ORIGINAL ARTICLE

Role of cytokine receptor-like factor 1 in hepatic stellate cells and fibrosis

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Abstract

AIM: To elucidate the role of cytokine receptor-like factor 1 (CRLF1) in hepatic stellate cells and liver fibrosis.

METHODS: Rat hepatic stellate cells (HSCs) were isolated by Nykodenz gradient centrifugation and activated by culturing *in vitro*. Differentially expressed genes in quiescent and culture activated HSCs were identified using microarrays. Injections of carbon tetrachloride (CCl$_4$) for 4 wk were employed to induce liver fibrosis. The degree of fibrosis was assessed by Sirius red staining. Adenovirus expressing CRLF1 was injected through tail vein into mice to achieve overexpression of CRLF1 in the liver. The same adenovirus was used to overexpress CRLF1 in quiescent HSCs cultured *in vitro*. Expression of CRLF1, CLCF1 and ciliary neurotrophic factor receptor (CNTFR) in hepatic stellate cells and fibrotic livers was analyzed by semi-quantitative reverse transcription-polymerase chain reaction and Western blotting. Expression of profibrotic cytokines and collagens was analyzed by the same method.

RESULTS: CRLF1 is a secreted cytokine with unknown function. Human mutations suggested a role in development of autonomous nervous system and a role of CRLF1 in immune response was implied by its similarity to interleukin (IL)-6. Here we show that expression of CRLF1 was undetectable in quiescent HSCs and was highly upregulated in activated HSCs. Likewise, expression of CRLF1 was very low in normal livers, but was highly upregulated in fibrotic livers, where its expression correlated with the degree of fibrosis. A cofactor of CLRF1, cardiotrophin-like cytokine factor 1 (CLCF1), and the receptor which binds CRLF1/CLCF1 dimer, the CNTFR, were expressed to similar levels in quiescent and activated HSCs and in normal and fibrotic livers, indicating a constitutive expression. Overexpression of CLRF1 alone in the normal liver did not stimulate expression of profibrotic cytokines, suggesting that the factor itself is not pro-inflammatory. Ectopic expression in quiescent HSCs, however, retarded their activation into myofibroblasts and specifically decreased expression of type Ⅲ collagen. Inhibition of type Ⅲ collagen expression by CRLF1 was also seen in the whole liver. Our results suggest that CLRF1 is the only component of the CRLF1/CLCF1/CNTFR signaling system that is inducible by a profibrotic stimulus and that activation of this system by CLRF1 may regulate expression of type Ⅲ collagen in fibrosis.

CONCLUSION: By regulating activation of HSCs and expression of type Ⅲ collagen, CRLF1 may have an ability to change the composition of extracellular matrix in fibrosis.

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INTRODUCTION

Cytokine receptor-like factor 1 (CRLF1) is a secreted protein belonging to the family of cytokine type I receptors and has homology to two other secreted receptors: IL12B and EBI3[1,2]. Both of these receptors are implicated in regulating immune response by T-lymphocytes. Expression of CRLF1 is induced by tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and γ-interferon and it is expressed in lymphatic tissue, placenta, stomach and lungs[3]. CRLF1 forms a heterodimer with cardiotrophin-like cytokine factor 1 (CLCF1, also known as B-cell stimulating factor 3) and this complex binds to the ciliary neurotrophic factor receptor (CNTFR)[4]. CNTFR is a non-signaling component of the heterotrimeric receptor composed of CNTFR, interleukin 6 signal transducer (IL6ST) and LIFR[5]. The IL6ST and LIFR subunits of this heterotrimeric receptor are also components of IL-6 receptor. However, while IL-6 receptor binds IL-6, CNTFR/IL6ST/LIFR receptor binds either CLRF1/CLCF1 dimer or ciliary neurotrophic factor (CNTF)[6]. Like IL-6 receptor, the CNTFR/IL6ST/LIFR receptor signals to activate STAT/ERK pathway[7]. CNTF is required for development of central nervous system and has neurotrophic activity for motor neurons. Knock out of *CNTF* gene results in progressive muscular atrophy and loss of motor neurons[8]. Elson *et al*[4] have shown that CLRF1/CLCF1 dimer is a competitive inhibitor for binding of CNTF to CNTFR/IL6ST/LIFR receptor.

The physiological role of CRLF1 is unknown. Mutations in *CRLF1* gene were associated with cold sweat syndrome type 1 and Crisponi syndrome[9-14]. Cold sweat syndrome type 1 is characterized by profuse sweating induced by cold and craniofacial deformities[12,14]. Crisponi syndrome is associated with dismorphic facial features, facial muscle contractions, scoliosis and hyperthermia[9,13]. Interestingly, mutation of CLCF1 causes cold sweat syndrome type 2, which is similar to cold sweating syndrome type 1[15], suggesting the common signaling defect of the CLRF1/CLCF1 pathway. These syndromes implicated the role of CLRF1/CLCF1 in the function of the autonomic nervous system. Mice lacking the *CLRF1* gene were unable to suckle and died from starvation few

days after birth[16]. No craniofacial deformations were observed and the reason for suckling defect is unknown.

