CXCL6 promotes human hepatocyte proliferation through CXCR1-NFκB pathway and inhibits collagen I secretion by hepatic stellate cells

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Complete List of Authors:	Li, Zhenghong; Shanghai General Hospital zhang, Qidi; Shanghai General Hospital zhang, Qingqing; Shanghai General Hospital Xu, Mingyi; Shanghai General Hospital Qu, Ying; Shanghai General Hospital Cai, Xiaobo; Shanghai General Hospital Lu, Lungen; Shanghai General Hospital
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CXCL6 promotes human hepatocyte proliferation through CXCR1-NFκB pathway and inhibits collagen I secretion by hepatic stellate cells

Zhenghong Li, Qidi Zhang, Qingqing Zhang, Mingyi Xu, Ying Qu, Xiaobo Cai, Lungen Lu*

Department of Gastroenterology & Hepatology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 20080, China

***Corresponding author:** Prof. Lungen Lu, M.D, Department of Gastroenterology & Hepatology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 100, Hai Ning Road, Shanghai, China 200080. Tel: +86-21-63240090; Fax: +8621-63241377; E-mail: lungenlu1965@163.com

Running title: CXCL6 promotes hepatocyte proliferation

Abstract

Hepatocyte proliferation and collagen I (COLI) secretion are important processes during liver regeneration. This study aimed to investigate the role of CXCL6 in hepatocyte proliferation and COLI secretion. Serum CXCL6 level in chronic hepatitis B (CHB) patients were examined and the effects of CXCL6 on the proliferation of L02 hepatocytes and the secretion of COLI from LX2 human hepatic stellate cells were evaluated. We found that serum CXCL6 level increased gradually with disease progression of CHB, and there was positive correlation between serum CXCL6 level and alanine transaminase (ALT) and aspartate transaminase (AST). In vitro, CXCL6 promoted L02 proliferation but this was blocked upon CXCR1 knockdown. The level of phospho-I κ B α was upregulated by CXCL6 but downregulated by CXCR1 siRNA in L02 cells. CXCL6 inhibited the secretion of COLI by LX2 cells, dependent on CXCR1 and CXCR2. Taken together, these data suggest that increased expression of CXCL6 during CHB could promote hepatocyte proliferation through CXCR1/NF- κ B pathway and inhibit the secretion of COLI by hepatic stellate cells.

Keywords: CXCL6, hepatocyte, hepatic stellate cells, proliferation, COLI

Introduction

Liver fibrosis is an inevitable stage during the course of chronic hepatitis B (CHB), and leads to the development of cirrhosis and hepatocellular carcinoma. Because liver fibrosis is potentially reversible, effective suppression of hepatitis B virus (HBV) replication and subsequent acceleration of liver recovery are essential to the outcome of this disease. Hepatocyte proliferation and collagen I (COLI) degradation are important processes during liver regeneration (Oyanagi et al. 2014).

Inflammatory mediators, such as the CXC chemokines, are essential in the response to hepatic injury (Clarke et al. 2009). CXC chemokines are subdivided into two subsets based on the presence or absence of a Glu-Leu-Arg (ELR) motif at the amino terminus. ELR-positive CXC chemokines include epithelial neutrophil-activating protein (ENA-78), macrophage inflammatory protein-2 (MIP-2), granulocyte chemotactic protein-2 (GCP2/CXCL6) and interleukin 8 (IL8), which possess the ELR motif to the CXCR1 and CXCR2 receptors (Mantovani et al. 2006). CXCR1 and CXCR2 are expressed in neutrophils, endothelial cells, and hepatocytes (Cao et al. 2000, Murphy 2002). CXC chemokines bind CXCR1/CXCR2 and promote hepatocyte proliferation (Colletti et al. 1998, Ren et al. 2003). In addition, CXCR1 promotes liver repair by facilitating recovery after ischemia/reperfusion (I/R) injury (Kuboki et al, 2008). In virus-induced liver injury, CXCL6 was upregulated in liver specimens of chronic hepatitis C patients (Asselah et al, 2005). However, the underlying mechanism remains unknown.

