

Materials and Methods

Reagents

The plasmid Pgenesil-1 was purchased from Genesil Biotechnology (Wuhan, China). Antibody against Bcl-2 (sc-509) was purchased from Santa Cruz (Santa Cruz, USA). RPMI 1640 and fetal calf serum were purchased from Gibco (Gaithersburg, USA). Ara-C was purchased from Suzhou Unite Pharmaceutical Corp. (Suzhou, China). In our lab, one effective siRNA sequence targeting against the region at 195–215 nt (GenBank accession No. M13995) of *Bcl-2* mRNA has been identified by the synthesis of transcription *in vitro*. The Bcl-2 shRNA expression plasmid and a negative control hairpin RNA encoded a hairpin structure has been constructed and preserved in our lab [10].

Cell culture and transfection with shRNA expression vector

The Raji cell line was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum at 37°C under 5% CO₂ in a humidified incubator. Raji cells in the exponential phase of growth were grown for 24 h, then transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocols. The ratio of Lipofectamine 2000 to plasmid was 1 µl/µg. In addition to medium control, cells were transfected with negative control shRNA. At 48 h of transfection, silencing of *Bcl-2* gene was examined. At 4 h of transfection, Ara-C was added with final concentration of 10 µg/ml.

Assay of *Bcl-2* mRNA by RT-PCR

Total RNA was isolated by using Trizol total RNA isolation system (Invitrogen). One microgram of total RNA was reverse transcribed into first-strand cDNA by using cDNA synthesis kit (Promega, Madison, USA). PCR was carried out with a final volume of 50 µl for each reaction containing specific primers synthesized by Sangon (Shanghai, China) targeting at human *Bcl-2* gene (forward, 5'-CGACGACTTCTCCCGCCGCTACCG C-3'; reverse, 5'- CCGCATGCTGGGGCCGTACAG TTCC-3') and human glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*; forward, 5'- GTCA GTGG TGGACCTGACCT -3'; reverse, 5'- TGAGGAGGGG AGATTCAGTG-3'). *GAPDH* was used as an internal control. After 95°C for 3 min, the experimental reaction

consisting of 30 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s, and the PCR products were analyzed by gel electrophoresis. The relative expression of *Bcl-2* mRNA was quantitatively measured using densitometry analysis by gene expression analysis software.

Quantification of Bcl-2 protein using immunofluorescence by flow cytometry

Transfected cells with shRNA and untreated Raji cells were collected by centrifugation (2000 g) for 5 min. The cells were washed twice and fixed in 4% formaldehyde (Sigma, St Louis, USA) for 30 min on ice, followed by permeabilization with 0.1% Triton X-100 in phosphate-buffered saline containing 1% albumin for 10 min at 4°C. Cells were then washed in cold phosphate-buffered saline (PBS) before being added to 10 µl of mouse anti-human Bcl-2 monoclonal antibody (1:100) for 30 min at room temperature in the dark. After washing twice with PBS, 10 µl of Cy3-conjugated goat anti-mouse second antibody IgG was added. The secondary antibody (monoclonal antibody; Zhongshan Bio Corp., Zhongshan, China) was also used at a 1:100 dilution. After washing twice with PBS, the cells were examined by flow cytometry (BD, USA) and the percentage of cells with positive staining for Bcl-2 protein was determined.

Assay of cell viability

To determine whether Bcl-2 shRNA could enhance sensitization of Raji cells to Ara-C, the effects of Ara-C on Raji cells were estimated after Bcl-2 shRNA transfection by MTT assay [11]. The Raji cells were plated in 96-well plates at a density of 1×10^5 cells/well in 200 µl RPMI 1640 and with 10% fetal calf serum. Four hours after transfection, Ara-C was used at 10 µg/ml. At 24, 48, and 72 h, 20 µl of MTT solution (5 mg/ml; Sigma) was added. After 4 h of incubation, the samples were centrifuged at 2000 g for 5 min. After removal of the supernatant, 200 µl of dimethyl sulfoxide (Sigma) was added to dissolve the crystals. Cell viability was calculated from the absorbance at 490 nm. The inhibition rates of cell growth were calculated according to the formula: inhibition rate (%) = $(1 - \text{mean absorbance of treated group} / \text{mean absorbance of untreated group}) \times 100\%$.

