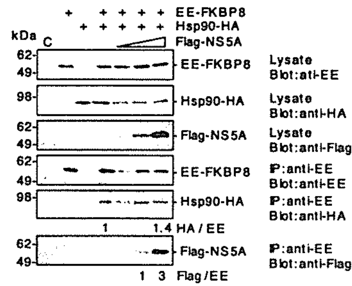
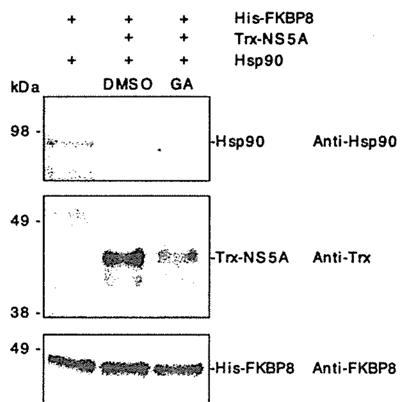


Supplementary Figure 3. Interaction of NS5A, FKBP8 and Hsp90
EE-FKBP8 was co-expressed with Hsp90-HA and Flag-NS5A in 293T cells and immunoprecipitated with anti-EE antibody. Precipitates were analyzed by Western blotting by anti-EE, -HA or -Flag antibody. Effect of increase of Flag-NS5A expression on the association of FKBP8 with Hsp90 was examined by transfection with 0.1, 0.2 or 0.4 μg of Flag-NS5A expression plasmid.



Supplementary Figure 4. Disruption of NS5A/FKBP8/Hsp90 complexes by geldanamycin

Purified His-FKBP8, Hsp90 and/or Trx-NS5A were mixed with DMSO or geldanamycin (GA) (100 nM) and subjected to immunoprecipitation with anti-FKBP8 antibody. Precipitates were immunoblotted with antibody to Hsp90, thioredoxin, or FKBP8.

Supplementary materials and methods

Preparation of monoclonal antibody to FKBP8

Glutathione-S-transferase-fused human FKBP8 (GST-FKBP8) was expressed in *Escherichia coli* strain JM109 transformed with pGEX-4T3 containing FKBP8 gene. GST-FKBP8 was purified with Glutathione-conjugated Sepharose Affinity Matrix (Amersham Pharmacia Biotech, Franklin Lakes, NJ). Purified GST- FKBP8 was immunized to *Balb/c* mouse. Lymphonodus cells were obtained after 5 boost immunizations and were fused to mouse myeloma PAI cells. The resulting hybridomas were screened by enzyme-linked immuno-sorbent assay using GST and GST-FKBP8. The selected clones were further screened by flow cytometry using 293T cells expressing HA-FKBP8 (O'Reilly et al., 1998). Among several positive clones, two clones strongly reactive to human FKBP8 were designated as KDM-11 and 19 (IgG2b). Antibodies were purified from supernatants of cell culture by Protein G Sepharose 4B beads (Amersham).

Preparation of recombinant proteins

His_6-tagged FKBP8 (His-FKBP8) and thioredoxin-fused NS5A (aa 25-213, domain I) (Trx-NS5A) were generated from recombinant *Escherichia coli.* Either pET30a encoding FKBP8 or pET32a encoding NS5A (aa 25-213) was introduced into *E. coli* strain BL21(DE3). Ten milliliter of overnight culture was added into 1 L of 2 x YT medium and was incubated at 37℃. When the absorbance of culture supernatant indicated 0.4 OD_{600}, isopropyl beta-thiogalactoside (IPTG) was added at final concentration of 0.4 mM and was then incubated at 20℃ overnight. After centrifugation, the cell pellet was washed once with 10 ml phosphate buffered saline (PBS). The washed cell pellet was suspended in 40 ml lysis buffer (50mM phosphate buffer [pH 8.0] containing 150mM NaCl, 1% Triton X-100 and 0.2 μg/ml lysozyme) and was incubated at 4℃ for 2h. After freezing and thawing, the mixture was sonicated at 4℃ for 5 min and was treated with 0.02 mg/ml of DNase at room temperature for 5 min. The cell lysates were centrifuged at 10,000 x g for 5 min. The resulting supernatant

was mixed with 0.5 ml of Nickel agarose beads (Sigma, St. Louis, MO) and was rotated at 4°C for 60 min. The Nickel beads were washed twice with PBS containing 10 mM imidazole. The recombinant protein was eluted from Nickel beads with PBS containing 0.25 M imidazole. Bovine Hsp90 was purchased from Sigma. Bovine Hsp90-α shares 99.5% amino acid identity to human Hsp90-α.

