

Original Research Article

Effect of entecavir on IL-6/STAT3/SOCS3 pathway in patients with chronic hepatitis B-induced liver fibrosis

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Abstract

Purpose: To evaluate the impact of entecavir (ENT) on patients with liver fibrosis resulting from chronic hepatitis B (CHB) and its association with the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3)/suppressor of cytokine signaling 3 (SOCS3) pathway.

Methods: Thirty-one patients with liver fibrosis received ENT at a dose of 0.5 mg/day for 48 weeks. Relevant protein levels in patient's serum before and after treatment were assayed using enzyme-linked immunosorbent assay (ELISA). Furthermore, human hepatic stellate cells (HSCs) were cultured in vitro and divided into three groups: control, transforming growth factor beta 1 (TGF- β 1) induction (TGF- β 1 group), and ENT treatment (TGF- β 1 + ENT group). Protein levels in the supernatant were assayed using ELISA, while the expression levels of related genes were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Expression of α -SMA was visualized using immunofluorescence assay and the relevant protein levels were determined by Western blotting.

Results: Treatment with ENT significantly decreased ($p < 0.01$) IL-6 and STAT3 expression, increased SOCS3 expression and significantly reduced ($p < 0.01$) the concentrations of hyaluronic acid (HA), type IV collagen (IVC), laminin (LN) and pro-collagen type III (PCIII) in patients with liver fibrosis. TGF- β 1 significantly ($p < 0.01$) elevated IL-6, STAT3 and Col-I expressions and a tissue inhibitor of metalloproteinases-1 (TIMP-1) suppressed the expression of SOCS3 in human HSCs and induced fibrosis. Entecavir mitigated TGF- β 1-induced fibrogenesis in HSCs ($p < 0.01$).

Conclusion: Entecavir has a positive effect on liver fibrosis resulting from CHB by regulating IL-6/STAT3/SOCS3 pathway. Future research will focus on conducting larger clinical trials to further validate these findings and explore the long-term effects of ENT on liver fibrosis progression and patient outcomes.

Keywords: Entecavir, Chronic hepatitis B (CHB), Liver fibrosis, IL-6/STAT3/SOCS3 pathway

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INTRODUCTION

Fibrotic diseases are major contributors to deaths worldwide, accounting for 45 % of total fatalities in developed countries [1]. These diseases are characterized by the buildup of

excessive extracellular matrix that disrupts the normal structure of tissues [2]. The causes of tissue fibrosis can be difficult to identify and can occur in various organs due to various stimuli and inflammatory responses [3]. Chronic hepatitis B (CHB) is a global health issue,

affecting 240 million people mainly in Africa and Asia [4]. CHB is responsible for an estimated 340,000 and 310,000 deaths each year due to liver cancer and liver decompensation, respectively [5,6]. The CHB patients are also prone to developing liver fibrosis.

Antiviral therapy plays a crucial role in the treatment of CHB patients [7-9], with the potential to significantly improve patient outcomes. It has been observed that even patients with liver cirrhosis show improvement in liver fibrosis after antiviral therapy [10,11]. Entecavir (ENT) is an important first-line drug for treating CHB patients with decompensation. The oral administration of ENT is well tolerated by CHB patients and has been shown to significantly improve liver function after 12 months of treatment [12]. Despite the widespread use of ENT in CHB treatment, its mechanism of action remains unclear. This study aims to better understand ENT's impact on liver fibrosis in CHB patients and to shed more light on the molecular mechanism of ENT's ability to alleviate liver fibrosis.

METHODS

Clinical data

The study comprised 31 patients who fulfilled the inclusion criteria, which included a diagnosis of liver fibrosis resulting from CHB, an age range of 18 to 65 years, no prior antiviral treatment within the past 6 months and the absence of any other serious illnesses. The exclusion criteria include unclear clinical data, infection by viruses other than hepatitis B, other liver diseases and pregnancy or lactation. The study was approved by the Ethics Committee of The First People's Hospital of Linping District Hangzhou, China (approval no. Y-01-203) and also complied with the international guidelines for human studies. All patients gave informed consent to participate.

