Intrahepatic IFN-alpha expression in liver specimens from HBV-infected patients with different outcomes

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Abstract. – BACKGROUND: Interferon-alpha (IFN-α), an active cytokine, plays an important role in antiviral host responses, including protection against hepatitis B virus (HBV) infection. This study was designed to investigate the correlation between intrahepatic IFN-α expression levels and disease severity using liver biopsy specimens from HBV-infected patients with different outcomes.

PATIENTS AND METHODS: Immunohistochemistry (IHC) was performed to detect intrahepatic IFN-α expression in liver biopsy specimens obtained from 69 HBV-infected patients with different outcomes (including 23 cases with chronic hepatitis B [CHB], 18 cases with severe hepatitis B [SHB], and 28 cases with liver cirrhosis [LC]). In situ hybridization (ISH) was carried out to measure the levels of HBV DNA in liver samples. In addition, the liver specimens of 33 healthy liver transplant donors without detectable liver diseases comprised a normal control (NC) group.

RESULTS: The intrahepatic expression levels of IFN-α were higher in the HBV-infected patients than the NC group (p = 0.001). Intrahepatic IFN-α expression was also significantly higher in the SHB and CHB groups compared to the NC group (p = 0.001 and p = 0.001, respectively), while the intrahepatic HBV DNA levels of the SHB patients were higher than those of LC patients (p = 0.013). Furthermore, intrahepatic IFN-α expression was positively correlated with serum alanine aminotransferase (ALT) levels in CHB patients; no significant correlations were discovered between intrahepatic IFN-α expression and intrahepatic HBV DNA levels in all other subgroups.

CONCLUSIONS: Intrahepatic IFN-α expression may correlate with liver inflammation after hepatitis B virus infection, and IFN-α may play a vital role in the occurrence of SHB.

Key Words: Hepatitis virus B, Interferon-alpha, Chronic hepatitis B, Severe hepatitis B, Liver cirrhosis.
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Patients and Methods

Study Subjects

The liver biopsy specimens were collected from 69 patients with HBV infection, including 23 chronic hepatitis B (CHB) cases, 18 severe hepatitis B (SHB) cases, and 28 cases of liver cirrhosis (LC). All patients were positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Exclusion criteria included the following: co-infection of human immunodeficiency virus (HIV) or other hepatitis viruses (hepatitis A, C and E viruses), autoimmune hepatitis, drug-induced hepatitis and liver injury caused by other etiologies, HCC, portal vein thrombosis or cardiovascular comorbidities. In addition, 33 liver specimens were collected from healthy liver transplant donors with undetectable liver diseases to serve as the normal control (NC group). The detailed characteristics of these subjects are described in Table I.

Patients were informed of the aims of the study and written consent was acquired. This study was approved by the West China Hospital Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Preparation of Liver Biopsy Samples and Integrated Tissue Slice Slides and Histologic Scoring System

Percutaneous liver biopsies were performed using a 16-gauge biopsy needle under ultrasound guidance. Biopsy specimens were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at 3 µm thickness. The slices were stretched at 40°C and mounted on a glass slide at a marked region. Then the integrated tissue slide was accomplished with 12 tissue slices on one slide.

Finally, positive IHC signals were indicated by yellow, brown or tan staining; positive ISH signals were indicated by purple-blue or blue-black staining. Two experienced pathologists examined the integrated slides without information regarding patients. The percentage of positive cells and the intensity of positive staining were scored according to the Axiots score standard based on evaluation of five randomly chosen fields at 400-fold magnification17. If the histology scores of the two pathologists were different for the same sample, we chose the mean as the final result.

Detection of Intrahepatic IFN-α Expression by Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded liver samples were incubated with a goat anti-human IFN-α polyclonal antibody at a dilution of 1:200 (Santa Cruz Biotechnology; CA, USA) at 4°C overnight. This step was followed by three washes with phosphate-buffered saline (PBS), and staining with biotin-labelled rabbit anti-goat IgG at a dilution of 1:100 (Zhongshan Goldenbridge Biotechnology; Beijing, China) at room temperature for 1 h. Finally samples were stained with a 3,3′-Diaminobenzidine kit (Wuhan Boster Biological Technology; Wuhan, China), and counterstained with hematoxylin. Otherwise, samples were incubated with PBS instead of IFN-α polyclonal antibody as a negative control.

Detection of HBV DNA in Liver Tissue by in Situ Hybridization (ISH)

ISH was carried out as previously described by Long et al18. The levels of HBV DNA in the liver biopsy samples were detected with a 3.2 kb HBV DNA probe labelled with digoxin with the use of a DIG-High prime kit (Roche; Basel, Switzerland). Briefly, paraffin sections were de-paraffinised, rehydrated, incubated with H2O2, digested with protease K and repaired with super-

Table I. Characteristics of normal controls and patients with HBV infection.

