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Hepatic expressions of cannabinoid receptors CB1 and CB2 correlate with the fibrogenesis in patients with chronic hepatitis B

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Abstract

Aim: The endocannabinoid system is involved in the pathogenesis of liver fibrosis. However, most of the findings come from experiment researches on animal model or clinical trial on chronic hepatitis C. The roles of cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) in hepatofibrosis on patients with chronic hepatitis B(CHB) have not been studied universally. This study aimed to explore the relationship between liver fibrosis and expressions of CB1 and CB2 on patients with CHB.

Methods: Eighty liver biopsy on patients with CHB (male 52, female 28) were enrolled. Fibrosis was staged on a scale of 1 to 4 (F1 to F4, F4 defining as cirrhosis). There were 20 samples in each fibrosis stage. The expressions of hepatic alpha-smooth muscle actin(α-SMA), CB1 and CB2 were detected by immunohistochemistry.

Results: Hepatic CB1 and CB2 were expressed in all patients with CHB. The degree of fibrosis was associated with significantly increased CB1 and CB2 expressions in CHB. Furthermore a significant increase of positive cells of both CB1 and CB2 was detected in stage 3 and stage 4 compared to stage 1 and stage 2. There was a strong positive association between CB1 expression and α-SMA expression. Moreover, double immunofluorescence staining of CB1 and α-SMA demonstrated that the activated hepatic stellate cells(HSCs) express CB1.

Conclusions: The hepatic expressions of CB1 and CB2 play important roles during the progression of fibrosis induced by CHB. Endogenous activation of CB1 receptors in patients with CHB enhances fibrogenesis by direct effect on activated HSCs.

Key words cannabinoid receptor 1; cannabinoid receptor 2; hepatic stellate cells; liver fibrosis; chronic hepatitis B
1. Background

Marijuana has been used for its psychoactive and medicinal properties for millennia. Understanding of pathways involved in the pharmacological properties of cannabinoids has only emerged with the identification of an endocannabinoid system that comprises at least two specific G-protein coupled receptors [cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2)], their endogenous lipidic ligands (endocannabinoids), and enzymes involved in endocannabinoid synthesis and degradation.1 CB1 receptors are the most abundant receptors in the mammalian brain, but are also expressed at lower levels in a large number of peripheral tissues, including various cell types of the liver.2–6 CB2 receptors are predominantly expressed by immune cells, and have also been detected in the liver in certain pathological states.7, 8 Additional CB receptors may exist, 9 but their potential roles in liver biology are unknown.

Chronic liver diseases are characterized by prolonged liver injury resulting in the chronic activation of an altered wound-healing with progressive accumulation of fibrosis in the liver parenchyma, eventually leading to liver cirrhosis, portal hypertension and liver failure. Progression of fibrosis combines enhanced production of extracellular matrix by hepatic myofibroblasts and impaired matrix turnover.10 Studies have revealed the major impact of the endocannabinoid system in the regulation of liver fibrogenesis. CB1 receptors have been implicated in the pathogenesis of liver fibrogenesis. In contrast, stimulation of hepatic CB2 receptors is emerging as an overall protective pathway with antifibrogenic properties.11 However, most of the findings come from experiment researches on animal model or clinical trial on chronic hepatitis C.12, 13 The roles of CB1 and CB2 receptors in liver fibrosis on patients with chronic hepatitis B(CHB) have not been studied universally.
Patients with CHB have up to a 15% to 40% risk of cirrhosis, hepatocellular carcinoma, and hepatic decompensation in their lifetime. The fibrogenic process involves both the activation and recruitment of hepatic stellate cells (HSCs) in the injured liver tissue. The present study was therefore undertaken to investigate whether the hepatic endocannabinoid receptors in patients with CHB play roles in pathogenesis of liver fibrosis.

2. Materials and methods

2.1. Patients.

Eighty consecutive patients were selected from 455 patients with chronic HBV infection who underwent liver biopsy at the Fifth Hospital of Shijiazhuang between November 2008 and June 2009. All subjects were HBsAg positive with or without HBeAg positive and the detectable HBV DNA measured by real-time polymerase chain reaction (Daan Gene Co., Ltd. of Sun Yat-sen University, China) was among the range of 2.70–8 log10 copies/mL. Serum HBV markers were measured by enzyme-linked immunosorbent-assay (Shanghai Kehua Bio-engineering Ltd, China). All of the patients had no co-infection with hepatitis C virus or human immunodeficiency virus. The study protocol was approved by the Ethics Committee of the Fifth Hospital of Shijiazhuang, and the informed written consents were obtained from all participants.

