Inhibitor RNA blocks the protein translation mediated by hepatitis C virus internal ribosome entry site in vivo

Xue-Song Liang, Jian-Qi Lian, Yong-Xing Zhou, Mo-Bin Wan

INTRODUCTION

Hepatitis C virus (HCV) is the primary causative agent of parenterally transmitted non-A, non-B hepatitis and affects a significant part of the world population. HCV infection frequency leads to chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma. The genome of HCV is a single-stranded, plus-polarity RNA. The 5' untranslated region (UTR) of HCV RNA is approximately 340 nt long, and contains multiple AUG codons. The 5' UTR is highly conserved among different strains of HCV. Nucleotides 40 to 370 of the 5' UTR of HCV have been shown to contain an internal ribosome entry site (IRES)[1-3]. The presence and stability of IRES play an important role in virus life cycle, so the region has become the target of antiviral gene therapy[4-16]. Coward and Dasgupta found that gene expression mediated by polio virus (PV) IRES was inhibited by one 60 nt long RNA which is called inhibitor RNA (IRNA). Because HCV and PV IRES elements bound to similar polypeptides[16-20], it was reasoned that IRNA might also interfere with HCV IRES-mediated translation. Using transient transfection of hepatoma cells and a hepatoma cell line constitutively expressing IRNA, we demonstrated specific inhibition of HCV IRES-mediated translation by IRNA in vivo.

MATERIALS AND METHODS

Materials

Vectors pcRz-IRNA and pcRz-mRNA were constructed by our laboratory, which introduced the sequences of 5' and 3' cis-self cleavage ribozyme into both sides of IRNA or mRNA sequence[21]. pcMVMNCRluc contain full sequence of HCV 5' UTR and 66 nt core gene, and was fused with lucerase gene (generous gift of professor Alt). pcHCVcluc was constructed by our laboratory containing full sequence of HCV 5' UTR and partial sequence of core region, and could express in cells stably.

Methods

Cell culture Human hepatocarcinoma cell (HHCC) HepG2 was grown in RPMI1640 medium supplemented with 100 mL/L newborn calf serum.

Plasmid construction By using subcloning methods, IRNA and mRNA sequence were cloned into the pcDNA3 vector, yielding pcRz-IRNA and pcRz-mRNA which introduced the ribozyme sequence over both sides of IRNA and mRNA sequence to generate the correct side of IRNA and mRNA[16]. In brief, by using PCR methods the sequences of target RNA were generated from pGRz-IRNA or pGRz-mRNA which was constructed by our laboratory. Then the PCR product was cloned into the BamHI-ApaI sites of the pcDNA3 vector.

Establishment of stable hepatoma cell line expressing IRNA or cloning HCV replicon Plasmids pcRz-IRNA, pcRz-mRNA, pcDNA3 and pcHCVcluc were transfected into HHCC respectively by using Lipofectamine 2000 reagent (GIBCO) and screened for neomycin resistance with 300 µg/mL of geneticin (G418 ) (Invitrogen) per milliliter for 4 weeks. The antibiotic-resistant cell clones were harvested and further screened by dilution titer.

RESULTS: Transient expression of IRES specific IRNA could significantly inhibit the expression of reporter gene and viral antigen mediated by HCV IRES by 50% to 90% in vivo, but mRNA lost its inhibitory activity completely. The lucerase gene expression mediated by HCV IRES was blocked in the HHCC constitutively expressing IRNA. At 48 h after transfection, the expression level of reporter gene was decreased by 20%, but cap-dependent lucerase gene expression was not affected. IRNA could inhibit the HCV replicon expression 24 h after transfection and the highest inhibitory activity was 80% by 72 h, and the inhibitory activity was not increased until 7d after transfection.

CONCLUSION: IRNA can inhibit HCV IRES mediated gene expression in vivo.