In this study we aimed to investigate the role and mechanism of CXCL6 in CHB. We collected the serum from 50 patients with CHB, detected serum CXCL6 level and analyzed clinical data of these patients. Next, we explored the effect and mechanism of CXCL6 on hepatocyte proliferation and the secretion of COLI from hepatic stellate cells.

Methods

Patients

Fifty CHB patients (age range 27–59 years, male-to-female ratio 37:13) who were HBsAg positive for at least 6 months were treated at Shanghai General Hospital between 2008 and 2010. Patients with the following characteristics were excluded: HIV or HCV co-infection, consumption of more than 30 g alcohol per day, having received anti-viral treatment, or insufficient liver biopsy tissue (a minimum length of 1.0 cm with at least six portal tracts was required). CHB patients with end-stage liver disease were excluded. This study was approved by our institutional ethics committee and informed consent was obtained from all patients prior to participation. All patients underwent percutaneous liver biopsy to determine fibrotic stage and inflammation grade, according to Scheuer's classification (Scheuer 1995).

Cell culture

L02 human hepatocyte cell line was purchased from the Cell Bank of Shanghai Institutes of Life Sciences, and LX-2 human hepatic stellate cell line was kindly provided by the Laboratory Diagnostic Division of Shanghai Changzheng Hospital. Hepatocytes were maintained in 1640 medium (Gibco) containing 10% fetal bovine serum (FBS), and HSCs were grown in DMEM medium (Gibco) containing 10% FBS.

ELISA

Serum CXCL6 protein and COLI of cell culture supernatant were examined using enzyme-linked immunosorbent assay (ELISA) kits (WESTANG BIO-TECH CO, China) according to the manufacturer's instructions.

Real-time PCR

Total RNA was extracted from cells using Trizol and was reverse-transcribed using an iScript cDNA kit (TAKARA, Japan). Real-time PCR was performed on an iCycler system using SYBR Green Master Mix (TAKARA). Primer specificity was confirmed by sequencing PCR products. GAPDH was used as the internal control. Data were presented according to the $\Delta\Delta C_t$ method.

Western blot analysis

Cultured cells were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors (Sigma, USA). Cell lysate was separated on 10% Bis-Tis gradient gel (Invitrogen, USA), transferred to a PVDF membrane, then incubated with antibody to CXCL 6 (1:500, Abcam, USA), CXCR1 (1:500, Abcam), CXCR2 (1:500, Abcam), phospho-I κ B α (1:1000, Cell Signaling Technology, USA), STAT3 (1:1000, Cell Signaling Technology), pSTAT3 (1:2000, Cell Signaling Technology) and GAPDH (1:1000, Cell Signaling Technology) overnight at 4°C, followed by incubation with HRP conjugated secondary antibodies. Membranes were incubated with chemiluminescent substrate (Pierce, USA) and exposed to radiographic film. Bands were quantified by Scion Image software, version 4.0.3. GAPDH was used as loading control.

Immunocytofluorescence

Cells were grown on glass slides and treated as indicated. Then the cells were stained by the antibody to albumin (Abcam) and secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Representative images were captured with an Olympus IX70.

Cell transfection

siCXCR1 (forward: CAUCGAUGAAGGAAUAUCUdTdT; reverse: AGAUUUCCUUCAUCGAUGdTdT), siCXCR2 (forward: CAAGAUUCUAGCUAUACAUDdTdT; reverse: AUGUAUAGCUAGAAUCUUGdTdT) and siControl were chemically synthesized (BioTend, Shanghai, China). The cells were transfected with 50 nM of siCXCR1, siCXCR2, or siControl using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions.

