Inhibition effect of Chinese herbal medicine on transcription of hepatitis C virus structural gene in vitro

Jun Dou, Qian Chen, Jing Wang

AIM: To investigate the inhibitory effect of Chinese herbal medicine on the transcription of hepatitis C virus (HCV) structural gene in Hela D cells.

METHODS: Hela cell line was transfected with recombinant pBK-CMV-HCV containing HCV structural gene by Lipofectamine. RT-nested-PCR and Western blot assay were used to testify the HCV gene expression in Hela cells. The Hela cells expressing HCV structural protein were named Hela D cells. Prescriptions of Xiao chaihu Decoction (XCHD), Fufang Huangqi (FFHQ) and Bingganling (BGL) were respectively added to Hela D cells in various concentrations. Semi-quantitative RT-nested-PCR product analysis was performed according to the fluorescent density between HCV DNA band and GAPDH DNA band in gel electrophoresis after screened.

RESULTS: Recombinant pBK-CMV-HCV could correctly express the HCV structural gene in Hela D cells. After co-culture of Hela D cells with three prescriptive different concentrations for 48 h respectively, the transcription of HCV gene decreased with increasing of the concentration of each prescription. The lightness ratio of HCV product bands to GAPDH product bands was 0.24, 0.10 and 0.12 in Hela D cells incubated with 0.1 g/mL of XCHD, FFHQ and BGL respectively and the lightness ratio HCV product bands to GAPDH product bands was 0.75, 0.67 and 0.61 respectively in the control cells.

CONCLUSION: The prescriptions of XCHD, FFHQ and BGL partly inhibit the transcription of HCV structural gene in Hela D cells.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.
Total RNA was isolated from Hela D cells with TRIzol reagent. Before extraction, 100 µL reaction buffer (10 mmol/L Mgc2+, 0.1 mmol/L DTT, 1 u/mL RNasin, RNase-free DNaseI) was added to 2×10^7 Hela D cells and stored at 37 °C for 1 h to remove all contaminated DNA and then the extraction process was performed according to the TRIzol protocol[10].

RT-nested-PCR and semiquantitative RNA analysis
cDNA was prepared by reverse transcription of 2 µg total RNA and then was amplified by nested PCR with two different primers to detect the transcriptions of HCV structural and GAPDH gene. In PCR amplified products, the GAPDH gene was used as an internal control of HCV gene[12]. PCR products were checked by gel electrophoresis and semiquantitative RNA analysis was performed according to the fluorescent density between HCV DNA band and internal control GAPDH gene band in gel electrophoresis.

Western blot
Whole cell extract was prepared by protein extraction buffer (Novagen, Germany) according to the manufacturer’s protocol. Western blot was performed after 12% SDS-polyacrylamide gel electrophoresis using Western Breeze kit (Invitrogen, CA). Briefly, proteins (15 µg/lane) were electrot-transferred onto nitrocellulose membrane. The membrane was blocked with blocking solution 30 min on a rotary shaker and washed twice with water and subsequently incubated with primary antibodies for 1 h. The membrane was washed thrice for 5 min with antibody washing solution. The rest of the steps were performed according to the kit’s protocol.

RESULTS
Expression of HCV structural gene in Hela D cells
Total RNA was isolated from Hela D cells and Hela cells respectively with TRIzol reagent and cDNA was then amplified by nested PCR with two different primers. HCV structural gene was expressed in Hela D cells because there was a 144 bp band in the extracts from Hela D cells (Figure 1). However, there was no band in the extracts from Hela cells. Under similar conditions, there was no protein expression in Hela cells transfected with blank plasmid or Hela cells without any transfection.

Cytotoxicity to Hela D cells and inhibition effect of Chinese herbal medicine on HCV gene transcription
Each kind of Chinese herbal medicine was put into Hela D cell culture medium for 72 h separately and the final concentration was 1.0, 0.8, 0.6, 0.4 and 0.2 g/mL respectively. The cytotoxicity of Chinese herbal medicine to Hela D cells was observed under microscope and by MTT methods[10]. After Hela D cells were incubated for 10 h, 200 µL of various concentrations of Chinese herbal medicine (0.1, 0.01, 0.001 g/mL) was respectively added to the culture medium in a six well plate for an additional 48 h incubation and 200 µL PsBS was added to Hela D cells as control.

Total RNA extraction
Total RNA was isolated from Hela D cells with TRIzol reagent. Before extraction, 100 µL reaction buffer (10 mmol/L Mg2+, 0.1 mmol/L DTT, 1 u/mL RNasin, RNase-free DNaseI) was added to 2×10^7 Hela D cells and stored at 37 °C for 1 h to remove all contaminated DNA and then the extraction process was performed according to the TRIzol protocol[10].

