Original Article

Inhibitory effects of short hairpin RNA against caspase-8 on apoptosis of murine hepatoma Hepa1-6 cells

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Summary **Caspase-8 plays an important role in death-receptor-mediated apoptosis of hepatocytes. We constructed short hairpin RNAs (shRNAs) against caspase-8 and investigated the effects of caspase-8 targeting shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α. Oligonucleotides coding for four shRNAs against caspase-8 were cloned into mammalian expression vector Pgenesil-1 containing U6 promoter, which were then introduced into Hepa1-6 cells using liposome-mediated transfection. Effects of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α were detected by PI apoptosis detection kit. Effects of caspase-8-shRNAs on caspase-8 mRNA expression in apoptosis Hepa1-6 cells induced by TNF-α were detected by real-time fluorescent RT-PCR. Of the four caspase-8-shRNAs, Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 were successfully constructed. The apoptosis of Hepa1-6 cells induced by TNF-α was significantly inhibited by either Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 ($p < 0.05$). Caspase-8 mRNA expression levels in apoptosis Hepa1-6 cells induced by TNF-α were significantly decreased by either Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 ($p < 0.05$). This study suggested that shRNAs against caspase-8 could effectively inhibit apoptosis of Hepa1-6 cells induced by TNF-α by suppressing caspase-8 mRNA expression.**

Keywords: Caspase-8, RNA interference, apoptosis, Hepa1-6 cells

1. Introduction

Acute liver failure (ALF) is a dramatic clinical syndrome with high mortality rates in which a previously normal liver fails within days or weeks. A major concern in ALF therapy is protection of hepatocytes to prevent apoptosis and maintain liver function (*1*). In ALF, signals released from the cell membrane of hepatocytes trigger suicide pathways, leading to the activation of caspase cascades that subsequently kill hepatocytes through apoptosis. Death receptors such as Fas trigger death signals when bound to their natural ligands, leading to recruitment of the adaptor protein, Fas-associated death domain, which in turn recruits caspase-8 zymogens to form death-inducing signaling complexes (DISCs) (*2,3*). Caspase-8 molecules become activated at DISCs and subsequently activate proapoptotic downstream molecules (*4*). Hence caspase-8, a downstream target of all known death receptors, may be a more suitable target than Fas to achieve future successful ALF therapy.

RNA interference (RNAi) is a potent gene silencing mechanism conserved in all eukaryotes, in which double-stranded RNAs suppress the expression of cognate genes by inducing degradation of mRNAs or by blocking translation of mRNAs (*5-7*). Short hairpin RNAs (shRNAs) are RNA sequences that makes a tight hairpin turn that can be used to silence gene expression *via* RNAi (*8,9*). Conditional gene silencing in mammalian cells, *via* the controlled expression of shRNAs, is an effective method for studying gene function, particularly if the gene is essential for cell survival or development (*10*). Also, efficient delivery of small interference RNAs (siRNAs) into hepatocytes

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in vivo and *in vitro* has already been reported (*11,12*). Since activation of caspase-8 is the central event in the hepatocyte apoptotic pathway, shRNA against caspase-8 might be useful to control the apoptosis of hepatocytes. In this study, we constructed shRNA eukaryotic expression vector for caspase-8 and investigated the inhibitory effects of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by tumor necrosis factor (TNF)-α.

2. Materials and Methods

2.1. *Materials*

The plasmid vector Pgenesil-1, which contains U6 promoter and the reporter gene of enhanced green fluorescence protein (EGFP), was purchased from Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China. TRIzoL reagents and SuperScript™ First-Strand Synthesis System for RT-PCR were obtained from Invitrogen, Carlsbad, CA, USA. Magnetic bead plasmid extract kit, reverse transcription kit, restriction enzyme *BamH* I, *Hind* III, T4 DNA ligase, and Taq DNA polymerase were obtained from Promega, Madison, WI, USA. Annexin V-FITC Apoptosis Detection Kit was purchased from IMGENEX Corporation, CA, USA. DNA marker was obtained from Takara Bio, Shiga, Japan. Murine hepatoma Hepa1-6 cells were obtained from Mount Sinai Medical Center, Miami Beach, FL, USA.

