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Inhibition of Fas-mediated Apoptosis in Yac-1 Cell via Anti-Fas Ribozyme

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Abstract To detect a new and more effective way against apoptosis mouse lymphomatic cell line-Yac-1 in which *fas* gene was expressed highly was used as a model for studying the effects of anti-Fas ribozyme on Fas-mediated apoptosis. A hammerhead ribozyme gene targeting the *fas* mRNA was synthesized and its *in vitro* transcription vector was constructed, which was transfected into Yac-1 cells using electroporation. Rz596 expression was detected using RT-PCR, and Fas expression in Yac-1 cells was detected using RT-PCR, Western blot and flow cytometry. After treated with anti-Fas antibody (JO_2), Yac-1 cell viability was measured with MTT assay, caspase-3 proteolytic activity was detected, and cell apoptosis was measured according to annexin V apoptosis detecting kit. Anti-Fas ribozyme could cleave *fas* mRNA efficiently *in vivo* and *in vitro*. Fas expression in Yac-1 cells transfected with anti-Fas ribozyme was decreased remarkably and correlated with resistance to Fas-mediated apoptosis as determined by flow cytometry and caspase-3 proteolytic activity. Anti-Fas ribozyme was detected in cells transfected with pU6-RZ596 and pU6-dRZ596 and could remarkably decrease the Fas expression in Yac-1 cells, which made Yac-1 cells get rid of Fas-mediated apoptosis. Because of wide expression of *fas* in organs and tissues, our research was very useful for studying the inhibition of apoptosis of many organs and tissues in the future.

Key words transplantation immunology; ribozyme; Fas; Yac-1; apoptosis

As an RNA molecule with catalytic activity, ribozyme can inhibit gene expression via binding and cleaving target RNA in a sequence specific way [1–3]. Now hammerhead ribozyme is widely used in gene therapy because of its many superiorities [4–6], which include small molecular weight, easy to design and synthesize, and confirmative roles [7–9]. Anti-Fas hammerhead ribozyme was designed and synthesized to aim directly at position 596 of the *fas* RNA, and cloned into eukaryotic vector, which was transfected into Yac-1 cells (a mouse lymphoma cell line) highly expressing *fas* via electroporation [10,11], then the effects of anti-Fas ribozyme on Fas expression and apoptosis of Yac-1 cells were investigated.

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Materials and Methods

Materials and reagents

Mouse lymphoma cell line Yac-1 cell was purchased from Wuhan University Culture Center. *E. coli* DH5α was a kind gift from Department of Immunology, Tongji Medical College. Prokaryotic vector pBSKU6 and green fluorescent protein plasmid pEGFPC1 were saved in our lab. All restriction endonucleases and T_4 DNA ligase were products of Promega Company. Mini plasmid DNA extraction kit, gel DNA purification kit, and DL-2000 DNA marker were purchased from TaKaRa Company. Reverse transcriptional kit, dNTP, *Taq* DNA polymerase, and pUC mix marker were products of MBI Company, USA. Fluorescein isothiocyanate (FITC)-conjugated annexin V kit was product of Bender Company (purchased from

Jingmei Company). FITC-conjugated anti-mouse Fas antibody (JO_2) and mouse $IgG_2\alpha$ isotype control were purchased from Pharmingen Company. Caspase-3 activity detection kit was product of Clontech Company. MTT reagent was purchased from Sigma.

