ISSN 1672-9145 Acta Biochimica et Biophysica Sinica 2004, **36**(8): 559–565 **CN** 31-1940/Q

Inhibiting Apoptosis of CTLL-2 Cells to Enhance Their GVL Effects via Anti-Fas Ribozyme

Min ZHANG, Fang LIU, Lin-Bo LIU, Yong YOU, Zhi-Chao CHEN, and Ping ZOU*

Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430022, China

Abstract To investigate the inhibition role of anti-Fas hammerhead ribozyme on *fas* expression and Fas-mediated apoptosis of CTL cell line CTLL-2 cells, the cDNA of an anti-Fas hammerhead ribozyme was synthesized, its expression plasmid was constructed and transfected into CTLL-2 cells by electroporation. *fas* expression of CTLL-2 cells was detected by RT-PCR and Western blot. CTLL-2 cell viability was measured using MTT assay when co-cultured with mouse T cell leukemia cell line EL4 cells that highly expressed Fas ligand (FasL). Meanwhile, caspase-3 proteolytic activity was detected, and cell apoptosis was measured by flow cytometry and Hochest-PI double staining. Killing activity of CTLL-2 cells was detected by lactate dehydrogenase (LDH) releasing assay *in vitro*. Results showed that the expression of both Fas mRNA and protein in CTLL-2 cells were decreased after transfection of anti-Fas ribozyme. Compared with mock-transfected group and mutant ribozyme-transfected group, viability of CTLL-2 cells co-cultured with EL4 cells was increased significantly and cells killing activity was enhanced after transfected with anti-Fas ribozyme, while the caspase-3 activity and apoptosis rate was significantly decreased. The results demonstrated anti-Fas ribozyme could efficiently cleave Fas and inhibit Fas-mediated apoptosis of CTLL-2 cells to improve their viability. Our study made a basis for enhancing CTLL-2 cells anti-leukemia effect in DLI.

Key words Hammerhead ribozyme; anti-Fas; CTLL-2 cells; apoptosis; tumor immunity

Donor lymphocyte infusion (DLI) is an adoptive immunotherapy to achieve particular therapy aims for patients accepting allogenetic hemopoietic stem cell transplantation [1–3]. Recently, many researches have testified that the graft-versus-leukemia effect (GVL) of donor lymphocytes after transplantation plays a basic and important role in curing leukemia. Experiments *in vitro* have conformed that T lymphocytes ($CD4^+$ and $CD8^+$ T cells) are very important for GVL effect induced by DLI [4,5]. The expression of Fas and Fas ligand (FasL) were increased in activated T cells [6,7], and leukemia cells could express high level of FasL , therefore induce the apoptosis of T cells through Fas-FasL way [8,9], so that the GVL effect of T cells was inhibited and leukemia cells escaped from immune response, which was the so-called "Fas counterattack" phenomenon [10,11].

Thus, reducing *fas* expression in CTL cells can inhibit T cell apoptosis and enhance GVL effect via Fas-FasL way. At present, there are many methods for inhibiting gene expression, including antibody, soluble receptor, corresponding ligand, antisense RNA and ribozyme [12,13]. Ribozyme is praised because of its specificity, confirmative effect and mature technique [14], in which the hammerhead ribozyme is widely used because of its many superiorities, including lower molecular weight, easy to design and synthesize [15,16].

Materials and Methods

Cell lines and cell culture

Mouse CTL cell line, CTLL-2, was from Shanghai Cell Bank, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium supplemented with 100

Received: April 15, 2004 Accepted: June 28, 2004

This work was supported by a grant from the National Natural Science Foundation of China (No. 30240022)

*Corresponding author: Tel, 86-27-85726880; E-mail, zhangmin35@yahoo.com.cn

IU/ml human recombinant IL-2 and 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Mouse lymphoma cell line Yac-1 and mouse T cell leukemia cell line EL4 were from Wuhan University Cultures Center, and the former was maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics, the latter was cultured in DMEM medium containing 10% FBS and antibiotics. All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

Bacteria and reagents

E. coli DH5α was a kind gift from Department of Immunology, Tongji Medical College. Prokaryotic vector pBSKU6 and green fluorescent protein (GFP) expression plasmid pEGFPC1 were saved in Lab of Institute of Hematology, Wuhan Union Hospital. All restriction endonucleases and T4 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 were products of MBI Company. Fluorescein isothiocyanate (FITC)-conjugated AnnexinV kit was product of Bender Company. FITC-conjugated anti-mouse Fas antibody (JO_2), FITC-conjugated anti-mouse FasL and mouse $IgG_2\alpha$ isotype control were from PharMingen Company. Rabbit anti-mouse Fas antibody and goat anti-rabbit IgG were purchased from Santa Cruz Company. Caspase-3 activity detection kit was a product of Clontech Company. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent was purchased from Sigma Company. Human recombinant IL-1α, IL-2 and IFN-γ were purchased from Biosource Company.