Reagents and consumables

Human hepatic stellate cells (HSCs) were sourced from ATCC, the antiviral drug ENT manufactured by Jiangsu Chiatai Tianqing Pharmaceutical Co., Ltd., transforming growth factor beta 1 (TGF- β 1) provided by PeproTech, Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12), fetal bovine serum (FBS), trypsin and ethylenediaminetetraacetic acid (EDTA) and phosphate-buffered saline (PBS) were all obtained from Gibco. ELISA kits were purchased from TSZ, while antibodies for alpha-smooth muscle actin (α -SMA), phosphorylated signal transducer and activator of transcription 3 (p-

STAT3), suppressor of cytokine signaling 3 (SOCS3) and β -actin were acquired from Abcam.

Treatments

All the participants in the study ingested ENT orally at a dose of 0.5 mg per day for a period of 48 weeks. Prior to and after the treatment, blood samples were collected for the analysis of protein expression levels.

Culture of LX-2 cells

After thawing, the LX-2 cells were seeded in 24-well plates and cultured in a mixture of DMEM/F12 containing 15 % FBS in an incubator with 5 % CO₂ at 37 °C. The cells were divided into three groups: the Control group, the TGF- β 1 induction group (TGF- β 1 group) and the ENT treatment group (TGF- β 1 + ENT group). Cells in the TGF- β 1 group were cultured in a medium containing 10 ng/mL TGF- β 1 for 48 h, while those in the TGF- β 1 + ENT group were cultured in a medium containing both 10 ng/mL TGF- β 1 and 5 μ g/mL ENT for 48 h.

Serum protein determination

The isolated serum was subjected to evaluation of protein expression levels and liver fibrosis by utilizing ELISA kits for interleukin-6 (IL-6), STAT3, SOCS3, hyaluronic acid (HA), pro-collagen type III (PCIII), laminin (LN) and type IV collagen (IVC). The procedures were carried out according to the kit manufacturer's guidelines.

Total ribonucleic acid (RNA) extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were harvested from culture medium and mixed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). The mixture was left to stand for 5 min, then 200 μ L of chloroform was added and shaken evenly. The mixture was then centrifuged at 4 °C and 12,000 rpm for 10 min. The supernatant was mixed with an equal volume of isopropanol, left at room temperature for 10 min and then centrifuged again at 4 °C and 12,000 rpm for 15 min. The resulting precipitate was washed twice with freshly prepared 75 % alcohol and dissolved in an appropriate volume of DEPC-treated water (Beyotime, Shanghai, China). The RNA concentration was measured using a NanoDrop spectrophotometer.

The primers for IL-6, STAT3, SOCS3 and GAPDH were designed online using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sangon Biotech

(Shanghai, China) Co., Ltd. and are listed in Table 1. The cDNAs for IL-6, STAT3, SOCS3 and GAPDH were synthesized from the total RNA using random primers from the RT Master Mix kit. The qRT-PCR was performed using the SYBR-Green Real-Time PCR Master Mix and ABI 7500 sequence detection system according to the manufacturer's protocols. The transcription level was evaluated using the cycle threshold (Ct) value and the levels of target genes were normalized to the endogenous reference using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequences of IL-6, STAT3, SOCS3 and GAPDH

Gene	Sequence of primers
IL-6 (Forward)	5'-CCACCGGGAACGAAAGAGAA-3'
IL-6 (Reverse)	5'-GAGAAGGCAACTGGACCGAA-3'
STAT3 (Forward)	5'-CTTCCCGGTTTTGCCTTCA-3'
STAT3 (Reverse)	5'-GCTTCCAACCTTTGGCAGATT-3'
SOCS3 (Forward)	5'-GCTCCTTTGTGGACTTCACG-3'
SOCS3 (Reverse)	5'-CCCGAATCGAAGTCTCCGTC-3'
GAPDH (Forward)	5'-TCAGACACCATGGGGAAGGT-3'
GAPDH (Reverse)	5'-TCCCGTTCTCAGCCATGTAG-3'

Cell immunofluorescence assay

Harvested cells were washed once with phosphate-buffered saline (PBS), fixed with 4 % paraformaldehyde for 10 min, washed once again with PBS and then treated with PBS containing 0.1 % TritonX-100. The cells were then washed twice with PBS, blocked with 10 % goat serum for an hour and incubated overnight at 4 °C with rabbit polyclonal primary antibody against alpha-smooth muscle actin (α -SMA) (dilution: 1:100). Thereafter, the cells were incubated for 1 h in a dark environment with a goat anti-rabbit secondary antibody at a 1:300 dilution at 37 °C, followed by three washes with PBS. The cell nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) and washed three times with PBS, before being mounted using an anti-fade fluorescence mounting medium. Finally, the cells were examined and photographed under a fluorescence microscope.