Synthesis and cloning of ribozyme

The secondary structure of the mouse *fas* RNA (GenBank accession No. M83649) was obtained with software that analyzes RNA folding by the Zucker-Stiegler [12], then the ribozyme gene was designed. GUA triplets located at 596 of the *fas* RNA were selected as the cleavage site, the cDNAs encoding the anti-Fas ribozyme were composed of two complementary strands about 50 nt, which were terminated with *Bam*HI and *Xba*I: a, 5′-TCTAGAGATATATAAACTGATGAGTCCGTGAGGACGAAACAAGTGGATCC-3′; b, 5′-GGATCCACTTGTTTCGTCCTCACGGACTCATCAGTTTATATATCTCTAGA-3′. No. 3 base near to cleavage site of ribozyme was mutated from G to A, which was named as dRZ596, all cDNAs were synthesized by Shanghai Sangon Company. The ribozyme was cloned into the *Bam*HI and *Xba*I sites of the pBSKU6 and named U6-RZ596 and U6-dRZ596 respectively, which were digested and sequenced to be correct, then these recombinant plasmids were used as templates to get fragments including U6 promoter and ribozyme using PCR. The fragments were subcloned into the *Mlu*I site of the pEGFPC1 and named pU6-RZ596 and pU6-dRZ596 respectively, which were testified to be correct.

Cloning of *fas* cDNAs and *in vitro* ribozyme-mediated cleavage reactions

The plasmid was a kind gift from Prof. Pastori, School of Medicine in University of Miami, which was named pFas-596 [13]. Two recombinant plasmids and pFas-596 were linearized using *Sma*I and transcribed into RZ596 RNA, dRZ596 RNA (464 nt) and *fas* RNA (1130 nt) with T7 RNA polymerase respectively. *In vitro* cleavage reactions were performed under different temperatures and different molecular ratios of transcriptional products. After electrophoresis on 6% polyacrylamide gel autoradiography was performed to analyze results.

Cell culture and stable transfection of ribozyme

Yac-1 cells were grown in RPMI 1640 supplemented with 10% FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin, in the atmosphere containing 5% CO_2 at 37 °C. Cells were collected and grouped as following: (1) empty control; (2) cells transfected with pEGFPC1; (3) cells transfected with pU6-RZ596; (4) cells transfected with pU6-dRZ596. Cells were adjusted to a density of 3×10^6 cells/ml, the electroporation was performed under the condition of 500 V, 60 μs and shock once (electroporational apparatus was purchased from Eppendorf Company), after cultured for 48 h, the stable transfected cells were then selected by culturing in medium containing G418 (800 μg/ml).

Detecting expression of anti-Fas ribozyme in Yac-1 cells using RT-PCR

Total RNA was extracted from all above-mentioned groups using Trizol reagent. After cDNA was synthesized PCR was performed and β-MG was used as control. Sense primer of pU6-RZ596 was 5′-TCGAGGTCGTCGGTATCGAT-3′, and anti-sense primer 5′-ATGAGTCCGTGAGGACGAAAG-3′; β-MG sense primer was 5′-ATCTTCAAACCTCCATGATG-3′ and anti-sense primer 5′-ACCCCCACTGAAAAAGATGA-3′. Reaction conditions for PCR were as following: pre-denaturation 4 min at 95 °C; 30 cycles (denaturing 40 s at 95 °C; annealing 50 s at 57 °C; extension 40 s at 72 °C); extension 3 min at 72 °C. PCR products were analyzed on 2 % agarose gel.

Detection of *fas* mRNA expressed in Yac-1 cell using RT-PCR

RNA extraction and cDNA synthesis were the same as above, β-MG was used as control. *fas* gene sense primer was 5′-GCTGCAGACATGCTGTGGATC-3′, anti-sense primer 5′-TCACAGCCAGGAGAATCGCAG-3′. Reaction conditions for PCR were as following: pre-denaturation 3 min at 95 °C; 35 cycles (denaturing 30 s at 95 °C; annealing 45 s at 58 °C; extension 45 s at 72 °C); extension 3 min at 72 °C. PCR products were analyzed on 1.5% agarose gel.

Detection of Fas protein expressed in Yac-1 cells by using Western blot

Four groups cells were collected regularly and lysed in solubilization buffer, then the proteins were measured as 0.5 μg/μl, which were electrophoresized in 10% SDS-polyacrylamide minigels. The first antibody was rabbit anti-mouse-Fas antibody (1:200), and the second antibody was goat anti-rabbit-IgG (1:5000). At last the color was developed by ECL system.

