

Expression of Bcl-2 inhibited Fas-mediated apoptosis in human hepatocellular carcinoma BEL-7404 cells

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ABSTRACT

Apoptosis plays an important role in embryonic development, tissue remodeling, immune regulation and tumor regression. Two groups of molecules (Bcl-2 family and "Death factor" family) are involved in regulating apoptosis. In order to know about the effect of Bcl-2 on apoptosis induced by Fas, a typical member of "Death factor" family, the transfection experiments with expression vectors pcDNA3-fl and pcDNA3-bcl-2 were performed in BEL-7404 cells, a human hepatocellular carcinoma cell line which expresses endogenous Fas, but not FasL and Bcl-2. The data showed that the expression of FasL in pcDNA3-fl transfected hepatoma cells obviously induced the apoptosis of the cells. However, the overexpression of Bcl-2 in pcDNA3-bcl-2 transfected 7404/b-16 cells counteracted pcDNA3-fl transient transfection mediated apoptosis. Further study by co-transfection experiments indicated that Bid but not Bax (both were pro-apoptotic proteins of Bcl-2 family) blocked the inhibitory effect of Bcl-2 on Fas-mediated apoptosis. These results suggested that Fas-mediated apoptosis in human hepatoma cells is possibly regulated by Bcl-2 family proteins via mitochondria pathway.

Key words: *Apoptosis, FasL, Bcl-2, Bid, Bax, human hepatocellular carcinoma cells.*

INTRODUCTION

Apoptosis, or programmed cell death, is an innate mechanism by which the organism eliminates unwanted cells. It plays an important role in embryonic development, tissue

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Bcl-2 inhibited Fas-mediated apoptosis

remodelling, immune regulation and tumor regression. Cells undergoing apoptosis show a sequence of morphological changes including membrane blebbing, cellular shrinkage and condensation of chromatin. In addition, activated DNA endonuclease in apoptotic cells can cleave genomic DNA into internucleosomal fragments, giving “DNA ladder” in agarose gel.

Higher organisms have developed several mechanisms to eliminate cells by apoptosis. An important mechanism to regulate apoptosis is the signaling pathway mediated by “Death factors” including TNFR1, Fas and TRAILR1 and their cognate ligand (TNF α , Fas Ligand and TRAIL) [1]. Fas (also called APO-1, CD95), the first identified member of “Death receptors”, is a 45 kDa type I glycoprotein expressed on cell surface. Crosslinking of Fas by its ligand FasL binding leads to conformational changes of Fas, which results in formation of DISC (death induced signaling complex) followed by activation of caspase-8. Then activated caspase-8 activates itself and other caspases. These activated caspases induce apoptosis by cleaving their substrates. It is found that Fas is expressed in many tumor cell lines and may mediate apoptosis induced by FasL or agonistic anti-Fas antibody.

Members of Bcl-2 family are also involved in regulation of apoptosis. Bcl-2 is an inhibitor of apoptosis whose expression can inhibit apoptosis induced by many signals such as ultra-violet radiation, anti-tumor drugs and deprivation of growth factors[2]. Effects of Bcl-2 expression on Fas-mediated apoptosis are different in several lymphoma cell lines[3],[4]. Scaffidi C. et al. classified them into two kinds of cells (Type I and Type II cells)[5]. Ectoexpression of Bcl-2 inhibited Fas-mediated apoptosis in Type II but not Type I cells. However, there are not many studies on this issue in solid tumor cell lines.

The aim of this study is to examine the effect of Bcl-2 on Fas-mediated apoptosis in human hepatoma cells. In our study, we used a human hepatocellular carcinoma cell line BEL-7404 which expressed Fas antigen but not FasL and Bcl-2. By transient transfection with expression plasmid pcDNA3- fl, we constructed a model of Fas-mediated apoptosis. We also found that expression of Bcl-2 in BEL-7404 cells was able to inhibit Fas-mediated apoptosis. Further study suggested that overexpression of Bid but not Bax blocked the inhibitory effect of Bcl-2 on Fas-mediated apoptosis.