2.2. Liver Histopathology.

Percutaneous liver biopsy was performed in all subjects. The paraffin-embedded hepatic tissue sections (4 μm) were stained with hematoxylin-eosin (H&E) and Argyrophilic+ Manson staining. All biopsies were diagnosed by the two liver pathologists. Inflammation and fibrosis were graded according to the Scheuer scoring system. Hepatic inflammatory activity grade (G) was divided into G0 (no hepatic necroinflammation) and G1-G4, and liver fibrosis stage (S) was divided into S0
(no fibrosis) and S1-S4. (Tab.1). No patient had clinical evidence of hepatic decompensation and received anti-viral therapy at the time of biopsy.

2.3. **Immunohistochemistry of α-SMA, CB1 and CB2.**

Sections were deparaffinized and antigen retrieval was performed by microwave in sodium citrate buffer (pH 6.0) for 15 min. After blocking of endogenous peroxidase by 0.3% H2O2 and unspecific binding by 2% goat serum, sections were incubated with antibodies of α-SMA, CB1 and CB2, respectively, for overnight at 4°C. Application of the secondary antibodies was followed by incubation with biotinylated anti-rabbit (Golden Bridge Biotechnology, Beijing, PR China) at 1:200 dilution. Sections incubated with Streptavidin peroxidase were visualized with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin. The primary antibodies were used at the following dilutions, rabbit anti-human of CB1 and CB2: 1:150 and 1:200 (Cayman chemical company, USA), rabbit anti-human of α-SMA monoclonal antibody: 1:400 (Golden Bridge Biotechnology, Beijing, PR China).

2.4. **Double immunofluorescence staining of CB1 and α-SMA.**

After endogenous peroxidase activity was quenched, the sections were incubated with mixed antibodies of rabbit anti-human CB1 and mouse anti-human α-SMA for overnight at 4°C. After Dylight 488 goat anti-rabbit IgG (1:300, Earthox, USA) was applied, the sections were incubated with the Dylight 594 goat anti-mouse IgG (1:300, Earthox, USA). The pictures were taken under Olympus BX5 fluorescence microscope.

2.5. **Quantification analysis of α-SMA, CB1 and CB2.**

Positive cells of α-SMA, CB1 and CB2 in hepatic lobules were counted in 10 fields at 400× magnification in each slide.

2.6. **Statistical Analysis.**
Statistical analysis was performed with the SPSS software (SPSS Standard version 13.0, SPSS Inc., Chicago, IL). Data for normal distribution were expressed as mean± standard deviation and compared by one-way ANOVA among groups; data for non-normal distribution were presented as median with interquartile range(M, QR) and analyzed with the U-Mann-Whitney test. Categorical variables were reported as number and percentage and compared by x² test or Fisher’s exact test where appropriate. Linear correlation analysis was performed to determine the correlation between two indexes. A two-sided P value <0.05 is considered statistically significant.

3. Results

**General characteristics of the study population.** Table 1 showed the clinic characteristics of the 80 patients. The patients in the four groups had comparable age, gender, tobacco use, alcohol intake, body mass index(BMI), steatosis, fasting glucose level <6.1 mmol/L, ALT, HBV genotype, HBeAg-positive rate and viral load. But grade of portal inflammation increased with increased fibrosis stage(P <0.001). Representative morphologic changes of liver fibrosis were showed as Fig. 1.

