ISSN 1009-3095 *Journal of Zhejiang University(SCIENCE)* V.2, No.1, P.100-103, Jan.-Mar., 2001
http://www.chinainfo.gov.cn/periodical; http://www.zju.edu.cn;
http://lib.zju.edu.cn/English; http://www.zjupress.com

IN VITRO STUDY ON RIBOZYME AGAINST HEPATITIS C VIRUS

CHEN Zhi (陈 智)[1], LIU Yong (刘 勇)[1], LIU Ke-zhou(刘克洲)[1],
Dennin R.H[2]., Dou Jun(窦 骏)[1], Reinhard U[2]

([1] *Institute of Infectious Diseases, Medical College, Zhejiang University, Hangzhou 310003, China*)

([2] *Institute of Microbiology, Lubeck Medical University, Germany*)

Received Aug. 18, 1999; revision accepted July 5, 2000

Abstract: Objective: To determine the effective nucleotide sites of ribozymes against HCV, and obtain a highly effective, nontoxic and inexpensive antisense ribozyme specific for HCV. Methods: Two effective ribozymes, targeted to HCV 5'NC region and C region, were designed and synthesized. Eukaryotic expression vectors, pSV2-gpt.CD-SR (, containing either HCRZNC or HCRZC were constructed and transfected into MT-2 cells, which had been infected by HCV. Quantitative PCR and hybridization were used to determine the effect of inhibition of HCV by ribozymes. Results: HCRZNC and HCRZC suppressed the replication of HCV by 54.7% and 62.1%, respectively. Furthermore, when both ribozymes were cotransfected into cells, they suppressed replication by 78.8%. Conclusion: Two specific antisense ribozymes have strong inhibitory effects on the replication of HCV in cultured cells, and have better effect when used together.

Key words: ribozyme, inhibition, hepatitis C virus, MT-2 cells
Document code: A **CLC number:** R575

INTRODUCTION

Ribozyme was first described by Cech and Altman in 1981. This form of RNA had highly specific endonuclease activities to RNA (Kruger et al., 1982). Ribozymes have three structures: hammerhead, hairpin and axe ribozymes. Among them, the hammerhead ribozyme has a simpler secondary structure, including upstream and downstream sequences, which can be combined with the target RNA, and the hammerhead domain can cleave target RNA at a certain position(Sakamoto et al., 1996). Ribozyme can reconstruct its secondary structure to avoid being degraded itself and then be used in recycle. Natural ribozyme in some RNA viruses may affect the three dimension structure of the viruses in a cis-effect style, which may benefit viral replication. Haseloff and Gerlach (1988) constructed a specific antisense ribozyme, which was thought to have some potential activities for application in the treatment of viral diseases and malignancies.

Sarver et al. (1990) reported that ribozyme inhibited the replication of HIV in cultured cells. Ribozyme has been used therapeutically to a limited extent (Ohkawa et al., 1995). However, the following problems must be solved before ribozyme can be used in clinical trials: how to choose the best cleavage site, how to obtain highly effective, nontoxic and inexpensive specific antisense ribozyme, how to improve the efficiency of ribozyme's transfection into target cells as well as the problem of stability. Based on the HCV infected cell model which we developed, the inhibition of HCV replication by specific hammerhead ribozyme is reported here and the mechanisms are discussed.

METHODS

1. The HCV-infected MT-2 cell model

The MT-2 cell line was kindly supplied by Dennin RH. The HCV infected MT-2 cells were harvested every 3 to 4 days after the infection. Total RNA was extracted and the positive strand and negative strand of HCV were measured. The cells left were incubated with fresh medium. The PCR products were identified by HCV probe after their Southern transfer.

2. Recombination and expression of pSV2-gpt. CD-SR α with ribozyme against HCV

Ribozymes against the NC region and C region of HCV and their antisense primers synthesized according to the protocol of Sakamoto N. et al. (1996), were hybridized with template ribozyme genes , and extended with Taq DNA polymerase. This target DNA was ligated to the clone vector in a TA kit, and then transferred to TOP 10F' strain and screened by ampicillin and X-gal/IPTG, after which ampicillin-resistant white transformants were selected . After being digested by EcoR I and recovered from 4% agarose gels by Agrose Gel DNA Extraction Kit (Boehring Mannheim, Cat No: 1696505), the target DNA was subcloned into pSV2-gpt. CD-SR α vectors. Transformants with one point mutation in the active region of the ribozyme were constructed at the same time so that we could determine if the cleavage of ribozyme or the blockage of antisense nucleotide inhibits the replication of HCV. The colonies were all screened by hybridization, and named HCRZNC (NC region), HCRZC (C region) and HCRZCM (point mutation).