CLRF1 was found to be expressed at high levels in osteoarthritic human knee cartilage and was upregulated by stimulating mouse chondrocytes by transforming growth factor-β (TGF-β)[17]. CRLF1/CLCF1 complex promoted the proliferation of chondrocyte precursors and suppressed the expression level of aggrecan and type II collagen[17]. This suggests that the CRLF1/CLC complex may disrupt cartilage homeostasis and promote the progress of osteoarthritis.

Ectopic bone formation can be induced by injection of bone morphogenetic protein-2 (BMP-2) into the muscle. When gene expression was analyzed in such ectopically induced bone, expression of CRLF1 was induced 5-fold at day 5 after BMP-2 injection. Temporally, CRLF1 induction preceded the stage of chondrogenesis in this model of endochondral osteogenesis[18].

Based on the results described above two roles of CLRF1 can be inferred; mediation of immune response and regulation of autonomic nervous system. However, association of bone deformities with CLRF1 mutation in humans, as well as regulation of CRLF1 by TGF-β and its induction by BMP-2, suggests a role in formation of the extracellular matrix. There are no reports on expression of CRLF1 in the liver or its association with liver fibrosis. Here we describe the expression of CRLF1 in hepatic stellate cells and fibrotic livers and the effect of CLRF1 overexpression in the liver.

MATERIALS AND METHODS

Isolation and culture of hepatic stellate cells
Hepatic stellate cells (HSCs) were isolated by perfusion of rat livers with pronase and collagenase, followed by centrifugation over Nycodenz gradient, as described[19]. The purity of cells was estimated to be > 95% by desmin staining. The cells were cultured in uncoated plastic dishes in Dulbecco's modified Eagle's medium/10% fetal bovine serum for 2 d and used as quiescent HSCs or were cultured for 7 d and used as activated HSCs.

Induction of liver fibrosis
Liver fibrosis was induced in rats by intraperitoneal injections of carbon tetrachloride (CCl4), 2 μL/g, twice a week for 4 wk. Livers were extracted, fixed in formalin and paraffin embedded sections were stained with Sirius red according to the standard procedure[20].

Adenovirus construction and injection
Adenovirus expressing human CRLF1 was constructed by cloning CRLF1 cDNA into pAdCMV TRACK vector and by subsequent recombination with pEasy vector in *Escherichia coli*, as described[21]. Viral particles were assembled and amplified in HEK 293 cells and purified by CsCl centrifugation, as described[22]. CRLF1 adenovirus also expressed green fluorescent protein (GFP) from an

Table 1 Primers used in reverse transcription-polymerase chain reaction analysis		
CRLF1-r	5′primer	GGACAATCTGGTGTGTCACG
	3′primer	GGGCCCACAGTGTGATATTC
CLCF1-r	5′primer	TCCACCTTCCATCTGGTCTC
	3′primer	GCAGAGGCAAGCTAACATCC
CNTFR-r	5′primer	ACACCACGGCTATCACCTTC
	3′primer	ATTCGAGAGCTCCACATGCT
COL3A1-m	5′primer	ACGTAAGCACTGGTGGACAG
	3′primer	AGCTGCACATCAACGACATC
KC-m	5′primer	TCGCCAATGAGCTGCGCTGTC
	3′primer	GCTTCAGGGTCAAGGCAAGCC
IL-1β-m	5′primer	GCCCATCCTCTGTGACTCAT
	3′primer	AGGCCACAGGTATTTTGTCG
IL-6-m	5′primer	AGTTGCCTTCTTGGGACTGA
	3′primer	TCCACGATTTCCCAGAGAAC
TNF-α-m	5′primer	CGTCAGCCGATTTGCTATCT
	3′primer	CGGACTCCGCAAAGTCTAAG
Actin-m	5′primer	CGTGCGTGACATCAAAGAGAAGC
	3′primer	TGGATGCCACAGGATTCCATACC
Actin-r	5′primer	CGTGCGTGACATTAAAGAGAAGC
	3′primer	TGCATGCCACAGGATTCCATACC
COL1A1-r	5′primer	TGAGCCAGCAGATTGAGAAC
	3′primer	TGATGGCATCCAGGTTGCAG
COL1A2-r	5′primer	CTCACTCCTGAAGGCTCTAG
	3′primer	CTCCTAACCAGACATGCTTG
COL3A1-r	5′primer	AGGCCAATGGCAATGTAAAG
	3′primer	GGCCTTGCGTGTTTGATATT
FIB-r	5′primer	GAAAGGCAACCAGCAGAGTC
	3′primer	CTGGAGTCAAGCCAGACACA
α-SMA-r	5′primer	ACAGAGAGAAGATGACGCAG
	3′primer	GGAAGATGATGCAGCAGTAG
CRLF1-h	5′primer	CCAGAGAAACCCGTCAACAT
	3′primer	GCCTCCACCCAGATCTCATA