Western blotting (WB)

The cells were lysed using radioimmunoprecipitation assay (RIPA) cell lysis buffer and centrifuged to extract total proteins. The protein concentration was then determined using the BCA protein assay. The proteins were separated by 8 % sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene fluoride membrane. The membrane was sealed in Tris-buffered saline with 5 % skim milk powder and 0.1 % Tween-20. It was gently shaken at 4 °C overnight with primary rabbit polyclonal antibodies for p-STAT3 (dilution: 1:500), SOCS3 (dilution: 1:500) and β -Actin (dilution: 1:500), respectively. The membrane was then incubated with secondary goat anti-rabbit (HRP) IgG antibody (dilution: 1/2000) and immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Foster City, CA, USA).

Statistical analysis

Data was processed statistically using the Statistic Package for Social Science (SPSS) 20.0 software (IBM, Armonk, NY, USA). The results were presented as mean \pm standard deviation (SD) and compared between groups using an independent-samples t-test. A *p*-value of less than 0.05 was indicative of statistically significant differences.

RESULTS

Effects of ENT on the expressions of serum IL-6, STAT3 and SOCS3 in patients

The concentrations of IL-6, STAT3 and SOCS3 in the serum of 31 patients treated with ENT were determined by ELISA. After 48 weeks of treatment, the concentrations of IL-6 and STAT3 in the serum showed a significant decrease compared to pre-treatment levels (*p* < 0.01) (Figure 1 A and B). Conversely, the concentration of SOCS3 showed a significant increase (*p* < 0.01) (Figure 1 C).

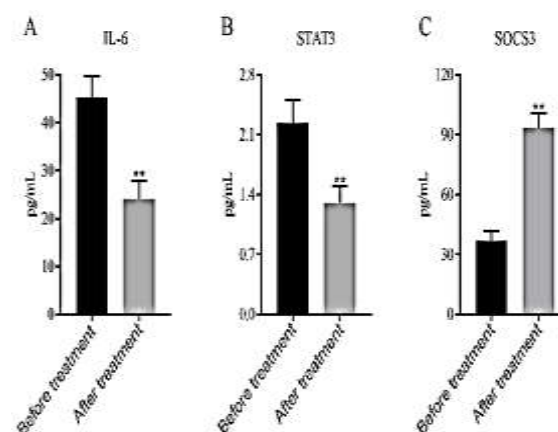


Figure 1: Expression levels of (A) serum IL-6, (B) STAT3 and (C) SOCS3 in patients before and after treatment. ***P* < 0.01

Influences of ENT on liver fibrosis indicators in patients

After treatment with ENT, the levels of liver fibrosis markers HA, LN, PCIII and IVC were assayed in the serum of 31 patients using ELISA. Results showed that the levels of HA and IVC decreased significantly when compared to pre-treatment levels ($p < 0.01$), as depicted in Figures 2 A and D. The levels of LN and PCIII also decreased, but to a lesser extent ($p < 0.05$), as shown in Figures 2 B and C.

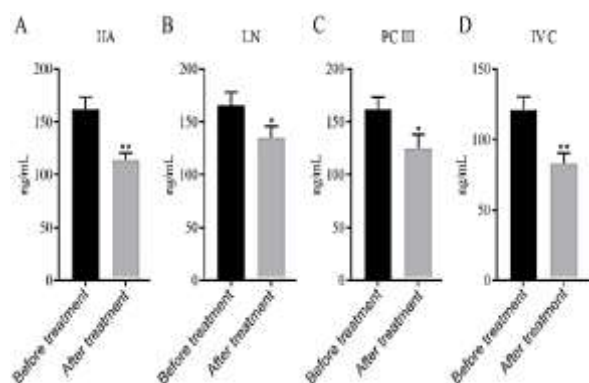


Figure 2: Concentrations of liver fibrosis indicators (A) HA, (B) LN, (C) PCIII, and (D) IVC in the serum of patients before and after treatment. * $p < 0.05$, ** $p < 0.01$