Assays of cell apoptosis

The transfected Raji cells were harvested after treated with Bcl-2 shRNA, followed by Ara-C, and fixed in methanol. Morphology was determined with Giemsa staining. The flow cytometry evaluation of the cell-cycle status and apoptosis was performed. Briefly, cells were

centrifuged, washed in PBS, and fixed in 70% ethanol. The tubes containing the cell pellets were stored at 20°C for at least 24 h. After that, the cells were centrifuged at 800 g for 15 min, and the supernatant was discarded to remove ethanol completely. The pellets were resuspended in 0.5 ml PBS and incubated with 20 µg/ml RNase A (Sigma) for 30 min at 37°C, then stained with propidium iodide solution (20 µg/ml; Sigma) for 30 min. The DNA content was analyzed using a FACScan flow cytometry. Cells with DNA content below G₁ phase were regarded as apoptotic cells. The percentage of cells in the apoptotic sub-G₁ was analyzed by Multicycle software.

Statistical analysis

Results are shown as the mean ± SD. Statistical comparisons were made by ANOVA. A value of $P < 0.05$ was considered to be statistically significant. All statistical analysis was carried out with SPSS 11.5.

Results

Inhibition of *Bcl-2* mRNA expression using shRNA expression vectors

The *Bcl-2* and *GAPDH* genes are 318 and 400 bp, respectively. The gene expression ratio of *Bcl-2*/*GAPDH* was calculated as the level of *Bcl-2* mRNA expression by the Bio-Rad Gel Doc1000 using Molecular Analyst software (Bio-Rad, Richmond, USA). RT-PCR experiments were run for three times and a representative result is shown in **Fig. 1**. As shown in **Fig. 1**, after 48 h of transient transfection, mRNA expression ratio (*Bcl-2*/*GAPDH*) was 0.73 ± 0.01 in the cells treated with Bcl-2 shRNA vector. In the cells treated with the negative control shRNA and pgenesil-1 empty vector, mRNA expression ratio (*Bcl-2*/*GAPDH*) was 0.98 ± 0.03 and 0.98 ± 0.08 , respectively. Compared with the control, *Bcl-2* mRNA expression was significantly reduced ($P < 0.05$), whereas the shRNA control and pgenesil-1 empty vector had no effect on the expression of *Bcl-2* mRNA.

Effect of Bcl-2 shRNA on Bcl-2 protein levels

To determine the effect of Bcl-2 shRNA on Bcl-2 protein levels, indirect immunofluorescence staining was performed. By flow cytometry assay, levels of Bcl-2 protein were (99.0 ± 0.7)% in untransfected Raji cells; levels of Bcl-2 protein were (74.7 ± 1.7)% at 48 h after transient transfection with Bcl-2 shRNA. Expression of Bcl-2 significantly decreased ($P < 0.05$) in the cells treated with Bcl-2 shRNA when compared with either

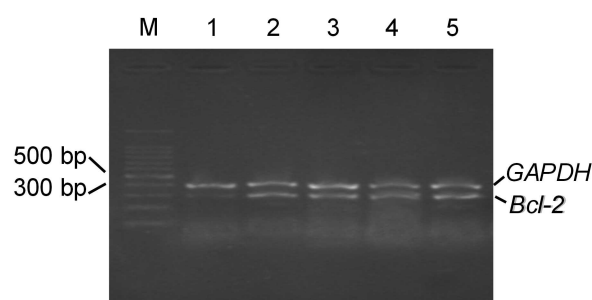


Fig. 1 The expression of *Bcl-2* mRNA after transfection with Bcl-2 shRNA at 48 h in Raji cells by RT-PCR. The expected size of the product of *Bcl-2* mRNA is 318 bp. GAPDH, the internal control is 400 bp. DNA marker is 100-bp standard DNA ladder. Results are from a representative experiment. Lanes 1 and 2 are from Bcl-2 shRNA and negative shRNA groups; Lanes 3, 4, and 5 are plasmid, Lipofectamine 2000, and blank groups, respectively.

negative control shRNA vector group (96.7 ± 2.0)% or pgenesil-1 empty vector group (98.6 ± 1.0)%. There was no difference in Bcl-2 levels among negative control shRNA group, pgenesil-1 empty vector, and untransfected cells ($P > 0.05$).

The effects of Ara-C on Raji cells transfected with Bcl-2 shRNA

To investigate whether Bcl-2 shRNA has the potential to sensitize Raji cells to Ara-C, a combination treatment with Bcl-2 shRNA and Ara-C was applied. After 4 h of transfection, the cells were exposed to Ara-C. A summary of experiments measuring cell death at 24, 48, and 72 h is shown in **Table 1**. Compared with treatments with either control shRNA plus Ara-C or Ara-C alone, the viability of the cells was significantly reduced by combining Bcl-2 shRNA and Ara-C in a time-independent manner. There was no difference on the viability of the cells between control shRNA combined Ara-C and Ara-C alone.