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RAPID COMMUNICATION

Risk factors for retinopathy associated with interferon α-2b and ribavirin combination therapy in patients with chronic hepatitis C

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Abstract

AIM: To elucidate the frequency and risk factors for retinopathy in patients with chronic hepatitis C who are treated by interferon-ribavirin combination therapy.

METHODS: We prospectively analyzed 73 patients with histologically confirmed chronic hepatitis C, who underwent combination therapy for 24 wk. Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy.

RESULTS: Fourteen patients (19%) developed retinopathy, which was initially diagnosed by the appearance of a cotton wool spot in 12 patients. Retinal hemorrhage was observed in 5 patients. No patient complained of visual disturbance. Retinopathy disappeared in 9 patients (64%) despite the continuation of combination therapy. However, retinopathy persisted in 5 patients with retinal hemorrhage. A comparison of the clinical background between the groups with and without retinopathy showed no significant differences in age, gender, viral genotype, RNA level, white blood cell count, platelet count, prothrombin time, complications by diabetes mellitus or hypertension, or pretreatment arteriosclerotic changes in the optic fundi. However, multiple logistic regression analysis revealed that complication by hypertension was observed with a high frequency in the group with retinopathy (P = 0.004, OR = 245.918, 95% CI = 5.6-10786.2).

CONCLUSION: Retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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Key words: Retinopathy; Ribavirin; Chronic hepatitis C; Interferon

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INTRODUCTION

Chronic hepatitis C, which affects more than 170 million people in the world[1], may eventually lead to cirrhosis and/or hepatocellular carcinoma. The main treatment for this intractable disease is interferon administration. Published guidelines recommend interferon-ribavirin combination therapy as a first-line treatment[2]. Interferon is also used in the treatment of other viral and neoplastic diseases.

Various adverse effects have been reported due to use of interferon[3]. An influenza-like syndrome, characterized by fever, chills, myalgias, arthralgias, and headache, is the most common adverse effect. Toxicities of the central nervous, hematopoietic, gastrointestinal, urinary, cardiovascular, musculoskeletal and endocrine systems have also been described. However, ocular toxicity was not reported before the use of interferon for chronic hepatitis[3].

After the introduction of interferon for the treatment of hepatitis, retinal complications have been reported. Hayakawa *et al* showed that 17 of 43 patients developed retinopathy during interferon monotherapy. They also showed that the prevalence of retinopathy was higher in patients with diabetes[4]. Subsequently, several papers have shown that a substantial proportion of patients undergoing interferon monotherapy develop retinopathy[5-7]. However, the prevalence of retinopathy is variable, which is

presumably attributed to the difference in the treatment regimen and/or background of patients.

As mentioned above, interferon-ribavirin combination therapy has become the standard treatment for chronic hepatitis C. Results from recent studies have suggested that the prevalence of retinopathy associated with combination therapy may be higher than that associated with interferon monotherapy, which should be further investigated[8-10].

In spite of the high prevalence, risk factors for interferon-associated retinopathy are still unclear. Diabetes mellitus and the patients' age were reported to be possible risk factors for retinopathy associated with interferon monotherapy[4]. In interferon-ribavirin combination therapy, diabetes, hypertension[8], and response to treatment[10] were considered possible risk factors. However, the results are not conclusive because of the small number of patients examined.

The aim of the present study is to elucidate the prevalence and risk factors for retinopathy associated with interferon-ribavirin combination therapy.

MATERIALS AND METHODS

Patients

Seventy-three consecutive patients with histologically confirmed chronic hepatit is C (47 males and 26 females; median age, 53.4 years; ranges 26-73 years) were enrolled in this study from 2002 to 2004. The clinical backgrounds of the enrolled patients are shown in Table 1. All patients were treated with recombinant interferon α-2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ, USA) combination therapy. All the patients were treated daily with interferon α-2b at 6 MU for 2 wk followed by three times a wk treatment with interferon α-2b at 6 MU for 22 wk in combination with ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for patients who weighed 60 kg or less and 800 mg for the remaining patients who weighed more than 60 kg for 24 wk.

All patients were assessed to determine the safety, tolerance, and efficacy of the treatment at the end of wk 1, 2, 4, and every 4 wk during the treatment. After the treatment was completed, patients were followed up on wk 4, 8, 12, and 24. The primary end point was indicated by a sustained loss of detectable HCV-RNA at 24 wk after the treatment.