3. Transfection of HCV infected MT-2 cells with pCD vector containing HCV ribozyme

The calcium phosphate-DNA suspension was transferred into the medium above the MT-2 cells on the fifth day of HCV infection. (1) Exponentially growing cells were harvested from the 1640 medium containing 10% bovine serum albumin , and then were washed twice with phosphate buffer (PBS pH 7.4), and finally suspended in 30 μl of 2 × HBS solution. (2) The calcium phosphate-DNA coprecipitate was prepared as follows : 407.5 μl of sterile water, 62.5 μl of $CaCl_2$ and 20 μl (1 μg/μl) recombinate vector were placed into a sterile microcentrifuge tube, after their being mixed well, and 500μl of 2 × HBS solution was added dropwise with gentle mixing. The mixture was incubated for 20 – 30 minutes at room temperature until a fine precipitate formed. (3) The calcium phophate-DNA suspension was gently mixed with MT-2 cells, and incubated for 30 minutes at room temperature. Then the mixture was transferred to an 8-well dish (NUNC), to which 8 ml 1640 medium containing 1 × RPMI (pH 7.4) was added, and after incubation for 12 hours, the suspension was centrifuged at 800 rpm for 5 minutes at room temperature. The supernatant was discarded , the pellet was washed once with PBS, and finally resuspended in 10 ml medium containing 10% FCS; (4) the cells were harvested 60 hours after transfection , and the pellet was resuspended with 1 ml XHTA-M selective medium containing 20% FCS, 250 μg/ml xanthine , 15 μg/ml hypoxanthine , 10 μg/ml thymine , 2 μg/ml methotrexate, 25 μg/ml mycophenolic acid, and 1 × RPMI 1640, pH 7.4. The positive clone was separated by limited dilution method.

4. Harvesting of cells and extraction of RNA

3×10^5 cells were harvested on the third, sixth and ninth day after transfection. Total RNA was extracted with Trizol Reagent (GIBCO BRL), and HCV RNA was detected by qualitative method. 100 base pair marker was used (GIBCO, Cat No 1721933). On the 9th day, HCV RNA was also detected by quantitative method.

5. Nucleic acid hybridization

The artificial fragments of ribozyme against the NC region and C region of HCV and one point-mutation of C region and their primers were all labeled with digoxinin by Dig Oligonucleotide 3'-End Labeling Kit (Boehring Mannheim) (according to the instructions) and then hybridizd and detected by Detection Kit.

6. Detection of HCV RNA by PCR

Quanlitative PCR for HCV RNA was used following Chen et al. (1995). Quantitative detection of HCV RNA was made with Quantitative Detection Kit (according to the instructions).

RESULTS

HCV RNA in MT-2 cells was detected from the 7th day to 28th day after the challenge. Negative strand of HCV was detected from the 10th day to 18th day. MT-2 cells cocultured with HCV negative serum had no HCV RNA during the whole culture time.

Qualitative PCR and hybridization showed that the positive trace in cells infected by HCRZNC or HCVZC was lower than that in cells infected by HCRZCM or pSV2-gpt. CD-SR α

containing no ribozyme (Fig 1). Fig 2 shows the results of quantitative PCR. The inhibition rate of HCRZCN, HCRZC and both of them were 54.7%, 62.1% and 78.8%, respectively.

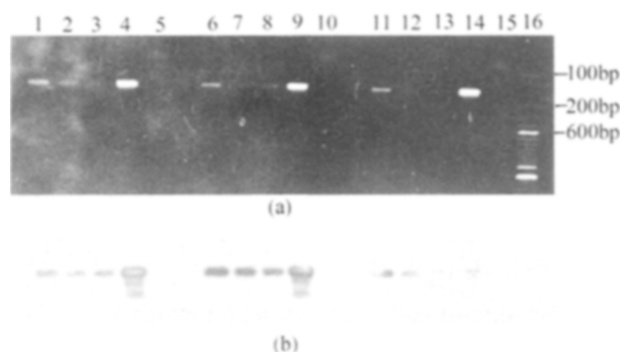

Fig.1 The qualitative RT-PCR of HCV in HCV infected MT-2 cells transfected with ribozymes. (a) shows the fragment of HCV on gels; (b) shows the result of hybridization. The cells were collected at 3rd (lane 1 – 5), 6th (lane 6 – 10) and 9th (lane 11-15) days. The cells infected by HCRZNC (lane 2, 7, 12) or HCRZC (lane 3, 8, 13) showed less positive trace than HCRZCM (lane1, 6, 11) or pSV2-gpt. CD-SR α containing no ribozyme (lane 4, 9, 14), and MT-2 cells without HCV (lane 5, 10, 15) used as negative control. Lane 16 is 100bp marker.

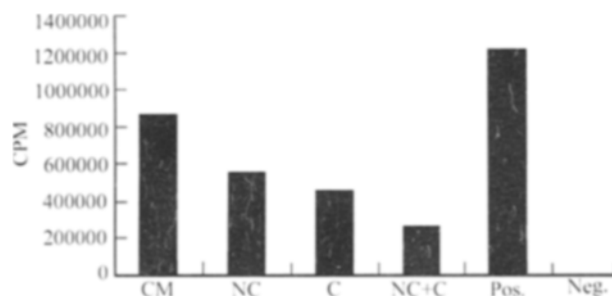

Fig.2 The quantitative RT-PCR of HCV in HCV infected MT-2 cells transfected with ribozymes. According to the positive (pSV2-gpt. CD-SR α containing no ribozyme) and negative controls (MT-2 cells without HCV), the inhibition rate of HCRZCN (NC), HCRZC (C) and both of them (NC + C) were 54.7%, 62.1% and 78.8%, respectively.