Cloning of anti-Fas ribozyme and its cleavage reaction *in vitro*

GUA triplets located at 596 bp of mouse Fas mRNA were selected as the cleavage site, the sequences of anti-Fas ribozyme cDNA strands were: a, 5′-TCTAGAGATATATAAACTGATGAGTCCGTGAGGACGAAACAAGTGGATCC-3′; b, 5′-GGATCCACTTGTTTCGTCCTCACGGACTCATCAGTTTATATATCTCTAGA-3′, which were annealed and named as RZ596. The No. 3 base near to the cleavage site of ribozyme was mutated from G to A, which was named as dRZ596. All cDNAs were synthesized by Shanghai Sangon Company. The mouse Fas cDNA plasmid pFas-596 was a kind gift from Prof. Pastori [17] (University of Miami School of Medicine). Construction of pU6-Rz and its cleavage reaction on Fas mRNA were performed as described previously, and two recombinants were named as pU6-RZ596 and pU6-dRZ596 individually [18].

fas expression in normal CTLL-2 cells and *fasL* expression on EL4 cells

CTLL-2 cells were cultured as mentioned above, *fas* expression was induced with 100 IU/ml IL-1α and 1000 IU/ml IFN-γ for 72 h. Then 10^6 CTLL-2 cells before and after treatment with cytokines were labeled with JO_2 for 30 min at 4 °C in dark, while EL4 cells of equal number were labeled with FITC-conjugated anti-mouse FasL at the same condition. The expression level of Fas and FasL were detected through FACScan (BD Company, America).

Stable transfection of ribozyme into CTLL-2 cells

The Fas^+ CTLL-2 cells successfully induced by cytokines were collected and grouped as following: cells transfected with pEGFPC1; cells transfected with pU6-RZ596 and cells transfected with pU6-dRZ596. The electroporation was performed with 2×10^6 cells/ml under the condition of 240 V, 40 μs, and shock once (electroporation instrument was purchased from Eppendorf Company) [19,20]. After 48 h culture, CTLL-2 cells were raised in medium containing 600 μg/ml G418 for 2 week. Then mouse lymphocytes separation solution was added into selected CTLL-2 cells, which were centrifuged at 2000 rpm for 20 min at room temperature to get active G418-resistant CTLL-2 cells. Then these cells were maintained in medium containing 200 μg/ml G418 for following study.

Detection of Fas mRNA in transfected CTLL-2 cells by using RT-PCR

Total RNA was extracted using Trizol reagent. PCR reaction was performed with synthesized cDNA as template and β-actin gene as internal control. The primers were as follows: *fas* sense primer 5′-GCTGCAGACATGCTGTGGATC-3′ and anti-sense primer 5′-TCACAGCCAGGAGAATCGCAG-3′; β-actin gene sense primer 5′-GACGATGATATTGCCGCACT-3′ and anti-sense primer 5′-GATACCACGCTTGCTCTGAG-3′. Reaction conditions for PCR were: pre-denaturation for 3 min at 95 °C; 30 cycles of denaturation for 30 s at 95 °C, annealing for 45 s at 58 °C, extension for 45 s at 72 °C, and extension for 3 min at 72 °C. PCR products were run on 2% agarose gel.

Detection of Fas protein in transfected CTLL-2 cells using Western blot

Cells were collected and treated with lysis buffer. The total protein concentration was measured to be 2 μg/μl, and separated on 10% SDS-polyacrylamide mini-gel. The

first antibody was rabbit anti-mouse-Fas antibody (1:200), and the rabbit anti-mouse β-actin (1:250) was used as control. The second antibody was goat anti-rabbit IgG (1:5000). The hybridized bands were detected with ECL and analyzed via Gel image analysis system.

