Stable transfection of extrinsic Smac gene enhances apoptosis-inducing effects of chemotherapeutic drugs on gastric cancer cells

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METHODS: After Smac gene was transferred into gastric cancer cell line MKN-45, subclone cells were obtained by persistent G_{418} selection. Cellular Smac gene expression was determined by RT-PCR and Western blotting. After treatment with mitomycin (MMC) as an apoptotic inducer, in vitro cell growth activities were investigated by trypan blue-staining method and MTT colorimetry. Cell apoptosis and its rates were determined by electronic microscopy, annexin V-FITC and propidium iodide staining flow cytometry. Cellular caspase-3 protein expression and its activities were assayed by Western blotting and colorimetry.

RESULTS: When compared with MKN-45 cells, the selected subclone cell line MKN-45/Smac had significantly higher Smac mRNA (3.12±0.21 vs 0.82±0.14, t = 7.52, P<0.01) and protein levels (4.02±0.24 vs 0.98±0.11, t = 8.32, P<0.01). After treatment with 10 μg/mL MMC for 6-24 h, growth inhibition rate of MKN-45/Smac (15.8±1.2-54.8±2.9%) was significantly higher than that of MKN-45 (5.8±0.4-24.0±1.5%), t = 6.42, P<0.01. Partial MKN-45/Smac cancer cells presented characteristic morphological changes of apoptosis under the electronic microscope with an apoptosis rate of 36.4±2.1%, which was significantly higher than that of MKN-45 (15.2±0.8%, t = 9.25, P<0.01). Compared with MKN-45, caspase-3 expression levels in MKN-45/Smac were improved significantly (3.39±0.42 vs 0.96±0.14, t = 8.63, P<0.01), while its activities were 3.25 times as many as those of MKN-45 (0.364±0.010 vs 0.112±0.007, t = 6.34, P<0.01).

CONCLUSION: Stable transfection of extrinsic Smac gene and its over-expression in gastric cancer cell line can significantly enhance cellular caspase-3 expression and activities, ameliorate apoptosis-inducing effects of mitomycin C on cancer cells, which is a novel strategy to improve chemotherapeutic effects on gastric cancer.

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Key words: Gastric cancer; Mitomycin C; Extrinsic Smac gene; Apoptosis; Transfection

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INTRODUCTION

Up to now, chemotherapy is still the important adjuvant treatment for postoperative and advanced gastric cancer. Its research focuses on how to reduce the side effects of chemotherapeutic drugs and improve the sensitivities of tumor cells to them\[4\]. A series of researches indicate chemotherapeutic drugs, radiotherapy and thermotherapy could all induce apoptosis to various extents by exerting their anti-tumor effects\[8\]. The second mitochondria-derived activator of caspases (Smac) or DIABLO gene is a recently identified and novel proapoptotic molecule, which is released from mitochondria into the cytosol when cell apoptosis undergoes, to enhance activities of caspase-3 through eliminating the functions of inhibitors of apoptosis proteins (IAPs)\[9\]. It was reported that the apoptosis-inducing effects of chemotherapeutic drugs on ovary cancer and leukemia could be significantly enhanced by gene transfer of Smac into cancer cells\[9,10\]. In this study, we investigated the effects of stable transfection of extrinsic Smac gene on apoptosis of gastric cancer cells induced by chemotherapeutic drugs, to explore a novel strategy to improve chemotherapeutic effects on gastric cancers.

MATERIALS AND METHODS

Genes and main reagents

Eukaryotic vector pcDNA3.1-Smac containing a full-length human Smac cDNA (719 bp) was kindly provided by Professor Xiao-Dong Wang (USA). Blank vector pcDNA3.1 was preserved by our central laboratory. Polyclonal rabbit anti-human Smac was a kind gift from Professor Emma (Ireland). Monoclonal mouse anti-human caspase-3 and its activity detection kit were purchased from Santa Cruz Biotechnology and Clontech Company respectively. Annexin V-FITC reagent kit was purchased from Jingmei Biotech Company. Liposome GeneSHUTTLE-40 was purchased from Q-bio Gene Limited Company. Newborn calf serum, RPMI 1640, Trizol™ reagent kit and G_{418} were all purchased from Gibco Company. Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were both purchased from Clontech Company. Mitomycin C (MMC) was a product from Japan Union Ferment Industry Company, which was prepared into 1 g/L stock solution with PBS, preserved at 4 ℃ and kept from light.