2.2. *Construction of caspase-8-shRNAs*

Following the rules of Tuschl T (*13*), shRNAs were designed according to Mus caspase-8 sequence in the GenBank (NM_009812), which showed no homology to any other sequences by a blast search. Eight oligodeoxyribonucleotides (66 bp) encoding four shRNAs against caspase-8 were synthesized by Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China. The shRNAs sequences are shown in Figure 1. The four DNA templates, named successively caspase-8-1 to caspase-8-4, contained *BamH* I and *Hind* III restriction sites which can ligate with Pgenesil-1 at the 5' extreme of positive-sense and antisense strands. The map of Pgenesil-1 vector containing the U6 promoter region is shown in Figure 2.

To construct caspase-8-shRNAs, two corresponding oligodeoxyribonucleotides encoding a shRNA were dissolved in annealing buffer, kept at 100°C for 5 min, and cooled gradually to room temperature to anneal. Pgenesil-1 was digested with *BamH* I and *Hind* III, blunt-ended with T4 DNA polymerase, and then ligated with the annealed oligodeoxyribonucleotides. Pgenesil-GAPDH and Pgenesil-HK were used as positive and negative controls, respectively. Then, 5 μL ligation mixtures (Pgenesil-caspase-8-1 to caspase-8-4) were transformed to *E. coli* DH5α. Positive kanamycin-resistant clones were selected and expanded. The recombinant plasmids were extracted according to the manufacturer's magnetic bead plasmid extract kit protocol and verified by *Sal* I digestion analysis. All the constructed plasmids were confirmed by DNA sequencing (Invitrogen).

2.3. *Transfection*

Hepa1-6 cells were transiently transfected using lipofectamine 2000 in accordance with the manufacture's protocol. In brief, cells at 80-90% confluence in a 6-well plate were incubated for 6 h with a mixture of 4 μg caspase-8-shRNAs or Pgenesil-GAPDH, or Pgenesil-HK, and 8 μL lipofectomine 2000 in serum-free DMEM medium (*14*). The transfection medium was then replaced with DMEM medium including 10% FCS. All transfections were performed in triplicate. The transfection efficiency was determined by calculating the rate of positive EGFP staining cells under fluorescence microscopy.

2.4. *Effect of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α*

To induce Hepa1-6 cell apoptosis, TNF-α was added to the cells to a final concentration of 20 ng/mL and normal saline was used as the negative control. Cells were then harvested and transfected with Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2. The cells were then cultured for 48 h and apoptosis levels were detected with Annexin V-FITC Apoptosis Detection Kit using FACScan® flow cytometer (Becton Dickinson, San Jose, CA, USA) and WinMDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

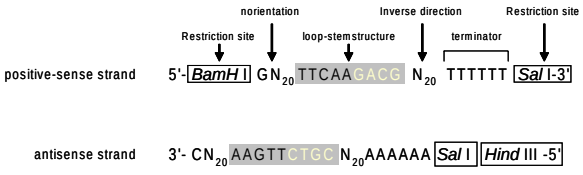

Figure 1. Structure of shRNAs oligonucleotide chains.

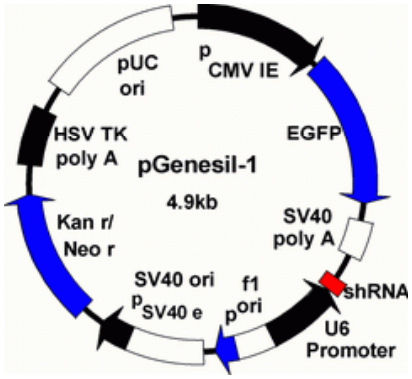

Figure 2. Map of Pgenesil-1 vector containing the U6 promoter region.