Detection of CTLL-2 cell apoptosis through fluorescein activated cell sortor (FACS) and Hochest33258-PI double staining

EL4 cells without proliferation ability were obtained by treatment with 30 μg/ml mitomycin, and co-cultured with CTLL-2 cell transfected with different plasmid (pEGFPC1, pU6-RZ596 and pU6-dRZ596) respectively at the ratio of 1:3 for 24 h to induce CTLL-2 cell apoptosis. Then the cells were conjugated with Annexin V-FITC and PI respectively and the apoptosis was analyzed by FACS. Cells were also stained with Hochest33258 and PI, and observed under fluorescence microscope.

Caspase-3 protease activity

Apoptosis was induced as above. Meanwhile the negative controls without apoptosis and apoptosis cells treated with DEVD-fmk (a caspase-3 inhibitor) were established. After centrifugation, cells were sufficiently treated with cell lysis buffer, and the supernatant containing protein needed was retained. Then the caspase-3 protease activity was detected according to the instruction of manufacturer of Caspase-3 activity detection kit. The absorbance was measured at 405 nm.

MTT assay

CTLL-2 cells were transfected and co-cultured with EL4 cells for 5 d, and cell mixture was collected every 24 h. 100 μl mixture per well were inoculated into 96-well plate at a concentration about 5×10^5 cells/ml. After incubation with 10 μl 0.5 mg/ml MTT for 4 h at 37 °C, the formazan crystals were dissolved with 100 μl DMSO, and the absorbance was measured at 490 nm.

Killing role of CTL *in vitro*

CTLL-2 cells of three groups were used as effect cells, and well-grown Yac-1 cells were used as target cells. Effect cells and target cells were mixed at the ratio of 20:1 and co-cultured for 8 h. Then, lactate dehydrogenase (LDH) activity of the culture medium was detected on Automatic biochemical analysis meter (Beckman LX20). The calculation for killing activity of CTL was as Formula (1):

$$KA = \frac{L_E - L_N}{L_M - L_N} \times 100\% \quad (1)$$

KA, killing activity of CTL; L_E, LDH activity of experimental group; L_N, LDH activity of natural releasing control group; L_M, LDH activity of maximum releasing group.

Statistical analysis

Each experiment was performed three times. All data were presented as mean±SD. The statistical significance of ratios were analyzed by χ^2 test, and $P<0.05$ was considered statistically significant.

Results

***fas* expression in CTLL-2 cells and *fasL* expression in EL4 cells**

fas expression level in normal CTLL-2 cells was very low [Fig. 1(A)], the treatment of IL-1α and IFN-γ increased the Fas level to 76.0% [Fig. 1(B)]. The *fasL* expression in EL4 cells was 87.0% [Fig. 1(C)].

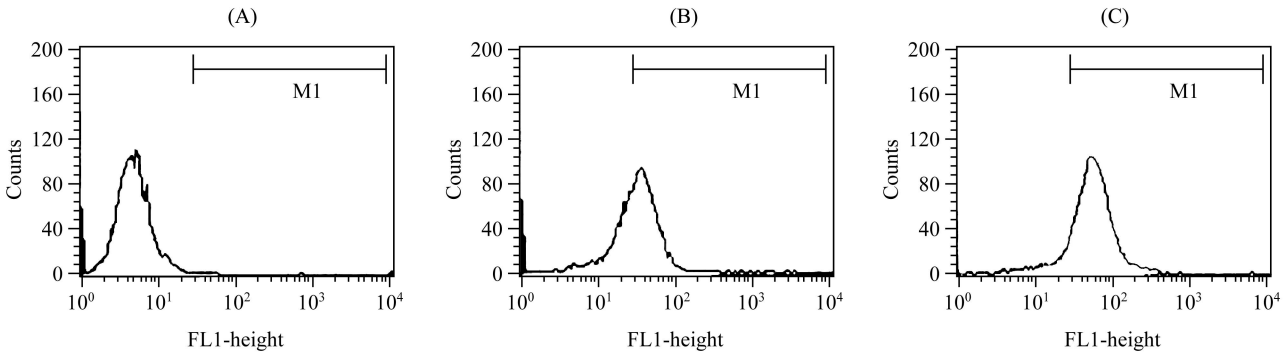

Fig. 1 *fas* expression on CTLL-2 cells and *fasL* expression on EL4 cells detected by using FACS

(A) *fas* expression on CTLL-2 cells was 3.5%. (B) *fas* expression on CTLL-2 cells treated with cytokines was 76.0%. (C) *fasL* expression on EL4 cells was 87%.