Cell culture

Human gastric cancer cell line MKN-45 was kindly provided by Professor Ji-Hua Dong, Department of Virology, Union Hospital...
in a humidified atmosphere of 50 mL/L CO₂ was repeated 3 times to draw the cell growth curve. 1640 at 37 °C was added into each well. Then cells were incubated with RPMI 2×10⁶ MKN-45, MKN-45/neo and MKN-45/Smac cells were seeded at 3×10⁴/L density into the 24-well chamber slides (each group had five wells). After incubated at 37 °C in a atmosphere containing 5 mL/L CO₂ for 48 h, the cells were digested with 0.01%EDTA, then seeded into a 6-well plate (35 mm in diameter) at 1×10⁵/L density, and then selected with 600 mg/L G418 for 2 wk. When most of the non-transfected cells were dead, the concentration of G418 was decreased to 300 mg/L and maintained for another 2 wk. After cellular clones were formed, subclones were chosen at random and amplified. The subclone cells expressing Smac and neo genes were named as MKN-45/Smac and MKN-45/neo respectively.

Detection of cellular Smac mRNA expression

Cellular Smac mRNA expression levels were assayed by reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA extraction was conducted with Trizol™ reagent kit, according to the protocol of the manufacturer. The reverse transcription was conducted at 42 °C for 30 min in 25 μL total volume containing 2 μg template RNA, 1 μL 10 mmol/L dNTP, 20 U RNasin, 1 μL Oligo dT₁₈, 200 μL AMV, 5 μL 5×AMV buffer. The PCR primers for Smac gene were designed by Primer 5.0 software: upstream 5’-CTGTGACGATTGGCCTT-G-3’, downstream 5’-GTGATT CCTGCGGTTAT-3’, which were synthesized by Shanghai GeneBase Company. The anticipated product length was 425 bp. α-tubulin was evaluated with a gel computer image system (MCIAAS-1000, Bio-Rad Company).

Detection of cellular Smac protein expression

Cellular Smac protein expression levels were assayed by Western blotting. The extraction, quantification and separation of proteins were conducted as previously described in Molecular Cloning. Blots were incubated sequentially with 1% nonfat dry milk, rabbit polyclonal anti-Smac antibody and goat radish peroxidase-conjugated immunoglobulin G, and evaluated using an ECL Western blotting kit. Smac protein band intensities were respectively amplified. The brightness ratio between Smac and α-tubulin was evaluated with a gel computer image system (MCIAAS-1000, Bio-Rad Company).

Cell growth curve

MKN-45, MKN-45.neo and MKN-45/Smac cells were seeded at 2×10⁴/L density into the 24-well chamber slides (each group had five wells). After cells were attached to the wells, 10 mg/L MMC was added into each well. Then cells were incubated with RPMI 1640 at 37 °C in atmosphere containing 5 mL/L CO₂ for 0, 6 h, 12 h, 18 h and 24 h respectively. Then cells were digested by 0.125% trypsinase + 0.01% EDTA, stained with trypsin blue and counted under an inversion microscope. For each well, cell count was repeated 3 times to draw the cell growth curve.

Detection of cellular chemotherapeutic sensitivity

MTT colorimetry was used. MKN-45, MKN-45/neo and MKN-45/Smac cells were seeded at 3×10⁴/L density into 96-well chamber slides. For each cell line, untreated control group, 0.1 mg/L MMC, 1 mg/L MMC and 10 mg/L MMC group were designed, with each group having five wells. After treating with MMC for 24 h, 20 μL MTT (5 g/L) was added into each well and cultured for another 4 h, the supernatant was discarded, then 100 μL DMSO was added. When the crystals were dissolved, the optical density A of the slides were read on the enzyme-labeled minireader II at the wavelength of 490 nm. Cell proliferation inhibitory rate (%) = (1 - average A of experimental group/average A of control group) × 100%. For each detection, the total procedure was repeated 3 times.

Cell ultrastructure observation

After treated with 10 mg/L MMC for 24 h, three kinds of cancer cells were collected, sequentially rinsed in PBS, fixed with 2.5% glutaraldehyde for 30 min, and then washed with PBS. After routine embedment and section, cells were observed under an electron microscope.

Detection of cell apoptosis

After treatment with 10 mg/L MMC for 24 h, apoptotic ratios of three kinds of cells were determined by annexin V-FITC and propidium iodide staining flow cytometry. Cells from the above groups were collected, washed twice with cold PBS, resuspended with 100 μL binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl2, pH7.4), and then incubated with annexin-V-FITC at room temperature for 10 min. After washing with binding buffer, the cells were resuspended with 400 μL binding buffer containing 10 μL propidium iodide (20 μg/mL), and incubated on ice for 15 min. Apoptosis was analyzed by flow cytometry (BD Company, USA) at a wavelength of 488 nm.