Ara-C enhances apoptosis of cells transfected with Bcl-2 shRNA

Two methods were used to assess apoptosis: Giemsa staining and FACS analysis. Raji cells presented characteristic of apoptosis (membrane blebbing, nuclear condensation, fragmentation, and formation of apoptotic bodies) (data not shown). Levels of apoptosis were (13.53 ± 2.10)% at 48 h after transfection with Bcl-2 shRNA (**Fig. 2**). However, a significant increase in apoptosis ($P < 0.05$) was observed after the combined treatment with both Bcl-2 shRNA and Ara-C (39.43 ± 2.30)%, the difference in an apoptotic rate was significant, compared

Table 1 Inhibition rate of Bcl-2 shRNA combined with Ara-C on Raji cells growth (%)

Group	24 h	48 h	72 h
Ara-C	60.19 ± 1.71	62.12 ± 3.32	66.65 ± 2.55
Negative shRNA +Ara-C	58.15 ± 3.86	62.31 ± 3.33	69.93 ± 4.12
Blank plasmid + Ara-C	60.70 ± 3.57	62.43 ± 2.52	61.59 ± 1.25
Bcl-2shRNA	40.90 ± 2.43	46.50 ± 4.20	42.18 ± 4.17
Bcl-2shRNA + Ara-C	82.82 ± 2.29*	86.19 ± 1.03*	76.06 ± 1.25*

The data were shown as the mean ± SD (n = 3).

*P < 0.05 vs. Ara-C + negative shRNA group and Bcl-2shRNA group.

with Ara-C (17.10 ± 0.20)% alone or control shRNA combined with Ara-C (17.32 ± 1.10)%, respectively (P < 0.05). No significant difference in apoptosis occurred between those cells treated with control shRNA plus Ara-C or Ara-C alone (P > 0.05).

Discussion

Bcl-2 is a prominent member of the Bcl-2 family proteins that regulate the induction of apoptotic cell death by a wide variety of stimuli. Increased expression of Bcl-2 in tumor is considered as the major determinants of

resistance to chemotherapeutic drugs and radiotherapy. Blocking of Bcl-2 expression and function may represent a relevant therapeutic strategy. Bcl-2 antisense oligonucleotides have succeeded in several preclinical and clinical studies. The currently ongoing Phase I and II clinical studies using antisense Bcl-2, known as G3139, aim at the treatment of patients with non-Hodgkin’s lymphoma, melanoma, and prostate cancer [12–14].

Lymphoma cell line Raji has been previously shown to produce large amounts of Bcl-2. Our previous studies showed that reduction in Bcl-2 expression by antisense oligonucleotides can induce apoptosis in Raji cells [15].