Detection of Fas protein expressed in Yac-1 cells

Stable clones for four group cells were collected and adjusted to a density of 10^6 cells/ml, which were labeled

with anti-Fas antibody (JO_2, 10 μg/ml), then the Fas expression was detected on FACS (product from BD Company).

Detection of caspase-3 protease activity

After treated with JO_2 (5 μg/ml) for 24 h in common growth condition, apoptosis of four group cells was induced, meanwhile the negative control without apoptosis and apoptosis cells treated with caspase-3 inhibitor (DEVD-fmk) were established. After centrifuged and collected, cells were treated with cell lysis buffer sufficiently, the supernatant was remained (including protein needed), then the operation was performed according to the instructions of caspase-3 activity detection kit. The absorbance (*A*) value was measured at 405 nm.

Cells viability

After grouped and treated as above, cells were inoculated in 96-well plates in a density of about 10^5 cells/ml (100 μl/well). After incubation with 10 μl MTT (0.5 mg/ml) for 4 h at 37 °C, the formazan crystals were solubilized with 100 μl DMSO, then the absorbance (*A*) value was measured at 570 nm.

Detection of apoptosis through FACS

Four group cells treated with JO_2 and negative control were labeled by annexin V-FITC and PI respectively, whose apoptosis was analyzed by flow cytometry.

Results

In vitro cleavage

As it was shown in Fig. 1, *fas* RNA containing the entire coding region of 1130 nucleotides was cleaved *in vitro* by both anti-Fas ribozymes pU6-RZ596 and pU6-dRZ596 (molar ratio of ribozyme to RNA is 3), autoradiography analysis displayed pU6-RZ596 could cleave *fas* RNA *in vitro* to degenerate fragments into 646 and 484 nucleotides, whose cleavage efficiency was 60%, but pU6-dRZ596 had no cleavage activity.

Expression of anti-Fas ribozyme in all group cells

Ribozyme and β-MG PCR amplification generated amplicons of 191 bp and 100 bp, ribozyme was detected in cells transfected with pU6-RZ596 and pU6-dRZ596, which was not detected in empty cells and mock-transfected cells (Fig. 2).

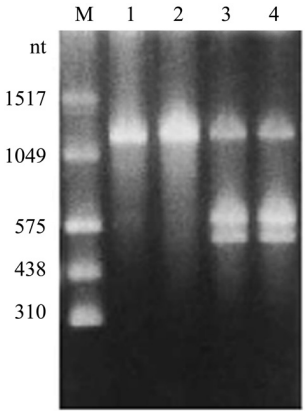

Fig. 1 *In vitro* cleavage of *fas* RNA

M, RNA marker; 1, *fas* RNA; 2, cleavage with pU6-dRZ596; 3, 4, cleavage with pU6-RZ596.

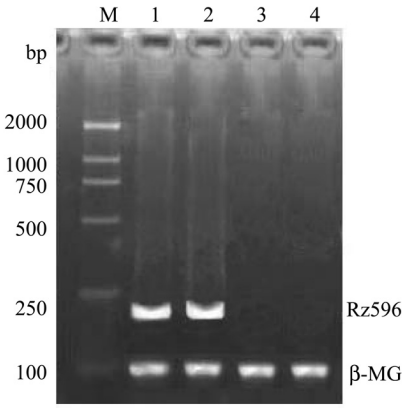

Fig. 2 Expression of Rz596 in four group cells

M, DL-2000 marker; 1, pU6-RZ596-transfected group; 2, pU6-dRZ596-transfected group; 3, mock-transfected group; 4, control group.

Effects of ribozyme on Fas expression

fas and β-MG PCR amplification generated amplicons of 419 bp and 100 bp respectively, through scanning the luminance and analyzing the ratio between *fas* and β-MG using gel image analysis system, the results were as following: negative control ratio is 1.1; Yac-1 transfected with pEGFPC1 ratio is 1.03; cells transfected with pU6-RZ596 ratio is 0.43; cells transfected with pU6-dRZ596 ratio is 0.75. It is clear that *fas* mRNA expression on cells transfected with anti-Fas ribozyme was lower than that on cells transfected with empty vector and mutation ribozyme (Fig. 3).