Methods

Optic fundi were examined before, and 2, 4, 12 and 24 wk after the start of combination therapy. Ophthalmological examinations were carried out before the start of treatment and 2, 4, 12 and 24 wk after the start of treatment until the completion of treatment or until the retinopathy disappeared. Fundus photographs were taken for documentation and comparison when retinal abnormalities were detected.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of our institutions.

Table 1 Profiles and initial laboratory data of patients with and without retinopathy during IFN-ribavirin combination therapy

	Total	Retinopathy (+)	Retinopathy (-)
Patients			
Number	73	14	59
Age (yr)	53.4 ± 10.9	56.3 ± 10.5	52.8±38.6
Gender (M/F)	47/26	10/4	37/22
Hypertension (Yes/No)ª	15/58	5/8	10/49
Diabetes mellitus (Yes/No)	2/71	1/13	1/58
Peripheral blood count			
Platelet count (×10⁴/mm³)	15.3 ± 6.0	12.5 ± 10.5	15.9 ± 38.6
White blood cell (×10³/mm³)	46.9 ± 12.6	46.5 ± 13.0	48.6 ± 10.9
Hemoglobin (×g/dL)	14.0 ± 1.3	14.0 ± 1.0	14.0 ± 1.4
Prothrombin time (%)	90.2 ± 13.3	87.1 ± 13.3	90.8 ± 13.3
ALT (IU/L)	109.4 ± 78.2	104.1 ± 41.0	110.4 ± 83.6
Viral factors			
Genotype (type 1/type 2)²	45/26	33/24	12/2
Viral load (kcopies/mL)	592.3 ± 271.2	505.6 ± 309.1	607.5 ± 271.2
Pretreatment/ Arteriosclerotic **changes in optic fundi (Yes/No)**	12/61	7/7	5/54
Response to therapy (SVR/non-SVR)	38/35	5/9	33/26

¹ Data are expressed as mean ± SD.
² Genotype could not be determined in 2 patients.
ªP = 0.004

RESULTS

Before the start of the combinaiton therapy, one patient had scars from laser coagulation of a previous interferon-associated retinopathy and another patient had retinal central vein occlusion. Arteriosclerotic changes of the optic fundi were observed in 12 patients.

After the start of interferon-ribavirin combination therapy, 14 out of 73 patients (19%) developed retinopathy. The clinical profiles and laboratory data of the patients with and without retinopathy are shown in Table 1.

We compared the characteristics of patients who developed retinopathy and those who did not. The two groups showed no statistical differences in age, gender, subtype of virus, RNA level, white blood cell count, platelet count, prothrombin time before treatment or prevalence of pretreatment fundic arteriosclerotic changes. The patients with retinopathy were more frequently complicated by hypertension (P = 0.004) (Table 1).

Logistic regression analysis of factors affecting retinopathy was also carried out. Hypertension was found to be a factor for predicting retinopathy (Table 2).

Table 3 shows the optic fundi findings of the 14 patients with retinopathy. Retinopathy was initially diagnosed by the appearance of a cotton wool spot in 12 patients. In three of the 12 patients, retinal hemorrhage was also observed simultaneously or sequentially. Two of the 14 patients who developed retinopathy were diagnosed by retinal hemorrhage without a cotton wool spot. No patient complained of the visual disturbance.

Table 2 Logistic regression analysis of factors associated with retinopathy

Factor	*P*	Odds ratio	95% confidence interval
Sex	0.68	1.699	0.1-21.0
Age	0.203	1.099	1.0-1.3
Genotype	0.776	1.621	0.1-45.5
Levels of HCV RNA	0.114	1.006	0.99-1.0
Hypertension	0.004	246.32	5.5-10977.8
Diabetes mellitus	0.211	0.122	0.1-3.3
Abnormal findings in pretreatment optic fundi	0.904	1.192	0.1-20.3
Platelet	0.059	1.391	1.0-1.9
Prothrombin time	0.747	0.982	0.9-1.1
ALT	0.992	1	0.98-1.0
WBC	0.964	1.027	0.4-2.9
Response to therapy (SVR or non-SVR)	0.123	0.016	0.0-3.1