α-SMA: α-smooth muscle actin; CNTFR: Ciliary neurotrophic factor receptor; COL1A1: Collagen, type Ⅰ, α 1; COL1A2: Collagen, type Ⅰ, α 2; COL3A1: Collagen, type Ⅲ, α 1; CRLF1: Cytokine receptor like factor 1; CLCF1: Cardiotrophin-like cytokine factor 1; IL: Interleukin; TNF-α: Tumour necrosis factor-α; KC: Chemokine; FIB: Fibronectin; -m: Mouse; -r: Rat; -h: Human.

independent transcription unit, GFP served as a marker of viral transduction. Control virus expressed only GFP. Viruses were injected through tail vein into mice at a dose of 1×10^7 PFU/g. HSCs in culture for two days after isolation were transduced with the adenovirus at multiplicity of infection of 500 and culturing continued until day seven after isolation, when the cells were collected for analysis.

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from HSCs using RNAeasy kit from Sigma. From livers, total RNA was isolated by homogenization of liver in 6 mol guanidine-isothiocyanate solution, followed by phenol/chlorophorm extraction and ethanol precipitation. Reverse transcription-polymerase chain reaction (RT-PCR) was performed by our well established procedure[23-26]. Briefly, reverse transcription was done with a primer that was specific for each individual RNA and subsequent PCR amplification was done in the presence of ^{32}P-dCTP to label the PCR products, which were resolved on a sequencing gel. The

identity of products was confirmed by the expected size and by sequencing. To avoid saturation, the number of cycles in the PCR step was kept at a minimum, sufficient to give signals. The results obtained by this method are in excellent agreement with the results obtained by real time RT-PCR, as shown in our previous work[27]. The primers used are shown in Table 1.

Western blotting

Proteins were extracted from the liver by homogenization in Rippa buffer and 200 μg of total liver proteins was analyzed. For analysis of cellular medium, 100 μL of cellular medium collected from confluent HEK293 was directly loaded on the gel. Anti-CRLF1 antibody was purchased from Santa Cruz Biotechnology (sc-100297) and used at 1:1000 dilution.

RESULTS

HSCs are the main cells responsible for synthesis of extracellular matrix in liver fibrosis[28,29]. HSCs undergo activation from quiescent state into myofibroblasts upon a fibrogenic stimulus. Similar activation can be achieved by culturing HSCs *in vitro*[30]. To identify genes associated with activation of HSCs we used microarrays to compare the gene expression profile of quiescent rat HSCs and rat HSCs activated by culturing *in vitro*[31]. In two independent experiments we found that *CLRF1* gene was expressed dramatically higher in activated HSCs than in quiescent HSCs (Figure 1A). The genes encoding *LIFR*, *CNTFR*, *CTF1*, *IL6ST* and *CNTF* were constitutively expressed and showed no change in expression. Expression of CNTF was found to be only moderately increased in activated compared to quiescent HSCs using microarrays (Figure 1A). Expression of IL-6 was greatly increased in activated HSCs, but the level of IL6 receptor, which together with IL6ST and LIFR forms IL-6 receptor, was unchanged. This suggested that only IL-6 and CRLF1 are dramatically upregulated in the activation of HSCs, while the rest of the signaling machinery seems to be constitutively expressed.

To verify the microarray data we assessed the expression of *CLRF1*, *CLCF1* and *CNTFR* genes in quiescent and activated rat HSCs by RT-PCR (Figure 1B). In these experiments HSCs were only subjected to culture activation and were not stimulated with TGF-β or platelet-derived growth factor. We used our radio-labeled RT-PCR method which is in excellent agreement with the results obtained by real time RT-PCR, as demonstrated in our earlier work[27]. Expression of CNTFR and CLCF1 was similar in quiescent and activated HSCs and this result is in good agreement with the microarray data (Figure 1B). The expression of CRLF1 was dramatically increased in activated HSCs compared to quiescent HSCs, where it was undetectable (compare lanes 1 and 2). This is in excellent agreement with microarray data, which showed the logarithm of the signal ratio between activated and quiescent cells of 6 (Figure 1A). The results obtained by
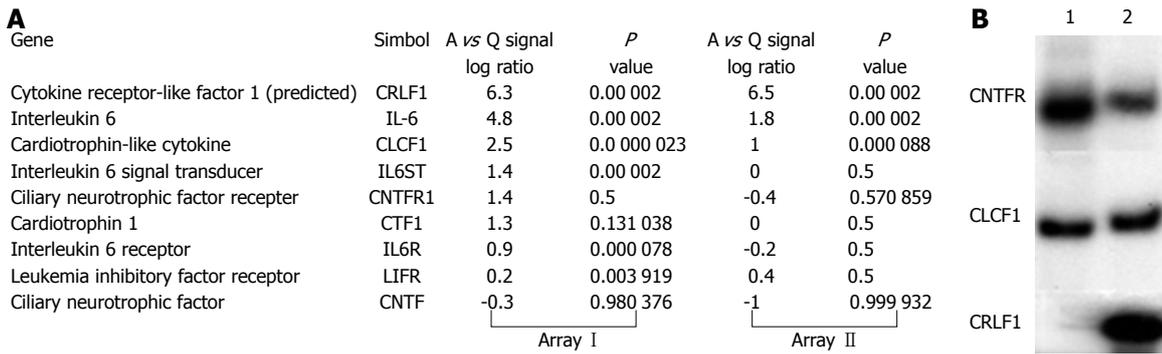
A