MATERIALS AND METHODS

Construction of expression plasmid

The 900 bp fas ligand cDNA fragment containing intact ORF was digested by XbaI from pBX-fasl vector (gift of Professor Nagata S), and inserted into the XbaI site of pcDNA3.0 (Invitrogen) to generate an expression plasmid pcDNA3- fl. The correct orientation was confirmed by SacI digestion. 1.6 kb Bcl-2 cDNA (gift of Doctor Yin DL) and 600 bp bax cDNA (gift of Professor Korsmeyer SJ) fragment was inserted into EcoRI site of pcDNA3.0 to generate expression vectors pcDNA3- bcl-2 and pcDNA3- bax respectively. bid cDNA was amplified by RT-PCR from human normal liver and inserted into EcoRI and BamHI sites of pcDNA3.0 to generate pcDNA3- bid.

Cell culture and establishment of cell lines

Human hepatocellular carcinoma cell line BEL-7404 (Shanghai Cell Bank, Chinese Academy of Sciences) was maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10 % newborn calf serum (Evergreen, Hangzhou, P.R.China), 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were subcultured at 70-80 % confluence and culture medium was changed every 2 or 3 d to maintain good growth condition. To establish BEL-7404 cells with highly expressed Bcl-2 protein, BEL-7404 cells were transfected with pcDNA3-bcl-2 by calcium phosphate precipitate method[6]. The cells were cultured for 3 weeks in selection medium (400 μ g/ml Geneticin, SIGMA) to select stable transfectants. BEL-7404 cells were also transfected with pcDNA3.0 to obtain control cells. Individual clones were picked up at random and amplified. The expression of Bcl-2 protein was determined by western blot analysis.

Transient transfection (by electroporation method)

The apparatus used in the experiments was Gene PulserTM (BIORAD). Cells growing to exponential stage were pooled, rinsed twice with D-Hanks saline solution and suspended in D-Hanks at $0.5-1.0 \times 10^7$ cells/ml. Plasmid was added to final concentration 50 μ g/ml and incubated on ice for 10 min. Then cells were electroporated one time at 600 V, 25 μ F and 100 Ω and incubated on ice for another 10 min. Appropriate amount of cells were inoculated into DMEM and grew at 37 °C, 5 % CO₂. For co-transfection, the ratio of two plasmids was 1:1.

RT-PCR analysis

Total RNA isolation and RT-PCR was based on Zhao's method[7]. In brief, 2 μ g total RNA extracted from cells was reverse transcribed using 200 ng 6-mer random primers in a volume of 20 μ l for 1 h at 37 °C water bath. Then 1 μ l reverse transcription products was used for PCR. In a total of 25 μ l reactive mixture, 10 pmol forward primer and 10 pmol reverse primer, 1 \times reaction buffer and 1 unit Taq polymerase were mixed. The reaction was run for 30 cycles, each consisted of denaturation at 94°C for 30 S, annealing at 55°C for 30 S, and extension at 72 °C for 60 S and Final extension prolonged for 10 min at 72°C. The primer for fas sequence was: forward, 5' -GAT TGC TCA ACA ACC ATG-3'; reverse, 5' -TTT CAC TCT AGA CCA AGC TTT G-3' (1029bp). The primer for fasl sequence was: forward, 5' -CGA GAG TCT ACC AGC CAG ATG C-3'; reverse, 5' -TCC CAA AGT GCT TCT CTT AGA GC-3' (450bp). The primer for β -actin sequence was: forward, 5' -ACA CTG TGC CCA TCT ACG AGG GG-3'; reverse: 5' -ATG ATG GAG TTG AAG GTA GTT TCG TGG AT-3' (360bp). The primer for bid sequence was: forward, 5' -ATG GAC TGT GAG GTC AAC-3'; reverse, 5' -AGT CCA TCC CAT TTC TGG-3' (587bp). The primer for bax sequence was: forward, 5' -CAC CAG CTC TGA GCA GAT G-3'; reverse, 5' -GCG AGG CGG TGA GCA CTC C-3' (516bp).