Detection of caspase-3 protein expression and activities

After treatment with 10 μg/mL MMC for 24 h, cellular caspase-3 protein expression levels were assayed by Western blotting (as the same method above). The caspase-3 expression levels were analyzed with the computer imaging system. 2×10⁶ cells from above groups were respectively collected, added into 50 μL cellular lysis buffer, then incubated on ice for 10 min. After centrifugation (12 000 r/min) at 4 °C for 3 min, the supernatant was collected and added sequentially into 50 μL 2×reaction buffer, 5 μL 1.0 mmol/L caspase-3 substrate DEVD-pNA, and incubated at 37 °C for 1 h. After transferred into 96 wells, the optical density A of the slides were read on the enzyme-labeled minireader II at the wavelength of 405 nm (A₄₀₅ nm), which stood for the relative activities of caspase-3.

Statistical analysis

Data was expressed as mean±SD and analyzed using SPSS10.0 statistical software.

RESULTS

Establishment of Smac stably-transfected subclone cells

All the untransfected MKN-45 cells were dead after G₄₁₈ (600 μg/mL) selection for 1 wk. The pcDNA3.1 and pcDNA3.1-Smac transfected cells were continuously selected with G₄₁₈ for 4 wk, until magnificent clones could be observed. The clones were respectively amplified. The subclone MKN-45.neo and MKN-45/Smac cells were obtained, stably expressing neo and Smac genes respectively.
Cellular Smac mRNA expression
As shown in Figure 1A, after electrophoresis of RT-PCR products, Smac (425 bp) amplification bands could be observed in MKN-45, MKN-45/neo and MKN-45/Smac cells. There were only weak bands in MKN-45 and MKN-45/neo cells, and brighter bands in MKN-45/Smac cells, amplified with the same amount of RNA template. MGIAS-1000 gel computer image system proved that the Smac/α-tubulin ratio in MKN-45/Smac was 3.8 times, 3.7 times as many as that of MKN-45 (3.12±0.21 vs 0.82±0.14, t=7.52, P<0.01), MKN-45neo (3.12±0.21 vs 0.84±0.11, t=8.26, P<0.01) respectively. The brightness of Smac bands between MKN-45 and MKN-45/neo had no significant difference (P>0.05).

Cellular Smac protein expression detected by Western blotting.
As shown in Figure 1B, after treated with 10 mg/L MMC for 6-24 h, the apoptotic rates of MKN-45, MKN-45/neo and MKN-45/Smac cells were all decreased. The growth inhibitory rates were 5.8±0.4-24.0±1.5%, 7.1±0.6-26.8±1.2% and 15.8±1.2-54.8±2.9% respectively. The differences in growth activities between MKN-45 and MKN-45/neo cells were not significant (P>0.05), while the growth activities of MKN-45/Smac cells were reduced by 10.0±0.9-30.8±1.5% (t=6.42, P<0.01), when compared with those of MKN-45 cells (Figure 2).

Cellular sensitivity to MMC
After treated with 0.1 mg/L, 1 mg/L, 10 mg/L MMC, the growth activities of MKN-45, MKN-45/neo and MKN-45/Smac cells were reduced in a time- and dose-dependent manner. After treatment with 10 mg/L MMC for 24 h, the inhibitory rate of MKN-45 cells was 21.85±1.64%, while that of MKN-45/Smac cells reached 43.71±3.12%, and the difference between these two groups was significant (t=7.56, P<0.01). The difference in growth inhibitory rate of MMC between MKN-45 and MKN-45/neo cells was not significant (P>0.05) (Figure 3).

Cellular morphological features
After treating with 10 mg/L MMC for 24 h, some cells had characteristic morphological changes of apoptosis under an electron microscope, such as cellular volume reduction, nuclear shrinkage, chromatin congregation around the nuclear membrane and integrity of cellular membranes (Figure 4).

Cell apoptosis detection
After treated with 10 mg/L MMC for 24 h, the apoptotic rates of MKN-45 and MKN-45/neo cells were 15.2±0.8% and 16.5±1.1% respectively, with no significant difference (P>0.05). The apoptotic MKN-45/Smac cells increased and the apoptotic rate reached 36.4±2.1%, with a significant difference compared to that of MKN-45 (t=9.25, P<0.01) and MKN-45/neo (t=7.72, P<0.01) (Figure 5).

