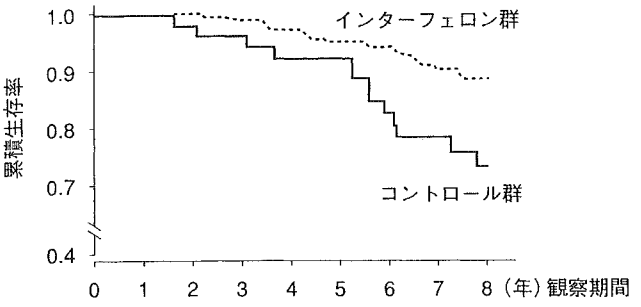

図　高齢C型肝炎患者におけるインターフェロン群およびコントロール群の累積生存率
(文献4より引用)

低下します．しかしながら，70歳を一応の目安として高齢C型肝炎患者においても，特に肝線維化の進んだ発がんリスクの高い患者においては，肝疾患以外の合併症を検索したうえで，1型高ウイルス量症例においてはペグインターフェロン・リバビリン併用療法を考慮すべきであると考えられます．また，1型高ウイルス量症例以外の肝線維化進行例においては，6カ月間のペグインターフェロン・リバビリン併用療法あるいはIFN単独療法による著効率が高く，合併症を十分検索したうえで，積極的にこれらの治療の導入を考慮すべきであると思われます．また，IFN治療により著効が得られない症例においても，発がん抑制を目指した少量長期のIFN治療が試みられています．

3) Kasahara A, Tanaka H, Okanoue T, *et al.*: Interferon treatment improves survival in chronic hepatitis C patients showing biochemical as well as virological responses by preventing liver-related death. *J Viral Hepat* **11**: 148-156, 2004
4) Imai Y, Kasahara A, Tanaka H, *et al.*: Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response. *J Gastroenterol* **39**: 1069-1077, 2004

KEY WORD 解説

ペグインターフェロン・リバビリン併用療法： ペグインターフェロンはIFNαにポリエチレングリコールを結合させたもので，半減期が長く週1回の投与で持続的なIFN血中濃度を維持でき，従来のIFNと比べ発熱，全身倦怠感が軽度である．リバビリンは単独では抗ウイルス効果がなく，IFNと併用することでIFNの抗ウイルス効果を高める．溶血性貧血が主たる副作用である．ペグインターフェロンとリバビリンの併用療法1年間投与で，genotype I型高ウイルス量例でも約50%の著効が得られるようになった．genotype I型高ウイルス量例以外でも，6カ月投与で約80%の著効率である．

ADVICE

高齢C型肝炎患者，特に65歳以上の患者においては，高血圧，糖尿病，虚血性心疾患，脳血管障害といった合併症をIFN治療前に十分に検索しなければなりません．一方，高齢C型肝炎患者においても死亡原因の半数以上が肝がんを含めた肝疾患です．明らかな合併症がなく，肝線維化の進んだ症例では肝細胞がんにて死亡する確率が高いと考えられます．高齢患者ほど個々の患者の合併症，予想される発がん率，IFN治療による著効率を検討したうえで，ペグインターフェロン・リバビリン併用療法あるいはIFN単独療法の適応を決定すべきです．

Journal of Viral Hepatitis, 2005, **12**, 130–138 doi:10.1111/j.1365-2893.2005.00576.x

Enhanced expression of suppressor of cytokine signalling-1 in the liver of chronic hepatitis C: possible involvement in resistance to interferon therapy

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Received April 2004; accepted for publication May 2004

SUMMARY. Interferon-α (IFN-α) is widely used in the treatment of chronic hepatitis C (CHC). The suppressor of cytokine signalling (SOCS) family has been implicated in the regulation of JAK–STAT signalling, including IFN signalling. The negative effect of SOCS expression on the response of CHC to IFN-α is demonstrated here. The transcriptional levels of SOCS-1 and -3 in the livers of 21 patients with CHC and eight controls were investigated by quantitative reverse transcription-polymerase chain reaction. We established stable transfectants of SOCS-1 in a human hepatoma cell line, PLC/PRF/5 and analysed the effects of SOCS-1 on the phosphorylation of IFN-α-induced STAT-1 tyrosine by immunoblotting and the expression of antiviral genes by Northern blot. A prospective cohort study on SOCS-1 expression and clinical outcome was carried out in 77 patients with CHC who received IFN therapy. SOCS-1, but not SOCS-3, transcripts in the livers of CHC were significantly higher than controls ($P < 0.005$). IFN-α-induced STAT-1 phosphorylation and the expression of antiviral genes were inhibited in SOCS-1-transfected cells. Patients showing high SOCS-1 expression in the liver had a significantly lower rate of sustained virological response (SVR) to IFN therapy than those with low SOCS-1 expression ($P = 0.0014$). A multivariate analysis performed with host factors revealed that SOCS-1 staining in the liver can serve as a significant predictor for IFN SVR ($P = 0.004$). SOCS-1 expression is enhanced in the livers of CHC patients and might be involved in resistance to IFN therapy.

Keywords: antiviral gene, hepatitis C, IFN signalling, SOCS.

INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases worldwide. Interferon-α (IFN-α) with or without the guanosine analogue, ribavirin is widely used in the treatment of chronic hepatitis C (CHC). However, a sustained virological response (SVR) as a result of therapy is limited [1– 5]. The HCV genotype and baseline levels of viraemia have been reported to be the most important predictive factors for responsiveness to IFN therapy [4– 6]. Several host factors, namely a young age, female sex and a low degree of fibrosis on liver biopsy are correlated with a greater likelihood of a sustained response [7, 8]. However, very little is known concerning the issue of whether differences in intracellular IFN signalling in individuals contribute to the outcome of IFN therapy.