RT-nested-PCR and semiquantitative RNA analysis
cDNA was prepared by reverse transcription of 2 µg total RNA and then was amplified by nested PCR with two different primers to detect the transcriptions of HCV structural and GAPDH gene. In PCR amplified products, the GAPDH gene was used as an internal control of HCV gene[12]. PCR products were checked by gel electrophoresis and semiquantitative RNA analysis was performed according to the fluorescent density between HCV DNA band and internal control GAPDH gene band in gel electrophoresis.

Western blot
Whole cell extract was prepared by protein extraction buffer (Novagen, Germany) according to the manufacturer’s protocol. Western blot was performed after 12% SDS-polyacrylamide gel electrophoresis using Western Breeze kit (Invitrogen, CA). Briefly, proteins (15 µg/lane) were electrot-transferred onto nitrocellulose membrane. The membrane was blocked with blocking solution 30 min on a rotary shaker and washed twice with water and subsequently incubated with primary antibodies for 1 h. The membrane was washed thrice for 5 min with antibody washing solution. The rest of the steps were performed according to the kit’s protocol.

RESULTS
Expression of HCV structural gene in Hela D cells
Total RNA was isolated from Hela D cells and Hela cells respectively with TRIzol reagent and cDNA was then amplified by nested PCR with two different primers. HCV structural gene was expressed in Hela D cells because there was a 144 bp band in the extracts from Hela D cells (Figure 1). However, there was no band in the extracts from Hela cells. Under similar conditions, there was no protein expression in Hela cells transfected with blank plasmid or Hela cells without any transfection.
**Cytotoxicity to Hela D cells in various concentrations**

When the concentration of Chinese herbal medicine gradually decreased, its cytotoxicity to Hela D cells was also decreased by degrees. When the concentration of Chinese herbal medicine was maintained at 0.2 g/mL in Hela D cells culture medium for 72 h, the survival rate of Hela D cells reached more than 99% (Table 1).

| Herbs   | 1 (g/mL) | 0.8 (g/mL) | 0.6 (g/mL) | 0.4 (g/mL) | 0.2 (g/mL) |
|---------|----------|------------|------------|------------|------------|
| XCHD    | 22.57    | 48.63      | 73.25      | 89.86      | 99.97      |
| FFHQ    | 25.36    | 57.34      | 76.28      | 92.57      | 99.98      |
| BGL     | 21.67    | 47.35      | 79.24      | 94.31      | 99.97      |

**Inhibitory effect of Chinese herbal medicine on HCV gene transcription in Hela D cells**

When the concentration of Chinese herbal medicine was increased, the transcription of HCV gene gradually decreased and its lightness of HCV band was weak, but the lightness of GAPDH band had no change, suggesting that XCHD, FFHQ and BGL could inhibit the transcription of HCV gene and had no effect on GAPDH in Hela D cells (Figure 2).

**Figure 2** Inhibition of HCV structural gene transcription in Hela D cells by various concentrations of XCHD (A), FFHQ (B), and BGL (C). Lane 1: GAPDH as internal control; lane 2: 0.001 g/mL; lane 3: 0.01 g/mL; lane 4: 0.1 g/mL; lane 5: PBS as control; lane 6: DNA molecular standard.

**DISCUSSION**

It is very difficult to establish a culture system in vitro for HCV replication because most cell lines are not sensitive to HCV infection and HCV in cell line cannot be cultured for a long time. The problem of successive incubation of normal liver cells is still not solved. Cell models transfected with HCV gene or part of gene become an effective method in research of HCV replication, gene transcription, protein expression and drug screening for treating hepatitis C. Some cell lines can be used as important tools to investigate structural and functional properties of HCV core protein and may be useful in evaluating gene therapeutic strategies against HCV\[^{[3,4]}\]. Some researches have focused on Hela cell in recent years\[^{[5,6]}\]. Ide et al\[^{[7]}\] established a Hela cell model which can successfully express HCV NS5A gene. It was reported that Hela cells transfected with non-structural gene and structural gene are used in research of the function of HCV proteins\[^{[7,11]}\]. Based on these research reports, we used the Hela cell line as a cell model for selection of Chinese herbal medicine against HCV.

XCHD, FFHQ and BGL are effective prescriptions of Chinese herbal medicine in treating hepatitis C patients\[^{[14]}\]. These prescriptions, however, lack laboratory evidence to support their clinical therapeutic effect on HCV. We employed molecular biological methods to establish Hela D cell model by transfection of HCV structural gene, which could stably express most HCV structural genes.