<table>
<thead>
<tr>
<th>Fibrosis Staging</th>
<th>Stage I (n=20)</th>
<th>Stage II (n=20)</th>
<th>Stage III (n=20)</th>
<th>Stage IV (n=20)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Male/female</td>
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<td>13/7</td>
<td>14/6</td>
<td>13/7</td>
<td>0.932</td>
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<tr>
<td>Average age</td>
<td>34.52± 10.65</td>
<td>41.95± 14.73</td>
<td>33.90± 11.67</td>
<td>44.44± 13.25</td>
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<td>BMI(kg/m²)</td>
<td>23.15± 2.16</td>
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<td>24.85± 2.89</td>
<td>24.65± 3.09</td>
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<td>Tobacco use, n (%)</td>
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<td>Alcohol intake, n (%)</td>
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<td>2(10.0)</td>
<td>1(5.0)</td>
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<tr>
<td>Parameter</td>
<td>Stage 1</td>
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<td>Stage 3</td>
<td>Stage 4</td>
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<td>-----------------------------------</td>
<td>---------</td>
<td>---------</td>
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<td>Steatosis, n (%)</td>
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<td>2(10.0)</td>
<td>4(20.0)</td>
<td>3(15.0)</td>
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<tr>
<td>Fasting glucose level &lt;6.1 mmol/L, n (%)</td>
<td>1(5.0)</td>
<td>1(5.0)</td>
<td>2(10.0)</td>
<td>1(5.0)</td>
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<td>ALT (IU/mL)</td>
<td>54.67±49.23</td>
<td>102.33±164.01</td>
<td>139.13±163.50</td>
<td>60.64±54.54</td>
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<td>HBV genotype (B/C)</td>
<td>2/18</td>
<td>1/19</td>
<td>1/19</td>
<td>0/20</td>
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<tr>
<td>Positive HBeAg</td>
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<td>14</td>
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<td>15</td>
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<td>HBV DNA</td>
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<tr>
<td>(log_{10} IU/mL)</td>
<td>6.42±2.19</td>
<td>5.38±2.19</td>
<td>5.22±2.19</td>
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<td>Portal Inflammation Grade (M, QR)</td>
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<td>2.50, 0.88</td>
<td>2.00, 1.00</td>
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</tr>
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</table>

**Fig.1** Representative photomicrographs of liver biopsy on patients with CHB were stained with Argyrophilic +Masson staining showing degree of fibrosis tissue.

Mild proliferation of fibrous tissue in the portal area was observed in the stage 1 (a). Enlarge portal area induced by proliferation of fibrous tissue both in the portal area and paraportal area was observed in the stage 2 (b). Lobules were divided by proliferative fibrous tissue in the portal area and bridging fibrous septa in the form of delicate bands in the stage 3(c). Pseudolobules were
formed in the stage 4. Bar(a,b)=80um; Bar(c,d)=160um.

The increased expression of $\alpha$-SMA in hepatic lobules is accompanied by the increased fibrosis stage. In addition to the smooth muscle of arterioles in portal area, the expression of $\alpha$-SMA on S1 was also observed in hepatic sinus. The expression of $\alpha$-SMA in hepatic sinus was significantly induced with fibrosis progression (Fig.2). Positive cell counts of hepatic sinus on S1, S2, S3 and S4 were 13.84±5.88, 21.16±11.64, 26.08±11.29 and 39.12±17.76 cells/high power field (HPF), respectively. Positive cell counts on S2, S3 and S4 were significantly higher than those on S1 ($P<0.05$), and the counts of S4 were significantly more than those of S2 and S3 (Table 2).

Fig.2 Representative photomicrographs of immunohistochemical finding for $\alpha$-smooth muscle actin($\alpha$-SMA) in hepatic lobule. Positive cells of $\alpha$-SMA in hepatic lobule was increased accompanied by the increased fibrosis stage. (Immunohistochemistry 40×) Bar=50um. a: stage 1; b: stage 2; c: stage 3; d: stage 4.

| Table 2  Positive cells counts of $\alpha$-SMA, CB1 and CB2 in hepatic lobules |
|-----------------|-----------------|---------------|-----------------|---------------|
| stage | case | $\alpha$-SMA ($\mu$±s) | CB1(M, QR) | CB2(M, QR) |
| S1 | 20 | 13.84±5.88 | 18.00, 18.00 | 18.00, 9.00 |
The increased expression of CB1 in hepatic tissue is accompanied by the increased fibrosis stage. CB1 immunoreactivity showed in sinus, portal area and fibrotic septa (Fig.3). Cell counts of CB1 in hepatic lobules on S1, S2, S3 and S4 were 18.00, 18.00, 25.00, 27.00; 30.00, 27.00; 41.00, 31.25 cells/HPF, respectively. Positive cell counts on S2, S3 and S4 were significantly higher than those on S1 (P<0.05), and the counts of S4 and S3 were significantly more than those of S2 (Table 2).