Western blot analysis

Protein extraction and western blot was based on Oltvai's method[8]. In brief, cells were washed twice with D-Hanks and lysed in following buffer with freshly added protease inhibitors (142.5 mM KCl, 5 mM MgCl₂, 10 mM HEPES [pH 7.2], 1 mM EGTA, 0.2 % NP-40, and 0.2 mM PMSF) for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 12000 g for 10 min. Protein concentration was determined by Bradford's method. For western blot, 10 μ g of total protein was subjected to 12 % SDS-PAGE and electrotransferred at 4°C for 2 h onto nitrocellulose membrane. After blocking at 37 °C for 2 h with TBST (Tris-buffered saline containing 0.5 % Tween-20) containing 5 % nonfat milk, membrane was incubated with anti-Bcl-2 antibody (1:100, Sinoamerica Biotech) at 37 °C for 2 h. Membrane was subsequently incubated with HRP-conjugated second antibody (1:3000, Vector) at room temperature for 2 h. Finally, membrane was developed with diazobenzidine (Amersico) and H₂O₂. Membrane was washed with TBST for 4

Bcl-2 inhibited Fas-mediated apoptosis

times between each step.

PI staining and flow cytometric analysis

Cells were washed twice with D-Hanks and fixed in sodium citrate at 4 °C for 30 min. 5 ml staining solution (propidium iodide 50 μ g/ml, RNase 10 μ g/ml) was added to 10^6 cells for 15 min at 4 °C in dark. Then cells were examined in fluorescence microscope or analysed by flow cytometric with FACS calibur (Becton Dickinson) and analysis software CELLQest (Becton Dickinson).

DNA fragmentation analysis

Isolation of low molecular weight DNA was based on Lowe' s method[9]. In brief, adherent and nonadherent cells were pooled, washed with D-Hanks, and resuspended in solution I (0.15 M NaCl, 10 m M Tris-Cl [pH7.4], 2 m M MgCl₂, 1 m M DTT, and 0.5 % [v/v] NP-40) for 30 min on ice. Nuclei were isolated by centrifugation at 5000 g for 5 min, resuspended in solution II (0.35 M NaCl, 10 m M Tris-Cl [pH7.4] 2 m M MgCl₂ and 1 m M DTT) for 20 min on ice. The nuclei were then removed by centrifugation at 5,000 g for 5 min, and the supernatant was extracted with phenol and chloroform. The low molecular weight DNA was recovered by ethanol precipitation. Samples were resuspended in TE buffer and treated with RNAase A for 30 min prior to electrophorsis on 1.4 % agarose gels.

RESULTS

Transfection with pcDNA3-fl induced apoptosis in BEL-7404 cells

Using primers specific for fas and fasl sequences shown in materials and methods, we have detected the expression of fas mRNA but not fasl mRNA in human hepatocellular carcinoma BEL-7404 cells by RT-PCR (Fig 1A). There was a predicted 1029bp fas fragment in BEL-7404 cells (Lane 1), however fasl product was undetectable (Lane 2). In later experiments, BEL-7404 cells were transiently transfected with plasmid pcDNA3 or pcDNA3- fl by electroporation. Total RNA of cells transfected with pcDNA3 or pcDNA3- fl was extracted and used for RT-PCR analysis. As shown in Fig 1B, a specific 450 bp band was only seen in the cells transfected with pcDNA3- fl (Lane 4). This suggested

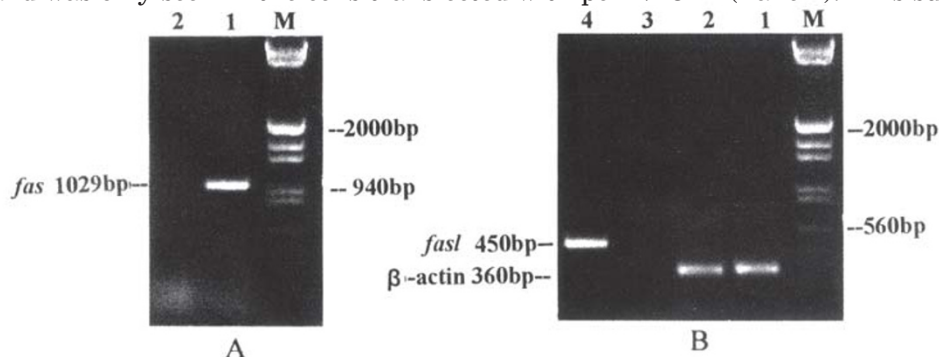


Fig 1. A, Detection of fas and fasl mRNA expression by RT-PCR in BEL-7404 cells. Lane 1, amplification of fas. Lane 2, amplification of fasl. **B,** Detection of fasl mRNA expression by RT-PCR in BEL-7404 cells transfected with pcDNA3- fl. Lane 1, amplification of β -actin in cells transfected with vector pcDNA3. Lane 2, amplification of β -actin in cells transfected with vector pcDNA3- fl. Lane 3, amplification of fasl in cells transfected with control vector pcDNA3. Lane 4, amplification of fasl in cells transfected with vector pcDNA3- fl.

that fasl expressed in BEL-7404 cells after being transfected with pcDNA3- fl.