Table 3 Optic fundi findings of patients with retinopathy

No	Age	Sex	Underlying disease		Optic fundi before treatment		Optic fundi after treatment	
			Hyper tension	Diabetes mellitus	H	S	Cotton wool spot	Retinal hemorrhage
1	38	M	+	+	0	0	4 wk-	4 wk-
2	52	M	+	-	1	0	4-12 wk	-
3	40	M	-	-	0	0	6-36 wk	-
4	62	F	-	-	0	0	4-36 wk	-
5	61	M	+	-	0	0	12 wk-	-
6	58	M	-	-	1	1	12 wk-	-
7	73	M	-	-	2	2	4-28 wk	-
8	65	F	+	-	0	0	24-36 wk	-
9	59	F	+	-	2	2	2 wk-	4-24 wk
10	40	M	-	-	0	0	4-20 wk	-
11	62	F	-	-	1	2	2 wk-	4 wk-
12	65	M	-	-	1	1	2-24 wk	-
13	40	M	-	-	0	0	-	8-16 wk
14	40	M	-	-	0	0	-	2-4 wk

Retinopathy disappeared in 9 of the 14 patients despite the continuation of combination therapy. However, it continued in three patients with retinal hemorrhage and two without retinal hemorrhage.

Ocular manifestations other than retinopathy (e.g., ocular pain, a mild watery eye and conjunctivitis) were not observed in any patients.

DISCUSSION

Interferon associated retinopathy was first recognized in 1990 when Ikebe and associates reported a 39-year-old patient who developed retinal hemorrhages and cotton wool spots following intravenous administration of interferon[11].

The exact mechanism of interferon-induced-retinopathy is not known but is presumably related to the disturbance in retinal microcirculation[12]. Therefore, preexisting arteriosclerosis that affects microcirculation may promote interferon-induced retinopathy.

Our study shows that hypertension is a more frequent complication in patients with interferon-induced-retinopathy. Chronic hypertension is associated with the thickening of the walls of the arteries and small arterioles[13]. Therefore, systemic hypertension predisposes patients to interferon-induced-retinopathy. The fact that hypertensive retinopathy induces the formation of flame-shaped hemorrhages and white cotton wool spots, which are also seen in interferon-induced-retinopathy, implies that systemic hypertension and interferon-induced-retinopathy may be related each other.

Statistical analysis did not indicate pretreatment optic fundic changes or diabetes as predictive factors of retinopathy. This may be attributed to the following reasons: (1) pretreatment changes in the optic fundi as a predictive factor are included in hypertension; and (2) the number of patients with diabetes is too small. Regardless of these reasons, systemic hypertension is an important risk factor for interferon-related retinopathy.

The frequencies of interferon-induced retinopathy associated with interferon monotherapy and interferon-ribavirin combination therapy are reported to be 24%-58%[4-7,14,15] and 16%-64%[8-10,16], respectively. The frequency in the present study (20%) was lower than that in previous reports. Furthermore, the ocular side effects of ribavirin, which include a mild watery eye and conjunctivitis, were not seen in this study. Therefore, the frequency of induced retinopathy associated with combination therapy may be considered as high as that associated with interferon monotherapy.

Retinopathy developed by 12 wk in most (13/14, 93%) of the patients after the start of combination therapy and disappeared in majority (10/14, 71%) of the patients during the 4-8 wk period, in which the patients were receiving the treatment. This suggests that treatment can be continued despite the development of retinopathy in many patients. However, two patients who developed cotton wool spots early in the therapy (2 wk) therafter suffered from retinal hemorrhage in a prolonged manner. Therefore, patients who develop cotton wool spots early in the therapy should be carefully monitored. However, as reported in previous studies[4,8,17], most of the patients with retinopathy in this study were asymptomatic. Therefore, combination therapy may be continued in most patients.

The fact that retinopathy occurred more frequently in patients with hypertension, suggests that these patients should be carefully monitored. With periodic examination of the optic fundi, major bleeding that causes visual symptoms may be prevented or detected at an early stage. Therefore, patients who undergo interferon-ribavirin combination therapy, particularly those with hypertension, should undergo periodic examination of the optic fundi. To conclude, retinopathy associated with combination therapy of interferon α-2b and ribavirin tends to develop in patients with hypertension.