Gene	Simbol	A vs Q signal log ratio	P value	A vs Q signal log ratio	P value
Cytokine receptor-like factor 1 (predicted)	CRLF1	6.3	0.00 002	6.5	0.00 002
Interleukin 6	IL-6	4.8	0.00 002	1.8	0.00 002
Cardiotrophin-like cytokine	CLCF1	2.5	0.0 000 023	1	0.000 088
Interleukin 6 signal transducer	IL6ST	1.4	0.00 002	0	0.5
Ciliary neurotrophic factor recepter	CNTFR1	1.4	0.5	-0.4	0.570 859
Cardiotrophin 1	CTF1	1.3	0.131 038	0	0.5
Interleukin 6 receptor	IL6R	0.9	0.000 078	-0.2	0.5
Leukemia inhibitory factor receptor	LIFR	0.2	0.003 919	0.4	0.5
Ciliary neurotrophic factor	CNTF	-0.3	0.980 376	-1	0.999 932
		Array I		Array II	

B

Figure 1 Genes upregulated in activation of hepatic stellate cells. A: Rat hepatic stellate cells (HSCs) were isolated and cultured for 2 d (quiescent HSCs) or for 7 d (activated HSCs) and total RNA was analyzed for gene expression by microarrays in two independent experiments. The fold increase in transcript level and P values for the selected genes is shown; B: Expression of cytokine receptor-like factor 1 (CRLF1), cardiotrophin-like cytokine factor 1 (CLCF1) and ciliary neurotrophic factor receptor (CNTFR) in quiescent and activated HSCs was estimated by reverse transcription-polymerase chain reaction. Q vs A: Quiescent HSCs vs activated HSCs; IL-6: Interleukin-6; IL6ST: Interleukin 6 signal transducer; IL6R: Interleukin 6 receptor; CNTF: Ciliary neurotrophic factor.

both of these techniques indicated that CRLF1 is dramatically upregulated during activation of HSCs.

To assess if CRLF1 expression is also upregulated in fibrotic livers we induced liver fibrosis by repeated injections of carbon tetrachloride CCl4 into rats. Sirius red staining of one normal liver (N1) and two livers extracted from the CCl4 treated animals are showed in Figure 2A. One of these livers showed a moderate degree of fibrosis (F1) and the other showed more massive bridging fibrosis (F4). Expression of CRLF1, CLCF1 and CNTFR mRNA in three normal livers, including the N1 liver (lanes 1-3), and five fibrotic livers, including the F1 and F4 livers (lanes 4-8) is showed in Figure 2B. CLCF1 was constitutively expressed in the liver and its expression did not change with induction of liver fibrosis. This is in agreement with its equal expression in quiescent and activated HSCs. Expression of CNTFR appeared to be slightly lower in fibrotic livers than in control livers (Figure 2B; compare lanes 4-8 to lanes 1-3), but CNTFR was clearly constitutively expressed. CRLF1 mRNA was detected at low levels in normal livers and its expression was increased in all fibrotic livers. There was a correlation between the degree of fibrosis and CRLF1 expression. For example, liver F1 had less fibrosis than liver F4 (Figure 2A) and expression of CRLF1 in liver F1 is lower than in liver F4. However, it is still higher than in all normal livers (Figure 2B). Thus, CRLF1 is again the only component that is upregulated in liver fibrosis, while CLCF1 and CNTFR are constitutively expressed. Since activation of HSCs is responsible for development of liver fibrosis and activated HSCs dramatically upregulate expression of CRLF1, it is likely that increased CRLF1 expression in fibrotic livers originates from the activated HSCs.

Since it was reported that CRLF1 can modulate immune response, we assess if overexpression of CRLF1 in the liver can induce changes in expression of proinflammatory genes. To achieve overexpression of CRLF1 in the liver we constructed an adenovirus expressing human CRLF1 and injected the adenovirus into tail vein of three mice. Human CRLF1 protein shares 95% identity to mouse CRLF1. Control adenovirus, expressing only GFP was injected into two mice. No liver fibrosis was induced in these animals, only overexpression of CRLF1 was achieved by adenoviral injections. Injection of adenovirus into circulation results in the clearance of the virus by the liver and transduction of all cell types in the liver. We measured the expression of CRLF1 protein (Figure 3A, upper panel) and mRNA (lower panel) in the livers of these animals two days after the viral injection. There was high level of expression in all three CRLF1 adenovirus injected animals. There was also a very good correlation between expression of CRLF1 protein and mRNA. This was in sharp contrast to CRLF1 expression in control livers, which was detect at very low levels. Next, we examined if high CRLF1 level in the liver can stimulate expression of four proinflammatory cytokines. We chose chemokine (KC) (mouse homolog of gro-α)[32,33], IL-1, IL-6 and TNF-α, as the most common cytokines. Expression of KC, IL-1β, IL-6 and TNF-α showed some variation between the animals but neither cytokine was upregulated in the CRLF1 overexpressing livers (Figure 3). The measurement of aminotransferases in plasma of these animals showed normal levels (not shown). From these results we concluded that high levels of CRLF1 in the liver do not stimulate production of proinflammatory cytokines nor they cause liver damage.