The effect of anti-Fas ribozyme on *fas* expression

RT-PCR amplification generated amplicons of 419 bp and 186 bp (for Fas and β-actin respectively) (Fig. 2). The Fas/β-actin ratio was 1.06±0.09 in control, 0.41±0.03 in pU6-RZ596-transfected cells, and 0.89±0.08 in pU6-dRZ596-transfected cells as determined by analyzing the luminescence using Gel image analysis system. It was clear that the differences of Fas mRNA expression among these groups were significant ($P<0.05$ or $P<0.01$).

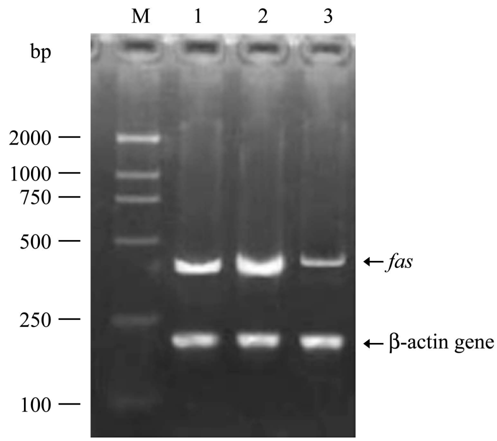

Fig. 2 *fas* gene transcription in three groups
M, DL-2000 DNA marker; 1, mutant ribozyme-transfected; 2, mock-transfected group; 3, ribozyme-transfected group.

Western blot analysis showed the ratios of Fas/β-actin in three groups were 1.00±0.09, 0.39±0.03 and 0.88±0.08 relatively (Fig. 3). It was obvious that Fas protein expressed

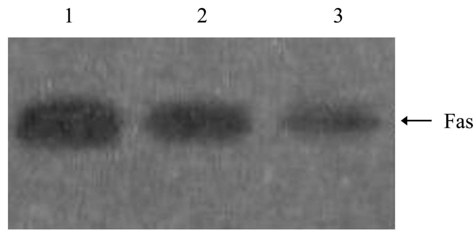

Fig. 3 Fas protein expression in three groups
1, mock-transfected group; 2, pU6-dRZ596-transfected group; 3, pU6-RZ596-transfected group.

in ribozyme-transfected cells was much lower than that in control and mock-transfected cells ($P<0.01$), which was consistent with the results of RT-PCR.

Inhibition of anti-Fas ribozyme on Fas-mediated CTLL-2 cell apoptosis

Cell apoptosis rate was detected by FACS, which was (85.0±7.2)%, (38.0±3.0)% and (58.0±4.9)% in turn for three groups of CTLL-2 cells (Fig. 4). Apoptosis rate of pU6-RZ596-transfected group was much lower than those of other two groups ($P<0.01$), and the difference between pU6-RZ596-transfected and pU6-dRZ596-transfected group was significant ($P<0.05$). Morphology of cell apoptosis was observed using Hochest33258-PI double staining (Fig. 5). Mock-transfected group showed many apoptosis cells (stained as blue) and some necrosis cells (stained as red). pU6-RZ596-transfected group showed no necrosis cells, pU6-dRZ596-transfected group showed many necrosis cells. Apoptosis cells were much smaller than necrosis cells and showed cellular shrinkage, while necrosis cells were swelling.