Caspase-3 protein expression and activities
As shown in Figure 6, after treated with 10 mg/L MMC for 24 h, 17 ku (p17) and 20 ku (p20) protein bands, subunits of caspase-3, could all be detected in MKN-45, MKN-45/neo and MKN-45/Smac cells. Computer image system indicated that there was a significantly higher p20 expression in MKN-45/Smac compared to MKN-45 (3.39±0.42 vs 0.96±0.14, t=8.63, P<0.01) and MKN-45/neo (3.39±0.42 vs 0.9±0.11, t=9.43, P<0.01). There was no significant difference in p20 expression levels between MKN-45 and MKN-45/neo cells (P>0.05). The A405 values of MKN-45, MKN-45/neo and MKN-45/Smac cells were 0.055±0.008, 0.052±0.012 and 0.060±0.011 respectively, while differences among them were not significant (P>0.05). After treatment with 10 μg/mL MMC for 24 h, the A405 value reached 0.364±0.010 in MKN-45/Smac cells, which was 2.35 times as many as that in MKN-45 cells (0.112±0.007, t=6.34, P<0.01) (Figure 7).
Gastric cancer is one of the most common malignant neoplasms in alimentary tract. Because of the side effects of chemotherapy on normal cells and drug resistance of tumor cells, it has been a research focus on how to ameliorate the chemotherapeutic effects on gastric cancer\cite{11}. Recent researches indicate that abnormal blockage of apoptosis is an important factor for the occurrence and development of cancer\cite{12-14}. To understand apoptosis mechanisms is hopeful for improving the sensitivities of tumor cells to chemotherapeutic drugs, and overcoming drug resistance\cite{15,16}.

The mechanisms of apoptosis are highly conserved in all sorts of species, including a series of processes. Apoptosis usually has three phases: initiation, effectors and execution\cite{17-19}. When external stimuli induce cell apoptosis by different pathways, such as death receptor-mediated and stress-dependent pathways, imbalance between activators and inhibitors of apoptosis occurs, thus activating caspase family and changing mitochondrial outer membrane permeability, finally resulting in cell apoptosis\cite{20}. Mitochondria are regarded as the key regulation element of cell death and the target of many proapoptotic signal pathways\cite{21,22}.

Some researches demonstrate that there are inhibitors of apoptosis proteins (IAPs) in mammalian cells, which suppress apoptosis by inhibiting procaspase activation and catalytic activity of mature caspases\cite{23,24}. IAPs, including MIHA (mammalian IAP homolog A, or called XIAP), c-IAP1, c-IAP2 and survivin, could bind directly to caspase-3, caspase-7 and caspase-9, and inhibit their activities, which are the downstream effectors during cascades of caspase family. MIHA could also suppress apoptosis induced by chemotherapeutic agents, UV-irradiation and Bax\cite{25-27}. It is suggested that cellular IAPs accumulation is one of the reasons for cancer cells to escape...
the killing effects of anti-cancer agents[29]. Smac or DIABLO gene is a proapoptotic molecule, which is released from mitochondria into the cytosol, along with cytochrome C (cyt-C) during apoptosis. As an important apoptotic modulator, Smac functions as eliminating the caspase-inhibitory properties of IAPs[30]. Some researches have found that Smac could promote apoptosis via two pathways, which are dependent on the interaction between Smac and IAPs[31]. One hydrolyzes caspase-3 protein, and the other enhances the catalytic activity of mature caspase-3. McNeish et al[32] constructed the adenoviral vector for Smac gene, and transfected it into ovarian cancer cells. They found that apoptotic cancer cells increased remarkably, and cell apoptosis mediated by Smac was not dependent on cyt-C and Bcl-2, but realized through Caspase-9. Jia et al[33] stably transfected full-length (FL) and mature (MT) Smac genes into K562 and CEM leukemic cell lines, and they concluded that both FL and MT Smac transfection could promote the sensitivity of leukemic cells to UV light, and activate cellular caspase-9 and caspase-3.

In this study, an extrinsic Smac gene was transfected into gastric cancer cells which induced its over-expression. We found that Smac overexpression could enhance the apoptosis-inducing effects of MMC, by electronic microscopy and annexin V-FITC and propidium iodide staining flow cytometry. These results are consistent with those recently reported by Guo et al[34] (2002) and Fulda et al[35] (2002), in which Smac could enhance apoptosis in leukemia and malignant neuroglioma cells induced by chemistry or immunology in vivo. Western blotting and colorimetry were sequentially used to assay the cellular caspase-3 protein expression and its activities, and it was found that stable transfection of an extrinsic Smac gene could increase the cellular activity levels of caspase-3 after treating with MMC, which accords with the functional mechanisms of Smac. These results provide a novel strategy to improve chemotherapeutic sensitivity in gastric cancer patients and reduce their side effects, thus establishing a basis for further exploring the roles of Smac gene in apoptosis regulation of gastric cancer.

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