Activated HSCs express high level of CRLF1, while its expression in quiescent HSCs is undetectable (Figure 1). However, quiescent HSCs express CLCF1 and CNTFR, therefore they may be responsive to CRLF1 stimulation. We decided to overexpress CRLF1 in quiescent HSCs and assess what effects it may have on activation of HSCs and collagen expression. To verify that adenovirus transduced cells can secrete CRLF1 we infected HEK293 cells and measured the CRLF1 in the cellular medium by western blot. High level of secreted CRLF1 was found in the medium of CRLF1 adenovirus transduced cells, while it was undetectable in control cells
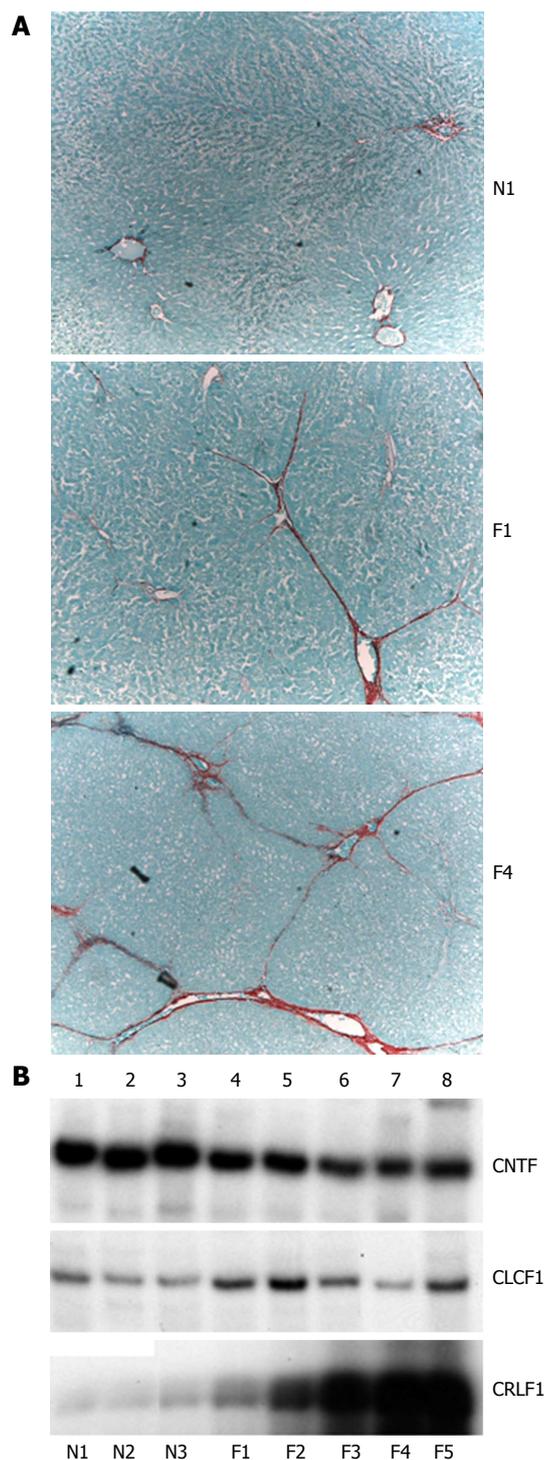
Figure 2 Expression of cytokine receptor-like factor 1 in fibrotic livers. A: Sirius red staining of liver slices from three representative animals; N1: Normal liver; F1: Liver with moderate degree of fibrosis induced by CCl₄; F4: Liver with high degree of fibrosis induced by CCl₄; B: Reverse transcription-polymerase chain reaction analysis of expression of cytokine receptor-like factor 1 (CRLF1), cardiotrophin-like cytokine factor 1 (CLCF1) in three normal livers (N1-N3) and five fibrotic livers (F1-F5).

Figure 3 Overexpression of cytokine receptor-like factor 1 in normal liver does not increase expression of profibrotic cytokines. A: Overexpression of cytokine receptor-like factor 1 (CRLF1). Control adenovirus was injected into tail vein of two mice and adenovirus expressing cardiotrophin-like cytokine factor 1 (*CLCF1*) gene was injected into three mice. After three days liver proteins and RNA were extracted and expression of CLRF1 protein was analyzed by Western blot (top panel) and CLRF1 mRNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (bottom panel); B: Expression of profibrotic cytokines. Expression of the indicated profibrotic cytokines was analyzed by RT-PCR in the control (CON) and CRLF1 overexpressing livers. PROT: Protein; CNTFR: Ciliary neurotrophic factor receptor; IL: Interleukin; TNF-α: Tumour necrosis factor-α; KC: Chemokine.

