The expression profile of Fc Receptor-like Y in B lymphocytes with hepatitis B virus induced diseases

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Abstract: The Fc Receptor-like Y (FcRY) molecule is preferentially expressed by B lymphocytes and has recently been considered as a potential therapeutic target for B cell malignancies. In this study, we investigated the correlation between FcRY expression profile, B lymphocytes population and different HBV infection disease status. The FcRY expression level on B lymphocytes and the number of B lymphocytes population from peripheral blood in 27 healthy controls (HC) and 65 patients with HBV-induced diseases, including chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC), were analyzed using quantitative real-time PCR and flow cytometry. The results showed the level of FcRY expression and frequency of germinal center (GC) B lymphocytes from peripheral blood were significantly correlated with the HBV-related disease status, which was highest in HCC and LC patients, lowest in healthy donors, and in the middle in patients with CHB. Our study indicates that there is a significant correlation between FcRY expression profile, B lymphocytes population and HBV-induced diseases. However, the roles of FcRY and B lymphocytes in HBV-induced diseases are unclear and need further investigation. (Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 3, pp. 405–409)

Key words: Fc Receptor-like Y, B lymphocytes, germinal center B lymphocytes, hepatitis B virus, gene expression

Introduction

Hepatitis B virus (HBV) infection is highly prevalent worldwide, especially in the Asia-Pacific region, and is a major cause of morbidity and death. It is estimated that two billion people globally have been infected with HBV and tens of millions of new cases occur annually. Of those infected, 15% to 40% could develop into liver cirrhosis (LC) or hepatocellular carcinoma (HCC) [1–3]. Due to its significant economic and public health impact, the control and prevention of hepatitis B continues to be a major concern.

The cellular and molecular mechanisms of HBV infection have been investigated and it is now widely accepted that the adaptive immune responses, particularly the cellular immune response, play key roles in the development of immunity to HBV.

CD4 T cells, classically referred to as helper T cells, are robust producers of cytokines, while CD8 T cells go on to clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms, reducing the levels of circulating virus [4, 5]. Despite the cellular immune response being a major contributor to HBV clearance, humoral responses by B lymphocytes are also important in controlling HBV [6]. In addition to the production of antibodies, little is known about the role of B lymphocytes such as differentiation, maturation and function during the natural course of HBV infection.

Fc receptor-like (FCRL) molecules are a family of Fc receptor homologs comprising eight structurally related members [7–10]. Seven of the eight FCRL family members are preferentially expressed by B cells at different stages in their development, the excep-
The third group included 20 HBV-positive for more than six months and had histological evidence of chronic hepatitis. The second group was 25 CHB patients infected with HBV, were recruited from Wuxi Hospital of Infectious Disease and Jiangsu Institute of Nuclear Medicine. All donors gave written informed consent before blood was drawn. This study was conducted with the approval of the local Ethics Committee of Wuxi Hospital of Infectious Disease and Jiangsu Institute of Nuclear Medicine. All donors gave written informed consent before blood was drawn. Ninety-two subjects, including 27 healthy controls and 65 patients infected with HBV, were recruited from Wuxi Hospital of Infectious Disease between 2008 and 2009 in the Department of Infectious Disease.

Material and methods

Samples. This study was conducted with the approval of the local Ethics Committee of Wuxi Hospital of Infectious Disease and Jiangsu Institute of Nuclear Medicine. All donors gave written informed consent before blood was drawn. Ninety-two subjects, including 27 healthy controls and 65 patients infected with HBV, were recruited from Wuxi Hospital of Infectious Disease between 2008 and 2009 in the present study. The patients were divided into four groups according to the progression of HBV-induced diseases. The first group comprised 27 healthy controls with no previous history of B-cell responses [18–20]. Recent studies indicate that the activated level of B cells in human peripheral blood lymphocytes with HBV infection is higher than that of normal controls [21]. Based on that finding, we hypothesize that HBV infection might alter the proportion of B cells subpopulation and thus influence the expression level of FcRY. In this report, we investigated the correlation within the expression profile of FcRY, B lymphocytes population and HBV-induced diseases, especially chronic infection, LC and HCC, with peripheral blood. We believe that this report may provide valuable insights into HBV-induced disease pathology.

