

Original Article

Expression of caspase-3 gene in gastric adenocarcinoma cell line SGC-7901 via Ad-FasL

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Abstract: Objective: To investigate the relationship between caspase-3 expression and apoptosis in the gastric adenocarcinoma cell line SGC-7901 transfected with the adenovirus-mediated FasL gene. Methods: The human gastric adenocarcinoma cell line SGC-7901 was transfected with the adenovirus-mediated FasL gene. The expression of caspase-3 and apoptotic morphological changes were measured by flow cytometry, TUNEL, RT-PCR, and immunocytochemical assays. Result: As the results the expression of caspase-3 was low in the gastric adenocarcinoma cell line SGC-7901, but transfection of these cells with Ad-FasL enhanced the cellular expression of the caspase-3 gene and induced susceptibility to Fas/FasL-mediated apoptosis (25.4% vs. 7.2%, $P < 0.001$). Apoptosis exhibited a positive relationship with caspase-3 expression ($\gamma = 0.647$, $P < 0.01$). Conclusion: It can be inferred that the down-regulation of caspase-3 expression and resistance to Fas/FasL-mediated apoptosis in gastric adenocarcinoma cells were found to be related to the development and progression of the cells. FasL gene transfer may increase caspase-3 expression in gastric adenocarcinoma cells and enhance apoptosis in them.

Keywords: Membrane glycoproteins, caspases, gastric neoplasms/pathology, oncogenes, cell death

Introduction

Apoptosis is mediated by proteolytic enzymes known as caspases that trigger cell death by cleaving specific proteins in the cytoplasm and nucleus. Caspases exist in all cells as inactive precursors that are usually activated via cleavage by other caspases, producing a proteolytic caspase cascade. The activation process is initiated by either extracellular or intracellular death.

Signals that cause intracellular adaptor molecules to aggregate and activate procaspases. The Fas/FasL system transmits apoptotic signals from the surrounding environment into the cell. In this study, we investigated the caspase-3 expression in FasL-transfected gastric cancer cells and the relationship between caspase-3 expression and apoptosis in these cells [1, 2].

Materials and methods

Cell culture

Gastric cancer SGC-7901 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and maintained in 199 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS).

Recombinant adenoviral vector construction and transfection

The recombinant adenoviral plasmid Ad-FasL contains a cytomegalovirus (CMV) promoter, FasL CDNA (Jinmei, Shenzhen, China), and the SV40 poly (A) sequence in the E1 gene region of a replication-deficient adenovirus vector with E1-E3 deletion mutants. First, full-length FasL CDNA was cloned into the shuttle vector PAdCMV (kindly provided by Prof. Lu Daru,

Fudan University, Shanghai, China). The resultant plasmid was cotransformed into the adenoviral packaging cell line 293 together with the adenoviral packaging plasmid pJM17 (Microbix, Inc.), by using lipofectin (Gibco BRL). Individual plaques of Ad-FasL were selected; the virus was propagated, purified, and titrated as described previously [3]. As a control, Ad-LacZ was constructed with a structure similar to that of Ad-FasL. For in vitro adenoviral gene transfer, 10^5 SGC-7901 cells were incubated with viral supernatant and 8 $\mu\text{g/mL}$ polybrene (Sigma) at 37°C for 2-3 h; subsequently, the culture medium was replaced with fresh medium, and the cells were further incubated for 48 h. The infected cells were passaged in a medium containing G418 (Gibco BRL) at a final concentration of 1 mg/mL , and G418-resistant colonies were selected.

Determination of transduction efficiency

Cells were incubated with Ad-FasL at various multiplicities of infection (MOIs). After 48 h, the medium was discarded, and the cells were fixed in 10% formaldehyde for 6-8 h, treated with X-gal dye solution (1 mg/mL X-gal in 0.1 mol/L phosphate-buffered saline (PBS), 1.3 m mol/L MgCl_2 , 3 m mol/L $\text{K}_3\text{Fe}(\text{CN})_6$, and 3 mmol/L $\text{K}_4\text{Fe}(\text{CN})_6$), and incubated at 37°C for 2 h or overnight. The percentage of blue-stained cells was calculated.