2.5. *Effects of caspase-8-shRNAs on caspase-8 mRNA expression levels in Hepa1-6 cells*

Levels of caspase-8 mRNA expression levels before and after caspase-8-shRNA treatment in apoptosis of Hepa1-6 cells induced by TNF-α were detected by quantitative RT-PCR (*15*). Briefly, after TNF-α was added to induce apoptosis, Hepa1-6 cells were transfected with caspase-8-shRNAs. Total RNA was isolated from Hepa1-6 cells using TRIzoL reagents according to the manufacturer's protocol. Isolated total RNA was first reverse transcribed into cDNA using random primers and SuperScript™ II reverse transcriptase. Then cDNA was used as the template in real-time RT-PCR. The sequences of the primers were as follows: caspase-8-primer, sense: 5'-GCC ACA GGG TCA TGC TCT TTA-3', antisense: 5'-TGC CAG CAT GGT CCT CTT CT-3'. beta-Actin-primer, sense: 5'-CAT CAT GAA GTG TGA CGT TGA CAT-3', antisense: 5'-GCT CAG GAG GAG CAA TGA TCT T-3'. Cycle threshold (Ct) values of samples were analyzed by Sequence Detection System software during the PCR procedure. Inhibition ratios were calculated by the following equation: inhibition ratio (shRNA on caspase-8 mRNA) = [1 – experiment group $2^{Ct\ (GAPDH)-Ct\ (caspase-8)}$/ control group $2^{Ct\ (GAPDH)-Ct\ (caspase-8)}$]·100.

2.6. *Statistical analysis*

SPSS 11.5 software was used for data analysis. The experimental results were recorded as mean ± S.D. Differences between group means were analyzed by *t* test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. *Construction of caspase-8-shRNAs*

The multiclone sites of plasmid Pgenesil-1 were as follows: 5'-*Hind* III-ShRNA-*BamH* I-U6 Promoter-EcoRI-*Sal* I-XbaI-DraIII-3'. A *Sal* I site for plasmid Pgenesil was designed in the inserted fragments between the sites of *BamH* I and *Hind* III. Correct insertion was evaluated by a production of about 400 bp-band by *Sal* I digestion. As shown in Figure 3, digestion of the four recombinant DNA vectors with restriction endonucleases gave fragments at the expected position. However, by means of automated DNA sequencing, the insert sequence was verified in two shRNAs, Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2, but not in the other two (data not shown).

3.2. *Transfection of caspase-8-shRNAs*

The transfection rates of Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 were evaluated under fluorescence microscopy. A typical example of EGFP positive cells is shown in Figure 4. The transfection rates were about 35%-40% according to the expression of EGFP in Hepa1-6 cells.

3.3. *Inhibition of Hepa1-6 cells apoptosis by caspase-8-shRNAs*

The effect of caspase-8-shRNAs on apoptosis of Hepa1-6 cells induced by TNF-α was examined (Figure 5). Apoptotic rates of Hepa1-6 cells were calculated using WinMDI 2.8 software. TNF-α successfully induced Hepa1-6 cells' apoptosis and the apoptotic rate was significantly increased from 1.20 ± 0.32% (panel *A*) to 17.40 ± 2.21% (panel *B*; $p < 0.05$). After the Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 treatment, apoptotic rates were significantly decreased from 17.40 ± 2.21% (panel *B*) to 4.70 ± 0.89% (panel *C*; $p < 0.05$) and 10.23 ± 2.56% (panel *D*; $p < 0.05$), respectively. This result suggests that Pgenesil-caspase-8-1 and Pgenesil-caspase-8-2 can significantly inhibit the apoptosis of Hepa1-6 cells induced by TNF-α. The inhibitory effect was more obvious in the Pgenesil-caspase-8-1 group than in the Pgenesil-caspase-8-2 group ($p < 0.05$).

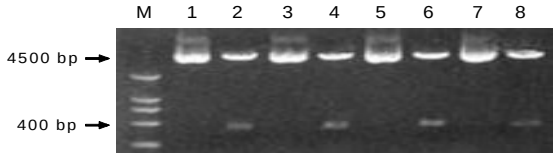

Figure 3. Restriction digestion analysis of eukaryotic recombinant vectors Pgenesil-caspase-8-1 through caspase-8-4. M, DNA marker; 1, 3, 5, and 7, negative control; 2, 4, 6, and 8, Pgenesil-caspase-8-1 through caspase-8-4, respectively.