Table 1. Clinical characteristics of the subjects enrolled in the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HC (n = 27)</th>
<th>CHB (n = 25)</th>
<th>LC (n = 20)</th>
<th>HCC (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35.9 ± 7.2</td>
<td>37.2 ± 10.3</td>
<td>44.5 ± 10.1</td>
<td>40.2 ± 9.2</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/6</td>
<td>14/11</td>
<td>7/3</td>
<td>6/4</td>
</tr>
<tr>
<td>Serum bilirubin [mg/mL]</td>
<td>0.6 ± 0.4</td>
<td>3.5 ± 10.7</td>
<td>22.4 ± 10.1</td>
<td>30.1 ± 18.6</td>
</tr>
<tr>
<td>Albumin [g/dL]</td>
<td>4.5 ± 1.9</td>
<td>4.2 ± 3.0</td>
<td>6.3 ± 3.9</td>
<td>5.5 ± 2.1</td>
</tr>
<tr>
<td>AST [IU/L]</td>
<td>19.3 ± 3.2</td>
<td>96.6 ± 45.3</td>
<td>116.9 ± 55.7</td>
<td>104.2 ± 62.7</td>
</tr>
<tr>
<td>ALT [IU/L]</td>
<td>15.4 ± 2.8</td>
<td>99.8 ± 28.2</td>
<td>98.8 ± 43.3</td>
<td>116.4 ± 50.3</td>
</tr>
</tbody>
</table>

HC — healthy controls; CHB — chronic hepatitis B; LC — liver cirrhosis; HCC — hepatocellular carcinoma; AST — aspartate aminotransferase; ALT — alanine transaminase

HBV-related LC patients, and the fourth group was 20 HBV-related HCC patients clinically and histologically diagnosed. Clinicalopathological data were retrospectively reviewed, which included age, sex, serum bilirubin, albumin, AST and ALT (Table 1).

Cell preparation. Heparinized peripheral blood samples (10 ml) collected from the donors were diluted in PBS and layered on Isopaque-Ficoll separation medium and centrifuged for 30 min at 400 × g. Peripheral blood mononuclear cells (PBMC) accumulating at the interface were separated and resuspended at RPMI 1640. CD19 B cells were isolated from PBMC using direct MACS kit (StemCell, Vancouver, BC, Canada) according to the manufacturer’s instruction. The purity of cells was > 95% as determined by flow cytometry.

Real-time PCR. Total RNA was extracted from sorted cells using Trizol according to the manufacturer’s recommendations and first-strand cDNA synthesis using PrimeScript™ RT reagents Kit (Takara, Otsu, Shiga, Japan). Real-time polymerase chain reaction (RT-PCR) was performed with the cDNA using SYBR green PCR Master Mix (Takara, Otsu, Shiga, Japan). Each amplification reaction underwent denaturation at 95°C for 30 s, amplification for 40 cycles at 95°C for 5 s, annealing and extension at 60°C for 20 s using LightCycler sequence detection system. Human FcRY gene-specific primers used for real-time PCR amplification were 5’GGG AGC ACC CGT CAG TGA 3’and 5’CTG GCG CAT AGG GCA CTT 3’. The gene-specific primers of human housekeeping gene GAPDH were 5’AAC AGC CTC CAT AGG GCA CTT 3’. Only those samples with a positive GAPDH amplification were used for this study.