As shown in Fig 2, no apparent change of cell morphology occurred in the cells transfected with control vector pcDNA3 (A), but most of the cells transfected with pcDNA3- fl became round and detached from the surface of dishes and underwent apoptosis (B). By PI staining, we also found that the nuclei of cells transfected with pcDNA3- fl were smaller than control group and fragmented into small pieces, which were typical characteristics of apoptosis (compare C with D). In order to study the time effect of transfection with pcDNA3- fl on cell death, cells at different time after transfection were recovered to analyse by flow cytometry. “Sub-G1” cells, which had less DNA than normal cells in flow cytometric analysis, were used to represent apoptotic cells. Results shown in Fig 3 indicated that apoptotic cells occurred 12 h (11.9 %) after transfection. Cell death reached to a maximum 24 h (26.2 %) and decreased 48 h after transfection (15.7 %). In addition, typical “DNA ladder” appeared in the DNA extracted from cells 12 h after transfection.

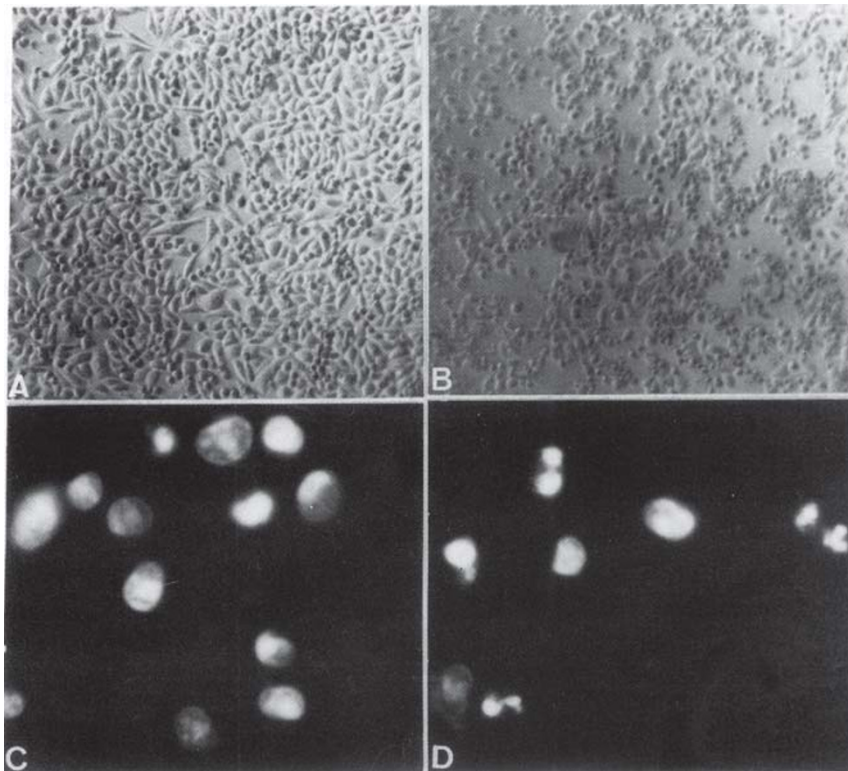


Fig 2. Morphology comparison of BEL-7404 cells and its nuclei at 24 h after transfection with pcDNA3 or pcDNA3- fl. A) cells transfected with pcDNA3. B) cells transfected with pcDNA3- fl. C) nuclear morphology of cells transfected with pcDNA3. D) nuclear morphology of cells transfected with pcDNA3- fl.

Bcl-2 inhibited Fas-mediated apoptosis

The DNA fragmentation was obviously more serious 24 and 48 h after transfection with pcDNA3- fl as compared with control (Fig 4). Taken together, all above data suggested that transfection with pcDNA3- fl induced apoptosis in BEL7404 cells.