The main pathway of IFN-α/β signalling requires two receptor subunits. The binding of type I IFN to their receptors, IFNAR1 and IFNAR2, activates JAK1 and Tyk2. The activated JAK phosphorylate specific tyrosine residues on STAT1 and STAT2 in response to IFN-α/β and heterotrimerize with IRF-9. These are then translocated into the nucleus to initiate gene transcription by binding to IFN-stimulated response elements (ISREs).

The suppressor of cytokine signalling (SOCS) family, which consists of SOCS-1 to 7 and CIS, has been

Abbreviations: 2′-5′-OAS, 2′-5′-oligoadenylate synthetase; PKR, double-stranded RNA-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; PBS, phosphate-buffered saline.

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implicated in the negative feedback regulation of the JAK–STAT pathway, which is induced by cytokine signalling [9]. SOCS-1, also referred to as the STAT-induced STAT inhibitor (SSI-1) or the JAK-binding protein (JAB), was first identified by its ability to inhibit the differentiation of macrophages in M1 cells in response to interleukin-6 (IL-6) [10– 12]. It has been reported that the levels of several cytokines, including IL-6, IFN-γ and tumour necrosis factor (TNF)-α are elevated in patients with CHC [13– 17]. A low responder rate to IFN therapy has been reported in patients with high serum IL-6 levels [14]. It has been shown that IFN-γ induces SOCS-1 and IL-6 induces SOCS-3 in the mouse liver [18]. The induction of SOCS-3 and the SH2-containing protein tyrosine phosphatase 2 (SHP2) by TNF-α has recently been reported [19]. It was also recently reported that the HCV core protein may impair IFN-α-induced signal transduction via the induction of SOCS-3 expression [20]. Therefore, SOCS-1 and -3 may regulate IFN signalling in the livers of CHC subjects.

The objective of this study was to examine the expression levels of SOCS-1 and -3 gene in the livers of CHC patients and to clarify their relationship with the clinical outcome of IFN therapy by means of a prospective cohort study.

MATERIALS AND METHODS

Cells culture and transfections

PLC/PRF/5 human hepatoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained in DMEM (Sigma, St Louis, MO, USA) containing 10% foetal bovine serum (FBS) (Trace Scientific Ltd, Melbourne, Australia) at 37 °C in 5% CO_2 and 95% air. The mouse SOCS-1 cDNA, a gift from Dr M. Narazaki (Department of Molecular Medicine, Grad. School of Med., Osaka University) [21] was ligated into pcDNA3 (Invitrogen, Groningen, the Netherlands) digested with *Bam*HI/*Age*I generating pSOCS-1-pcDNA3. PLC/PRF/5 cells were transfected with pSOCS-1-pcDNA3 using FuGENE^{TM}6 (Roche, Mannheim, Germany) according to the manufacturer's protocol and SOCS-1-expressing colonies were selected by 800 μg/mL G418 (Nacalai Tesque, Inc. Kyoto, Japan). Transfection with pcDNA3 alone generated mock transfectants.

Reagent and antibodies

A murine anti-mouse SOCS-1 monoclonal antibody, raised against the oligopeptide, THFRTFRSHSD, was provided by Dr M. Narazaki [21]. A mouse monoclonal antibody raised against human phosphotyrosine-STAT-1 (Tyr^{701}) (sc-8394) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The recombinant human IFN-α was purchased from Pepro Tech Ec Ltd (London, UK).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay

Biopsied liver specimens, obtained prior to IFN treatment, from 21 patients (14 males and seven females) with CHC were each divided into two blocks: one was frozen for use in RNA extraction and the other was used for a histological analysis. Histologically, normal liver tissues from eight patients with gall stones, colon cancer and metastatic liver tumour served as controls. Written informed consent was obtained from all patients prior to the study. For analysing SOCS-1 and -3 expression in the liver tissues, total RNAs were isolated using the Trizol® reagent. First-strand cDNAs generated by a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) were amplified with HotstarTaq DNA polymerase (Qiagen, Hilden, Germany) for SOCS-1 or *Taq* DNA polymerase (Applied Biosystems) for SOCS-3. The specific sets of primers for SOCS-1 were 5′-CGC CAG CGC CGC GTG CGG CC-3′ and 5′-CTG CGG CCT CGT CTC CAG CC-3′, and for SOCS-3 were 5′-AGC CTG CGC CTC AAG ACC-3′ and 5′-GGA GGG TTC AGT AGG TGG-3′ [20]. The PCR protocol for SOCS-1 consisted of 35 cycles and one cycle was composed of 94 °C/30 s, 68 °C/3 min with an initial denaturation of the sample cDNA at 94 °C for 1 min prior to PCR. The PCR procedure for SOCS-3 consisted of 21 cycles and one cycle was composed of 94 °C/30 s, 60 °C/30 s, 70 °C for 1 min with an initial denaturation of the sample cDNA at 94 °C for 1 min before PCR. The PCR products were stained with SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and analysed as described [22– 24]. PCR products were quantitated to confirm that they were in the linear range of amplification. Amplification of the housekeeping gene, β-actin served as an internal control.

Immunoprecipitation and Western blotting analysis

Immunoprecipitation and Western blotting with anti-STAT-1 or antiphosphotyrosine-STAT-1 was performed as described [25, 26]. Cells were stimulated with IFN-α for 10 min at 37 °C after they were serum starved overnight to avoid the effects of various cytokines included in the serum [27].