Reverse transcriptase polymerase chain reaction

Reverse transcription (RT) of RNA from sample tissues followed by polymerase chain reaction (PCR) was used to detect *FasL* and caspase-3 expression. Total RNA was isolated from the infected cells by using Trizol reagent according to manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed according to the manufacturer's protocol. All primers were synthesized by Shanghai Sangon Biotechnology Co., and the sequences of the primers used are as follows.

FasL primers: sense 5'-CTGAATTCTGACTCACC-AGCTGCCATGC-3'; anti-sense 5'-TACTCGAGCT-ATTAGAGCTTATATAAGCCG-3'. Caspase primers: sense 5'-TGGCCCTGAAATACGA-AGTC-3'; anti-sense 5'-GGCAGTAGTCGACTCTGAAG-3'.

The PCR conditions for exogenous *FasL* were as described previously. In the case of cas-

pase-3, 50 μL PCR reaction mixture contained 2 mL of the RT product as template, 5 μL $10\times$ PCR buffer, 1 μL 10 mol/L dNTPs, 3 μL 25 mol/L MgCl_2 , 1.5 μL 0.1 mol/L primers, and 0.5 μL *Taq* DNA polymerase. β -actin was used as an internal control as reported previously. The PCR conditions for caspase-3 were as follows: initial denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then final extension at 72°C for 10 min. We performed 25 PCR cycles for β -actin. PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide (EB). The target bands were densitometrically analyzed using a gel imaging system and gel analyzing software (Bio-Rad), and the results were calculated as the ratio of the optical density (OD) value relative to β -actin.

Immunohistochemical staining

The expression of caspase-3 in *FasL*-transfected SGC-7901 cells was immunohistochemically examined with a commercial kit according to the manufacturer's instructions. Briefly, cells grown on coverslips were washed, fixed with 4% paraformaldehyde, and incubated with primary antibodies (1:1000) for 1 h. After washing in PBS, the cells were covered with secondary antibodies (1:1000). The stained cells were visualized by the application of diaminobenzidine (DAB) substrate chromogen solution and by hematoxylin counterstaining.

Flow cytometry assay

The expression of *FasL* and caspase-3 was determined by a flow cytometry assay. For membrane *FasL*, the infected SGC-7901 cells were trypsinized and incubated with fluorescein isothiocyanate (FITC)-*FasL* monoclonal antibodies (Jackson Immuno Research Lab) at 4°C for 1 h in the dark and then resuspended in 1% paraformaldehyde. For caspase-3, the cells were incubated with 10 mg/L anti-caspase-3 monoclonal antibodies at 4°C for 1 h in the dark, followed by incubation with goat anti-mouse FITC-IgG (1:50) and fixing in 1% paraformaldehyde. Flow cytometry was performed using a FACS Vantage flow cytometer (Becton Dickinson). A minimum of 10,000 cells were assayed in each sample. Data were analyzed using Cell Quest software (Becton Dickinson). For the apoptosis assay, the cells were stained

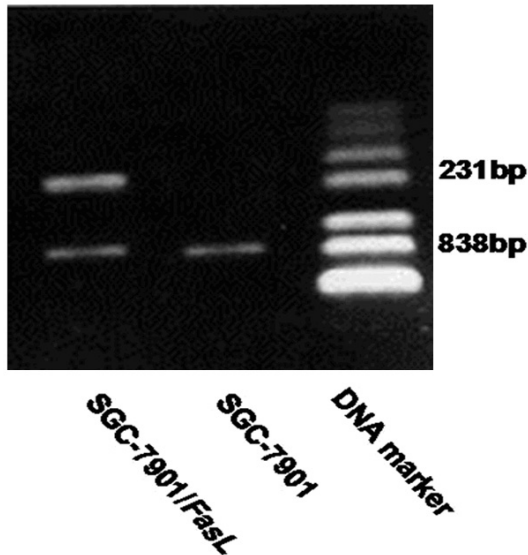


Figure 1. FasL expression in gastric cancer cells as determined by RT-PCR.