<table>
<thead>
<tr>
<th></th>
<th>CHB</th>
<th>SHB</th>
<th>LC</th>
<th>NC</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Total number</td>
<td>23</td>
<td>18</td>
<td>28</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Age (year)</td>
<td>35.4 ± 8.3</td>
<td>44.1 ± 10.9</td>
<td>43.6 ± 9.3</td>
<td>38.1 ± 7.7</td>
<td>0.002</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>29.0 (13-101)</td>
<td>79.5 (19-2448)</td>
<td>46.0 (6-381)</td>
<td>33.0 (6-61)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum HBV DNA (copies/mL)</td>
<td>4.7E+06</td>
<td>2.0E+05</td>
<td>6.1E+04</td>
<td>NA</td>
<td>0.065</td>
</tr>
<tr>
<td>IFN-αΨ</td>
<td>1.67 ± 0.65</td>
<td>1.86 ± 0.80</td>
<td>1.32 ± 0.76</td>
<td>1.02 ± 0.72</td>
<td>0.001</td>
</tr>
<tr>
<td>HBV DNAΨ</td>
<td>0.67 ± 0.58</td>
<td>0.86 ± 0.74</td>
<td>0.41 ± 0.68</td>
<td>NA</td>
<td>0.027</td>
</tr>
</tbody>
</table>

ALT: alanine aminotransferase; BLQ: below limit of quantification; NA: not applicable. Ψ: ISH scores of intrahepatic IFN-α expression and HBV DNA in liver samples.
After being denatured, the slices were put into -20°C anhydrous ethanol and the hybridization solution containing the DIG-HBV DNA probe was put into 0°C ice water; then these were incubated together at 56 °C overnight. This step was followed by three washes and an incubation with anti-Dig-alkaline phosphatase Fab fragments at a dilution of 1:200 (Roche; Basel, Switzerland) at 37°C for 1 h. Finally, the hybridized signal was detected with a NBT/BCIP kit (Boehringer Mannheim GmbH; Mannheim, Germany). Each sample was counterstained with 1% methyl green before being mounted.

**Statistical Analysis**

The histological scores of intrahepatic IFN-α expression and HBV DNA level in liver tissue were presented as the mean ± standard deviation of each sample. The Kruskal-Wallis test was used to evaluate the differences among more than 2 groups, while the Mann-Whitney U test were used to compare variables between 2 groups. The correlation analysis was performed by using non-parametric correlation analysis (Spearman). Data were analyzed with SPSS statistical software package, version 16.0 (SPSS Inc., Chicago, IL, USA). A difference with a p value less than 0.05 was considered statistically significant.

**Results**

The data reported in the study were obtained from CHB, SHB, LC and healthy liver transplant donors. Detail characteristics of these subjects are presented in Table I.

**Intrahepatic Expression of IFN-α in Liver Samples from HBV-infected Patients and Normal Controls**

Intrahepatic IFN-α expression was detected by IHC in all 69 samples from HBV-infected patients as well as 33 samples from healthy liver transplant donors. Expression of IFN-α in liver samples was apparent in the cytoplasm, and representative images are showed in Figure 1.

As presented in Figure 2A, the expression level of IFN-α was higher in the HBV-infected group (n=69) than that in the NC group (n=33; p = 0.001) (Figure 2A). According to the statistical analysis.
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Figure 2B. Intrahepatic IFN-α expression in liver samples. A, Intrahepatic IFN-α expression in HBV-infected patients (n=69) and normal controls (n=33). B, Intrahepatic IFN-α expression in HBV-infected patients with different outcomes.

Levels of HBV DNA in Liver Samples from HBV-infected Patients with Different Outcomes

The presence of HBV DNA in liver biopsy samples was observed in the nucleus/cytoplasm (Figure 3A, B, C). There was significant difference in the levels of HBV DNA in the 69 HBV-infected patients ($\chi^2 = 7.198, p = 0.027$). The levels of HBV DNA in the SHB group were higher than those observed in the LC group ($p = 0.013$) (Figure 3D); however, there was no statistically significant difference when comparing with the CHB group ($p = 0.489$), and this result was similar to the intrahepatic IFN-α expression of the SHB group. Moreover, we observed a statistically significant difference between expression levels of HBV DNA between the CHB and LC groups ($p = 0.043$) (Figure 3D).