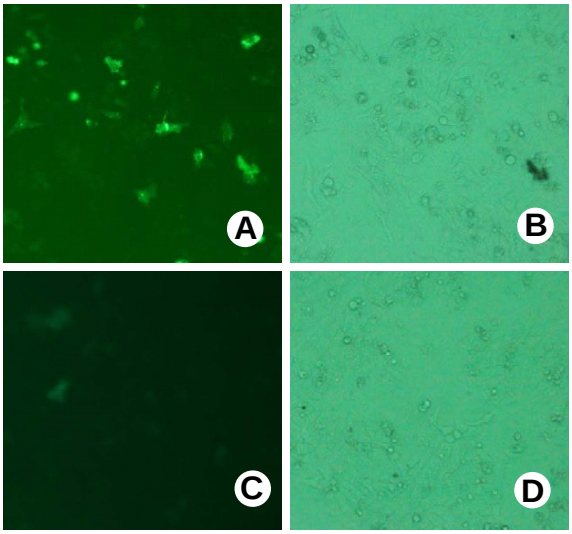

Figure 4. EGFP expression in Hepa1-6 cells transfected by eukaryotic expression vectors. A, Hepa1-6 cells transfected by Pgenesil-Caspase-8; B, Distribution of Hepa1-6 cells observed by light microscope in the same area as A; C, Hepa1-6 cells that were not transfected by Pgenesil-Caspase-8; D, Distribution of Hepa1-6 cells observed by light microscope in the same area as C. (×200)

3.4. *Inhibition of caspase-8 mRNA expression by caspase-8-shRNAs*

The effect of caspase-8-shRNAs on caspase-8 mRNA expression in Hepa1-6 cell apoptosis induced by TNF-α was measured by fluorescence RT-PCR. The results showed that the expression of caspase-8 mRNA was significantly increased from 0.050 ± 0.006 to 0.286 ± 0.063 ($p < 0.05$) after the treatment with TNF-α (Table 1). After transfection by Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2, caspase-8 mRNA expression was significantly decreased to 0.098 ± 0.037 ($p < 0.01$) and 0.127 ± 0.043 ($p < 0.05$), respectively. The inhibitory effect was more obvious in the Pgenesil-caspase-8-1 group than in the Pgenesil-caspase-8-2 group ($p < 0.05$).

4. Discussion

Due to its high efficiency and specificity, RNAi is now being widely used as a method to knock down target genes, to study gene function, or to explore experimental treatments for certain diseases (*16-18*). The siRNAs can be produced by four different ways: chemical synthesis, *in vitro* transcription, enzymatic digestion of dsRNAs, and transfection of DNA vectors encoding siRNAs or shRNAs, which are converted to siRNAs in cells (*19*). Of the four ways, transfection of DNA vectors offers advantages in silencing longevity, delivery options, and cost. Since the central event in the hepatocyte apoptotic pathway is the proteolysis activation of caspase-8 (*20,21*), in the present study, we constructed four shRNAs against caspase-8 and against