Construction of BEL-7404 cell lines with highly expressed Bcl-2 protein

Recombinant expression plasmid pcDNA3- bcl-2 was transfected into BEL-7404 cells using calcium phosphate precipitate method. Vector pcDNA3 was used as control plasmid for transfection. Through G418 selection, most cells died in 10-14 d, but some cells later formed G-418-resistant cell foci. By random picking, cell amplification and identification by western blot, two cell clones expressing bcl-2 protein were obtained, designated as 7404/b-13 and 7404/b-16 respectively. We also obtained one empty vector transfected clone, named as 7404/neo. Expression of Bcl-2 protein detected by western blot was shown in Fig 5. A specific 26 kDa band was only seen in cell lysates from 7404/b-13 and 7404/b-16 cells (Lane 3 and 4). It indicated that 26 kDa Bcl-2 was expressed in 7404/b-13 and 7404/b-16 cells but not in parent BEL-7404 and 7404/neo cells (Lane 1 and 2).

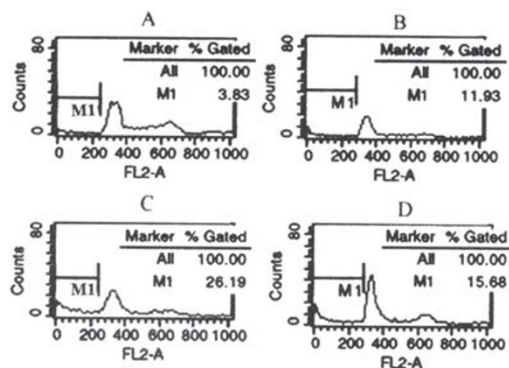


Fig 3. Percentage of “sub-G1cells” in BEL-7404 cells at different time after transfection with pcDNA3- fl. The bar ‘M1’ was referred “tosub-G1” cells. A, 0 h after transfection. B, 12 h after transfection. C, 24 h after transfection. D, 48 h after transfection.

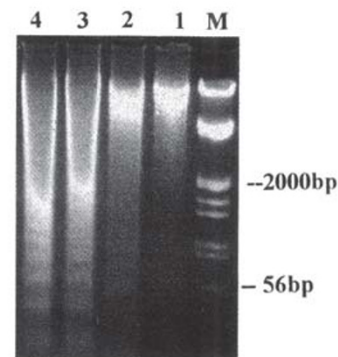


Fig 4. Analysis of low molecular weight DNA from BEL-7404 cells transfected with pcDNA3- fl. Lane 1, DNA of control cells. Lane 2, DNA of cells 12 h after transfection. Lane 3, DNA of cells 24 h after transfection. Lane 4, DNA of cells 48 h after transfection.

Bcl-2 protein inhibited Fas-induced apoptosis in BEL-7404 cells

In order to examine the effect of Bcl-2-expression on Fas-mediated apoptosis, BEL-7404, 7404/neo and 7404/b-16 cells were transiently transfected with pcDNA3- fl or pcDNA3 respectively. Cells were pooled for flow cytometric analysis 24 h after transfection. As shown in Fig 6, the percentage of apoptotic cells in BEL-7404 and 7404/neo cells trans-

fectured with pcDNA3- fl (25.4 % and 26.1 %, respectively) was significantly higher than that transfected with pcDNA3 (7.1 % and 4.9 %, respectively). However, there was no apparent change on the percentage of apoptotic cells between 7404/b-16 cells transfected with pcDNA3 (13.1 %) or pcDNA3- fl (14.3 %). These results suggested that expression of Bcl-2 protein was able to inhibit Fas-mediated apoptosis in BEL-7404 cells.

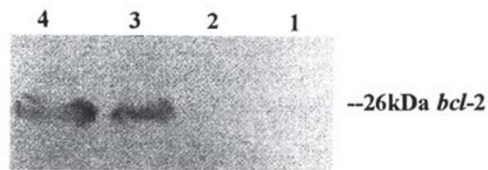


Fig 5. Detection of 26 kDa Bcl-2 protein by western blot in BEL-7404 cells after being transfected with pcDNA3- bcl-2. Lane 1, BEL-7404 cells. Lane 2, 7404/neo cells. Lane 3, 7404/b-13 cells. Lane 4, 7404/b-16 cells.