The cytotoxicity assay of XCHD, FFHQ and BGL to Hela D cells showed that the concentration of 0.2 g/mL Chinese herbal medicine had no cytotoxicity to Hela D cells after incubated for 72 h. The inhibitory effect of Chinese herbal medicine on HCV gene expression showed that the three prescriptions can partly inhibit the transcription of HCV structural gene because the transcription quantity of HCV gene is decreased as their concentration is increased. The efficient inhibitory effect was achieved at the highest dose of 0.1 g/mL of different Chinese herbal medicine.

The mechanism of XCHD, FFHQ and BGL underlying inhibition of HCV gene expression is still not clear. Based on the traditional Chinese medicine theory, we consider that Chinese herbal medicine can change the internal environment of cells, where recombinant pBK-CMV-HCV does not make use of synthetic enzymes and proteins for HCV structural gene mRNA transcription. There might be some pathways that inhibit HCV mRNA transcription in Hela D cells.

In conclusion, Hela D cell model is very useful for observation of HCV transcription, protein expression and for screening new drugs against HCV.

**REFERENCES**

1. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; 122: 366-375
2. Dou J, Liu K, Chen Z, Wo J, Liu Y, Xu C, Chen M, Jin J, He N. Experimental study of immunization of mice with hepatitis C virus genetic vaccine constructs. *Zhonghua Neike Za Zhi* 1999; 38: 390-392
3. Moradpour D, Gosert R, Egger D, Penin F, Blum HE, Bienz K. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. *Antiviral Res* 2003; 60: 103-109
4. Li FC, Wei JB. Survey of Xiao Chai Hu Decoction in clinical application. *Res Traditional Chinese Med* 1998; 14: 60-62
5. Ma SY, Pei ZG. Treatment 46 cases of hepatitis C with method of integrated traditional and western medicine. *Chin J Integrated Traditional Western Medi* 1996; 16: 558-560
6. Peng WP, Zhou ZG, Wu PL, Li B. Treatment 13 cases of hepatitis C with Bing Gan Ling. *Information Traditional Chin Medi* 1997; 14: 23-25
7. Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus
replicate efficiently in cultured Huh7 cells. *J Virol* 2002; 76: 2997-3006

8 **Dou J**, Wu MY. Effect of si jun zi tang on the macrophage cytotoxic activity in mice. *Zhongxiyi Jiehe Zazhi* 1990; 10: 612-613, 582

9 **Sun BS**, Pan J, Clayton MM, Liu J, Yan X, Matskevich AA, Strayer DS, Gerber M, Feitelson MA. Hepatitis C virus replication in stably transfected HepG2 cells promotes hepatocellular growth and tumorigenesis. *J Cell Physiol* 2004; 201: 447-458

10 **Mossman T**. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55-63

11 **Dou J**, Liu K, Chen Z, Wo J, He N, Liu Y, Zhang M, Wang X, Xu C. Effect of immunization in mice with recombinant DNA encoding the hepatitis C virus structural protein. *Chin Med J (Engl)* 1999; 112: 1036-1039

12 **Kim JS**, Ryu J, Hwang SB, Lee SY, Choi SY, Park J. Suppression of ceramide-induced cell death by hepatitis C virus core protein. *J Biochem Mol Biol* 2004; 37: 192-198

13 **Moradpour D**, Englert C, Wakita T, Wands JR. Characterization of cell lines allowing tightly regulated expression of hepatitis C virus core protein. *Virology* 1996; 222: 51-63

14 **Yamaguchi R**, Momosaki S, Gao G, Hsia CC, Kojiro M, Scudamore C, Tabor E. Truncated hepatitis C virus core protein encoded in hepatocellular carcinomas. *Int J Mol Med* 2004; 14: 1097-1100

15 **Mizuno M**, Yamada G, Tanaka T, Shimotohno K, Takatani M, Tsuji T. Virion-like structures in HeLa G cells transfected with the full-length sequence of the hepatitis C virus genome. *Gastroenterology* 1995; 109: 1933-1940

16 **Hassan M**, Ghozlan H, Abdel-Kader O. Activation of RB/E2F signaling pathway is required for the modulation of hepatitis C virus core protein-induced cell growth in liver and non-liver cells. *Cell Signal* 2004; 16: 1375-1385

17 **Ide Y**, Tanimoto A, Sasaguri Y, Padmanabhan R. Hepatitis C virus NS5A protein is phosphorylated *in vitro* by a stably bound protein kinase from HeLa cells and by cAMP-dependent protein kinase A-alpha catalytic subunit. *Gene* 1997; 201: 151-158