There was no significant change of HCV RNA in HCV infected MT-2 cells by HCRZCM. The suppression was 28.3%. It was thought that the inhibition was related to the specific combination site of ribozyme against HCV.

Compared to negative control, cells transfected by ribozyme had no difference in morphology and growth. The results showed there was no toxic effect of ribozyme in the dose used here on cultured cells.

DISCUSSION

Persistent hepatitis C (HCV) infection often lead to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma. IFN is the major medicine for it. But only 20 – 30% patients had satisfactory result (Hagiwara et al., 1993, Mitta et al., 1994, Kasahara et al., 1995). There is a need to develop new antiviral approaches for the treatment of this disease. There is much progress in investigation of ribozyme as an antiviral method.

Weizacker et al. (1992) constructed and expressed a multitarget ribozyme against three regions near the HBV core region in vitro, and found it could cleave HBV RNA effectively, which may decrease the possibility of virus escape from attack by mutation. Larsson S. et al. (1996) constructed a hammerhead ribozyme with a 6 + 7-nt antisense specificity for the nef 9016 – 9029 site and transfected it into the human T-cell line HUT78, and observed a dose-dependent repression of the expression of pol and gag. Welch PJ et al. (1996) constructed two effective hairpin ribozymes that target HCV RNA 5' UTR and capsid gene regions, and demonstrated they can inhibit the expression of a cotransfected reporter gene containing HCV RNA target sequences in vitro. They found that transduction of human hepatoma cells, HepG2, with retroviral vectors carrying those two ribozymes enabled the cells to resist the infection by retroviral particles containing HCV target sequences.

Because synthesis of RNA in vitro is expensive, and RNA is easily degraded by RNAse, the strategy of ribozyme gene therapy should consider gene expression. When the RNA transcribed from ribozyme into the cells, it may be protected by the natural modification, and keep stable in the cells. In this study eukayronic expression vector pSV2-gpt. CD-SR α containing two antisense ribozymes against the NC region and C region of HCV were constructed, and transfected into HCV infected MT-2 cells under conditions similar to those in nature. The results showed that HCRZCN and HCRZC and both of them

combined, could suppress HCV by 54.7%, 62.1% and 78.8%, respectively. Both of the two ribozymes significantly suppress HCV in vitro, and have better effect when used together. Compared to antisense nucleic acid, ribozyme has stronger and longer effect and fewer side effects against HCV.

References

Chen, Z, Dennin R.H., He, N. X. et al., 1995. Detection of minus strand RNA of HCV in liver tissues of patient with hepatitis C and hepatocellular carcinoma by RT-PCR. *Chinese J. of Microbiology and Immunology*, **15**(2): 116 - 118 (in Chinese, with English abstracct).

Hagiwara, H., Hangashi, N., Mitta, E. et al., 1993. Quantitative analysis of hepatitis C virus RNA in serum during interferon therapy. *Gaseroenterology*, **104**: 877.

Haseloff, J., Gerlach, W. L., 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature*. 18, **334**(6183):585.

Kasahara, A., Hagiwara, H., Hayasi, N., et al., 1995. Ability of prolonged interferon treatment to suppress ralapse after cessation of therapy in patients with chronic hepatitis C a multicenter randomized controlled trial. *Hepatology*, **21**:291 - 297.

Kruger, K, Grabowski, P. J, Zaug A. J. et al., 1982. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of tetrahymena. *Cell*. **31**(1):147.

Mitta, E., Hayashi, N., Hagiwara H, et al., 1994. predicting interferon therapy efficacy from hepatitis C virus genometype and RNA titer. *Dig. Dis Sci*; **39**:977

Ohkawa, J., Koguma, T., Kohda, T., et al., 1995. Ribozymes: from mechanistic studies to applications in vivo. *J Biochem (Tokyo)*. **118**(2):251.

Sakamoto, N., Wu, C. H., Wu, G. Y., 1996. Intracellular cleavage of hepatitis C virus RNA and inhibition of viral protein translation by hammerhead ribozymes. *J Clin Invest*. **98**(12):2720.

Sarver, N., Cantin, E. M., Chang P. S. et al., 1990. Ribozymes as potential anti-HIV-1 therapeutic agents. *Science*. **247**(4947):1222.

Weizsacker I. F., Blum H. E., Wards G. R., 1992. Cleavage of hepatitis B virus RNA by three ribozymes transcribed from a single DNA template. *Biochem Biophys Res Commun*. **189**: 243.

Welch, P. J., Tritz, R., Yei. S., et al., 1996. A Polential therapeutic application of hairpin ribozymes: *in vitro* and *in vivo* studies of gene therapy for hapatitis C virus infeetion. *Gene ther*. **3**(11): 994 - 1001.