MTT assay

Cells were plated in 96-well culture plates (1×10^3 cells per well) and incubated for 1 to 3 days. Then, 20 μ l of 5 mg/ml of MTT solution was added to each well and incubated for 4 h at 37°C. The media were removed from each well, and the resultant MTT formazan was solubilized in 150 μ l of DMSO. The absorbance values at 492 nm were measured using a micro-plate reader (Bio-Rad). The experiment was repeated three times and each experiment had six replicate wells.

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation and were representative of at least three separate experiments. Comparisons were performed using ANOVA and correlation was determined using Pearson's correlation coefficient. A p-value of less than 0.05 was considered significant.

Results

CXCL6 level is high in the serum of CHB patients

The demographic characteristics and histologic data for CHB patients are summarized in Table 1. A trend toward a higher serum level of CXCL6 in CHB patients was observed from S0 to

S4. Serum CXCL6 level was significantly higher in CHB patients with Scheuer fibrosis score S0-S1 than in those with S2-S4 ($P=0.001$, Fig. 1). Furthermore, we examined the association of serum CXCL6 level with alanine transaminase (ALT), aspartate transaminase (AST), platelets (PLT), international normalized ratio (INR), total bilirubin (TB), and direct bilirubin (DB) (Table 2). From stage S0 to S4, we observed significant association of serum CXCL6 level with ALT and AST.

CXCL6 promotes hepatocyte proliferation *in vitro*

We treated L02 human hepatocytes with different concentrations of CXCL6 and evaluated hepatocyte proliferation. MTT assay showed that CXCL6 stimulated hepatocyte proliferation in a time and concentration dependent manner (Fig. 2). In particular, the proliferation of L02 cells was significantly increased after treatment with 100 ng/ml CXCL6 for 48 h, compared to treatment with 1 ng/ml or 10 ng/ml CXCL6, while longer treatment (72 h) led to reduced proliferation of L02 cells (Fig. 2). Therefore, we selected the concentration of CXCL6 at 100 ng/ml and treatment time at 48 h for the following experiments.

Next we detected the expression of CXCL6 receptors CXCR1 and CXCR2 in L02 cells by immunocytofluorescence. While we observed that hepatocyte marker albumin was stained as green in the cytoplasm, CXCR1 and CXCR2 were stained as red in the membrane, the cytoplasm and the nucleus (Fig. 3A). To investigate the role of CXCR1 and CXCR2 in mediating the effect of CXCL6 on hepatocyte proliferation, we employed siCXCR1 and siCXCR2 to knockdown CXCR1 and CXCR2 in L02 cells, as confirmed by real-time PCR analysis (Fig. 3B). MTT assay showed that L02 cells treated with CXCL6 in addition to siCXCR1 had no difference in proliferation capacity compared with L02 cells

treated with siCXCR1 alone ($P > 0.05$), but the proliferation capacity was weaker than L02 cells treated with CXCL6 alone ($P < 0.05$). However, the proliferation capacity of L02 cells treated with CXCL6 and siCXCR2 was stronger than that of cells treated only with siCXCR2 ($P < 0.05$) ($P < 0.05$; Fig. 3C).

Furthermore, after treatment with CXCL6 (100 ng/ml) L02 cells showed significantly increased level of phospho-I κ B α ($P < 0.05$), but the levels of total STAT3 and p-STAT3 showed no significant difference ($P > 0.05$; Fig. 4A). Meanwhile, the levels of phospho-I κ B α , STAT3, and p-STAT3 were decreased after the cells were treated with siCXCR1 (Fig. 4B). In contrast, in cells treated with siCXCR2, only p-STAT3 level was decreased while the levels of phospho-I κ B α and total STAT3 showed no significant difference (Fig. 4C). These data suggest that CXCR1 and CXCR2 have different role in mediating the effect of CXCL6 on hepatocyte proliferation.