Liang XS, Lian JQ, Zhou YX, Wan MB. Inhibitor RNA blocks the protein translation mediated by hepatitis C virus internal ribosome entry site in vivo. World J Gastroenterol 2004; 10 (5): 664-667

http://www.wjgnet.com/1007-9327/10/664.asp
Detection of IRNA in cell lines
IRNA or miRNA expression in the cells was measured by isolating total RNA from these cells and IRNA or miRNA were detected by reverse transcriptase (RT)-mediated PCR (RT-PCR) by using IRNA or miRNA specific oligonucleotide primers. One to 2 μg of total RNA isolated from the IRNA or miRNA expressing cells, and 2 μg total RNA from HHCC control cells were reversely transcribed by murine leukemia virus RT using random hexamer primers in 20 μL reaction mixture according to the TaKaRa RNA PCR kit protocol. Twenty pmol of each primer (corresponding to 5’ nt 1 to 20 and 3’ nt 1 to 20 of the IRNA or miRNA sequence) was used to amplify the 60-nt fragment in 100 μL PCR reaction. The cycling parameters were as follows: denaturation at, 95 °C for 1 min, annealing at, 65 °C for 1 min, extension at, 72 °C for 1 min, a total of 50 cycles, then total extension at, 72 °C for 10 min. Twenty microliters of each reaction product were loaded onto 20 g/L gel and visualized by ethidium bromide staining.

Detection of HCV core protein expression in HHCC
HCV core protein was detected by using indirect immune fluorescence method. HCV replicon cells were plated on a cover glass and fixed with pure ethanol for 10 min. Monoclonal antibody of HCV core protein was properly diluted (1:100) and covered on the glass with HCV replicon cells for 1 h at 37 °C, and then the glass was washed 3 times with PBS (10 min each). Then FITC labeled second antibody was covered on the glass at 37 °C for 1 h and the glass was washed 3 times again with PBS. At last the cells were examined by using fluorescence microscopy or laser confocal microscopy.

DNA transfection
For each transfection assay, 1×10⁶ HHCC cells in 30-mm-diameter plates were transfected with 15 μL of lipofectin (GIBCO) and 2 to 5 μg of plasmid DNA. At 16 h post transfection, cell lysates were prepared according to the luciferase assay kit protocol (Promega) and assayed for both β-galactosidase (β-Gal) and luciferase expression.

RESULTS
Inhibitory effect of IRNA transient expression on HCV IRES-mediated translation
To test the possibility that IRNA interfered with HCV IRES-mediated translation, human hepatocellular carcinoma cells (HepG2) were transiently cotransfected with three plasmids: a reporter gene expressing luciferase programmed by the HCV IRES element (pCMVNCrluc), pSV-β-galactosidase to measure transfection efficiency, and the plasmid expressing IRNA (pcRz-IRNA). All transfections were done in triplicate and contained equal amounts of the luciferase reporter and β-Gal plasmid. Increasing concentrations of plasmid pcRz-IRNA were used in various reactions, and the total amount of DNA in each reaction was kept constant by addition of an appropriate amount of a nonspecific DNA (pcDNA3). Following transfection, luciferase activity was measured in cell extracts at 48 h. At the lowest concentration of the IRNA plasmid, inhibition of luciferase activity from plasmid pCMVNCrluc was approximately 50% compared to the control. However, at the highest concentration, 92% of luciferase activity was inhibited. Translation of luciferase from a control plasmid (pCDNA-luc) without HCV IRES was not significantly inhibited by IRNA. (P>0.05, Table 1).

In order to determine the inhibitor effect of IRNA on HCV IRES-mediated translation further, the HHCCs were transfected with pCMVNCrluc or cotransfected with pcRz-IRNA and pCMVNCrluc. Following transfection, the HCV core protein programmed by HCV IRES was detected by using laser confocal microscopy. HCV core protein could express efficiently in the HHCC cells as shown in Figure 1. But IRNA plasmid cotransfection could inhibit HCV core protein expression. The pels density of HCV core protein was different between the two groups (58.05±42.24 vs 15.56±8.54). The inhibitory rate was plotted by 1-pels density of IRNA transfection group/pels density of control x100%.

Table 1 Inhibitory effect of IRNA on HCV IRES mediated gene translation

| Sample  | pcRz-IRNA µg | Activity of luciferase programmed by cap-dependent mechanism (IU/U) | Activity of luciferase programmed by HCV IRES (IU/U) | Inhibitory rate (%) |
|---------|-------------|---------------------------------------------------------------|-------------------------------------------------|------------------|
| 0       |             | 55.5±3.11                                                   | 52.49±2.31                                    | pCDNA-luc        |
| 2       |             | 60.1±2.31                                                  | 27.0±0.740                                   | 0                | 50       |
| 4       |             | 58.3±1.89                                                  | 14.7±0.380                                   | 0                | 72       |
| 6       |             | 52.4±2.12                                                  | 4.03±0.120                                   | 4                | 92       |

Figure 1 Stable expression of IRNA and miRNA in HHCC cells. 1: DL2000 DNA marker; 2, 3: IRNA RT-PCR; 4: miRNA RT-PCR; 5: IRNA PCR.