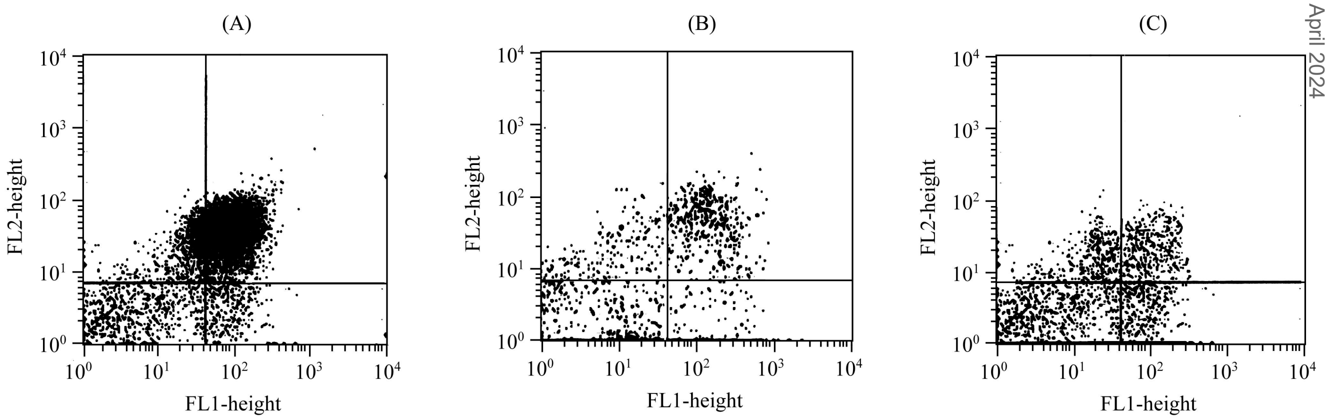

Fig. 4 Detection of apoptosis of CTLL-2 cells through flow cytometry
(A) Apoptosis rate of mock-transfected group was 85.0%. (B) Apoptosis rate of pU6-RZ596-transfected group was 38.0%. (C) Apoptosis rate of pU6-dRZ596-transfected group was 58.0%.

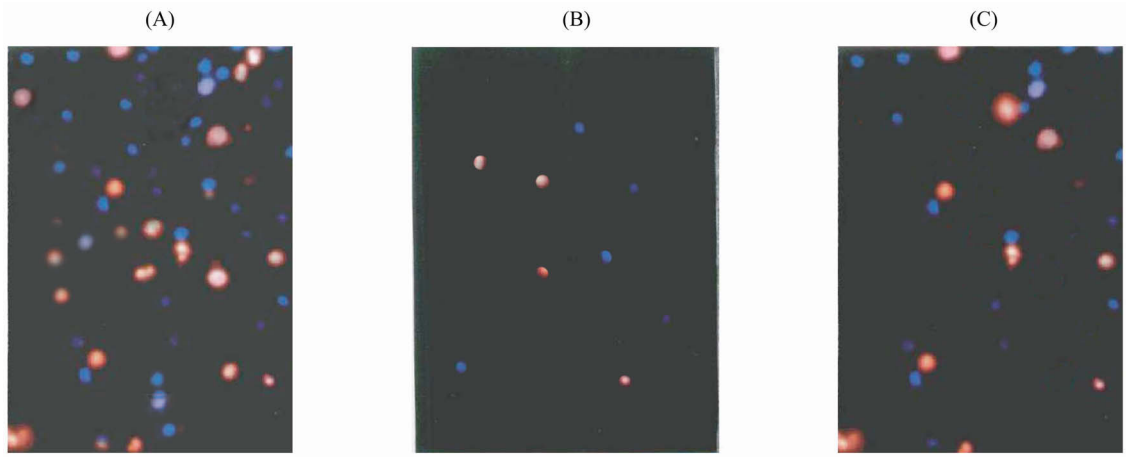

Fig. 5 Detection of apoptosis of CTLL-2 cells by using Hochest33258-PI double staining

(A) Apoptosis of mock- transfected cells. (B) Apoptosis of pU6-RZ596-transfected cells. (C) Apoptosis of pU6-dRZ596-transfected cells. Apoptosis cells were stained as blue, and necrosis cells red.

The effect of anti-Fas ribozyme on caspase-3 activity of CTLL-2 cell

After induction of apoptosis, the caspase-3 activity of CTLL-2 cells was particularly inhibited by DEVD-fmk. Compared with empty control, the caspase-3 activity of mock-transfected, pU6-RZ596-transfected and pU6-dRZ596-transfected group was (97.0±8.2)%, (40.0±3.4)% and (72.0±6.8)% respectively. It was obvious that the differences of caspase-3 activity among three groups were much significant (P<0.01), and the difference between pU6-RZ596-transfected and pU6-dRZ596-transfected cells was significant (P<0.05).

The effect of anti-Fas ribozyme on cell viability

After co-cultured with EL4 cells for 5 d, CTLL-2 cells viability was detected by MTT assay. As shown in Fig. 6, cells viability of three groups were declining, but cells viability of ribozyme-transfected group was much stronger than those of mutant ribozyme-transfected group and mock-transfected group (P<0.05), and ribozyme-transfected group has the stronger ability against Fas-mediated apoptosis.