CXCL6 inhibits COLI secretion from human hepatic stellate cells

Next we aimed to investigate the effect of CXCL6 on collagen secretion by hepatic stellate cells. ELISA assay showed that after treatment with CXCL6, COLI level in the supernatant of LX2 cells was decreased ($P < 0.05$; Fig. 5A). To investigate the role of CXCR1 and CXCR2 in mediating the effect of CXCL6 on collagen secretion, we employed siCXCR1 and siCXCR2 to knockdown CXCR1 and CXCR2 in LX2 cells (Fig. 5B). Treatment with siCXCR1 or siCXCR2 resulted in no significant difference in the proliferation of LX2 cells compared with siControl group (Fig. 5C), but caused significant increase in COLI level in the supernatant of LX2 cells ($P < 0.05$; Fig. 5D). These data suggest that CXCR1 and CXCR2 play important role in mediating the effect of CXCL6 on collagen secretion from hepatic stellate cells.

Discussion

CXCL6 is an important chemokine involved in the pathogenesis of liver fibrosis. In this study we demonstrated that serum CXCL6 level increased along with liver disease progression in CHB patients. We also found a close association between serum CXCL6 level and biochemical markers of liver function. The potential mechanism is that CXCL6 promotes hepatocyte proliferation through CXCR1/NF- κ B signaling, while inhibits COL1 secretion from human hepatic stellate cells synergistically with CXCR1 and CXCR2.

Consistent with a previous study (Colletti et al. 1998), we found that CXCL6 (10–1000 ng/ml) promoted hepatocyte proliferation and hepatocyte proliferative capacity was the highest when cells were treated with 100 ng/ml CXCL6 for 48 h. However, when CXCL6 concentration was between 100 and 1000 ng/ml, proliferative capacity could not be increased further with the increase in CXCL6 concentration. This may be related to the availability of CXCL6 receptors. CXCL6 should bind with CXCR1 and/or CXCR2 to exert biological effects (Mantovani et al. 2006). If the receptors are saturated, even higher concentration of CXCL6 could not exert additional effects.

By using siRNA to knockdown CXCL6 receptors, we found that the effects of CXCL6 on hepatocyte proliferation could be mediated by CXCR1. Upon CXCR1 knockdown, CXCL6 could not promote hepatocyte proliferation. Meanwhile, even without CXCL6 stimulation, CXCR1 can regulate hepatocyte proliferation. In contrast, upon CXCR2 knockdown, CXCL6 could still promote hepatocyte proliferation. These results suggest that CXCR1 is the critical receptor for CXCL6 to promote hepatocyte proliferation, and hepatocytes can secrete other chemokines to maintain their proliferation through CXCR1.

NF- κ B and STAT3 are transcription factors critical to liver regeneration and are involved in signaling pathway downstream of CXCL6 and CXCR1 (Taub 2004, Terui and Ozaki 2005, Luedde and Trautwein 2006). Our results demonstrated that CXCL6 treatment led to increased phospho-I κ B α level, but could not increase the activation of STAT3. Furthermore, we found that siCXCR1 decreased not only phospho-I κ B α level but also total STAT3 and p-STAT3 levels. Considering these results, we postulate that CXCL6/CXCR1/NF- κ B signaling is the primary pathway through which CXCL6 promotes human hepatocyte proliferation, and CXCR1 may participate in STAT3 signaling pathway through other ligands.

Another interesting observation from our study is that CXCL6 inhibited COLI secretion from human hepatic stellate cells. Inhibition of COLI secretion is the key factor in promoting hepatocyte proliferation in vivo (Iimuro et al. 2003). HSC is the major source of COLI (Issa et al. 2003). Therefore, we examined the impact of CXCL6 on COLI secretion by LX2 cells. We found that CXCL6 (100 ng/ml) significantly inhibited the secretion of COLI from LX2 in vitro. However, after CXCR1 or CXCR2 knockdown, CXCL6 could not inhibit COLI secretion by LX2 cells. These data indicate that CXCL6 could inhibit the secretion of COLI from HSC through CXCR1 and CXCR2 pathways. Interestingly, a recent study using LX2 cells showed that HSC could promote hepatocellular carcinoma cell invasion through SDF-1/CXCR4 pathway (Li et al. 2014). These results suggest that CXC chemokine family members play diverse role in HSC.