Flow cytometry. All blood samples were processed on the day of collection and the antibodies used in this study were purchased from BD Biosciences and R&D Systems. For the detection of natural GC B cells, PBMC were stained with peridinin chlorophyll protein (PerCP)-conjugated anti-CD19, fluorescein isothiocyanate (FITC)-conjugated anti-
-CD38 and phycoerythrin (PE)-conjugated anti-IgD and gating was on CD19 B cells. For the detection of FcRY positively expressed B cells, PBMC was first fixed and permeabilized using 4% paraformaldehyde and ice-cold methanol and then stained with PerCP-conjugated anti-CD19 and FITC-conjugated anti-FcRY. After staining, cells were washed twice and resuspended in fluorescence-activated cell sorting solution, fixed in PBS containing 1% paraformaldehyde, and analyzed in FACSCalibur system. Mouse isotype-matched FITC, PE and PerCP were used as negative controls.

**Statistical analysis.** Demographic and clinical data were presented as mean ± SD deviation. Differences between variables were compared by the unpaired Student’s t-test, and differences in proportions were compared by Fisher’s exact test as appropriate. A p-value of < 0.05 was considered to be significant. Analysis of data was performed using SPSS 13.0 (Chicago, IL, USA).

**Results**

**Association of B cell frequency with HBV-induced diseases**

PBMC from healthy controls and HBV patients was analyzed using flow cytometry for evidence of chronic antigenic stimulation. First, the percentage of B cells from PBMC in the four studied groups was investigated by staining with anti-CD19 and anti-CD3. In controls, the frequency of CD19 B cells ranged from 3.22% to 15% of gated lymphocytes. The range in HBV patients was much greater, with CD19 B cell frequencies ranging between 2.52% and 30.1% of gated lymphocytes. The variance of the HBV patients’ B cell frequency data was significantly different from that for the healthy controls (p < 0.05) but there was no significant difference among patients of different groups (Table 2).

To further investigate possible alteration of GC B cells in different HBV-induced disease status, PBMC were stained for anti-CD38 and anti-IgD of gated CD19 B cells. A small proportion of GC B cells (CD38+ IgD−) was found circulating in peripheral blood and the percentage of GC B cells was significantly increased in the patients compared to healthy controls (p < 0.01). We next questioned whether the percentage of GC B cells would vary within patients of different disease status. We found that the proportion of GC B cells of CHB patients was significantly different compared to the other two groups (p < 0.05), but there was no significant difference between LC and HCC patients (Table 2).

**Expression profile of FcRY in different HBV-induced diseases**

FcRY mRNA expression was detectable by quantitative real-time PCR in 24 of 27 (89%) normal samples and 63 of 65 (97%) patient samples. Relative expression of FcRY mRNA levels in B cells from patients with HBV-induced diseases and healthy controls was calculated by normalizing against GAPDH. Significantly higher levels were found in the HBV patients compared to healthy donors. Furthermore, we found FcRY expression in CHB patients was about 20-fold higher (p < 0.05). However, in patients with HBV-associated LC and HCC, FcRY was about 500-fold higher (p < 0.01), compared to healthy controls. In addition, there was a significant difference for expression of FcRY gene between CHB patients and the other two groups (p < 0.05), but no significant difference between LC and HCC patients (Figure 1).

Flow cytometric analysis of intracytoplasmic expression of FcRY protein was performed in B cells from 87 clinical samples including 24 healthy controls and 63 HBV-infected patients (Figure 2). As expected, FcRY protein expression was observed in 87 samples. The FcRY levels on the CD19 B cells in HCC (45.0% ± 15.1%) and LC (41.7% ± 13.3%) patients were significantly higher compared to CHB patients (11.4 ± 4.1%, p < 0.05) and healthy controls (2.5 ± 1.2%, p < 0.01). Overall, the protein expression pattern was similar to that obtained by real-time PCR.