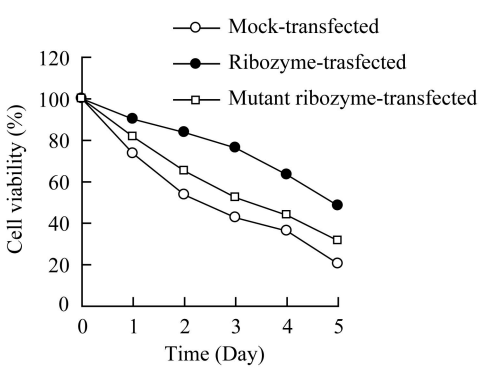

Fig. 6 Detection of CTLL-2 cells viability co-cultured with EL4 cells for 5 d by MTT assay

In vitro killing activity of CTL

Yac-1 cells were used as target cells. Killing activity of three groups was (26.0±2.0)%, (67.0±5.4)% and (41.0±3.5)% in turn. The killing activity of ribozyme-transfected group was stronger than those of other groups (P<0.05).

Discussion

Activated T cells express more Fas and FasL, which can kill leukemia cells via Fas-FasL pathway. But *in vivo*, leukemia cells can also express FasL and induce apoptosis of activated T cells expressing Fas, in addition, apoptosis can even be induced among activated T cells (such as one expressing Fas and the other expressing FasL) [21]. Beside antigen recognition signal and synergic stimulation signal [22,23], cytokines are essential for T cell activation [24,25]. Through imitating microenvironment *in vivo*, high-level *fas* expression in CTLL-2 cells was successfully induced by culturing in medium containing IL-2, IL-1α and IFN-γ. CTLL-2 cell could be used to study the effects of anti-Fas ribozyme, which was aiming at position 596 of Fas mRNA, on *fas* expression and Fas-mediated cell apoptosis. Synthesized anti-Fas ribozyme and its mutant

were cloned into pEGFPC1, then the recombinant plasmids were transfected into Fas^+ CTLL-2 cells using electroporation, meanwhile mock-transfected group were established. *fas* expression in cells transfected with ribozyme, detected by RT-PCR and Western blot, was obviously decreased. Different from other studies, EL4 cells expressing much FasL were selected as stimulating cells and co-cultured with Fas^+ CTLL-2 cells to induce CTLL-2 cell apoptosis, which imitated the process of Fas counterattack of leukemia cells *in vivo*. This model was established to detect the roles of anti-Fas ribozyme in Fas^+ CTLL-2 cells. After co-culturing with EL4 cells, apoptosis rate of ribozyme-transfected group was remarkably lower than those of mutant ribozyme-transfected and mock-transfected groups. Accordingly, caspase-3 activity of ribozyme-transfected group was much lower than those of other groups, but cell viability was stronger.

Apoptosis activates a series of proteases to induce programmed death of target cells through two ways, death receptor way and mitochondria way [26–28]. Eventually, caspase-3 is activated and its activity is increased when apoptosis rate enhances [29,30]. With the results above, it is clear that hammerhead ribozyme targeting position 596 of *fas* gene in this research can efficiently cleave mouse Fas mRNA, which inhibits not only the *fas* expression but also the function of Fas. In existence of factors inducing apoptosis as FasL, ability of CTLL-2 cells transfected with ribozyme against apoptosis was remarkably enhanced and cell viability was increased, which was consistent with the results of Klein *et al.*[31].

In addition, mouse lymphoma cell line-Yac-1 cells expressing more Fas were used as target cells to study killing activities of three groups cells *in vitro*. Results showed the killing activity of ribozyme-transfected group was much stronger than those of other groups. All these clarified that Fas-blocking T cells could avoid apoptosis induced by Fas^+ or $FasL^+$ T cells. The mutant ribozyme could inhibit *fas* expression in CTLL-2 cells and enhance their ability against apoptosis at some extent, but the effect was weaker. The reason for the difference between two ribozymes is that mutant ribozyme only blocks the gene expression just at the antisense RNA level, while ribozyme can not only block the gene expression but also cleave the RNA. That is to say, ribozyme is more efficient than mutant ribozyme on blocking gene expression.