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Bacterial flagellin inhibits T cell receptor-mediated activation of T cells by inducing suppressor of cytokine signalling-1 (SOCS-1)

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Summary

Flagellin, the structural component of bacterial flagella, is secreted by pathogenic and commensal bacteria, and is recognized by Toll-like receptor (TLR) 5. Flagellin is a common bacterial antigen present on most motile bacteria and is speculated to contribute to the activation of CD4⁺ T cells in the intestine. However, molecular mechanisms by which flagellin regulate T cell activation remains to be determined. Using Jurkat T cells or human primary T cell, we showed that flagellin stimulation induced tyrosine phosphorylation of TLR5 and activation of both mitogen-activated protein kinases and nuclear factor κB. In addition, stimulation by flagellin did not induce nuclear factor of activated T cells (NFAT) activation, while stimulation via the T cell receptor (TCR) leads to activation of NFAT. However, TCR-mediated NFAT activation and tyrosine phosphorylation of zeta-associated protein 70 (Zap-70) were inhibited in cells pre-stimulated by flagellin. Furthermore, flagellin stimulation induced suppressor of cytokine signalling-1 (SOCS-1), which formed a complex with Zap-70 after stimulation via TCR, and inhibition of SOCS-1 expression by SOCS-1-specific small interfering RNA reinstated TCR-mediated activation of NFAT in cells pre-stimulated with flagellin. These results collectively indicate that bacterial flagellin inhibits TCR-mediated activation of T cells by inducing SOCS-1.

Introduction

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and mediate the production of cytokines necessary for the development of effective immunity (Aderem and Ulevitch, 2000; Brightbill and Modlin, 2000; Means *et al.*, 2000; Medzhitov and Janeway, 2000). Flagellin, the structural component of bacterial flagella, is secreted by pathogenic and commensal bacteria, and is recognized by the innate immune system in organisms as diverse as flies, plants and mammals (Samakovlis *et al.*, 1992; Ciacci-Woolwine *et al.*, 1999; Wyant *et al.*, 1999a,b; Gomez-Gomez and Boller, 2000; McDermott *et al.*, 2000; Steiner *et al.*, 2000). Specifically, flagellin is a common bacterial antigen present on most motile bacteria in the intestine (Winstanley and Morgan, 1997). Flagellin is an extremely potent inducer of cytokine and nitric oxide production (McDermott *et al.*, 2000; Steiner *et al.*, 2000; Eaves-Pyles *et al.*, 2001a,b; Gewirtz *et al.*, 2001a,b; Moors *et al.*, 2001; Ogushi *et al.*, 2001; Sierro *et al.*, 2001). Flagellin induces signalling via TLR5 in a variety of cell types including human and murine monocytes, dendritic cells, epithelial cell lines and TLR5-positive intestinal epithelial cells (Gewirtz *et al.*, 2001a; Hayashi *et al.*, 2001; McSorley *et al.*, 2002a; Mizel and Snipes, 2002; Didierlaurent *et al.*, 2004).

As flagellin is ubiquitously present in the intestine and can be transported across intestinal epithelia by some pathogens (Gewirtz *et al.*, 2001b), it may also contribute to the activation of CD4⁺ T cells in the intestine (McSorley *et al.*, 2002a). Flagellin enhances the clonal expansion of naive CD4⁺ T cells *in vivo* and induced production of interferon-γ (IFN-γ) *in vitro* (McSorley *et al.*, 2002a). Marked reactivity against flagellin has also been seen in mesenteric and splenic T cell cultures from colitic animals, and flagellin-specific T cells were able to induce colitis when adoptively transferred into immunodeficient animals (Lodes *et al.*, 2004). Flagellin was also found to be a target of CD4⁺ T cells during murine *Salmonella typhimurium* infection, and antigenic responses against flagellin are protective in *Salmonella* infections in mice (Cookson and Bevan, 1997; McSorley *et al.*, 2000; 2002b). Given the activity of flagellin as a specific ligand for TLR5, these data provide a potentially important link between adaptive and innate immune responses.

T cells express a relatively small number of TLRs, while macrophages or dendritic cells express wide varieties of TLRs. TLR4 is expressed in murine CD3⁺ T lymphocytes, and more specifically in particular subsets of

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γδ T cells (Matsuguchi *et al.*, 2000; Mokuno *et al.*, 2000). Expression of TLR4, TLR5, TLR7 and TLR8 in regulatory T cell subsets have been reported (Caramalho *et al.*, 2003). Recently, it was also reported that effector memory T cells are stimulated by ligands of TLR5, TLR7 and TLR8 (Caron *et al.*, 2005). In addition, exposure to lipopolysaccharide (LPS) markedly increases activity of regulatory T cells and their proliferative response does not require antigen-presenting cells (APC). This response is augmented by T cell receptor (TCR) triggering, and synergizes with IL-2 stimulation (Caramalho *et al.*, 2003). Furthermore, flagellin synergized with TCR-dependent stimulation [anti-CD3 monoclonal antibody (Ab)] upregulates proliferation and cytokine productions in $CD4^+$ T lymphocytes (Caron *et al.*, 2005). However, the effects of flagellin stimulation in these T cells remain to be determined.