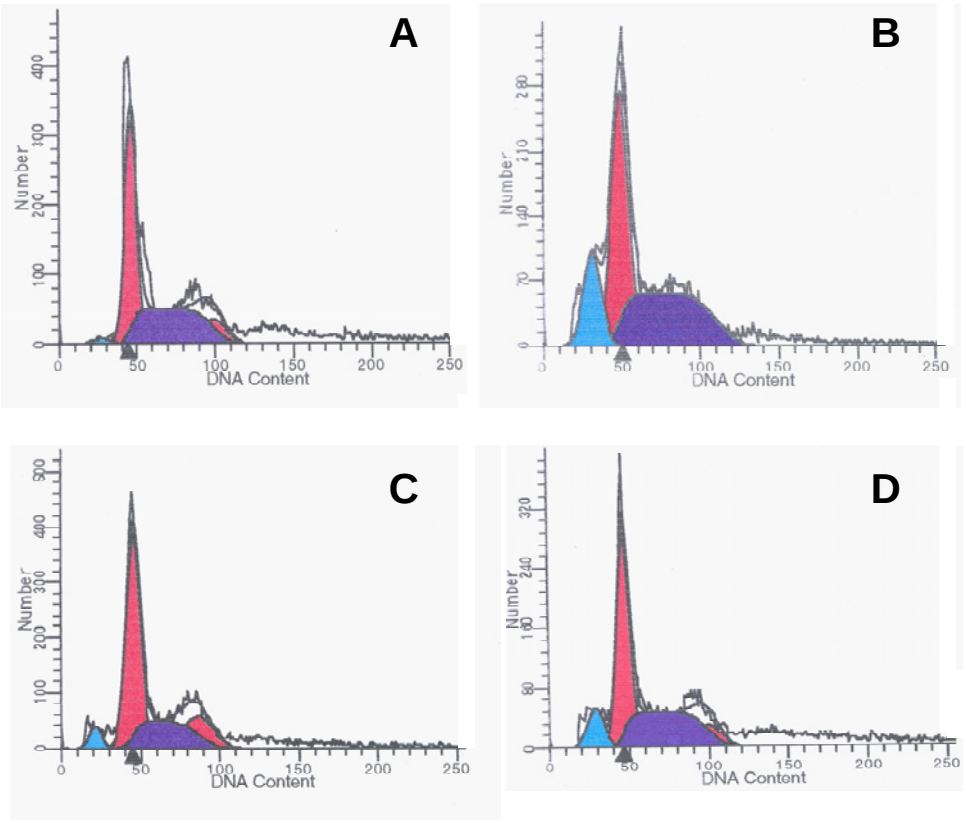

Figure 5. The effect of caspase-8-shRNAs on Hepa1-6 cells apoptosis induced by TNF-α. A, untreated Hepa1-6 cells; B, Hepa1-6 cells treated with TNF-α; C, Hepa1-6 cells treated with TNF-α followed by Pgenesil-caspase-8-1 transfection; D, Hepa1-6 cells treated with TNF-α followed by Pgenesil-caspase-8-2 transfection.

Table 1. Effects of caspase-8-shRNAs on caspase-8 mRNA expression levels in Hepa1-6 cells induced by TNF-α

Pre-treatment	Transfection	Caspase-8 mRNA levels (mean ± S.D.)	Inhibitory rates (%)
None	None	0.050 ± 0.006	–
TNF-α	None	0.286 ± 0.063[a]	–
TNF-α	Pgenesil-caspase-8-1	0.098 ± 0.037[b,d]	76
TNF-α	Pgenesil-caspase-8-2	0.127 ± 0.043[c]	52

[a] $p < 0.05$, compared with Hepa1-6 cells without TNF-α pre-treatment; [b] $p < 0.01$, [c] $p < 0.05$, compared with Hepa1-6 cells with TNF-α pre-treatment; [d] $p < 0.05$, compared with Hepa1-6 cells with Pgenesil-caspase-8-2 treatment.

Pgenesil-caspase-8-1 through caspase-8-4.

One problem with using siRNA to knock down gene expression is target sequence selection: siRNAs target different sites of the same gene, producing different effects varying from strong to no inhibition of gene expression. The mechanism of this selection is not well known. Therefore, the design of the most effective siRNAs is still empirical, although some principles have been put forward and some software products have been developed to facilitate the selection process (*22,23*). In the present study, four target sites were chosen according to these criteria, but only half of them turned out to be effective; these two effective shRNAs against caspase-8 were used in subsequent experiments.

Since TNF-α and TNF-related apoptosis-inducing ligand are important mediators of apoptosis in hepatocytes (*24*), we used TNF-α to induce apoptosis of mouse Hepa1-6 cells in this study. It was shown that TNF-α could significantly increase the apoptosis rate and caspase-8 mRNA expression of Hepa1-6 cells. After introducing the Pgenesil-caspase-8-1 or Pgenesil-caspase-8-2 into Hepa1-6 cells, both the apoptosis rate and caspase-8 mRNA expression were significantly decreased. This result suggests that caspase-8-shRNAs could effectively inhibit apoptosis of murine hepatoma Hepa1-6 cells induced by TNF-α. Further studies are needed to verify if the two shRNAs can also reduce caspase-8 protein levels efficiently and lead to growth arrest and/or apoptosis of hepatic cells *in vivo*.

In summary, this study suggested that the two shRNAs against caspase-8 could effectively inhibit apoptosis of Hepa1-6 cells induced by TNF-α by suppressing caspase-8 mRNA expression. The results of our study provide a basis for future research to utilize RNAi in induction of hepatocytes apoptosis.

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