In short, the ribozyme used in this study could significantly decrease *fas* expression in Fas^+ CTLL-2 cells and enhance cell ability resistant to apoptosis, so to increase the survival rate of CTLL-2 cells and enhance the killing activity *in vitro*, then reinforce the GVL effects. However, it is necessary to further study the roles and side effects of anti-Fas ribozyme in animals and humans [32,33].

Acknowledgements

We thank Dr. Ling-Bo LIU and Dr. Wen-Shu CHENG for their kind assistances in this work.

References

1 Kircher B, Wolf M, Stevanovic S, Rammensee HG, Grubeck-Loebenstein B, Gastl G, Nachbaur D. Hematopoietic lineage-restricted minor histocompatibility antigen HA-1 in graft-versus-leukemia activity after donor lymphocyte infusion. J Immunother, 2004, 27: 156–160

2 van der Mast BJ, van den Dorpel MA, Drabbels JJ, Class FH, Weimar W. Transfusion-associated graft vs. host disease after donor-specific leukocyte transfusion before kidney transplantation. Clin Transplant, 2003, 17: 477–483

3 Kumar S, Chen MG, Gastineau DA, Gertz MA, Inwards DJ, Lacy MQ, Tefferi A *et al*. Lymphocyte recovery after allogeneic bone marrow transplantation predicts risk of relapse in acute lymphoblastic leukemia. Leukemia, 2003, 17: 1865–1870

4 Ji YH, Weiss L, Zeira M, Abdul-Hai A, Reich S, Schuger L, Slavin S. Allogeneic cell-mediated immunotherapy of leukemia with immune donor lymphocytes to upregulate antitumor effects and downregulate antihost responses. Bone Marrow Transplant, 2003, 32: 495–504

5 Mapara MY, Kim YM, Marx J, Sykes M. Donor lymphocyte infusion-mediated graft-versus-leukemia effects in mixed chimeras established with a nonmyeloablative conditioning regimen: Extinction of graft-versus-leukemia effects after conversion to full donor chimerism. Transplantation, 2003, 76: 297–305

6 Toth B, Ludanyi K, Kiss I, Reichert U, Michel S, Fesus L, Szondy Z. Retinoids induce Fas (CD95) ligand cell surface expression via RARgamma and nur77 in T cells. Eur J Immunol, 2004, 34: 827–836

7 Lawson BR, Baccala R, Song J, Croft M, Kono DH, Theofilopoulos AN. Deficiency of the cyclin kinase inhibitor p21(WAF-1/CIP-1) promotes apoptosis of activated/memory T cells and inhibits spontaneous systemic autoimmunity. J Exp Med, 2004, 199: 547–557

8 Grullich C, Richter M, Exner S, Finke J. Fas ligand is not constitutively expressed in low-grade B-cell lymphoma and B-lymphoblastoid cells. Eur J Haematol, 2003, 71: 184–188

9 Hashimoto MN, Kimura EY, Yamamoto M, Bordin JO. Expression of Fas and Fas ligand on spleen T cells of experimental animals after unmodified or leukoreduced allogeneic blood transfusions. Transfusion, 2004, 44: 158–163

10 Nagarkatti N, Davis BA. Tamoxifen induces apoptosis in Fas^+ tumor cells by upregulating the expression of Fas ligand. Cancer Chemother Pharmacol, 2003, 51: 284–290

11 Groneberg C, Pickartz T, Binder A, Ringel F, Srock S, Sieber T, Schoeler D *et al*. Clinical relevance of CD95 (Fas/Apo-1) on T cells of patients with B-cell chronic lymphocytic leukemia. Exp Hematol, 2003, 31: 682–685

12 Alvarez-Salas LM, Benitez-Hess ML, Dipaolo JA. Advances in the development of ribozymes and antisense oligodeoxynucleotides as antiviral agents for human papillomaviruses. Antivir Ther, 2003, 8: 265–278

13 Kim K, Umamoto S, Trang P, Hai R, Liu F. Intracellular expression of engineered RNase P ribozymes effectively blocks gene expression and replication of human cytomegalovirus. RNA, 2004, 10: 438–447

14 Oshima K, Kawasaki H, Soda Y, Tani K, Asano S, Taira K. Maxizymes and small hairpin-type RNAs that are driven by a tRNA promoter specifically cleave a chimeric gene associated with leukemia *in vitro* and *in vivo*. Cancer Res, 2003, 63: 6809–6814