Suppressor of cytokine signalling-1 (SOCS-1) is a member of the protein family regulating cytokine signalling pathways via inhibition of key tyrosine phosphorylation events on cytokine receptors and signalling molecules such as JAK family members. SOCS-1 interacts with JAK tyrosine kinases and inhibits kinase activity, thereby suppressing cytokine signal transduction (Yasukawa *et al.*, 2000; Kubo *et al.*, 2003). Furthermore, SOCS-1 expression is rapidly induced by LPS and negatively regulates LPS signalling (Crespo *et al.*, 2000; Crespo *et al.*, 2002). In SOCS-$1^{-/-}$ mice, innate immunity is strongly enhanced, probably due to hypersensitivity of SOCS-$1^{-/-}$ mice to IFN-γ (Alexander *et al.*, 1999). SOCS-1 has been implicated in the hypo-responsiveness to cytokines, such as IFN-γ, after exposure of LPS to macrophages (Crespo *et al.*, 2002). SOCS-1-deficient mice are more sensitive to LPS shock than their wild-type littermates, and SOCS-1 overexpression suppresses LPS-induced inhibitory kappa B (I-κB) phosphorylation and NF-κB transcriptional activity (Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002). However, it was recently reported that SOCS proteins induced by TLR stimulation limit the extent of TLR signalling by inhibiting type I interferon signalling but not the direct NF-κB pathway via TLR (Baetz *et al.*, 2004; Gingras *et al.*, 2004). To our knowledge, no reports on whether SOCS proteins are induced by flagellin stimulation and whether they regulate TLR5-mediated signal transduction cascades have been published.

It was reported that a prior exposure to flagellin results in a subsequent state of flagellin tolerance in Jurkat T cells (Mizel and Snipes, 2002). In this study we examined whether Jurkat T cells or human primary T cells were activated by flagellin and whether pre-treatment of flagellin modulated TCR-mediated activation with particular focus on the role of SOCS-1.

Results

TLR5 surface expression in Jurkat T cells and TLR5 tyrosine phosphorylation by flagellin

To examine whether Jurkat T cells expressed TLR5 protein on the cell surface, we analysed surface expressions of TLR5 in Jurkat T cells by FACS (Mizel and Snipes, 2002). As shown in Fig. 1A, Jurkat T cells express TLR5 at the cell surface. We next examined whether flagellin itself induced tyrosine phosphorylation of TLR5. As shown in Fig. 1B, flagellin stimulation induced tyrosine phosphorylation of TLR5 within 1 min. To confirm that tyrosine-phosphorylation of TLR5 was specifically induced by flagellin stimulation, we examined whether stimulation

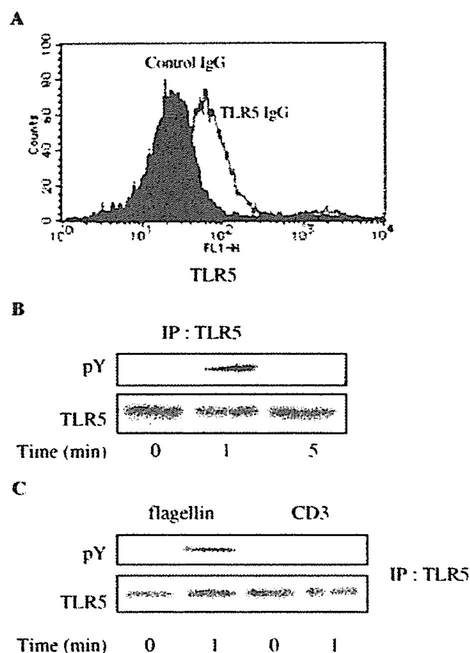

Fig. 1. TLR5 is expressed at the cell surface of Jurkat T cells and is tyrosine-phosphorylated by flagellin stimulation.
A. Jurkat T cells were stained with anti-TLR5 antibody (Ab) and FITC-conjugated donkey anti-goat secondary Ab. Cells expressed with TLR5 were examined by FACS analysis.
B. Jurkat T cells were stimulated with 10 ng ml^{-1} flagellin for the indicated times. Cell lysates were immunoprecipitated (IP) with TLR5 Ab and probed with anti-phosphotyrosine Ab, 4G10 (top), or anti-TLR5 (bottom).
C. Jurkat T cells were stimulated with 10 ng ml^{-1} flagellin or 1 μg ml^{-1} anti-CD3 Ab (UCHT1) for 1 min. Cell lysates were immunoprecipitated with TLR5 Ab and probed with anti-phosphotyrosine Ab, 4G10 (top), or anti-TLR5 (bottom).