Effect of ENT on the expressions of IL-6, STAT3 and SOCS3 genes in TGF- β 1-induced human HSCs

The qRT-PCR results showed that the gene expression levels of IL-6 and STAT3 in human HSCs significantly increased ($p < 0.01$) compared to the control group, as depicted in Figures 3 A and B, while the expression of SOCS3 gene was significantly decreased ($p < 0.01$) as shown in Figure 3 C in the TGF- β 1 group. Moreover, ENT has the potential to effectively reverse the effects of TGF- β 1.

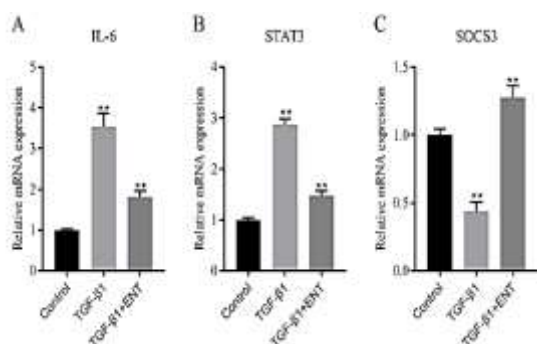


Figure 3: The expression of (A) IL-6, (B) STAT3 and (C) SOCS3 genes in human hematopoietic stem cells (HSCs). ** $P < 0.01$ vs. control

Influence of ENT on the TGF- β 1-induced fibrosis in human HSCs

qRT-PCR results showed that the gene expressions of collagen (Col)-I and tissue inhibitor of metalloproteinases-1 (TIMP-1) were significantly increased in the TGF- β 1 group compared to the Control group ($p < 0.01$) as depicted in Figures 4 A and B. Furthermore, the effect of TGF- β 1 was effectively reversed by ENT ($p < 0.01$).

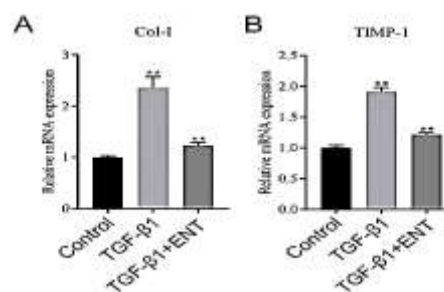


Figure 4: Impact of ENT on TGF- β 1-induced fibrosis in human HSCs was investigated. Gene expressions of (A) Col-I and (B) TIMP-1 were determined using qRT-PCR. ** $P < 0.01$ vs. control

Effect of ENT on the expression of IL-6/STAT3/SOCS3 pathway in human HSCs

ELISA results (Figure 5 A) showed that TGF- β 1 induction led to a significant increase in IL-6 expression ($p < 0.01$), but ENT significantly reduced the expression level of IL-6 ($p < 0.05$). Western blot analysis (Figure 5 B) showed that compared to control group, the protein expression of p-STAT3 was significantly elevated ($p < 0.01$) by TGF- β 1 induction, whereas the expression of SOCS3 was significantly decreased ($p < 0.01$). However, ENT treatment significantly reversed the effects of TGF- β 1 on protein expressions of p-STAT3 and SOCS3 ($p < 0.01$) as shown in Figure 5 C.

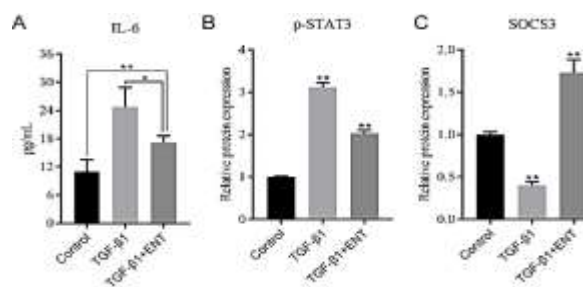


Figure 5: The effect of ENT on the expression of the IL-6/STAT3/SOCS3 pathway in human HSCs was evaluated. (A) The IL-6 expression levels were measured in the cell culture supernatant using ELISA. (B-C) Additionally, the relative expression levels of p-STAT3 and SOCS3 were analyzed. * $P < 0.05$, ** $p < 0.01$ versus control