Correlation of Intrahepatic IFN-α Expression with Serum ALT Levels, and with the Levels of HBV DNA in Liver Biopsy Samples

In this study, it was observed that there was a positive correlation between the intrahepatic expression of IFN-α and serum ALT levels in CHB patients ($r = 0.508, p = 0.014$) (Figure 4), but not in SHB, LC or NC patients ($r = -0.298, p = 0.229$; $r = 0.105, p = 0.594$; and $r = -0.0234, p = 0.189$; respectively). In addition, correlation analysis showed that there was no significant correlation between intrahepatic IFN-α expression and HBV DNA levels in liver biopsy samples in the CHB, SHB or LC groups ($r = -0.129, p = 0.558$; $r = -0.095, p = 0.708$; and $r = -0.013, p = 0.946$, respectively).

Discussion

The mechanism of HBV infection is complicated, as both the virus and the host immune response can have a direct impact on the natural course of HBV infection and the clinical outcome of liver disease. As an active cytokine, IFN-α can coordinate the immune response against viruses and other intracellular infections. Previous studies have shown that IFN-α can activate the expression of hundreds of genes, and generate some IFN-inducible proteins to establish the “antiviral state.” IFN-α also directly enhances NK cell-mediated cytotoxicity through activating NK cell receptors, and also regulates cytotoxic T cells differentiation. Both IFN-α and IFN-γ are required to maintain NK cell function. At present IFN-α is a candidate for the first-line treatment of patients with chronic hepatitis B, chronic hepatitis C and other viral infection.

Many studies have shown that IFN-α expression can directly inhibit HBV DNA replication and intracellular hepatitis B surface antigen se-
Intrahepatic IFN-α expression in liver tissue from patients with acute hepatitis B infection was significantly higher than patients with chronic hepatitis B infection. IFN-α also can induce apoptosis in hepatocytes through stimulating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway or non-TRAIL-induced apoptosis pathways in the liver. On the other hand, severe hepatitis caused by HBV infection can lead to large areas of necrosis of hepatocyte, and this process may involve IFN-α-mediated immune clearance response and hepatocyte apoptosis. Leifeld, et al. reported that intrahepatic IFN-α expression in patients with fulminant liver failure caused by a variety of etiologies was significantly higher than that observed in chronic liver disease cases (including chronic hepatitis B, chronic hepatitis C, primary...
biliary cirrhosis and chronic alcoholic hepatitis) and normal subjects. Our data showed that compared with normal subjects, intrahepatic IFN-α expression was significantly higher in HBV-infected patients, and intrahepatic IFN-α expression was higher in the CHB group (or SHB group) than in the NC group. It has been suggested that a modest rise in IFN-α expression may contribute to controlling the progression of HBV infection-related diseases, the immune response and preventing chronicity of HBV infection. IFN-α also has a potential role in promoting the inflammatory response through the tumor necrosis factor (TNF) signal pathway. In this study, there was a positive correlation between the expression levels of IFN-α in liver tissue from CHB patients and serum ALT levels. It is possible that the antiviral process accompanied by elevated intrahepatic IFN-α overexpression after HBV infection, promoted active inflammation and apoptosis mechanisms, and this led to the hepatocyte injury favouring the clearance of HBV infection. Moreover, the intrahepatic IFN-α expression levels in the SHB group was higher compared to the CHB group, demonstrating that elevated intrahepatic IFN-α expression may be involved in severe liver injury and occurrence of SHB; however, there was no statistically significant difference between the two group.

Kupffer cells, plasmacytoid dendritic cells, fibroblasts, and endothelial cells are the major source of intrahepatic IFN-α/β. In patients with liver cirrhosis, disruption of the normal liver architecture, the formation of pseudolobuli, encirled by fibrosis, and decreased numbers of IFN-α expression cells (such as Kupffer cells) are observed by liver histology. These changes may lead to reduced IFN-α expression levels in liver samples from patients with liver cirrhosis, and no statistically significant difference was observed in intrahepatic IFN-α expression between the LC and NC groups, this was also the case for the LC and CHB groups. Moreover, normally or slightly elevated ALT levels could be found incoincidentally in cirrhosis patients at times, and bilirubin enzyme separation phenomenon could be observed in SHB patients with the recurrence of condition. It is possible this is the reason why no correlation was found between intrahepatic IFN-α expression and serum ALT levels in the LC and SHB groups. Further work is required to understand the underline mechanisms of how IFN-α regulates the immune response after HBV infection.

Conclusions

Our study suggests that intrahepatic IFN-α expression may correlate with liver inflammation after hepatitis B virus infection, and plays an important role in the occurrence of SHB.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References


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