Northern blot analysis

A Northern blot analysis of 2′-5′-oligoadenylate synthetase (2′-5′-OAS), double-stranded RNA-dependent kinase (PKR) was performed as described [28] after stimulating overnight serum-starved cells with IFN-α for 24 h at 37 °C [29]. The 2′-5′-OAS cDNA fragment used as the probe was kindly provided by Dr N. Kato (Department Mol. Biol. Grad. School of Med. and Dent.Okayama University) [30]. The PKR and SOCS-1 probes were obtained by reverse transcription-polymerase chain reaction (RT-PCR) with the specific sets of primers [31]. The primers for SOCS-1 were the same as those described above.

Prospective cohort study

From June 1996 to December 1997, 77 patients with serologically and histologically proven CHC (Table 1) were consecutively registered by the Kansai Viral Hepatitis Research Group and treated with 10 MIU IFN α-2b (Intron A; Schering-Plough, Kenilworth, NJ, USA) daily for 4 weeks, followed by three treatments per week for 20 weeks [32, 33]. No patient had received IFN therapy before being enrolled in the study. Contraindications to IFN treatment included pregnancy, the presence of hepatitis B surface antigen and other types of liver disease, autoimmune disease and any other serious illness. Liver biopsies were performed at least 2 months before IFN treatment. The histological classification was conducted according to the European classification of chronic hepatitis [34]. The Histological Activity Index (HAI) score [35] was evaluated by two independent pathologists with no knowledge of the patients. The amount of baseline HCV-RNA was assessed by RT-nested competitive PCR with a lower sensitivity of 100 copies/mL (Otsuka Assay Laboratories, Diagnostic Division, Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan). Virological response in the prospective trials was assessed by a combined RT-PCR assay with a lower sensitivity of 100 copies/mL (Amplicor HCV; Roche Molecular Systems, Inc., Alameda, CA, USA). Patients with a negative serum HCV-RNA at 24 weeks after the completion of IFN therapy were defined as having a SVR and others were classified as having a nonresponse (NR).

Immunohistochemistry

Formalin-fixed paraffin-embedded liver tissue specimens were used. Antigen retrieval was performed by microwaving for 5 min at 90 °C using Dako target retrieval solution (Dako, Carpinteria, CA, USA) [36]. The immunohistochemistry associated with anti-SOCS-1 was performed as described previously [28]. For a negative control, the primary antibody was replaced with non-immune normal mouse IgG.

Table 1 Pretreatment characteristics of patients

Male/female	43/34
Age (years; mean ± SD)	48.7 ± 11.5
HCV genotype	
1b	49
2a	15
2b	11
2a + 2b	1
2	1
Histology	
CAH1B	1
CAH2A	54
CAH2B	21
CPH	1
HAI score (mean ± SD)	9.9 ± 3.9
HCV-RNA titre (10^6 copies/mL; mean ± SD)	10.1 ± 11.5
ALT level (U/L; mean ± SD)	102.4 ± 74.4

CAH, chronic active hepatitis; CPH, chronic persistent hepatitis.

Computed image analysis assessment of immunostaining

To obtain an independent and objective evaluation of SOCS-1 expression, the intensity value of SOCS-1 immunostaining was determined from light microscopic images (Provis AX

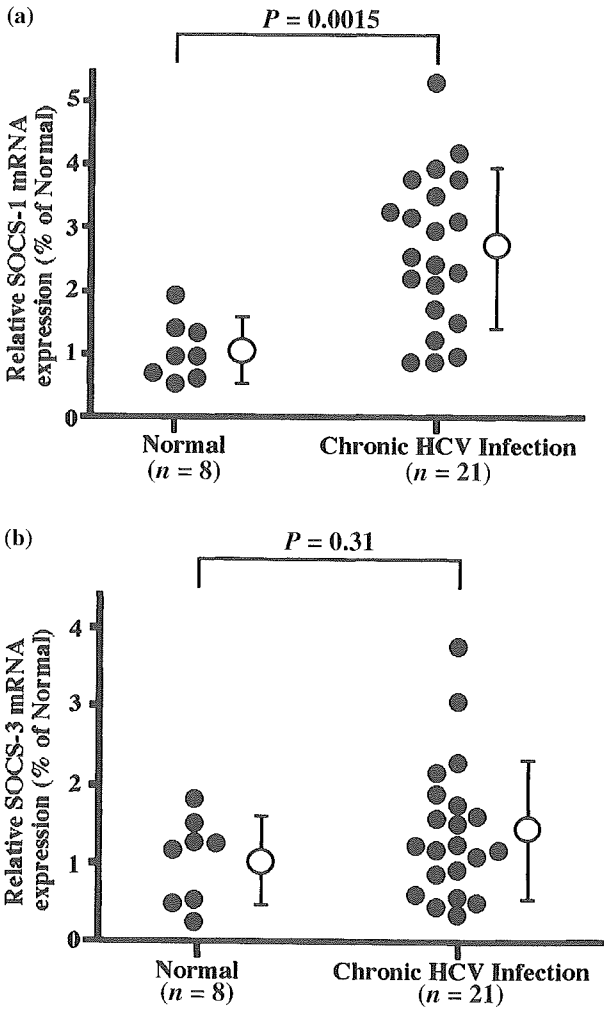

Fig. 1 Expressions of SOCS-1 and -3 in the livers of CHC. Total RNA was isolated from the livers of eight controls and 21 patients with CHC and then subjected to RT-PCR by using primers specific for human SOCS-1 and -3 and electrophoresed. PCR with β-actin primers served as an internal standard. A quantitative densitometric analysis of PCR products was carried out. The relative expression level of SOCS-1 (a) and SOCS-3 (b) was expressed as a ratio of SOCS-1 and -3 mRNA/β-actin mRNA normalized by the mean value of the controls. The bars indicate mean ± SD.