![Fig.3](image)

**Fig.3** Representative photomicrographs of immunohistochemical finding for CB1 in hepatic lobule. Increased positive cells of CB1 in hepatic lobule was accompanied by the increased fibrosis stage. (Immunohistochemistry 40×) Bar = 50μm. a: stage 1; b: stage 2; c: stage 3; d: stage 4.

The increased expression of CB2 in hepatic tissue is accompanied by the increased fibrosis stage. CB2 was expressed in all patients with CHB. The expression of CB2 was observed on sinus, portal area, fibrotic septa, inflamed necrotic foci of portal area. Although the expression patterns
were similar among S1 to S4, more positive cells on S2 and S3 were observed. Sinus was filled with much more positive cells on S4(Fig.4). Positive cell counts of CB2 in hepatic lobules were 18.00, 9.00; 30.00, 15.00; 34.50, 36.50; 44.00, 19.25 cells/HPF, respectively. Positive cell counts on S3 and S4 were significantly more than those on S1 (P<0.05) (Table 2).

**Fig.4** Representative photomicrographs of immunohistochemical finding for CB2 in hepatic lobule. Increased positive cells of CB2 in hepatic lobule was companied by the increased fibrosis stage. (Immunohistochemistry 40×) Bar=50um. a: stage 1; b: stage 2; c: stage 3; d: stage 4.

**Positive correlation between expressions of α-SMA and CB1.** The expressions of both α-SMA and CB1 went up companying with the increased fibrosis stage. There was a significantly positive relationship between α-SMA and CB1 (R=0.880, P<0.05).

**Activated HSCs expressed CB1.** α-SMA positive cells showed green fluorescence and CB1 showed red fluorescence in double immunofluorescence staining. Co-expression of α-SMA and CB1 showed yellow one (Fig.5). All of α-SMA positive cells in hepatic sinus expressed CB1, but CB1 positive cells were more diffuse than those of α-SMA. The result showed that activated HSCs expressed CB1.
4. Discussion

Liver fibrosis is a typical response to chronic liver injury that ultimately leads to further complications such as cirrhosis, liver failure, or hepatocellular carcinoma. The fibrogenic process involves the activation and recruitment of HSCs as well as hepatic myofibroblasts to the injured area, where they synthesize such factors as fibrogenic cytokines, growth factors and inhibitors of matrix degradation. HSCs constitute a large portion of the hepatic interstitium, representing 5%-8% of the total number of cells in the liver. HSCs are quiescent in physiological conditions, but can be activated by factors, including TGF β 1 and IFN γ , that are released by Kupffer cells (KC) and T cells after liver lesions. The activated HSCs could be detected by α -SMA immunostaining. We found that the increased expression of α -SMA in hepatic sinus is accompanied by the increased fibrosis stage. It indicated that activated HSCs involve in hepatic fibrosis progression in patients with CHB. It had been identified that hepatic myofibroblasts within fibrotic septa expressed CB1 receptor by double immunohistochemistry, using an anti-CB1 receptor antibody and an anti-smooth muscle actin antibody, CB1 receptors were detected in cultured hepatic myofibroblasts and in activated hepatic stellate cells. In order to determine the link between activated HSCs and CB1 expressions in liver fibrosis progression, a double immunofluorescence staining and a linear correlation analysis of CB1 and α-SMA were performed.

Fig.5  Double Immunofluorescence labeling

All of α-SMA(green) positive cells in hepatic lobules expressed CB1(red), CB1 positive cells were more than those of α-SMA(40×) Bar=50μm.
with the samples of liver fibrosis. The results showed that activated HSCs expressed CB1 and there was a strong positive association between expressions of CB1 and α-SMA. It has been demonstrated that CB1 was expressed in HSCs in cirrhotic livers during the process of their transformation into myofibroblasts. Therefore CB1 receptor may involved in the pathogenesis of liver fibrogenesis in patients with CHB. Genetic or pharmacological ablation of CB1 protected mice against liver injury, as reflected in the reduced expression of α-SMA and transforming growth factor-β. Regression of fibrosis be achieved by the pharmacological blockade of CB1 receptor antagonism even in an advanced stage of the disease when full-blown cirrhosis has already developed. It has also been shown that CB1 can mediate liver fibrosis through effects on apoptosis and the growth of hepatic myofibroblasts. We previously found that the CB1 receptor antagonist rimonabant can inhibit HSCs proliferation, collagen synthesis and induce HSCs apoptosis. We also demonstrated that rimonabant inhibits the expression of phosphorylated FAK and ERK, which may contribute to the inhibition of HSCs proliferation. Overall, these data suggest that endogenous activation of CB1 in patients with CHB enhances fibrogenesis by direct effect on activated HSCs.