Construction of hepatoma cell line expressing IRNA or miRNA
To determine the long-term effect of RNA expression in HepG2, the cell line constitutionally expressing IRNA was generated by using a pcDNA-based vector as described in Material and Methods. In order to obtain both the correct and stable sites of the expressed IRNA, ribozyme sequences were introduced into both sides of IRNA and miRNA. IRNA or miRNA was examined by RT-PCR using appropriate primers. IRNA and miRNA were expressed stably in the stable cell lines as shown in Figure 2.

Figure 2 Inhibitory effect of IRNA on luciferase expression mediated by different mechanism.
**HCV IRES-mediated gene expression in RNA expressing HepG2 cells**

IRNA or mRNA expressing HepG2 cells and empty vector pcDNA3 expressing cells or control cells were cotransfected with pCMVNCrluc and transfection efficiency control plasmid pSV-β-Gal. At 48 h post-transfection cell extracts were used to measure the activities of both luciferase and β-galactosidase. The result was plotted as percent of control after normalized for β-gal activity and protein concentration. The pcRz-IRNA cells showed approximately 80% inhibition of luciferase activity compared to the control. Both pcRz-mRNA cells and pcDNA3 cells showed less than 5% inhibition activity. No significant inhibition of cap-dependent translation from the pCDNA-luc construct was observed in cell lines expressing IRNA, (P=0.05, Figure 3).

**Construction of HCV replicon containing HCV IRES**

The results demonstrated that HCV core programmed by HCV IRES was positive in about 90% of HHCC cells (Figure 4).

**Interference of IRNA with HCV replicon translation**

To confirm the result obtained in IRNA expressing cells, HCV replicon containing HCV IRES was transfected with IRNA expressing plasmid, and luciferase activity was determined at different time following transfection. The result was that at 24 h HCV IRES-mediated luciferase gene translation decreases by 15% compared to the control HHCC cells, and along with time extending, the inhibitory effect of IRNA on HCV IRES-mediated luciferase gene translation increased and reached 80% at 72 h. On d 7, the inhibitory effect was still 80%. But mRNA and nonspecific short RNA did not show any inhibitory effect on HCV IRES-mediated gene translation (Figure 5).

**DISCUSSION**

IRES-dependent protein translation mechanism was first discovered in picornaviruses, including PV, rhinovirus and hepatitis A virus, as well as certain flaviviruses, such as hepatitis C virus[23-28]. Although there is very little sequence homology between these different IRES elements, structural similarity does appear to exist. In fact, in order to keep the activity of IRES, it was more important to maintain the secondary structure than to maintain the integrality of certain genome sequences[1-3,7,8]. IRES is the key structure for some viral RNA replication, so it has become the target for antiviral infection. We have constructed the self-cleavage plasmid of IRNA, and affirmed that IRNA can inhibit IRES-dependent protein translation in vitro[22]. In order to further confirm the effect of long-term expressing IRNA on cellular protein and viral protein translation, we established a HHCC line stably expressing IRNA, and confirmed that long-term expressing IRNA could significantly inhibit IRES mediated protein translation compared to the control cells and mRNA expressing cells. Das et al prepared the human hepatoma (Huh-7) cell lines expressing IRNA by using the similar methods. They found that HCV IRES-mediated cap-independent translation was markedly inhibited in cells constitutively expressing IRNA compared to control hepatoma cells[29].

