行政院國家科學委員會補助專題研究計畫成果報告

慢性C型肝炎患者細胞激素之研究
Study of cytokine profiles in patients with chronic hepatitis C

計畫類別：□個別型計畫　☑整合型計畫
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Background and Aim

Hepatitis C virus (HCV) is the major agent causing non-A, non-B hepatitis worldwide (1). It is responsible for 60-90% of post-transfusion hepatitis (2,3). In contrast to hepatitis A and B viral infection , HCV infection is characterized by an unusually high frequency of persistent infection after acute stage in otherwise healthy adults, and about 20% of these chronically infected patients eventually develop cirrhosis and even hepatocellular carcinoma two to three decades later (4). The mechanisms involved in the pathogenesis of chronic hepatitis C and in the outcome of patients are still not well clarified.

In the first year of this project, we found that the immunological responses to HCV proteins (NS3A) were mainly TH1 in cirrhotic patients, while were mainly TH2 in non-cirrhotic patients. The study material was peripheral blood mononuclear cells (PBMC) obtained from patients with chronic hepatitis C. In the second year of this project, we further studied intrahepatic cytokine profiles in patients with chronic hepatitis C who received interferon-α treatments. In the liver tissue, TH1/TH2 immunological responses in terms of the expression of IL-2, IFN-γ or IL-4, IL-5, IL-10, were quite prominent in all patients. No statistically significant difference in intrahepatic cytokine profiles was noted between responder patients and non-responder patients of interferon-α treatment.

Previous studies revealed that IL-1 might be related to the formation of fibrosis. Generally, synthesis of IL-1 is up-regulated in response to infection or tissue damage. IL-1 was synthesized by peripheral blood monocytes. Therefore, we tried to study peripheral blood monocytes to see whether synthesis of IL-1 was related to liver cirrhosis.

Materials and Methods

Materials

Totally 40 patients with chronic hepatitis C were enrolled for this study. Twenty of them were without liver cirrhosis, and 20 of them were in cirrhotic state. All of them were histologically verified.

Methods

1. Separation of PBMC from whole blood by using histopaque
2. Isolation of monocytes (immunomagnetic separation)
   PBMC were mixed with Dynabeads M-450 CD14 (Dynal, Inc., Lake Success, NY) in 1 ml PBS/FCS (5%) for 20 min under 4℃.
   Then, we used Dynal MPC (magnet) for 2~3 min to isolate Dynabeads-monocytes complexes. Resuspended rosetted cells in washing buffer (PBS / 5% FCS), and repeated previous two steps for 5 times. At last, we resuspended rosetted cells in 10 ml of PBS / 5% FCS
3. Confirmation of isolated monocytes
   FACS analysis

4. RNA extraction and RT-PCR

Total cellular RNA was extracted from isolated monocytes using TRIzol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's instructions. Single-stranded cDNA was then generated in a 20 μl RT reaction containing 1 μg of RNA as templated, random primers, and Super Scriot II Rnase H- reverse transcriptase (Life Tech ologies, Inc.). One ml of RT products was used for PCR amplification in a 50 μl reaction containing sense and anti-sense primers (400 nM), dNTPs (200 mM), 1X PCR buffer, and 1.6 units of DynaZyme II DNA polymerase (Finnzymes, Oy, Finland). All primer sequences used are listed in the Table 1. Amplification was carried out on a Perkin-Elmer Thermocycler for 30 cycles consisting of : 94℃, 1 min; 55℃, 1 min and 72℃ 2 min. PCR products were electrophoresed on 1.6% agarose gels and visualized with ethidium bromide staining under UV light. RNA extracted from healthy donw PBMCs stimulated with 5 μg/ml PHA was used as a positive control for cytokine and receptor transcripts. Negative controls used the products of RT reactions identical to the prositive control samples, hut without reverse transcriptase. Simultaneously, transcripts encoding b-actin were detected in all samples and served as internal controls.