In conclusion, we demonstrated that CXCL6 not only promotes hepatocyte proliferation through CXCR1/NF- κ B pathway, but also inhibits the secretion of COLI from HSC through the synergistic effects of CXCR1 and CXCR2. In the natural history of chronic

hepatitis B, why upregulated CXCL6 level could not improve the outcome of the disease is still not fully understood. One possibility is that with limited expression of CXCR1 and CXCR2 in hepatocytes and HSC, upregulated CXCL6 can not fully bind the receptors and exert maximum biologic effects. However, a previous study reported that CXCR1 expression was induced in hepatocytes during liver injury, suggesting that CXCR1 facilitates liver repair and regeneration after ischemia injury (Clarke et al. 2011). Another possibility is that only at relatively high concentration could CXCL6 inhibit collagen secretion. During the progression of CHB, CXCL6 may be induced to provide a protection mechanism to inhibit collagen secretion and reduce fibrosis. However, other pathological factors may interfere with the induction of CXCL6 expression in the liver tissues. Consequently, CXCL6 concentration in the liver is not high enough to inhibit collagen secretion and protect against fibrosis. Therefore, increasing CXCL6 level as well as promoting CXCR1 and CXCR2 expression in the liver may provide a new treatment option for patients with liver fibrosis.

Conflict of interest

None

Acknowledgments

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Figure Legends

Figure 1. The correlation of CXCL6 with histologic scores of inflammatory activity and fibrosis in CHB patients (n=10). (A) CXCL6 and Scheuer fibrosis score (0–4). Spearman's $\rho=0.781$, $P<0.05$; (B) CXCL6 and Scheuer inflammation score (0–4). $\rho=0.875$, $P<0.05$. Boxes represent interquartile ranges with medians; $P<0.01$: CHB patients with Scheuer fibrosis score 0-1 vs. CHB patients with Scheuer fibrosis score 2-4.

Figure 2. CXCL6 promotes hepatocyte proliferation. L02 cells were treated with CXCL6 (0–1,000 ng/ml) for 12–72 h. Cell proliferation was determined by MTT assay. * $P<0.05$ ** $P<0.01$ compared with the control group (n=5 for each group).

Figure 3. CXCL6 receptors regulate hepatocyte proliferation *in vitro*. (A) The expression of CXCR1 and CXCR2 in L02 cells was detected by immunocytofluorescence. Red: CXCR1 and CXCR2, Green: Albumin (Alb), a marker of hepatocytes and usually localized in the cytoplasm. Blue: the nucleus stained by DAPI. Original magnification: 200X. (B) L02 cells were treated with 50 nM siCXCR1, siCXCR2 or siControl for 24–72 h. CXCR1 and CXCR2 mRNA levels were determined by Real-time PCR with GAPDH as internal control. (C) MTT assay on the effects of the receptors on L02 cell proliferation *in vitro*. Data are expressed as the mean $\pm$ standard error (n=5).

Figure 4. Effects of CXCL6 on L02 proliferation are mediated through CXCR1/NF- κ B signaling. (A) After L02 cells were treated with CXCL6 (100 ng/ml) for 48 h, the protein levels of p-I κ B α , total STAT3 and p-STAT3 were detected by Western blot analysis. (B,C) L02 cells were treated with 50 nM siCXCR1, siCXCR2 or siControl, and the protein levels of p-I κ B α , total STAT3 and pSTAT3 were detected by Western blot analysis. Shown were representative blots from three experiments with similar results. Densitometry analysis of the blots were shown at the

right panles (n=3). * $P<0.05$. GAPDH was loading control.

Figure 5. CXCL6 inhibits the secretion of COLI by LX2 cells. (A) ELISA analysis of COLI level in the supernatants of LX2 cells. (B) Knockdown of CXCR1 and CXCR2 was confirmed by Real-time PCR analysis. (C) MTT assay on the effects of siCXCR1 and siCXCR1 on LX2 proliferation. Data are expressed as the mean $\pm$ standard error (n=5). (D) LX2 cells were treated with siCXCR1, siCXCR2 or siControl, and then treated with CXCL6 (100 ng/ml), COLI level in the supernatants of LX2 cells was detected by ELISA. Data are expressed as the mean $\pm$ standard error (n=5).