80 equipped with a HDTV system and a colour-chilled three charged coupling device camera; Olympus, Tokyo, Japan) using an image analysis system (MacScope version 2.55; Mitani, Fukui, Japan). Because of the triplicate nature of the arrays, three values of the intensity were obtained from every patient.

Statistical analysis

SOCS-1 and -3 transcripts were analysed by Mann–Whitney's *U*-test with StatView software. SOCS-1 protein expression in liver tissues was analysed using a chi-square test for independence or Fisher's exact probability test. Univariate and multivariate logistic regression analyses were performed with StatView software (SAS Institute Inc., Cary, NC, USA) and SAS software (SAS Institute Inc., Cary, NC, USA), respectively.

RESULTS

Expression of SOCS-1 and -3 in chronic liver disease specimens by RT-PCR

We investigated the expression of SOCS-1 and -3 in liver tissues from 21 patients with CHC and eight normal liver tissues by means of quantitative RT-PCR analysis. SOCS-1 and -3 transcripts were detected in all the liver tissues examined. The level of SOCS-1 expression was significantly higher in CHC livers than in normal livers ($P < 0.005$) (Fig. 1a). However, the level of SOCS-3 expression was not significantly higher in CHC livers than in normal livers ($P = 0.31$) (Fig. 1b).

Effect of SOCS-1 on STAT-1 phosphorylation induced by IFN-α in PLC/PRF/5

To analyse the effect of SOCS-1 on IFN-α-induced STAT-1 tyrosine phosphorylation and antiviral gene expression, we established stable transfectants of SOCS-1 in the human hepatoma cell line, PLC/PRF/5 which is frequently used in experiments of IFN signalling transduction [30, 37]. In addition, we confirmed that IFN-α-induced STAT-1 phosphorylation and antiviral gene expression was induced by IFN in PLC/PRF/5. Two clones (denoted as S5 and S7 in Fig. 2) with different expression levels of SOCS-1 mRNA (PLC/PRF/5/SOCS-1) were used in investigating the involvement of SOCS-1 in IFN-α signalling. The SOCS-1 protein has been shown to inhibit JAK tyrosine kinase activities and the phosphorylation of STAT-1 by directly binding to JAKs. As the phosphorylation of STAT-1 at Tyr^{701} is essential for IFN-α signalling by dimerizing and binding to DNA, phosphorylation at this site in SOCS-1-expressing cells was investigated by stimulation with IFN-α [38, 39]. Although IFN-α-induced STAT-1 phosphorylation in PLC/PRF/5 parental and mock cells, IFN-α-induced STAT-1 phosphorylation was markedly absent in SOCS-1-expressing cells (Fig. 2a): the extent of phosphorylation was slight in low-expressing cells (clone S7 in Fig. 2) and completely absent in high-expressing cells (clone S5 in Fig. 2). These

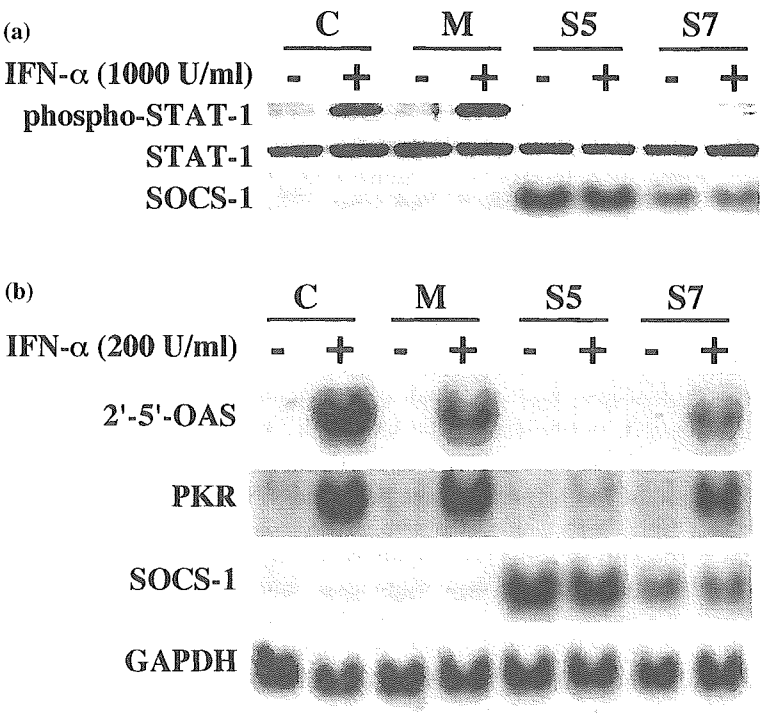

Fig. 2 (a) Overexpression of SOCS-1 markedly attenuated the STAT-1 phosphorylation. STAT-1 was phosphorylated when IFN-α (1000 U/mL) is present in parental and mock cells. However, it was completely or markedly inhibited in SOCS-1-transfected cells S5 and S7 respectively. (b) Northern blot analysis of IFN-inducible antiviral genes. 2′-5′-OAS and PKR gene expression induced by IFN-α (200 U/mL) was completely (S5) or markedly (S7) inhibited in the SOCS-1-overexpressing cells. GAPDH served as a control. C, parental cells; M, mock cells; S5 and S7, stable transfectants highly and moderately expressing SOCS-1, respectively.

results indicate that SOCS-1 may negatively regulate IFN-α-induced JAK–STAT signalling in the liver.