DISCUSSION

ENT, a novel analogue of 2'-deoxyguanosine, was found to suppress HBV replication in a dose-dependent manner in cells by transforming into active 5'-triphosphate [13]. Patients with CHB, who showed obvious fibrosis, experienced a significant improvement in liver fibrosis indicators following ENT treatment. The expression levels of IL-6, STAT3 and SOCS3 showed significant improvement after ENT treatment. The study by Ogata *et al* [14] showed that the deletion of SOCS3 in the liver resulted in over-activation of STAT3 and promotion of liver fibrosis in mice while the loss of SOCS3 gene enhanced the expression of TGF- β 1, a fibrosis mediator. Repression of TGF- β 1 expression was observed both *in vitro* and *in vivo* through the inhibition of STAT3 or the overexpression of SOCS3. These findings suggest that liver fibrosis is closely related to STAT3 and SOCS3 and that ENT is likely to target the STAT3/SOCS3 pathway to alleviate liver fibrosis. In a study by Wang *et al* [15], 82 CHB patients were treated with ENT, resulting an increase in platelets, effective improvement in liver fibrosis and a noticeable decrease in the collagen area percentage in patients with prominent fibrosis.

Studies have shown that IL-6, a cytokine with a protective effect on the liver, enhanced liver regeneration and protect against liver injury caused by alcohol and carbon tetrachloride poisoning [16-18]. IL-6 triggers intracellular pathways via the glycoprotein 130 (gp130) receptor, with the gp130/STAT3 signaling pathway mediating the protective effects of IL-6 [19]. Research suggests that STAT3 may be involved in mediating the effects of IL-6, as it has been observed that STAT3 does not respond to IL-6 activation during liver regeneration or injury [17,20]. In the present study, the expressions of IL-6 and STAT3 in patients were analyzed using serological tests, revealing a positive correlation with liver fibrosis that was significantly reduced after treatment with ENT. The results were consistent with *in vitro* cell assays. STAT3 has been identified as a vital and ubiquitous transcription factor that plays a role in fibrotic diseases like systemic sclerosis and liver fibrosis [21]. STAT3, initially activated by IL-6, is further activated by other cytokines, hormones and growth factors [22]. Therefore, ENT likely acts on the IL-6/STAT3 signaling pathway.

The activation of the Janus kinase (JAK)/STAT signaling pathway is triggered by cytokines such as IL-6 binding to their receptors, leading to dimerization and phosphorylation of the receptor-associated tyrosine kinase. This causes the

translocation of phosphorylated STATs into the cell nuclei in dimeric form, thereby activating the transcription of multiple target genes, including the SOCS genes. The JAK/STAT3 signaling pathway plays a key role in several physiological processes, such as the development and regeneration of the liver, prevention of liver injury caused by CCl₄ and antiviral defense in the liver [20]. The SOCS family consists of 8 proteins, including SOCS1-SOCS7 and cytokine-induced SH2-containing protein (CIS). Upon stimulation by various cytokines, SOCS1-SOCS3 and CIS are rapidly induced and inhibit JAKs to negatively regulate cytokine signaling. The study by Ogata *et al* [14] showed that SOCS3 expression is reduced not only in liver cancer but also in non-tumor regions, and the decline is more significant with the progression of liver fibrosis. The present study found that the expression of SOCS3 had a negative regulatory relationship with the expressions of IL-6 and STAT3. The expressions of IL-6 and STAT3 were elevated, while the expression of SOCS3 was decreased in patients with liver fibrosis following CHB. Treatment with ENT significantly increased the expression of SOCS3, while decreasing the expressions of IL-6 and STAT3.

CONCLUSION

This study provides evidence for the potential of ENT in the treatment of liver fibrosis in patients with CHB by modulating IL-6/STAT3/SOCS3 signaling pathway. Future research should focus on conducting larger clinical trials to further validate these findings and explore the long-term effects of ENT on liver fibrosis progression and patient outcomes.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

The study was approved by the Ethics Committee of The First People's Hospital of Linping District Hangzhou, China (approval no. Y-01-203).

Availability of data and materials

The datasets used and/or analyzed during the

current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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