene within the mitochondria can promote mitochondrial membrane stability, and block MPT opening and inhibiting cytochrome c release, thereby inhibiting the apoptosis of cells (35-37). Zhang et al (38) revealed that COX-2 inhibitors downregulated the Bel-2 gene in a colorectal HT-29 cancer cell line. Thus, Bel-2 may have regulatory functions in the process of apoptosis that is dependent on cytochrome c. Our data are in agreement with these findings. Bel-2 protein expression was downregulated with celecoxib treatment in a dose-dependent manner, reducing the inhibitory effect on apoptosis in a human BGC-823 gastric cancer cell line, thereby promoting apoptosis of cancer cells.

In conclusion, the present findings demonstrated that celecoxib treatment increased expression of Fas protein and decreased FasL protein expression, suggesting that these two molecules are important in apoptotic signaling. Bel-2 protein expression was downregulated by celecoxib treatment, suggesting that this, too, may be important in apoptotic signal transduction pathways that depend on cytochrome c. Regulation of apoptosis is complex, and non-COX-2 dependent pathways may exist. Therefore, further studies are warranted to investigate the molecular mechanism related to protein expression and of celecoxib-induced apoptosis.

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