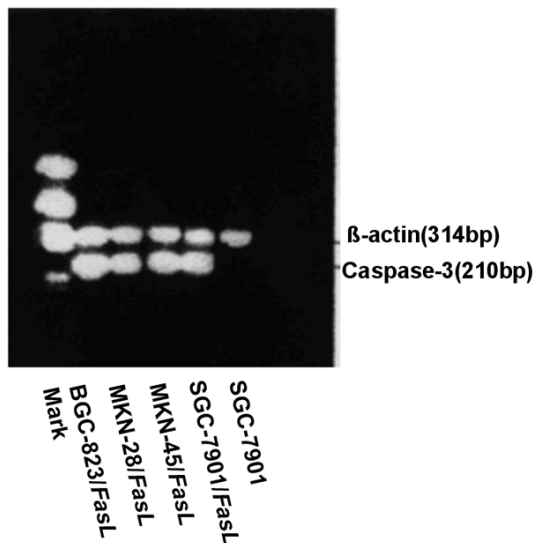


Figure 2. Caspase-3 expression in gastric cancer cells as determined by RT-PCR.

with a combination of FITC-annexin V and propidium iodide (PI).

Detection of DNA fragmentation by terminal dUTP nick end labeling assay

For the terminal DUTP nick end labeling (TUNEL) assay, we used the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's protocol with slight modifications. Briefly, gastric cancer specimens from nude tumor-bearing mice were fixed in 4% paraformaldehyde and

embedded in paraffin, and 5- μ m-thick sections were prepared. The sections were deparaffinized and rehydrated with graded alcohols. The specimens were treated with 0.02 mg/L pepsin (Sigma) for 20 min at room temperature, followed by incubation in 3% H_2O_2 to block endogenous peroxidase activity. The sections were then incubated in the dark at 37°C for 1.5 h with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TDT) and digoxigenin-labeled dUTP. They were then incubated with horseradish peroxidase (HRPase)-conjugated anti-digoxigenin antibodies in a humidified chamber at room temperature for 30 min, and the color was developed using DAB. TDT was replaced with deionized distilled water (ddH_2O) as a negative control. The apoptotic cells were identified by brown-yellow staining and the presence of combined apoptotic features including condensed and marginal nuclei, spheres of condensed chromatin, fragmented nuclei, and apoptotic bodies. The apoptotic index was defined as the ratio of apoptotic cells to the total cells counted. A minimum of 1000 cells were counted under the high-power lens of a microscope.

Statistical analysis

All values were expressed as mean \pm SD. Statistical Package for the Social Sciences (SPSS) 10.0 statistical software was used for statistical analysis. Analysis of variance (ANOVA), *t* test, and Q test were used to determine the significance of differences. A value of $P < 0.05$ was considered statistically significant.

Results

FasL expression was enhanced in FasL-transfected cells

SGC-7901 cells were infected with Ad-FasL. The FasL mRNA levels were analyzed by RT-PCR with primers specific for FasL. FasL mRNA was detected in the FasL-transfected SGC-7901 cells but not in the SGC-7901 cells, indicating that transfection leads to FasL expression (Figure 1).

Caspase-3 expression was enhanced after FasL transfection

Caspase-3 expression was detected in the Ad-FasL-transfected SGC-7901 cells but not in