(Figure 4A). This verified that transduction of HSCs will result in production of secreted CRLF1 capable of stimulating its receptor. Therefore, we transduced quiescent prima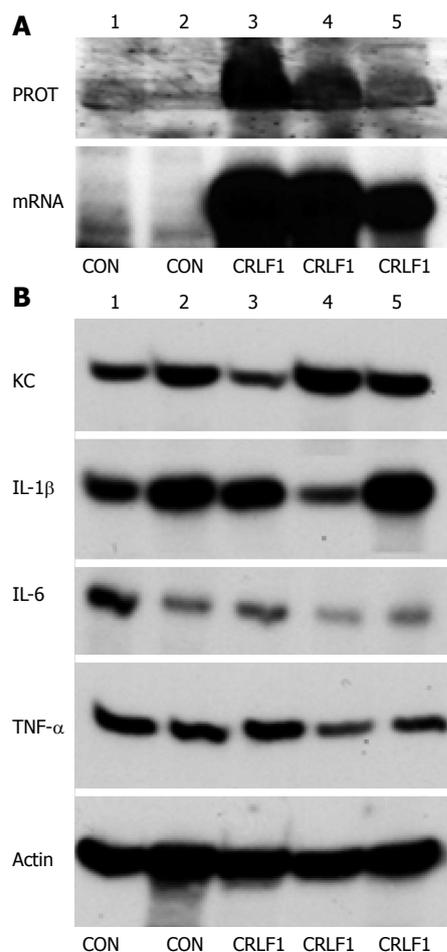ry rat HSCs with CRLF1 adenovirus two days after isolation, when they still have a quiescent phenotype[31] (Figure 1). We then analyzed the expression of α-smooth muscle actin (α-SMA), as the marker of activation[34], at day 6 (4 d after the viral transduction). The RT-PCR analysis (Figure 4B) revealed that expression of α-SMA was reduced in HSCs ectopically expressing CRLF1. This suggested that overexpression of CRLF1 in quiescent HSCs can slow down the activation process of isolated HSCs in culture. We also measured expression of collagen α1 (I) and α2 (I) mRNAs, encoding type I collagen, and collagen α1(Ⅲ) mRNA, encoding type Ⅲ collagen (Figuer 4B). There was a decrease in expression of collagen α1 (I) and α2 (I) mRNAs, which correlated with lower expression of α-SMA. This, again, suggested a retarded activation process of HSCs. The expression of collagen α1 (Ⅲ) mRNA seemed to be
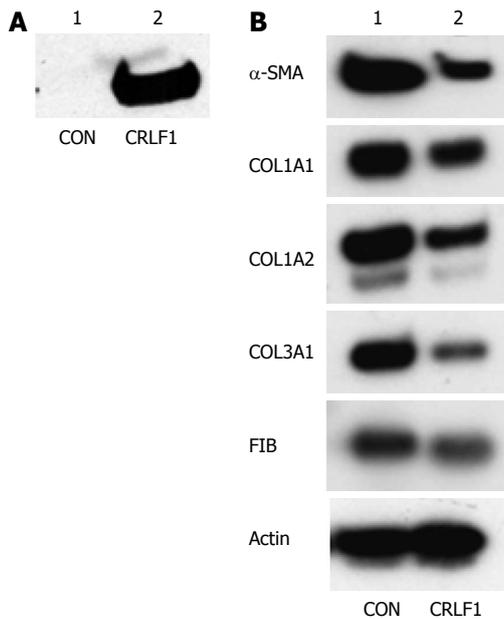
Figure 4 Cytokine receptor-like factor 1 decreases expression of type Ⅲ collagen by hepatic stellate cells. A. Expression of adenovirus delivered cytokine receptor-like factor 1 (CRLF1) in the cellular medium. HEK293 cells were transduced with control or CRLF1 expressing adenovirus. One day after transduction, CRLF1 protein was measured in the cellular medium by Western blotting; B: Stimulation of hepatic stellate cells (HSCs) with CRLF1. HSCs at day 2 after isolation were transduced with control or CRLF1 adenovirus. At day 7, expression of α-smooth muscle actin (α-SMA), collagen α1 (Ⅰ), collagen α2 (Ⅰ), collagen α1 (Ⅲ) and fibronectin (FIB) mRNAs was analyzed by reverse transcription-polymerase chain reaction. CON: Control; COL1A1: Collagen, type Ⅰ, α1; COL1A2: Collagen, type Ⅰ, α2; COL3A1: Collagen, type Ⅲ, α1.

Figure 5 Cytokine receptor-like factor 1 decreases type Ⅲ collagen expression in the whole liver. RNA was extracted from two control livers and two livers that overexpressed cytokine receptor-like factor 1 (CRLF1) after adenoviral delivery and expression of collagen α1 (Ⅲ) mRNA was analyzed by reverse transcription-polymerase chain reaction. CON: Control; COL3A1: Collagen, type Ⅲ, α1.

more downregulated than that of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs. This indicates that CRLF1, in addition to retarding activation of HSCs, may specifically inhibit the expression of collagen type Ⅲ.