Effect of SOCS-1 on IFN-α-induced antiviral gene expression in PLC/PRF/5

Interferon-α has been shown to elicit antiviral effects by inducing 2′-5′-OAS, PKR and MxA proteins through the activation of JAK/STAT signalling [37, 38]. 2′-5′-OAS and PKR mRNA expression was induced by IFN-α in parental and mock cells. However, in SOCS-1-expressing cells, the induced expressions of these proteins by IFN-α were markedly attenuated (Fig. 2b) in comparison with the levels of SOCS-1 expressed. These results suggest that SOCS-1 is capable of attenuating IFN-α-induced antiviral effects.

Relationship between SOCS-1 expression in the liver specimens of chronic hepatitis C and clinical outcome

To analyse the influence of SOCS-1 expression on IFN therapy, a prospective cohort study was initiated on SOCS-1 expression and clinical outcome. From June 1996 to December 1997, 77 patients (male 43 and female 34) completed IFN therapy. Profiles of those patients are shown in Table 1; 49 (63.6%) patients were of genotype 1b and 28 (36.4%) of genotype 2. Most patients had active hepatitis with a mean HAI score of 9.9. Based on the intensity of SOCS-1 expression by the automatic imaging analysis, we classified those patients into two groups: high-expression group (above 3 million pixel) with 61 patients; and low-expression group (below 3 million pixel) with 16 patients (Fig. 3). The SOCS-1 protein was expressed in the cytoplasm and hepatocyte membranes in the livers of CHC in a diffuse manner. Between these two groups, patient backgrounds were compared with analyse the factors contributing to SOCS-1 expression in the liver (Table 2). As a result, the SOCS-1-high group had a significantly lower rate of SVR to IFN therapy ($P = 0.0014$) and a significantly higher HCV-RNA titre ($P = 0.015$) than the SOCS-1-low group. A univariate logistic regression analysis was next performed to investigate factors contributing to responsiveness to IFN treatment (Table 3). Not only the HCV genotype (OR, 7.708; 95% CI, 2.705–21.964; $P = 0.0001$) and HCV-RNA titre (OR, 13.179; 95% CI, 3.807–45.624; $P < 0.0001$), but SOCS-1 expression was also found to be a significant predictor for SVR to IFN (OR, 6.150; 95% CI, 1.759–21.500; $P = 0.004$) (Table 3). There was no significant difference in the total IFN dose between patients who had SVR and patients who were nonresponsive (806 ± 119 *vs* 806 ± 144 MIU; $P = 0.234$). A multivariate analysis with stepwise logistic regression identified the gender (OR, 6.072; 95% CI, 1.176–31.353; $P = 0.031$), the HCV genotype (OR, 25.952; 95% CI, 4.315–156.098; $P < 0.0005$) and the HCV-RNA titre (OR, 27.810; 95% CI, 4.830–160.126; $P < 0.0005$) as independent factors contributing to SVR to IFN, in agreement with previously published reports [6]. In addition, patients with low SOCS-1 expression in the liver also tended to have SVR to IFN, but it was not statistically significant (OR, 4.730; 95% CI, 0.852–26.265; $P = 0.076$) (Table 4). Among the host factors including age, gender, degree of fibrosis, baseline alanine aminotransferase (ALT) level and SOCS-1 expression, the multivariate analysis demonstrated that a negative SOCS-1 expression was the

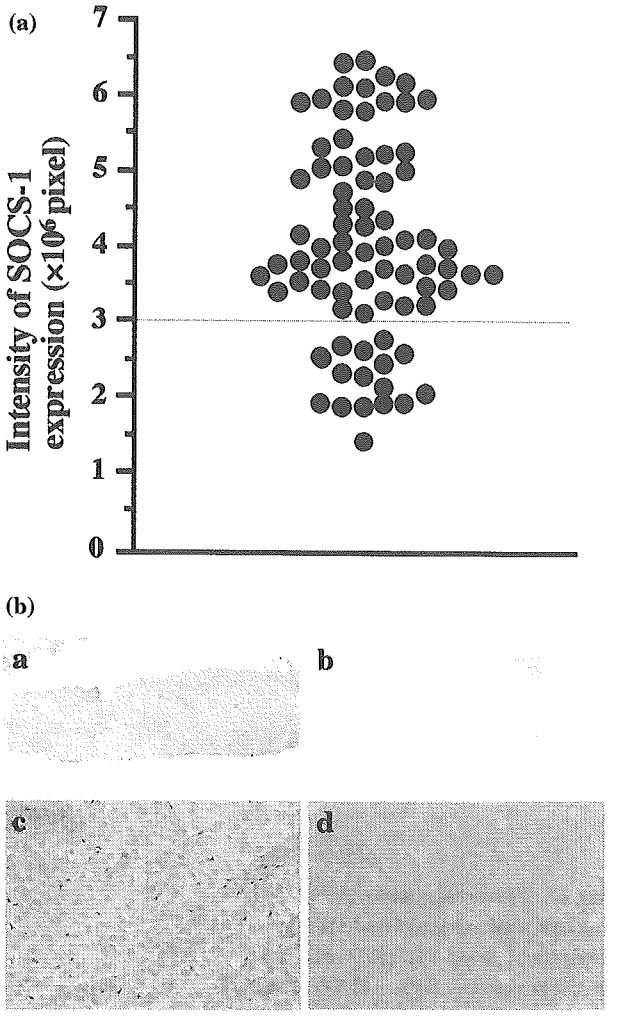

Fig. 3 (a) Computed image analysis assessment of immunostaining. Based on the automatic imaging analysis, SOCS-1 expression was grouped into two groups: low intensity of expression (below 3 million pixel) with 16 patients; high intensity of expression (above 3 million pixel) with 61 patients. (b) Representative immunohistochemical staining of SOCS-1 in the livers of chronic hepatitis C patients. (a, c) Representative case of SOCS-1 high intensity of expression was 4.2 million pixel. (b, d) Representative case of SOCS-1 low intensity of expression was 1.9 million pixel. Original magnification; (a, b) ×40 and (c, d) ×200. For a negative control, the primary antibody was replaced with nonimmune normal mouse IgG.