Western blot results showed that Fas protein level expressed on cells transfected with pU6-RZ596 was much lower than that expressed on control and cells transfected

with pEGFPC1 or pU6-dRZ596, which were coincident with the results of RT-PCR (Fig. 4).

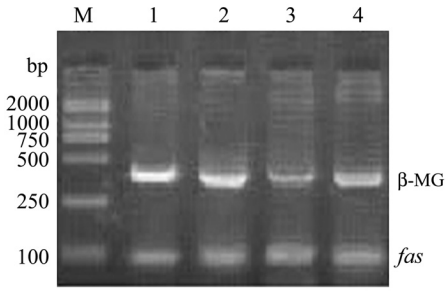

Fig. 3 *fas* gene transcription in four group cells

M, DL-2000 marker; 1, control group; 2, mock-transfected group; 3, pU6-RZ596-transfected group; 4, pU6-dRZ596-transfected group.

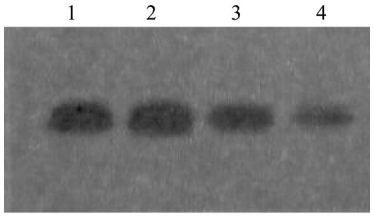

Fig. 4 Protein expression of *fas* in four group cells

1, empty control; 2, mock-transfected group; 3, pU6-RZ596-transfected group; 4, pU6-dRZ596-transfected group.

Through flow cytometry, Fas protein expression rates on four group cells were individually 98%, 96%, 50%, 76% (Fig. 5), which were coincident with the results of RT-PCR.

The effect of anti-Fas ribozyme on caspase-3 activity

After 24 h of stimulation with the JO_2, Yac-1 cells displayed caspase-3 activity that was specifically inhibited by a caspase-3 inhibitor. Compared with untransfected group, caspase-3 activities of other groups transfected with pEGFPC1, pU6-RZ596 and pU6-dRZ596 were 95%, 45%, and 74% respectively (Fig. 6).

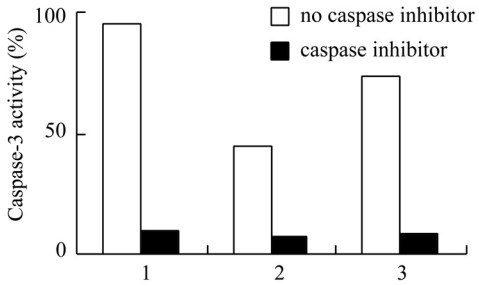

Fig. 6 Detection of caspase-3 activity in three transfected groups compared with untransfected group

After treated with JO_2 antibody (5 μg/ml) for 24 h, the activity in the presence or absence of the caspase-3 inhibitor (DEVD-fmk) was then detected. 1, mock-transfected; 2, pU6-RZ596; 3, pU6-dRZ596.