To assess if the effect on expression of type Ⅲ collagen can be reproduced in the whole liver, we analyzed expression of collagen α1 (Ⅲ) mRNA in two livers of mice injected with CRLF1 adenovirus. Again, no liver fibrosis was induced, only adenoviruses were injected. As shown in Figure 5, collagen α1 (Ⅲ) mRNA was expressed at lower level in CRLF1 overexpressing livers than in control livers, suggesting that CRLF1 can negatively regulate collagen type Ⅲ expression in the whole liver. Expression of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs was very low in control livers and we could not detect any decrease in CRLF1 livers (not shown). From these experiments we concluded that CRLF1 may control the activation of HSCs and specifically regulate expression of type Ⅲ collagen in the liver and HSCs.

DISCUSSION

This is the first report on expression of CRLF1 in liver fibrosis and in HSCs. Humans with the mutations in *CRLF1* gene suffer from cold sweat syndrome or Crisponi syndrome[9,10,12-14]. While these syndromes led to conclusion that CRLF1 is required for development
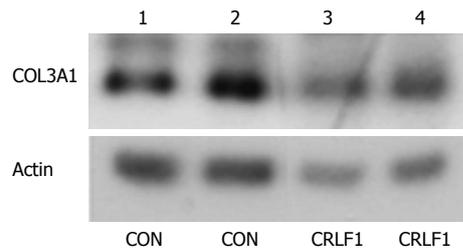
of peripheral nervous system, the craniofacial malformations of these patients suggest that CRLF1 may be important for proper formation of extracellular matrix. HSCs are the primary cells responsible for secretion of extracellular matrix in liver fibrosis. Upon fibrogenic stimulus HSCs undergo activation and dramatically up-regulate synthesis of collagens type Ⅰ and type Ⅲ[26]. Type Ⅰ collagen forms large diameter fibrils and is the major component of the sca[35], while type Ⅲ collagen forms fibrils of low diameter and is the main component of scar less wound healing[36]. Thus, the balance between the amount of type Ⅰ and type Ⅲ collagen can determine the extent and irreversibility of scarring.

We found that quiescent HSCs express CRLF1 at undetectable levels while its expression in activated HSCs is dramatically increased. Other proteins that participate in CRLF1 signaling, CLCF1 and CNTFR1, are constitutively expressed, therefore, it is highly likely that CRLF1 is the regulatory component of the signaling system in HSCs. CRLF1 is expressed at low levels in normal livers, however, its expression correlates with the degree of liver fibrosis and is dramatically increased in highly fibrotic livers. This suggests that it originates from activated HSCs. It has been reported that CRLF1 expression is activated by TGF-β[17], which is the main profibrotic cytokine[37,38]. Thus, TGF-β may not only stimulate HSCs to transdifferentiate into myofibroblasts, but it may be responsible for upregulation of CRLF1 production. Other liver cells are likely to produce small amounts of CRLF1, as evidenced by low level of expression in normal livers that do not contain activated HSCs.

High CRLF1 expression in the liver does not stimulate expression of proinflammatory cytokines. This was evidenced by adenoviral delivery of CRLF1 into normal livers. We did not observe an increase in expression of TNF-α, IL-1β or KC chemokine. There was also no evidence of increased hepatocellular damage, as assessed by measuring aspartate aminotransferase and alanine aminotransferase in plasma (not shown). Thus, expression of CRLF1 alone is not sufficient to induce proinflammatory changes in the liver.

Overexpression of CRLF1 in quiescent HSCs attenuated their activation in culture. When CRLF1 was

ectopically expressed in isolated quiescent HSCs, the expression of the marker of transactivation into myofibroblasts, α-SMA[31,34], was decreased. The expression of collagens type I and Ⅲ was also attenuated. The effect on multiple markers of activation suggested the general effect on this process. How CRLF1 may slow down culture activation of HSCs is not clear. Since CLCF1, CNTFR1, LIFR and IL6ST are constitutively expressed in HSCs, this may involve activation of this signaling pathway by the ectopical CRLF1. What downstream effectors of the pathway may be involved is not clear, but it is important to elucidate this, because it may shed light on the overall mechanism of HSCs activation.

The expression of collagen α1 (Ⅲ) mRNA in HSCs overexpressing CLRF1 was decreased more than that of collagen α1 (Ⅰ) and α2 (Ⅰ) mRNAs. This suggested that CLRF1 can specifically downregulate type Ⅲ collagen, in addition to inhibiting HSCs activation. This was confirmed in the whole liver. Type Ⅲ collagen was easily detectable in normal livers by RT-PCR, but when CLRF1 was overexpressed in two livers by adenoviral delivery, its expression was significantly decreased. The mechanism by which CRLF1 affects expression of type Ⅲ collagen in the liver is not known. It may involve association of CRLF1 with CLCF1 and their binding to CNTFR subunit of the receptor, because these components are constitutively expressed. The receptor activates suppressor of cytokine signaling (SOCS) family of transcription factor, in particular SOCS3, which is translocated into the nucleus and regulates transcription of its responsive genes[39]. However, it is not known if collagen α1 (Ⅲ) gene can be directly or indirectly regulated by SOCS transcription factors.