Table 2 Clinical characteristics of patients expressing SQOCS-1

Parameter	SOCS-1 expression		*P*-value
	High ($n = 61$)	Low ($n = 16$)	
Male/female	34/27	9/7	NS
Age (years; mean ± SD)	49.8 ± 10.7	44.6 ± 13.7	NS*
HCV genotype			
1b	40	9	
Others	21	7	NS
Histology			
CAH1B	1	0	
CAH2A	41	13	NS
CAH2B	18	3	
CPH	1	0	
HAI score (mean ± SD)	10.0 ± 3.8	9.4 ± 4.3	NS*
HCV-RNA titre (10^6 copies/mL; mean ± SD)	11.5 ± 12.1	4.7 ± 6.9	0.015*
Baseline ALT level (U/L; mean ± SD)	105.5 ± 79.7	90.5 ± 49.5	NS*
Week 24 ALT level (U/L; mean ± SD)	47.7 ± 45.5	29.9 ± 35.9	NS*
Week 48 ALT level (U/L; mean ± SD)	45.5 ± 34.3	36.4 ± 35.7	NS*
SVR/NR	18/43	12/4	0.0014**

SVR, sustained virological response; NR, nonresponse.
P-value was calculated by chi-square test for independence.
*Mann–Whitney's *U*-test; **Fisher's exact probability test.

sole significant predictor for SVR to IFN (OR, 7.472; 95% CI, 1.934–28.878; $P = 0.004$) (Table 5). Further multivariate analysis showed that a higher HCV-RNA titre ($>10^6$) was the only significant factor contributing to SOCS-1 expression in the liver (OR, 5.399; 95% CI, 1.489–19.584; $P = 0.010$).

DISCUSSION

Hepatitis C virus genotypes and viral loads have been shown to be the most important predictive factors for responsiveness to IFN therapy. Mutations in the nonstructural 5A (NS5A) protein and hypervariable regions in the HCV have also been reported as contributing factors to IFN responsiveness [40, 41]. In contrast to these viral indicators, very little is known concerning host factors that contribute to IFN responsiveness. The stage and duration of the disease have also been reported to influence responsiveness. Recently, several reports [13– 19] have suggested that SOCS-1 and SOCS-3 in the livers of CHC patients may regulate IFN signalling. The findings herein demonstrate that SOCS-1 mRNA in the livers of CHC patients is expressed at higher levels compared to controls and that the SVR rate was significantly lower in SOCS-1-positive patients compared with SOCS-1-negative patients, as estimated by immunohistochemical observation. Univariate analysis showed that the positive SOCS-1 staining in the liver represents a significant predictor of IFN responsiveness, in addition to HCV genotype and HCV-RNA titre. These results suggest that patients who are positive for SOCS-1 staining in the liver may be resistant to IFN therapy. The multivariate analysis performed with host factors in which a positive presence of SOCS-1 in the liver was the only significant host factor contributing to IFN responsiveness, supports this view.

As the SOCS family functions as a negative regulator for JAK–STAT signalling, it is possible that SOCS-1 expression may inhibit JAK–STAT signalling downstream from IFN-α/β receptors. Recently, the inhibition of IFN-α/β as well as IFN-γ signalling by SOCS-1 was reported in myelogenous leukaemia cells. JAK1 and Tyk2 phosphorylation induced by IFN-β was suppressed in SOCS-1-overexpressing cells. In addition, the phosphorylation of JAK1, Tyk2 and STAT-1, induced by IFN-α, was inhibited in IFN-α-resistant Daudi cells that overexpressed SOCS-1 [42]. Our findings show that STAT-1 phosphorylation induced by IFN-α was clearly inhibited by SOCS-1 overexpression in a dose-dependent manner by using two hepatoma clones expressing different levels of SOCS-1 are consistent with those findings. The inhibition of IFN signalling by SOCS-1 was further confirmed by investigating the transcriptional products of 2′-5′-OAS and PKR that are induced by IFN-α to elicit antiviral effects; the gene expression of 2′-5′-OAS and PKR was suppressed in SOCS-1-transfected cells in a dose-dependent manner. These results

Table 3 Univariate analysis of factors contributing to SVR

Parameter	*n*	OR	95% CI	*P*-value
Gender				
Female	34	1		
Male	43	1.996	0.784–5.083	0.147
Age (years)				
50≤	41	1		
<50	36	1.929	0.770–4.832	0.161
HCV genotype				
1b	49	1		
Others	28	7.708	2.705–21.964	0.0001
HAI (I + II + III)				
9–18	39	1		
4–8	26	2.437	0.699–8.500	0.162
1–3	12	1.950	0.545–6.975	0.304
HAI (IV)				
3–4	18	1		
1–2	59	2.194	0.694–6.940	0.181
HCV-RNA titre (10^6 copies/mL)				
10^6≤	55	1		
<10^6	22	13.179	3.807–45.624	<0.0001
Baseline ALT level (U/L)				
≤70	35	1		
70<	42	1.398	0.559–3.495	0.474
SOCS-1 expression				
High	61	1		
Low	16	6.150	1.759–21.500	0.004

Univariate logistic regression. Wald chi-squared *P*-value was calculated.