Table 1. Nucleotide sequences used for PCR and Southern hybridization analysis

Gene	Primer/ probe	Sequence (5'→3')	Length of product (bps)
IL-1β	Sense	TACGAATCTCCGACCACCACTACAG	295
	Antisense	TGGAGGTGGAGAGCTTTCAGTTCATATG	
	Probe	CAGACATCACCAAGCTTTTTTGCTG	
IL-2	Sense	TACAGGATGCAACTCCTGTCTTGCATTGAC	395
	Antisense	GTTGCTGTCTCATCAGCATATTCACACATG	
	Probe	CAGTGTCTAGAAGAAGAACTCAAACCTCTG	
IL-4	Sense	TGCCTCCAAGAACACAACTG	224
	Antisense	AACGTACTCTGGTTGGCTTC	
	Probe	TTGTGCCTGTGGAACTGCTGTG	
IL-5	Sense	CACCAACTGTGCACTGAAGAAA	213
	Antisense	CCACTCGGTGTTCATTACACCA	
	Probe	GGTACTGTGGAAAGACTATTC	
IL-10	Sense	CGCTTTCTAGCTGTTGAGCT	461
	Antisense	CACTGCAACTTCCATCTCCT	
	Probe	CACCATGTTGACCAGGCTGGTT	
IFN-γ	Sense	GCAGAGCCAAATTGTCTCCT	290
	Antisense	ATGCTCTTCGACCTCGAAAC	
	Probe	CTCCTTTTTCGCTTCCCTGTTTTAG	
β-actin	Sense	TTCTACAATGAGCTGCGTGT	636
	Antisense	GCCAGACAGCACTGTGTTGG	

5. Southern hybridization

RT-PCR products were transferred from the agarose gels described above onto a nitrocellulose membrane, following denaturation and neutralization. Then the blot was prehybridized for 2 h at 68℃, hybridized at 55℃ with an ologonujcleotide probe (probe sequences are listed in Table 2), and labeled at the 3' end with DIG (Boehringer Mannheim, Mannheim, Germany) for 16 h. The prehybridization and hybridization buffers contained 5X SSC (0.5 M NaCl plus 0.075 M sodium citrate), 2% blocking reagent (Boehringer Mannheim), 0.1% lauroyl sarkosine, 0.02% SDS, and 0.012% calf thymus DNA. After washing twice in 2X SSC followed by 0.1X SSC at 55℃, the blot was incubated with alkaline phosphatase-conjugated anti-DIG mAb at 30℃ for 1 h in a reaction mixture comprising 1% blocking reagent, 0.1 M maleic acid, and 0.15 M NaCl (PH 7.5). Thereafter, a chemiluminescent substrate (Boehringer Mannheim) was added, and the blot was exposed to X-ray film.

Results

Isolation of monocytes

By FACS analysis, the isolated monocyte solution was around 95% in purity.

Gene expression of various cytokines in monocytes obtained from two groups of patients with chronic hepatitis C

The gene3 expression of the above –mentioned cytokines is shown in Table 2. No statistically significant difference was noted between two groups of patients.

Table 2. Percentage of RNA Expression in Peripheral Blood Monocytes of Chronic Hepatitis C Patients

Cytokine	Percentage of RNA Expression in Peripheral Blood Monocytes of Chronic Hepatitis C Patients	
	Non-cirrhotic (1%)	Cirrhotic (1%)
IL-1β	40	52
IL-2	82	74
IFN-γ	85	82
IL-4	71	74
IL-5	54	60
IL-10	72	68

Discussions

It seems that the fibrogenesis of the liver under tissue damage is not a simple process. Instead, a complex system is involved in the formation of liver fibrosis, including hepatic stellate cell / sinusoidal endothelial cell, extracellular matrix (ECM) environment, and various cytokines, such as transformaing growth factor β, tumor necrosis factor α etc. (5, 6)

Further studies in the interaction of hepatitis C virus with various factors in the hepatic parenchyma are warranted.

References

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