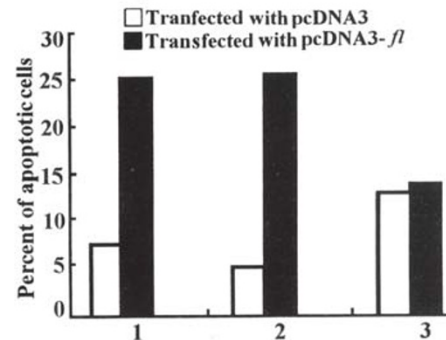


Fig 6. Effect of Bcl-2 on Fas-induced apoptosis in BEL-7404 cells. BEL-7404, 7404/neo and 7404/b-16 cells were transfected with pcDNA3 or pcDNA3-fl. 24 h after transfection, cells were recovered and analyzed by flow cytometry. 1, BEL-7404 cells. 2, 7404/neo cells. 3, 7404/b-16 cells.

Overexpression of Bid but not Bax counteracted the inhibitory effect of Bcl-2-expression on Fas-mediated apoptosis in 7404/b-16 cells

Bid and Bax protein are both pro-apoptotic members of Bcl-2 family. We wondered if they were involved in regulating Fas-mediated apoptosis in human hepatoma cells. By co-transfection experiments in 7404/b-16 cells, we examined the impact of overexpression of bid or bax on the inhibitory effect of Bcl-2 on Fas-mediated apoptosis. Fig 7A showed the percentage of apoptotic cells in 7404/b-16 cells 24 h after co-transfection. It was found that the apoptotic percentage of cells co-transfected with pcDNA3- fl plus pcDNA3- bid (Lane 3, 31.0 %) was much higher than that of cells co-transfected with double amount of pcDNA3 (Lane 1, 13.2 %) or pcDNA3 plus pcDNA3- fl (Lane 2, 14.1 %). However, there was no apparent difference on the apoptotic percentage of cells between control cells and cells co-transfected with pcDNA3- fl plus pcDNA3- bax (Lane 4, 14.5 %). bid and bax mRNA were detected in BEL-7404 cells by 35 cycles-RT-PCR analysis (Data not shown). Semi-quantitative RT-PCR analysis (25 cycles) proved overexpression of bid or bax in 7404/b-16 cells co-transfected with pcDNA3- fl plus pcDNA3- bid or pcDNA3- fl plus pcDNA3- bax (Fig 7B). These results indicated that overexpression of Bid but not Bax counteracted the inhibitory effect of Bcl-2 on Fas-mediated apoptosis in human hepatocellular carcinoma cells.

Bcl-2 inhibited Fas-mediated apoptosis

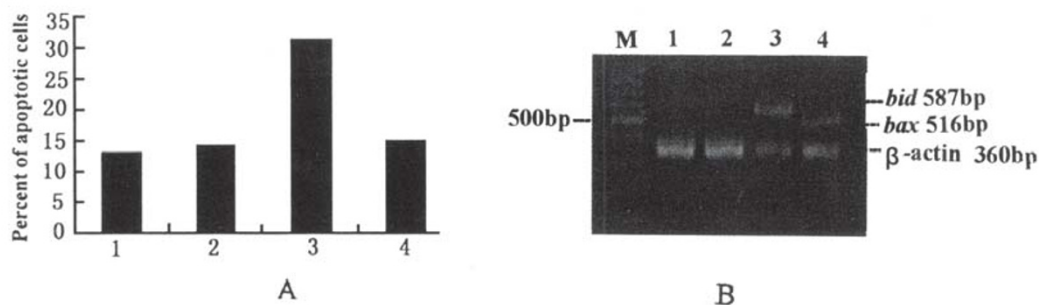


Fig 7. Overexpression of Bid but not Bax counteracted the inhibitory effect of Bcl-2 on Fas-mediated apoptosis in 7404/b-16 cells. A) 7404/b-16 cells were recovered and analyzed by flow cytometry 24 h after co-transfection with: 1, double amount of pcDNA3. 2, pcDNA3 plus pcDNA3- fl. 3, pcDNA3- fl plus pcDNA3- bid. 4, pcDNA3- fl plus pcDNA3- bax. B) RT-PCR (25 cycles for PCR) detection of *bid* and *bax* mRNA in cellular total RNA extracted from 7404/b-16 cells 24 h after co-transfection with: 1, double amount of pcDNA3. 2, pcDNA3 plus pcDNA3- fl. 3, pcDNA3- fl plus pcDNA3- bid. 4, pcDNA3- fl plus pcDNA3- bax. M was 1 Kb ladder marker.