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with anit-CD3 Ab induced tyrosine phosphorylation of TLR5. We demonstrated that stimulation with anti-CD3 did not induce tyrosine phosphorylation of TLR5 (Fig. 1C). These results indicate that TLR5 was tyrosine-phosphorylated by flagellin.

Flagellin activated NF-κB and mitogen-activated protein kinases, but not NFAT

As activation of NF-κB and mitogen-activated protein kinases (MAPKs) is the hallmark of TLR-mediated signalling cascades (Muzio *et al.*, 1997; 1998; Yu *et al.*, 2003; Khan *et al.*, 2004), we first examined whether flagellin induced NF-κB activation using a luciferase reporter assay. We demonstrated that the activity of NF-κB was increased in a dose-dependent manner, while *Salmonella* LPS did not activate NF-κB in Jurkat T cells (Fig. 2A). We then examined phosphorylation of MAPKs. Maximal phosphorylation of ERK, c-Jun N-terminal protein kinase (JNK) 1/2 and p38 was observed 15 min after flagellin stimulation (Fig. 2B). These results collectively indicate that flagellin stimulation induced activation of both NF-κB and MAPKs.

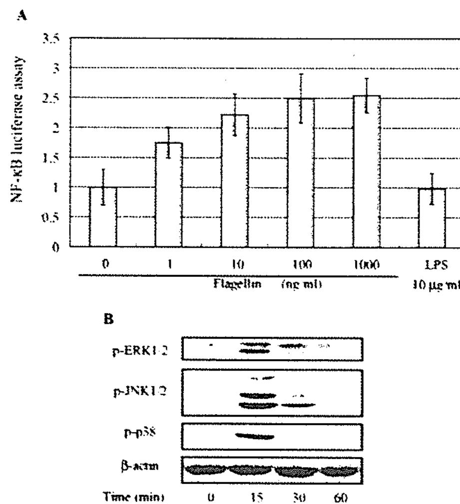

Fig. 2. Stimulation by TLR5 induced activation of Jurkat T cells.
A. A total of 1×10^7 Jurkat T cells were transiently transfected with 30 μg of NF-κB luciferase reporter construct along with 0.03 μg of pRL-TK by electroporation. Twenty-four hours after transfection, the cells were stimulated with the graded concentrations of flagellin or 10 μg ml^{-1} LPS. Six hours after stimulation, the cells were harvested and lysed, and the activities of control *Renilla* luciferase and *Firefly* luciferase (experimental) were measured in triplicate. After normalization according to the *Renilla* luciferase activity, the promoter activity was calculated as fold increase of the control. The data represent the mean ± SD of three independent experiments.
B. Jurkat T cells were stimulated with 10 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with a phosphospecific Ab for ERK1/2, JNK1/2 and p38, as indicated. Cell lysates were also blotted with β-actin Ab as a loading control.

Pre-treatment of flagellin suppressed TCR-mediated activation of NFAT

We attempted to examine whether pre-treatment of flagellin modulated the TCR-mediated activation of T cells. As NFAT is activated by TCR stimulation (Kane *et al.*, 2000), we analysed TCR-mediated NFAT activities using a luciferase assay in cells pre-treated with flagellin. NFAT activities were significantly suppressed in cells pre-treated with 100 ng ml^{-1} flagellin 6 or 18 h before stimulation with anti-CD3 (Fig. 3A). NFAT activities were also suppressed in a dose-dependent manner in cells pre-treated with graded concentrations of flagellin for 6 h and re-stimulated with anti-CD3 (Fig. 3B). However, TCR-mediated NFAT activities were reinstated 36 h after stimulation with 100 ng ml^{-1} flagellin (Fig. 3C). Furthermore, to exclude the possibility that stimulation with flagellin itself induces NFAT activation in Jurkat T cells, we examined flagellin-induced NFAT activation using a luciferase reporter assay. As shown in Fig. 3D, stimulation with flagellin alone did not activate NFAT, while treatment of PMA plus ionomycin or anti-CD3 Abs activated NFAT. These results suggest that pre-treatment of flagellin inhibited the TCR-mediated activation of T cells, as judged by NFAT activation. However, the inhibitory effect of flagellin on the TCR-mediated activation of T cells was reversible.