Our study demonstrated also that the presence of fibrosis was associated with significantly increased expressions not only CB1 but also CB2 in patients with CHB, and expressions of CB1 and CB2 increased with fibrosis stage. While both CB1 and CB2 expression is upregulated in hepatic myofibroblasts both in vivo and in vitro, activation of these receptors exerts opposing effects on the fibrogenic process, the CB1 being pro-fibrogenic and the CB2 being anti-fibrogenic. One experiment using CB2 genetic knockout mice demonstrated an augmented fibrogenic response to carbon tetrachloride-induced liver injury. In cirrhotic rats,
chronic treatment with the CB2 receptor agonist JWH-133 attenuated cellular markers of fibrosis and enhanced the regenerative response to acute liver injury. Activating CB2 on Th17 cells may be involved in inflammation and fibrosis of hepatic tissue. In CB2-deficient mice with bile duct ligation, intrahepatic Th17 cells and IL-17 levels were increased compared with wild-type mice, whereas the CB2 agonist JWH-133 reduced the differentiation and function of Th17 cells in vitro. Thus, activating CB2 on Th17 cells maybe effective for treatment of liver fibrosis.

Double immunohistochemistry, using an anti-CB2 receptor antibody and an anti-smooth muscle actin antibody, clearly identified smooth muscle actin-positive cells within fibrotic septa. CB2 receptors were detected in cultured hepatic myofibroblasts and in activated hepatic stellate cells. However, during the progression of chronic liver disease, the pro-fibrogenic CB1 signals seem to prevail over the anti-fibrogenic CB2 signals. It showed a significant relationship between use with cannabis daily and fibrosis progression in patients with ongoing CHC. Similar findings were reported in another cohort of patients. Furthermore, CB2 receptor may have a dual pro- and antifibrogenic function. Avraham’s results indicated that absence or antagonism of CB2 receptors increases fibrosis via activation of HSC directly. At the same time, CB2 antagonism or loss reduces fibrosis indirectly by attenuating inflammatory responses via apoptosis of T cells and decreasing their phagocytosis by HSCs. Furthermore, Coppola et al identified an independent association between the CB2-63 RR variant and more extensive liver cell necroinflammation in CHB. These data therefore suggest that CB1 signaling dominates over CB2 for exogenous cannabinoid ligands during chronic hepatitis C. Likewise, the roles of pro-fibrogenic CB1 maybe prevail over those of the anti-fibrogenic CB2 during the progression of CHB.

The present research was a pilot study. We have notified the different mechanisms of hepatic
fibrosis among chronic hepatitis B and C and other chronic liver diseases, for example, alcoholic hepatic injury and fatty liver disease. One limitation of this and other liver biopsy-based human studies relates to the cross-sectional nature of the data. Reports of this type can demonstrate significant associations, but inferences of cause and effect are always difficult. Further study is needed to demonstrate that CB1 and CB2 were directly induced by hepatitis B viral. Recently one study used a cell culture system to demonstrate a direct relationship between CB1 expression and hepatitis C viral infection, both in time course and static experiments. Moreover the quantitative analysis of CB1, CB2 and their ligands, AEA and 2-AG, in liver tissue is needed in greater number of patients with CHB. The other factors about affecting the expression of cannabinoid receptors such as liver tissue inflammation, viral load and genotype should be considered.

5. Conclusion

The expressions of both cannabinoid receptors CB1 and CB2 are up-regulated in liver fibrosis induced by CHB. The roles of pro-fibrogenic CB1 receptor maybe prevail over those of the anti-fibrogenic CB2 receptor during the fibrotic progression. CB1 receptor in the activated HSCs may link the fibrogenesis. These findings indicate that cannabinoid receptors CB1 and CB2 are important in the progression of fibrosis induced by CHB and may be new targets for pharmacotherapy in this disease.

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