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Table 1. Characteristics of CHB patients at different stages

	N (n=10)	S0 (n=10)	S1 (n=10)	S2 (n=10)	S3 (n=10)	S4 (n=10)
Age years	40 (27-55)	39 (28-52)	37 (21-56)	40 (28-56)	41 (24-54)	45 (30-59)
Gender	5:5	8:2	6:4	5:5	9:1*	9:1
Male: Female						
WBC 10 ⁹ /L	4.35±0.62	5.31±0.41	5.55±0.69	6.019±1.28	4.674±0.83*	4.225±1.37
PLT 10 ⁹ /L	152.8±12.64	177.2±19.64	181.5±50.15	198.5±65.28	143.3±32.78*	106.3±35.72★
ALT μmol/L	27.4±3.98	39.75±17.61	58.62±33.27	84.875±48.46	164±143.89	112.62±39.85
AST μmol/L	24.9±5.67	28.75±11.03	39±17.6	56.625±20.57	90.62±41.90	62.12±34.47
GGT U/L	28.4±6.73	27.87±11.47	19.375±7.75▲	35.625±19.98	88.5±72.69	92.25±45.36
ALP U/L	65±12.01	69.3±13.13	69.6±22.72	72±32.56	92.5±36.52	95.7±27.52
ALB g/L	45.3±2.43	43.525±4.57	45.35±4.04	42.15±2.94	43.77±5.62	40.25±6.31
TB μmol/L	14.1±7.54	16.975±6.77	13.614±5.54	10.8±3.19	18.16±4.68**	21.8±10.43
DB μmol/L	3.98±2.45	5.013±1.95	3.838±1.27	3.66±1.32	6.2±2.36**	6.77±2.09
Cholesterol mmol/L	1.13±0.08	1.06±0.24	1.27±0.48	1.49±0.62	1.25±0.24	1.25±0.44
Triglycerides mmol/L	3.23±1.01	5.62±1.00	4.68±1.01	3.67±0.24	4.06±0.49	3.77±1.02
INR	0.87±0.03	0.99±0.07	1.02±0.04	1.04±0.08	1.06±0.04	1.13±0.22

WBC, white blood cell count; PLT, platelets; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; ALB, albumin; TB, total bilirubin; DB, direct bilirubin; INR, international normalized ratio.

▲ $P<0.05$ S1 compared with S2; * $P<0.05$ ** $P<0.01$ S2 compared with S3; ★ $P<0.05$ S3 compared with S4.

Table 2. Associations of CXCL6 level with biochemical parameters in CHB patients at different stages

	ALT ($\mu\text{mol/L}$)	AST ($\mu\text{mol/L}$)	PLT ($10^9/\text{L}$)	INR	TB ($\mu\text{mol/L}$)	DB ($\mu\text{mol/L}$)
S0						
r	0.789	0.832	0.652	0.572	0.673	0.421
p	0.0211	0.0343	0.376	0.273	0.231	0.189
S1						
r	0.876	0.856	0.364	0.433	0.847	0.786
p	0.0097	0.0141	0.423	0.391	0.0335	0.431
S2						
r	0.797	0.779	0.403	0.224	0.534	0.321
p	0.0318	0.0388	0.321	0.67	0.227	0.945
S3						
r	0.821	0.747	0.722	0.797	0.523	0.403
p	0.0453	0.0376	0.151	0.121	0.183	0.322
S4						
r	0.95	0.685	0.53	0.678	0.548	0.457
p	0.013	0.0396	0.176	0.221	0.251	0.255

ALT, alanine transaminase; AST, aspartae transaminase; PLT, platelets; INR, international normalized ratio; TB, total bilirubin; DB, direct bilirubin

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