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**Table 2. Association of cell frequency with HBV-induced diseases**

<table>
<thead>
<tr>
<th>Trait</th>
<th>CD19+CD3+</th>
<th>CD19+CD38+IgD−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (%)</td>
<td>p</td>
</tr>
<tr>
<td>HC (n = 27)</td>
<td>6.2 ± 2.5</td>
<td>–</td>
</tr>
<tr>
<td>CHB (n = 25)</td>
<td>8.4 ± 5.1</td>
<td>0.042</td>
</tr>
<tr>
<td>LC (n = 20)</td>
<td>15.4 ± 9.3</td>
<td>0.017</td>
</tr>
<tr>
<td>HCC (n = 20)</td>
<td>14.6 ± 8.5</td>
<td>0.023</td>
</tr>
</tbody>
</table>

p values compared the patients group to the healthy controls group.
These results indicated that the protein expression level of FcR Y was indeed correlated positively with their corresponding mRNA expression level (Figure 3). The expression level of FcR Y was significantly associated with the progression of HBV-related diseases.

Discussion

HBV infection is a serious problem worldwide, especially in China. A substantial number of HBV carriers develop cirrhosis and HCC, but the mechanism underlying the different outcomes among patients is not clear [1–3]. In this study, we investigated the expression profile of FcR Y and B lymphocytes population in HBV-induced diseases to help clarify one aspect of the function of FcR Y, which was over-expressed in HBV-induced diseases. Previous studies failed to reveal any FcR Y mRNA expression in patients with autoimmune diseases. However, expression of FcR Y was found to be strongly enhanced in malignant and metastatic melanomas compared to that in melanocytic nevi [20]. To the best of our knowledge, this study is the first to report the correlation of FcR Y expression profile, B lymphocytes population from peripheral blood, and different stages of HBV infection.

Since FcR Y is preferentially expressed by B lymphocytes and largely limited to the germinal center B cells, we measured the frequency of B lymphocytes and germinal center B cell subpopulation of HBV-infected patients compared to healthy controls. The percentage of the HBV patients’ B cells in PBMC was significantly higher than that of the healthy controls. Furthermore, the germinal center B cell subpopulation was also increased in the course of HBV infection. The elevated presentation of this subpopulation might be the result of B cell activation in this disease. Then we measured the mRNA and protein expression level of FcR Y in 92 samples including HBV-related patients and healthy donors.

The results showed FcR Y expression increased as the HBV-induced disease progressed from chronic hepatitis to cirrhosis and HCC. In addition, up-regulation of FcR Y was indeed correlated positively with their corresponding mRNA expression level. The rarity of B cells expressing the FcR Y gene in the blood of healthy donors may be explained by the low percentage of GC B cells in the peripheral blood of healthy donors and an increased expression level of FcR Y may correlate with change of GC B cells in

Figure 1. mRNA expression of FcR Y on B lymphocytes from peripheral blood in patients with HBV-induced diseases and healthy controls; *p < 0.05; **p < 0.01

Figure 2. Protein expression of FcR Y on B lymphocytes from peripheral blood in patients with HBV-induced diseases and healthy controls. A: healthy control; B: CHB patient; C: LC patient; D: HCC patient

Figure 3. Comparison of mRNA and protein expression level of FcRY in 87 subjects. The real-time PCR results represent the relative values of FcRY mRNA expression to that of GAPDH multiplied by 100,000. The flow cytometry results are presented as percentage
peripheral blood under the pathological progression. However, the mechanism by which HBV antigen affects the function of B lymphocytes is unclear and needs to be addressed in future studies.

In conclusion, the results of our study suggest that B lymphocytes are more active in HBV-induced diseases because of the increased proportion of GC B cells. And the detection of increased FcR Y mainly expressed on GC B cells is associated with a high risk of HBV chronic infection development into cirrhosis and HCC. So FcR Y is perhaps one of the B cell activation markers.

However, it is not yet clear if activation of B lymphocytes and up-expression of FcR Y are the cause, or the outcome, of the disease progression. Additional studies are underway to elucidate the molecular mechanism and the more detailed function of FcR Y and B lymphocytes in HBV-induced diseases.

Acknowledgements

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References


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