15 Saksmerprome V, Burke DH. Structural flexibility and the thermodynamics of helix exchange constrain attenuation and allosteric activation of hammerhead ribozyme TRAPs. Biochemistry, 2003, 42: 13879–13886

16 de la Pena M, Gago S, Flores R. Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. EMBO J, 2003, 22: 5561–5570

17 Klein D, Denis M, Ricordi C, Pastori RL. Assessment of ribozyme cleavage efficiency using reverse transcriptase real-time PCR. Mol Biotechnol, 2000, 14(3): 189–195

18 Zhang M, Liu F, He W, You Y, Zou P, Liu LB. Inhibition of Fas-mediated apoptosis in Yac-1 cell via anti-Fas ribozyme. Acta Biochim Biophys Sin, 2004, 36(3): 199–205

19 Lai W, Chang CH, Farber DL. Gene transfection and expression in resting and activated murine CD4 T cell subsets. J Immunol Methods, 2003, 282: 93–102

20 Dayball K, Millar J, Miller M, Wan YH, Bramson J. Electroporation enables plasmid vaccines to elicit $CD8^+$ T cell responses in the absence of $CD4^+$ T cells. J Immunol, 2003, 171: 3379–3384

21 Fujii S, Shimizu K, Koji F, Kawano F. Malignant counterpart of myeloid dendritic cell (DC) belonging to acute myelogenous leukemia (AML) exhibits a dichotomous immunoregulatory potential. J Leukoc Biol, 2003, 73: 82–90

22 Conrad B. Potential mechanisms of interferon-alpha induced autoimmunity. Autoimmunity, 2003, 36: 519–523

23 Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of $CD4^+CD25^+$ T cell suppressor function. Eur J Immunol, 2004, 34: 366–376

24 Sugimoto T, Ishikawa Y, Yoshimoto T, Hayashi N, Fujimoto J, Nakanishi K. Interleukin 18 acts on memory T helper cells type 1 to induce airway inflammation and hyperresponsiveness in a naive host mouse. J Exp Med, 2004, 199: 535–545

25 Laurence A, Astoul E, Hanrahan S, Totty N, Cantrell D. Identification of pro-interleukin 16 as a novel target of MAP kinases in activated T lymphocytes. Eur J Immunol, 2004, 34: 587–597

26 Ryan L, O'Callaghan YC, O'Brien NM. Generation of an oxidative stress precedes caspase activation during 7beta-hydroxycholesterol-induced apoptosis in U937 cells. J Biochem Mol Toxicol, 2004, 18: 50–59

27 Knight MJ, Riffkin CD, Ekert PG, Ashley DM, Hawkins CJ. Caspase-8 levels affect necessity for mitochondrial amplification in death ligand-induced glioma cell apoptosis. Mol Carcinog, 2004, 39: 173–182

28 Deszcz L, Seipelt J, Vassilieva E, Roetzer A, Kuechler E. Antiviral activity of caspase inhibitors: Effect on picornaviral 2A proteinase. FEBS Lett, 2004, 560: 51–55

29 Eliner SG, Yoshida A, Bian ZM, Kindezelskii AL, Petty HR, Elner VM. Human RPE cell apoptosis induced by activated monocytes is mediated by caspase-3 activation. Trans Am Ophthalmol Soc, 2003, 101: 77–89

30 Chae IH, Park KW, Kim HS, Oh BH. Nitric oxide-induced apoptosis is mediated by Bax/Bcl-2 gene expression, transition of cytochrome c, and activation of caspase-3 in rat vascular smooth muscle cells. Clin Chim Acta, 2004, 341: 83–91

31 Klein D, Ricordi C, Pugliese A, Pastori RL. Inhibition of Fas-mediated apoptosis in mouse insulinoma betaTC-3 cells via an anti-Fas ribozyme. Hum Gene Ther, 2000, 11: 1033–1045

32 Hasegawa S, Jackson WC, Tsien RY, Rao J. Imaging Tetrahymena ribozyme splicing activity in single live mammalian cells. Proc Natl Acad Sci USA, 2003, 100: 14892–14896

33 Bok D. Gene therapy of retinal dystrophies: Achievements, challenges and prospects. Novartis Found Symp, 2004, 255: 4–12

Edited by
You-Xin JIN