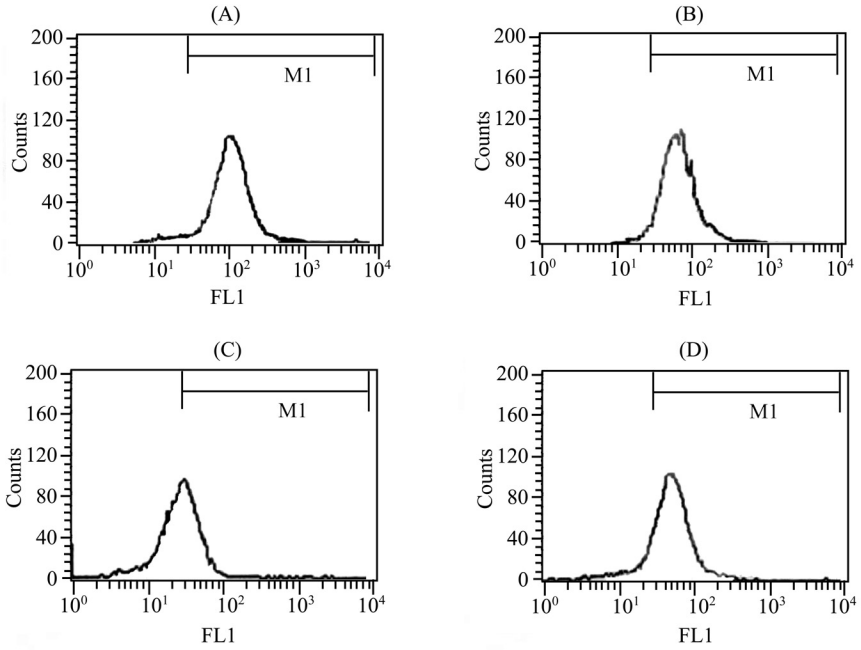

Fig. 5 Detection of Fas protein expression on four groups by flow cytometry

(A) Fas expression on control is 98%. (B) Fas expression on mock-transfected group is 96%. (C) Fas expression on pU6-RZ596-transfected group is 50%. (D) Fas expression on pU6-dRZ596-transfected group is 76%.

The effect of anti-Fas ribozyme on cell viability

After induction of apoptosis of Yac-1 cells through stimulating with JO_2, cell viability was detected by MTT assay. Compared with negative control, cell viability of other groups transfected with pEGFPC1, pU6-RZ596, and pU6-dRZ596 were 98%, 208%, and 145% respectively (Fig. 7).

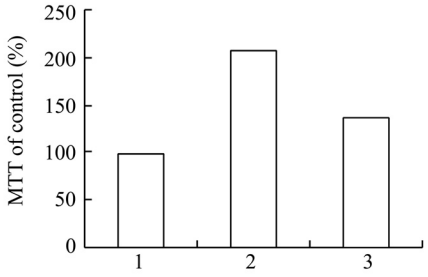

Fig. 7 After treated with JO_2 (5 μg/ml) for 24 h, cell viability of the groups transfected with mock-transfected, pU6-RZ596 and pU6-dRZ596 compared with untransfected group was measured by MTT respectively

1, mock-transfected; 2, pU6-RZ596; 3, pU6-dRZ596.

Inhibition of Fas-mediated apoptosis in Yac-1 cells

Cells apoptosis were induced just as above mentioned, apoptosis rates were detected using annexin V-FITC kit, which for four group cells were in turn 86%, 87%, 35% and 62% (Fig. 8).

Discussion

In the field of transplantation immunology, survival rate of grafts is an important factor that affects the success of transplantation. Apoptosis of grafts often decreases the survival rates of grafts to fail the organ transplantation [14–16]. It is well known, apoptosis includes two ways: one is death receptor way and the other is mitochondrial way, both can activate a series of cytosolic proteases to stir apoptosis of target cells [17–20]. Fas is one of the most important molecules in death receptor way and widely expressed in all kinds of tissues and organs *in vivo*. After tissues and organs were treated with anti-Fas antibody or Fas ligand (FasL), apoptosis is induced. So inhibiting Fas-mediated apoptosis is a new and effective way to improve