FasL gene transfer may increase caspase-3 expression in gastric

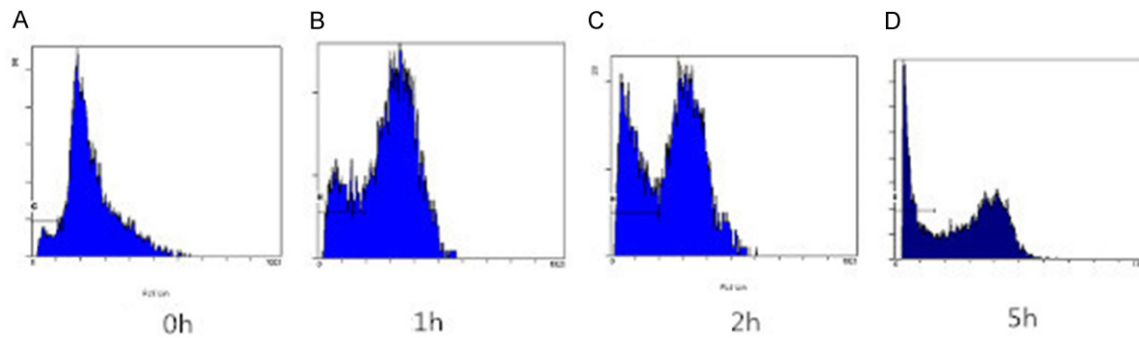


Figure 3. FasL expression in gastric cancer cells as determined by FACS.

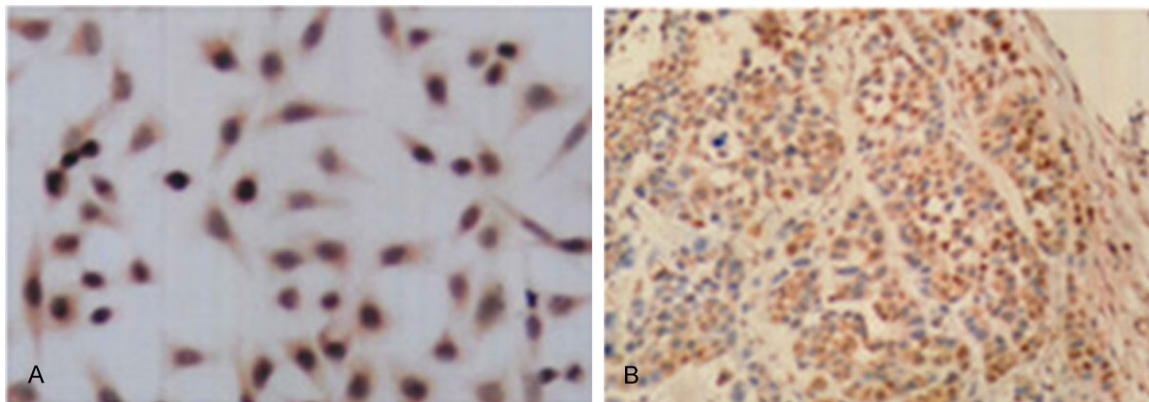


Figure 4. Immunohistochemical analysis of caspase-3 expression in FasL-transfected gastric cancer cells. A: Immunohistochemical analysis of FasL-transfected SGC-7901 cells, B: Immunohistochemical analysis of gastric cancer tissue obtained from nude mice.

the SGC-7901 cells, indicating that FasL signals activate caspase-3 expression (**Figure 2**).

Caspase-3 protein was detected in FasL-transfected cells

As shown in **Figure 3A**, no fluorescence signal of caspase-3 was detected in the SGC-7901 cells by fluorescein-activated cell sorter (FACS) analysis; however, it was detected in the FasL-transfected SGC-7901 cells (**Figure 3B-D**), indicating that caspase-3 protein was induced by FasL transfection.

Caspase-3 was mainly expressed in the cytoplasm and nucleus

No or low expression of caspase-3 was noted in the SGC-7901 and FasL-transfected SGC-7901 cells. However, caspase-3 protein was detected in both FasL-transfected SGC-7901 cells and xenotransplanted tumors induced by FasL-transfected SGC-7901 cells (**Figure 4A and 4B**).

FasL transfection increased apoptotic rate

The apoptotic rate in the FasL-transfected SGC-7901 cells (25.4%) was substantially higher than that in the SGC-7901 cells (7.2%), and the difference was significant ($P < 0.05$) (**Figure 5**).

Discussion

Caspase is an important recent discovery in the field of molecular biological research on apoptosis. Until now, at least 14 caspases that play important roles in Fas/FasL system-mediated and chemotherapeutically induced apoptosis have been identified. Caspase-3 is the most important effector proteinase, and it is also the most critical effector enzyme in the caspase cascade because when activated, it can cleave a large number of substrates [4-8] and induce apoptosis [9].