Alt et al designed the vector pCMVNCrluc fusing the luciferase gene to HCV core gene 66 nt site, and the gene expression was mediated by HCV 5’ UTR, so we could determine the inhibitor effect of new strategies on HCV 5’ UTR by examining the activity of luciferase. In this study, three plasmids pCMVNCrluc, pcRz-IRNA expressing IRNA and transfection efficiency control plasmid pSV-βGal were cotransfected into HHCC cells and luciferase activity (light units) was expressed as percentage of the control after normalized for β gal activity. When the effect of transfection efficiency and transient expression efficiency where excluded, the results of this study showed that IRNA could specifically inhibit HCV IRES mediated gene expression in vivo. The results of our study suggested that HCV 5’ UTR-mediated translation was specifically inhibited by IRNA transient expression in hepatoma cells (50% to 92%), whereas cap-dependent translation of luciferase from the control plasmid
lack of HCV IRES element was not significantly affected by IRNA. To confirm the result obtained by using transient transfection, the vector containing HCV IRES was transfected into human hepatoma cells expressing IRNA constitutively and the results demonstrated that stably expressing IRNA could inhibit HCV IRES-mediated translation. By using a bicistronic construct containing CAT and luciferase genes flanked by the HCV 5' UTR Das et al found that IRNA could significantly inhibit HCV IRES-mediated gene expression in *vivo*. Further, they studied the IRNA effect in *vivo* and obtained the similar result to our study.[29,30]

In order to determine the IRNA inhibitor effect in *vivo* further, we used the HCV replicon containing the full length of HCV 5' UTR to investigate the IRNA activity; the results demonstrated that IRNA could inhibit HCV 5' UTR mediated gene expression in *vivo*, but IRNA could not completely block HCV 5' UTR mediated gene expression.

To rule out the nonspecific effect of nonspecific short RNA regiment on HCV IRES –mediated gene expression, plasmids pCDNA3 and pCMVNC.Rluc were cotransfected into human hepatoma cells and the results showed that nonspecific RNA regiment didn’t have the inhibitor effect on HCV IRES–mediated translation.

In summary, IRNA can significantly inhibit HCV IRES–mediated translation.