Table 4 Multivariate analysis of factors contributing to SVR

Parameter	*n*	OR	95% CI	*P*-value
Gender				
Female	34	1		
Male	43	6.072	1.176–31.353	0.031
HCV genotype				
1b	49	1		
Others	28	25.952	4.315–156.098	<0.0005
HAI (IV)				
3–4	59	1		
1–2	18	3.858	0.691–21.541	0.124
HCV-RNA titre (10^6 copies/mL)				
10^6≤	55	1		
<10^6	22	27.810	4.830–160.126	<0.0005
Baseline ALT level (U/L)				
≤70	35	1		
70<	42	4.129	0.805–21.168	0.089
SOCS-1 expression				
High	61	1		
Low	16	4.730	0.852–26.265	0.076

Multivariate logistic regression. Wald chi-squared *P*-value was calculated.

Table 5 Multivariate analysis of host factors contributing to SVR

Parameter	*n*	OR	95% CI	*P*-value
Age (years)				
50≤	41	1		
<50	36	1.533	0.485–4.842	0.466
Gender				
Male	34	1		
Female	43	2.128	0.697–6.493	0.185
HAI (I + II + III)				
1–3	12	1		
4–8	26	1.455	0.276–7.674	0.556
9–18	39	2.117	0.380–11.788	0.392
HAI (IV)				
3–4	18	1		
1–2	59	2.714	0.616–11.960	0.187
Baseline ALT level (U/L)				
≤70	35	1		
70<	42	1.336	0.452–3.953	0.601
SOCS-1 expression				
High	61	1		
Low	16	7.472	1.934–28.878	0.004

Multivariate logistic regression. Wald chi-squared *P*-value was calculated.

suggest that SOCS-1 expression in the liver may lead to resistance to IFN therapy via the inhibition of IFN signalling.

The mechanism by which SOCS-1 is overexpressed in the livers of CHC is unknown. As mentioned above, the staining of SOCS-1 in the liver was the only significant host factor contributing to IFN responsiveness, as evidenced by a multivariate analysis performed with host factors. However, another multivariate analysis performed with factors that included viral indicators revealed that the staining of SOCS-1 in the liver could no longer be considered an independent predictor of IFN responsiveness. As the SOCS-1-high patients had a significantly higher HCV-RNA titre than SOCS-1 low patients ($P < 0.015$), SOCS-1-expression in the liver may be HCV-dependent. It was recently reported that the HCV core protein may impair IFN-α-induced signal transduction via the induction of SOCS-3 expression [20]. Therefore, it is possible that the HCV core protein may induce SOCS-1 as well as SOCS-3, leading to the inhibition of IFN-α signalling. An alternative possibility is that elevated SOCS-1 expression negatively regulates intrinsic IFN signalling, which may cause a higher HCV-RNA titre in the pretreatment characteristics of IFN-resistant patients. These results indicate that the cytokine-induced SOCS family may negatively regulate IFN signalling, thus leading to IFN resistance and that anti-cytokine or anti-SOCS family therapies may represent additional options for patients resistant to IFN therapy.

In conclusion, the expression of SOCS-1, a negative regulator for JAK–STAT signalling, is enhanced in the livers of CHC patients and might be involved in their resistance to IFN therapy.

ACKNOWLEDGEMENTS

We wish to thank Dr Masashi Narazaki and Dr Nobuyuki Kato for their generous gifts of the antibody and the cDNAs. We also thank Akito Yoshida for helpful discussions and suggestions regarding statistics and data analyses.