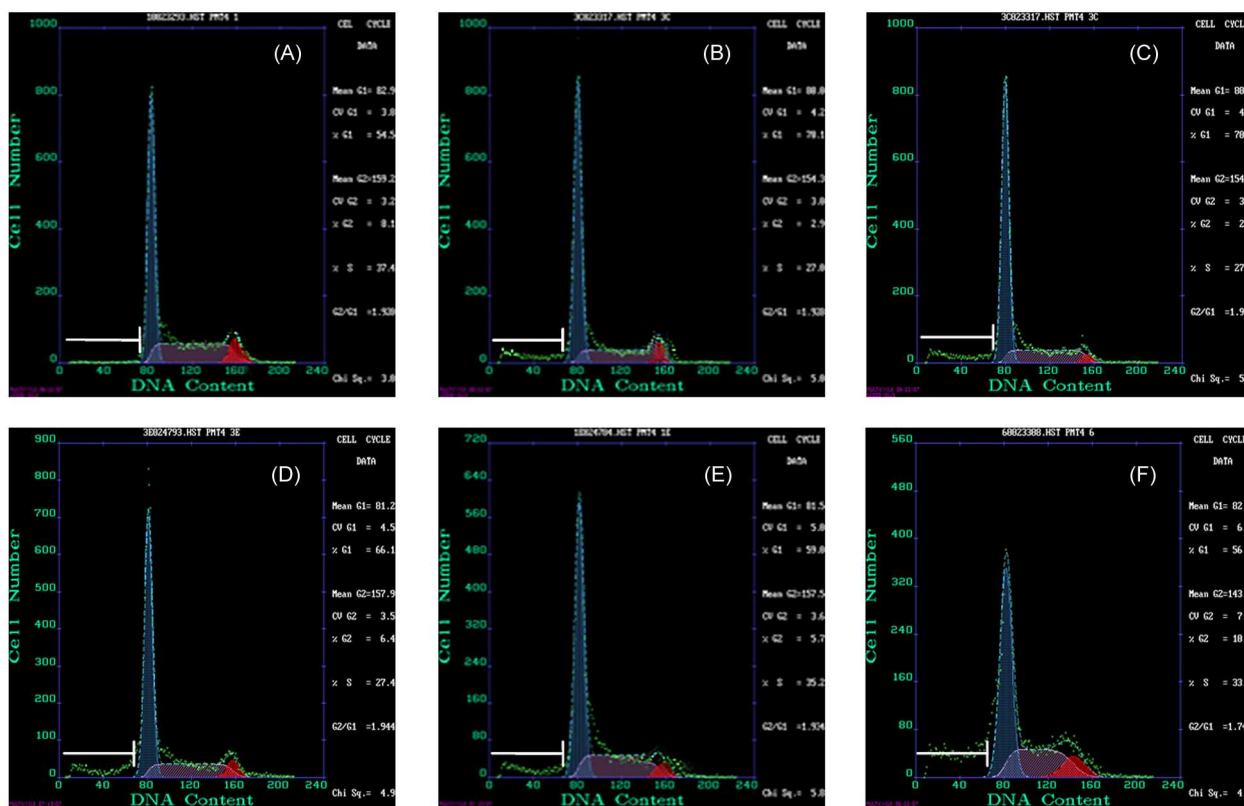


Fig. 2 Apoptosis rate of Bcl-2 shRNA combined with Ara-C in Raji cells Results are from a representative experiment. (A) Raji cells. (B) Ara-C group. (C) Negative shRNA with Ara-C group. (D) Blank plasmid with Ara-C. (E) Bcl-2 shRNA group. (F) Bcl-2 shRNA with Ara-C.

Recent investigations have shown the effectiveness of siRNA-mediated Bcl-2 inhibition in cancer cells [9,10,16,17]. The siRNA against Bcl-2 could increase sensitivity to chemotherapeutic agents [18,19]. However, whether shRNA against Bcl-2 could enhance Ara-C-induced apoptosis of lymphoma Raji cells remains uncertain.

In this study, as expected, Raji cells transfected with Bcl-2 shRNA showed a greater decrease in cell proliferation and Bcl-2 expression as compared with control shRNA. Moreover, knockdown of Bcl-2 by shRNA enhanced Ara-C inhibiting growth of Raji cells. This synergistic pro-apoptotic effect also leads to a concurrent reduction in cell number. Both the apoptotic effect and cell number reduction in the combination treatment are significantly greater than that of either Ara-C or Bcl-2 shRNA treatment alone. There was no difference in the viability of the cells and the apoptotic rate between those treated with control shRNA plus Ara-C and Ara-C alone.

Our results indicated that down-regulation of Bcl-2 protein by Bcl-2 shRNA increased Ara-C-induced apoptosis in Raji cells. Bcl-2 shRNA may be useful for the treatment of tumor with overexpression of Bcl-2. As previously reported, the selective blockade of Bcl-2 could trigger the apoptosis and suppress the transduction of survival signals, thus leading to cancer cell growth inhibition, enhancing anti-tumor activity of drug [20]. It has been shown that down-regulation of Bcl-2 expression by siRNA increased the effect of doxorubicin in a canine mammary gland tumor cell line [18]. Another study showed that Bcl-2 siRNA-mediated gene silencing strengthened the sensitivity to the chemotherapeutic drugs 5-fluorouracil and 10-hydroxycamptothecin in human hepatoblastoma HepG2 cells [19].

Our results showed that the combination of Bcl-2 shRNA and Ara-C produced a greater inhibitory effect on cell proliferation of Raji cells than the two treatments given separately, indicating that Bcl-2 knockdown caused a marked synergistic enhancement of Ara-C-induced apoptosis.

In our study, effectiveness of Bcl-2 gene silencing was low. We think that the lower inhibition rate in gene expression induced by Bcl-2 shRNA expression plasmid could be possibly explained by lower transfection efficiency of shRNA expression plasmid in suspension culture cells compared with that in siRNA delivery and/or in adherent culture cells. Further investigations should be carried out in shRNA delivery with high transfection efficiency.

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