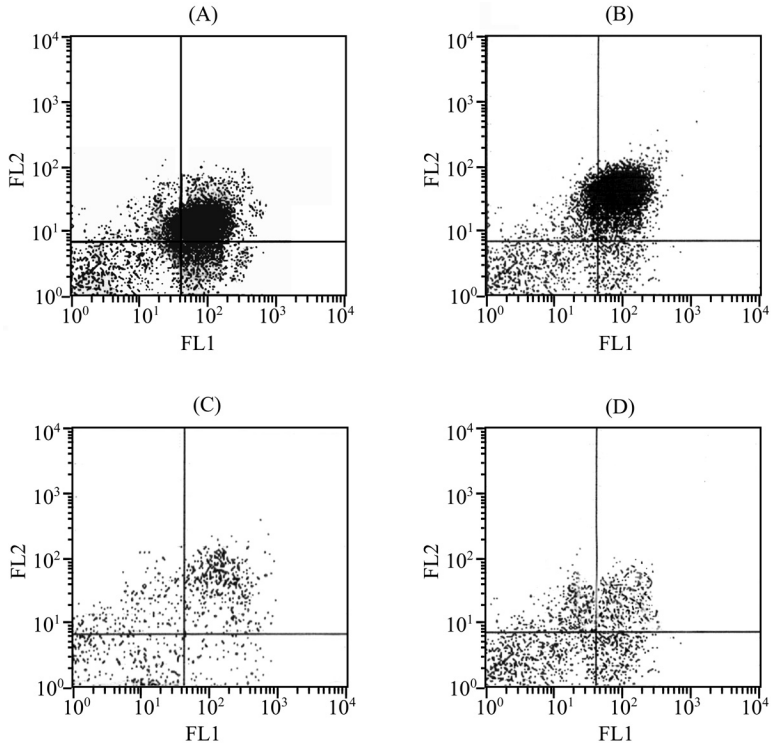

Fig. 8 Flow cytometry analysis of apoptosis induced by JO_2

(A) Empty control. (B) Apoptosis of mock-transfected cells. (C) Apoptosis of pU6-RZ596-transfected cells. (D) Apoptosis of pU6-dRZ596-transfected cells.

survival rate of grafts [21,22].

In view of this, the hammerhead ribozyme aiming at *fas* was designed and cloned into eukaryotic vector, which was then introduced into Yac-1 cells highly expressing Fas to study the effects of ribozyme on Fas-mediated apoptosis through electroporation-mediated transfection. Results showed that Rz596 was expressed on cells transfected with pU6-RZ596 and pU6-dRZ596, which was not expressed on control group and mock-transfected cells. Fas expression on cells transfected with anti-Fas ribozyme was remarkably lower than that on negative control and mock-transfected cells. After stimulation with apoptosis inducer JO_2 (imitating the role of FasL *in vivo*) for 24 h, apoptosis rate of cells transfected with anti-Fas ribozyme was obviously lower than that of negative control and mock-transfected cells, in accordance with, caspase-3 activity of ribozyme-transfected cells was much lower than that of other groups, but cells viability was higher than other two groups. It is thus clear that the hammerhead ribozyme aiming at 596 GUA triplets of *fas* RNA in this research can efficiently cleave *fas* mRNA, which decreases not only the Fas expression but also its function. In existence of apoptosis inducer, ribozyme-transfected cells' ability against apoptosis was enhanced. Anti-Fas hammerhead ribozyme designed by Dagmar *et al.* [23] can remarkably inhibit Fas-mediated apoptosis and improve the survival rates of islet cell grafts.

As for pU6-dRZ596 and pU6-RZ596, the difference between them is only that No. 3 base of the former near to the cleavage site was mutated from G to A. Although both can bind with target RNA in a complementation way, the cleavage activity of pU6-dRZ596 was much decreased, so the inhibition role of cells transfected with pU6-dRZ596 on Fas expression was lower than that of cells transfected with pU6-RZ596. After incubation with anti-Fas antibody, apoptosis rate induced by the former was much higher and caspase-3 activity was stronger, while the cells viability was lower. It is clear that hammerhead ribozyme can irreversibly cut the target site using its conservative hammerhead structure to inhibit gene expression, which is instead of a simple antisense blocking [24–26]. Compared with antisense RNA, ribozyme has two virtues at least: one is sequence specificity, the other is high efficiency and can be used repeatedly [27–29].

In short, anti-Fas ribozyme not only efficiently cleaves *fas* mRNA *in vitro*, but also inhibits Fas expression and Fas-mediated apoptosis to improve cells' survival rates *in vivo*. Because of widespread distribution of Fas in tissues [30,31], it is imagined that anti-Fas ribozyme can improve the survival rates of grafts and successful rates of organs transplantations through inhibiting the apoptosis of grafts in the future.

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