It has been shown that the disturbance of apoptosis control is also an important reason for

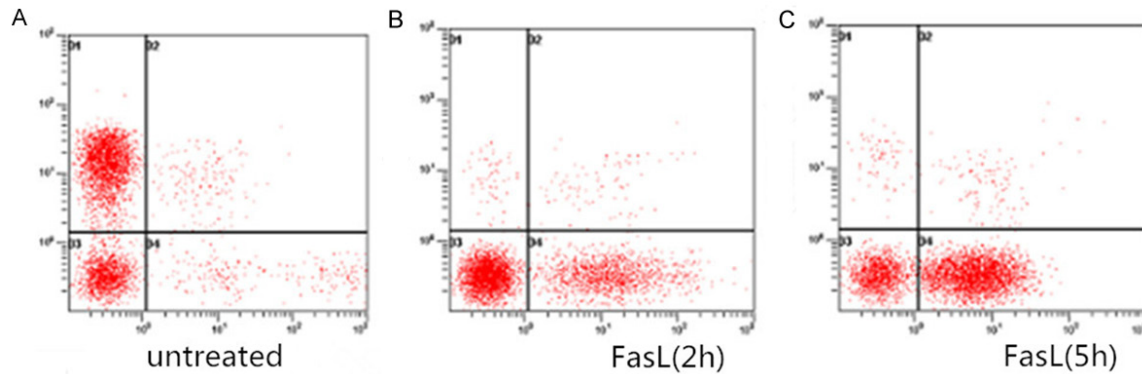


Figure 5. Apoptosis detected using FACS. A: Control, B: Early apoptosis, C: Late apoptosis.

excessive tumor cell proliferation. Caspase-3 is expressed in 87.5% of normal cells and in cells of paraneoplastic tissues [10]; thus, it is considered to probably be involved in the regulation of normal cell development and conversion of apoptosis. The failure of caspase-3 expression is very important in the process of tumorigenesis and possesses tissue heterogeneity [3, 11].

The present study demonstrated that caspase-3 expression in SGC-7901 gastric carcinoma cells was absent or low, which was consistent with the results in a previous report [3]. However, a significant increase in caspase-3 expression was noted in gastric carcinoma cells transfected with the *FasL* gene, indicating that *FasL* gene transfection induces apoptosis and that apoptosis signal transduction produced after the cells are transfected with the *FasL* gene is the effect of caspase-3. The Fas protein itself is involved in apoptosis signal transduction on the surface of the cell membrane. Fas binds to transfected *FasL* and forms a complex that subsequently conducts signals to caspase-8 and caspase-10. These in turn activate caspase-1 and caspase-4 and ultimately conduct signals to caspase-3 that enzymatically cleaves the substrate, thereby changing the shape of the cells and leading to DNA fragmentation [2, 3]. Recent studies have presented a clearer understanding of the apoptosis signaling pathway. There are 2 important apoptosis pathways in the cell: the mitochondrion-dependent Apaf-1/caspase-9 pathway and the mitochondrion-independent FADD/caspase-8 pathway. Different apoptosis stimuli induce different signaling pathways. FasL and

TNF- α mainly induce the FADD/caspase-8 pathway, whereas growth factor depletion and intracellular stress often activate the Apaf-1/caspase-9 pathway. However, there is crosstalk between the 2 pathways [11-13].

The present study demonstrated the mechanisms of *FasL*-induced apoptosis in gastric carcinoma cells. Apoptosis signals produced by the binding of Fas/FasL were transduced into the membrane from the external environment and reached the caspases via mediators that are involved in signal transduction in the cytoplasm. The activation of these proteins would ultimately lead to the apoptosis of gastric carcinoma cells. There have been no reports regarding this in our country. There are several other proteins involved in this pathway. Further investigation regarding the interaction among these proteins, the caspase-mediated signaling cascade, the complexity of its regulation, and other pathways that mediate apoptosis is required.

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Disclosure of conflict of interest

None.

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