REFERENCES

1 Lai MY, Kao JH, Yang PM *et al.* Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. *Gastroenterology* 1996; 111: 1307–1312.
2 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–965.
3 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–1069.
4 Tong MJ, Blatt LM, McHutchison JG, Co RL, Conrad A. Prediction of response during interferon alfa 2b therapy in chronic hepatitis C patients using viral and biochemical characteristics: a comparison. *Hepatology* 1997; 26: 1640–1645.
5 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C.. *N Engl J Med* 1998; 339: 1485–1492.
6 Stuyver L, Rossau R, Wyseur A *et al.* Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. *J Gen Virol* 1993; 74: 1093–1102.
7 Davis GL, Lindsay K, Albrecht J *et al.* Clinical predictors of response to recombinant interferon-alpha treatment in patients with chronic non-A, non-B hepatitis (hepatitis C). *J Viral Hepat* 1994; 1: 55–63.
8 Serfaty L, Giral P, Loria A, Andreani T, Legendre C, Poupon R. Factors predictive of the response to interferon in patients with chronic hepatitis C. *J Hepatol* 1994; 21: 12–17.
9 Naka T, Fujimoto M, Kishimoto T. Negative regulation of cytokine signaling: STAT-induced STAT inhibitor. *Trends Biochem Sci* 1999; 24: 394–398.
10 Naka T, Narazaki M, Hirata M *et al.* Structure and function of a new STAT-induced STAT inhibitor. *Nature* 1997; 387: 924–929.
11 Endo TA, Masuhara M, Yokouchi M *et al.* A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 1997; 387: 921–924.
12 Starr R, Willson TA, Viney EM *et al.* A family of cytokine-inducible inhibitors of signalling. *Nature* 1997; 387: 917–921.
13 Grüngreiff K, Reinhold D, Ansorge S. Serum concentrations of sIL-2R, IL-6, TGF-β1, neopterin and zinc in chronic hepatitis C patients treated with interferon-alpha. *Cytokine* 1999; 11: 1076–1080.
14 Malaguarnera M, Di Fazio I, Laurino A, Ferlito L, Romano M, Trovato BA. Serum interleukin 6 concentrations in chronic hepatitis C patients before and after interferon-alpha treatment. *Int J Clin Pharmacol Ther* 1997; 35: 385–388.
15 Koziel MJ. Cytokines in viral hepatitis. *Semin Liver Dis* 1999; 19: 157–169.
16 Sobue S, Nomura T, Ishikawa T *et al.* Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. *J Gastroenterol* 2001; 36: 544–551.
17 Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. *Hepatology* 1996; 24: 6–9.
18 Hong F, Jaruga B, Kim WH *et al.* Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. *J Clin Invest* 2002; 110: 1503–1513.
19 Hong F, Nguyen VA, Gao B. Tumor necrosis factor alpha attenuates interferon alpha signaling in the liver: involvement of SOCS3 and SHP2 and implication in resistance to interferon therapy. *FASEB J* 2001; 15: 1595–1597.
20 Bode JG, Ludwig S, Ehrhardt C *et al.* IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB J* 2003; 17: 488–490.
21 Narazaki M, Fujimoto M, Matsumoto T *et al.* Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling. *Proc Natl Acad Sci USA* 1998; 95: 13130–13134.
22 Sawai Y, Tamura S *et al.* Expression of ephrin-B1 in hepatocellular carcinoma: possible involvement in neovascularization. *J Hepatol* 2003; 39: 991–996.
23 Kamada Y, Tamura S, Kiso S, Fukui K *et al.* Angiotensin II stimulates the nuclear translocation of Smad2 and induces PAI-1 mRNA in rat hepatic stellate cells. *Hepatol Res* 2003; 25: 296–305.
24 Takano T, Amino N. Quantitative analysis of rat thyroglobulin messenger RNA in FRTL-5 cells by competitive polymerase chain reaction with human thyroglobulin messenger RNA. *Endocrine Res* 1991; 23: 119–130.
25 Oritani K, Medina KL, Tomiyama Y *et al.* Limitin: an interferon-like cytokine that preferentially influences B-lymphocyte precursors. *Nat Med* 2000; 6: 659–666.
26 Matsumura I, Ishikawa J, Nakajima K *et al.* Thrombopoietin-induced differentiation of a human megakaryoblastic leukemia cell line, CMK, involves transcriptional activation of p21 (WAF1/Cip1) by STAT5. *Mol Cell Biol* 1997; 17: 2933–2943.
27 Murphy D, Detjen KM, Welzel M, Wiedenmann B, Rosewicz S. Interferon-alpha delays S-phase progression in human hepatocellular carcinoma cells via inhibition of specific cyclin-dependent kinases. *Hepatology* 2001; 33: 346–356.
28 Sakuda S, Tamura S, Yamada A *et al.* Activation of signal transducer and activator transcription 3 and expression of suppressor of cytokine signal 1 during liver regeneration in rats. *J Hepatol* 2002; 36: 378–384.
29 Melen K, Keskinen P, Lehtonen A, Julkunen I. Interferon-induced gene expression and signaling in human hepatoma cell lines. *J Hepatol* 2000; 33: 764–772.

30 Naganuma A, Nozaki A, Tanaka T *et al.* Activation of the interferon-inducible 2′-5′-oligoadenylate synthetase gene by hepatitis C virus core protein. *J Virol* 2000; 74: 8744–8750.
31 Yokosawa N, Kubota T, Fujii N. Poor induction of interferon-induced 2′,5′-oligoadenylate synthetase (2–5 AS) in cells persistently infected with mumps virus is caused by decrease of STAT-1 alpha. *Arch Virol* 1998; 143: 1985–1992.
32 Hiramatsu N, Hayashi N, Kasahara A *et al.* Improvement of liver fibrosis in chronic hepatitis C patients treated with natural interferon alpha. *J Hepatol* 1995; 22: 135–142.
33 Fujiwara D, Hino K, Yamaguchi Y *et al.* Type I interferon receptor and response to interferon therapy in chronic hepatitis C patients: a prospective study. *J Viral Hepat* 2004; 11: 136–140.
34 De Groote J, Desmet VJ, Gedigk P *et al.* A classification of chronic hepatitis. *Lancet* 1968; II: 626–628.
35 Knodell RG, Ishak KG, Black WC *et al.* Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; 1: 431–435.
36 Pillai G, Roberts H, Gatter K, Pezzella F. p53 expression in normal paraffin-embedded tissue using different antibodies and antigen retrieval buffer systems. *Histopathology* 2003; 42: 83–87.
37 Hung WC, Chuang LY. Sodium butyrate enhances STAT 1 expression in PLC/PRF/5 hepatoma cells and augments their responsiveness to interferon-alpha. *Br J Cancer* 1999; 80: 705–710.
38 Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; 67: 227–264.
39 Haque SJ, Williams BR. Signal transduction in the interferon system. *Semin Oncol* 1998; 25(Suppl. 1): 14–22.
40 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
41 Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 1999; 285: 107–110.
42 Sakamoto H, Yasukawa H, Masuhara M *et al.* A Janus kinase inhibitor, JAB, is an interferon-gamma-inducible gene and confers resistance to interferons. *Blood* 1998; 92: 1668–1676.

APPENDIX

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平成17年度　厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業
「Ｂ型及びＣ型肝炎ウイルスの新たな感染予防法の確立のための
感染病態解明に関する研究」報告書

発行日：2006年３月31日

発行者：主任研究者　河田純男（国立大学法人山形大学医学部）

発行所：〒990-9585　山形市飯田西２-２-２　国立大学法人山形大学医学部
消化器病態制御内科学

印刷：コロニー印刷