What is the consequence of high CRLF1 expression in fibrotic livers? CRLF1 alone can not stimulate inflammation in the liver, although there is a possibility that it may potentiate the action of other cytokines. This, however, remains to be elucidated. CRLF1 seems to attenuate activation of HSCs *in vitro* and its expression is proportional to the degree of fibrosis *in vivo*, so it may regulate activation of HSCs in the liver. In addition, it may suppress expression of type Ⅲ collagen. This would favor deposition of type Ⅰ collagen over type Ⅲ collagen, what was observed in fibrotic livers[40] and in hypertrophic scarring[41]. In osteoarthritic cartilage, it was proposed that CRLF1 suppresses expression of type Ⅱ collagen[17]. So, altering extracellular matrix by changing the balance between different collagens may be a general property of CRLF1.

In conclusion, we show that CRLF1 is dramatically upregulated in liver fibrosis and in activated HSCs. Overexpression of CRLF1 does not stimulate expression of proinflammatory cytokines, but inhibits activation of HSCs *in vitro* and reduces expression of type Ⅲ collagen in the whole liver. By affecting the composition of extracellular matrix and activation of HSCs, CRLF1 may modulate the extent of fibrosis.

COMMENTS

Background
Hepatic stellate cells (HSCs) are the main cells responsible for development of liver fibrosis. In fibrosis, HSCs undergo activation from their quiescent state into myofibroblasts and dramatically upregulate expression of type Ⅰ and type Ⅲ collagens. Multiple cytokines have been implicated in this process, with transforming growth factor-β being the most potent profibrotic cytokine. The role of other cytokines in regulation of collagen expression is less well understood.

Research frontiers
Cardiotrophin-like cytokine factor 1 (CLCF1) is a cytokine implicated in development of central nervous system and is mutated in patients with cold sweat syndrome and Crisponi syndrome. Cytokine receptor-like factor 1 (CRLF1) is upregulated in damaged osteoarthritic cartilage. It acts as a dimer with CLCF1 by activating ciliary neurotrophic factor receptor (CNTFR). CNTFR in complex with two additional subunits, interleukin 6 signal transducer (IL6ST) and LIFR, signals by triggering STAT/ERK signal transduction pathway. CNTFR does not bind IL-6, although it shares IL6ST and LIFR subunits with IL-6 receptor. It is unclear how CRLF1/CLCF1/CNTFR activity regulates development of neurons or contributes to osteoarthritis and the rare congenital syndromes. There have been no reports on expression or a role of CRLF1 in fibrosis.

Innovations and breakthroughs
This is the first report on expression of CRLF1 in HSCs and liver fibrosis. CRLF1 is dramatically upregulated in activation of HSCs *in vitro* and *in vivo*. The other two specific components of the signaling pathway, CLCF1 and CNTFR, are constitutively expressed and do not change in fibrosis. This suggests that upregulation of CRLF1 triggers the pathway, activation of which contributes to the pathogenesis of fibrosis. A novel finding is also that CRLF1 decreases expression of type Ⅲ collagen. By changing the ratio of different collagens in the extracellular matrix, CRLF1 may promote fibrilogenesis. Thus, this work provided a new insight into molecular mechanisms of HSCs activation and fibrosis development.

Applications
By discovery of the role of CRLF1 in hepatic fibrosis, a possibility of new therapeutic intervention is suggested. CRLF1 is a secreted cytokine receptor-like molecule and by designing agonists or antagonists of its interaction with CLCF1 or CNTFR a novel strategy to modulate liver fibrosis may be initiated.

Terminology
CRLF1 is a 422 amino-acids secreted protein that represents a novel class of soluble cytokine receptor-like molecules. Its ligand is CLCF1 and the soluble CRLF1/CLCF1 dimer binds the membrane anchored receptor CNTFR. CNTFR can also bind another ligand, ciliary neurotrophic factor (CNTF) and a competition between CRLF1/CLCF1 dimer and CNTF for the CNTFR binding has been demonstrated. CNTFR is a non-signaling component of the heterotrimeric transmembrane complex composed of CNTFR, IL6ST and LIFR. The IL6ST and LIFR subunits of this heterotrimeric receptor are also components of IL-6 receptor. However, CNTFR does not bind interleukin-6.

Peer review
The authors provided evidence that expression of CRLF1 is highly upregulated in activation of HSCs, as well as in fibrotic livers. In the liver its expression is correlated with the degree of fibrosis. Interestingly, the other components of the signaling pathway, CLCF1 and CNTFR, are constitutively expressed in HSCs and livers. Therefore, CRLF1 regulates the activity of the pathway in fibrosis. CRLF1 attenuates the expression of type Ⅲ collagen. This may change the ratio of type Ⅰ to type Ⅲ collagen in the extracellular matrix, affecting its polymerization, crosslinking and turn over. This is the first report on the expression and effects of CRLF1 in fibrosis and represents a significant contribution to the understanding of the molecular mechanisms of hepatic fibrosis.

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