REFERENCES

1. Friebe P, Lohmann V, Krieger N, Bartenschlager R. Sequences in the 5'-nontranslated region of hepatitis C virus required for RNA replication. *J Virol* 2001; 75: 12047-12057
2. Jubin R. Hepatitis C IRES: translating translation into a therapeutic target. *Curr Opin Mol Ther* 2003; 3: 278-287
3. Reusken CB, Dalebout TJ, Eerligh P, Bredenbeek PJ, Spaan WJ. Analysis of hepatitis C virus' classical swine fever virus chimeric 5'NTR sequences within the hepatitis C virus IRES are required for viral RNA replication. *J Gen Virol* 2003; 84(Pt 7): 1763-1769
4. Klinck R, Westhof E, Walker S, Afsar M, Collier A. A potential RNA drug target in the hepatitis C virus internal ribosomal entry site. *RNA* 2000; 6: 1423-1431
5. Wang W, Previlles P, Morin N, Mounir S, Cai W, Siddiqui MA. Hepatitis C viral IRES inhibition by phenazine and phenazine-like molecules. *Bioorg Med Chem Lett* 2000; 10: 1151-1154
6. Shimazaki T, Honda M, Kaneko S, Kobayashi K. Inhibition of internal ribosomal entry site-directed translation of HCV by recombinant IFN-alpha correlates with a reduced La protein. *Hepatology* 2002; 35: 199-206
7. Gallego J, Varani G. The hepatitis C virus internal ribosome entry site: a new target for antiviral research. *Biochem Soc Trans* 2002; 30: 140-145
8. Vyasj, Elia A, Clemens MJ. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. *RNA* 2003; 9: 858-870
9. Kikuchi K, Umehara T, Fukuda K, Hwang J, Kuno A, Hasegawa T, Nishikawa S. RNA aptamers targeted to domain II of hepatitis C virus IRES that bind to its apical loop region. *J Biochem* 2003; 133: 263-270
10. Liang XS, Lian JQ, Zhou YX, Nie QH, Hao CQ. A small yeast RNA inhibits HCV IRES mediated translation and inhibits replication of poliovirus in vivo. *World J Gastroenterol* 2003; 9: 1008-1013
11. He Y, Yan W, Collo C, Li Y, Gale M Jr, Kalze MG. The regulation of hepatitis C virus (HCV) internal ribosome entry-site-mediated translation by HCV replicons and nonstructural proteins. *J Gen Virol* 2003; 84(Pt 3): 535-543
12. Otto GA, Lukavsky PJ, Lancaster AM, Sarnow P, Puglisi JD. Ribosomal proteins mediate the hepatitis C virus IRES-HeLa 40S interaction. *RNA* 2002; 8: 931-923
13. Das S, Ott M, Yamane A, Venkatesan A, Gupta S, Dasgupta A. Inhibition of internal entry site (IRES)-mediated translation by a small yeast RNA: a novel strategy to block hepatitis C virus protein synthesis. *Front Biosci* 1998; 3: D1241-D1252
14. Das S, Coward P, Dasgupta A. A small yeast RNA selectively inhibits internal initiation of translation programmed by poliovirus RNA: specific interaction with cellular proteins that bind to the viral 5'-nontranslated region. *J Virol* 1994; 68: 7200-7211
15. Kikuchi K, Umehara T, Fukuda K, Hwang J, Kuno A, Hasegawa T, Nishikawa S. Structure-inhibition analysis of RNA aptamers that bind to HCV IRES. *Nucleic Acids Res Suppl* 2003; 3: 291-292
16. Liang XS, Zhou Y, Lian J, Nie Q, Jia Z. Effect of inhibitor RNA on intracellular inhibition of viral gene expression in 5'-noncoding region of hepatitis C virus. *Zhonghua Neike Za Zhi* 2002; 41: 660-662
17. Das S, Kanen DJ, Bocskai D, Keene JD, Dasgupta A. Sequences within a small yeast RNA required for inhibition of internal initiation of translation: interaction with La and other cellular proteins influences its inhibitory activity. *J Virol* 1996; 70: 1624-1632
18. Isoyama T, Kornoshiba N, Yasui K, Iwai A, Shiroki K, Toyoda H, Yamada A, Takasaki Y, Nomoto A. Lower concentration of La protein required for internal ribosome entry on hepatitis C virus RNA than on poliovirus RNA. *J Virol* 1999; 73(Pt 8): 2319-2327
19. Gamarnik AV, Andino R. Interactions of virus-specific binding protein with the 5'-untranslated region of the poliovirus genome. *J Virol* 2000; 74: 2219-2226
20. Ray PS, Das S. La autoantigen is required for the internal ribosome entry site-mediated translation of Coxsackievirus B3 RNA. *Nucleic Acids Res* 2002; 30: 4500-4508
21. Ali N, Pruijn GJ, Kanen DJ, Keene JD, Siddiqui A. Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation. *J Biol Chem* 2000; 275: 27531-27540
22. Liang XS, Zhou YX, Lian JQ, Hao CQ, Wang LX. Structure modeling and construction recombinant plasmid of HCV IRES specific inhibitor RNA (hIRES1). *J Med Post* 2002; 15: 189-192
23. Jin J, Yang JY, Liu J, Kong YY, Wang Y, Li GD. DNA immunization with fusion genes encoding different regions of hepatitis C virus E2 fused to the gene for hepatitis B surface antigen elicits immune responses to both HCV and HBV. *World J Gastroenterol* 2002; 8: 505-510
24. Woytas RP, Petersen U, Moshage D, Brackmann HH, Matz B, Sauerbruch T, Spengler U. HCV-specific cytokine induction in monocytes of patients with different outcomes of hepatitis C. *World J Gastroenterol* 2002; 8: 562-566
25. Yu YC, Mao Q, Gu CH, Li QF, Wang YM. Activity of HDV ribozymes in trans-cleave HCV RNA. *World J Gastroenterol* 2002; 8: 694-698
26. Tang BZ, Zhuang L, You J, Zhang HB, Zhang L. Seven-years follow-up on trial of Interferon alpha in patients with HCV RNA positive chronic hepatitis C. *World J Gastroenterol* 2002; 6: 563-572
27. Li LF, Zhou Y, Xia S, Zhao LL, Wang ZX, Wang CQ. The epidemiologic feature of HCV prevalence in Fujian. *World J Gastroenterol* 2000; 6: 80
28. Kato J, Kato N, Morigiyo M, Goto T, Taniguchi H, Shiratori Y, Omata M, Otomo T, Okuda K, Moriyama M, Goto T, Taniguchi H, Shiratori Y. Interferons specifically suppress the translation from hepatitis C virus RNA: specific interaction with cellular proteins that bind to HCV IRES. *Nucleic Acids Res* 1999; 27: 563-572

*Edited by Gupta KM and Wang XL*