DISCUSSION

Not only cell proliferation but also programmed cell death (or apoptosis) is involved in the initiation and development of cancer. The elucidation of apoptotic mechanisms will make us to know more about carcinogenesis. The aim of this study is to investigate the effect of Bcl-2 on Fas-mediated apoptosis in human hepatoma cells. We used in this study a human hepatocellular carcinoma cell line BEL-7404, which expressed Fas antigen but not FasL and Bcl-2. It was found that transfection with pcDNA3- fl was able to induce cell death in these cells. Fragmentation of cellular nuclei (Fig 2) and formation of typical "DNA ladder" (Fig 4) as well as fasl expression by RT-PCR analysis in these human hepatoma cells transfected with pcDNA3- fl all suggested that true apoptosis was induced by the binding of FasL and Fas. Flow cytometry analysis indicated that percentage of apoptotic cells reached a maximum 24 h after transfection (Fig 3). This time point was selected as standard time in later experiments.

Bcl-2 is the mammalian homologue of Ced-9 protein, an apoptosis inhibitor in *C. elegans*. Its expression can protect cells from apoptosis induced by many signals. Since the expression of Bcl-2 protein was not detected in BEL-7404 cells, we transfected these cells with pcDNA3-bcl-2 and obtained a 7404/b-16 cell line with overexpressed Bcl-2 protein. Apparent apoptosis was induced in BEL-7404 and 7404/neo control cells, but not in 7404/b-16 cells after being transfected with pcDNA3- fl (Fig 6). Although percentage of apoptotic cells in 7404/b-16 cells transfected with control plasmid pcDNA3 (13.1 %) was a little higher than that in 7404 or 7404/neo control cells (7.1 % or 4.9 %) due to some unknown reasons, these results suggested that the expression of Bcl-2 could inhibit Fas-

mediated apoptosis in BEL-7404 cells. To date, the mechanism by which Bcl-2 protein inhibits apoptosis is still elusive. Expression of bcl-2 inhibits activation of caspase-8 and caspase-3, and this activation is necessary for Fas-mediated apoptosis[5]. Bcl-2 protein also regulates release of cytochrome c via mitochondria pathway[10].

Luo et al. and Li et al. both found that Bid, a member of Bcl-2 family, might be an intermediate link between Bcl-2 and Fas signaling pathway[11],[12]. Their study suggested that Bid can be cleaved into two parts by active caspase-8 in Fas-mediated apoptosis. The C-terminus of Bid containing BH3 domain has stronger capacity to induce release of cytochrome c, which can, in turn, activated caspase-3. To examine the effect of Bid on Fas-mediated apoptosis in human hepatoma cells, we cloned open reading frame of bid gene and constructed expression plasmid pcDNA3- bid. Transfection with pcDNA3- bid alone did not induce apoptosis in BEL-7404 cells (Data not shown). However, co-transfection with pcDNA3- bid and pcDNA3- fl induced apoptosis in 7404/b-16 cells (Fig 7), which expressed high level of Bcl-2 protein and was resistant to Fas-mediated apoptosis (Fig 6). Moreover we have also shown that overexpression of Bax did not affect Fas-mediated apoptosis (Fig 7), although it has been shown to be able to accelerate apoptosis induced by the deprivation of growth factors[7]. These suggested that overexpression of Bid but not Bax counteracted the inhibitory effect of Bcl-2 on Fas-mediated apoptosis. Bid and Bax both are pro-apoptotic proteins, Bid has only BH3 (Bcl-2 homologue 3) domain but Bax has all of four BH domains[13]. BH3 domain of Bid or Bax protein forms heterodimers with BH1 and BH2 domains of Bcl-2 protein[10]. C-terminus of Bid cleaved by caspases has stronger Bcl-2-binding capacity compared with full length Bid protein [12], but it has not been found that Bax protein can be cleaved by caspases. This may be the cause that overexpression of Bid but not Bax counteracted the inhibitory effect of Bcl-2 on Fas-mediated apoptosis. In addition, liver injury is not seen after injection with anti-Fas antibody in bid-deficient mice, also suggesting that Bid is essential to Fas-induced apoptosis[14].

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Bcl-2 inhibited Fas-mediated apoptosis

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