Flagellin stimulation regulated TCR signalling, but not TCR surface expression

We then analysed the molecular mechanisms by which pre-treatment of flagellin inhibited the TCR-mediated activation of T cells. First, we examined whether surface expression of TCR was downregulated by pre-treatment with flagellin using a FACS analysis. The levels of TCR expression in cells stimulated with flagellin for 1 or 6 h were almost the same as those in unstimulated cells (Fig. 4A), indicating that expression levels of TCR at the cell surface were unaffected by treatment with flagellin. Next, we compared TCR-mediated intracellular signalling cascade with NFAT activation in cells pre-treated with or without flagellin. Zeta-associated protein 70 (Zap-70) is a tyrosine kinase which is positioned just downstream of TCR in TCR-mediated signalling cascade and is pivotal for TCR-mediated T cell activation, including NFAT activation. Tyrosine phosphorylation of Zap-70 was observed clearly after TCR stimulation in control cells (Fig. 4B). However, pre-treatment with flagellin significantly reduced tyrosine phosphorylation of Zap-70 (Fig. 4B), indicating

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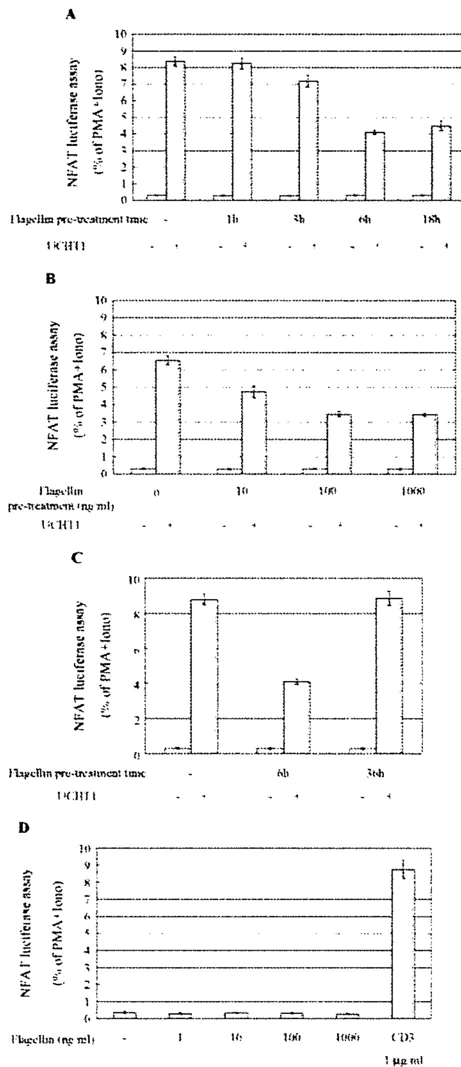

Fig. 3. Flagellin pre-treatment inhibited NFAT activation via T cell receptors.
A. Jurkat T cells transfected with NFAT reporter construct and pRL-TK were treated with 100 ng ml⁻¹ flagellin for the indicated times prior to anti-CD3 Ab (UCHT1) stimulation. Six hours after stimulation with anti-CD3 Ab, *Renilla* luciferase and *Firefly* luciferase (experimental) were measured in triplicate. After normalization according to the *Renilla* luciferase activity, the promoter activity was expressed as the percentage of response obtained with PMA plus ionomycin. The data represent the mean ± SD of three independent experiments.
B. Jurkat T cells transfected with the NFAT reporter construct and pRL-TK were treated with graded concentrations of flagellin for 6 h. Six hours after stimulation with anti-CD3 Ab, luciferase activities were measured as described in Fig. 3A.
C. Jurkat T cells transfected with NFAT reporter construct and pRL-TK were treated with 100 ng ml⁻¹ flagellin for 6 or 36 h prior to anti-CD3 Ab (UCHT1) stimulation. Six hours after stimulation with anti-CD3 Ab, NF-AT luciferase activities were measured as described in (A).
D. Jurkat T cells were transfected with the NFAT reporter construct and pRL-TK as an internal control and were stimulated with graded concentrations of flagellin, 1 μg ml⁻¹ anti-CD3 Ab (UCHT1) or PMA plus ionomycin as positive controls. Luciferase activities were examined as described in Fig. 2A.

that flagellin inhibited TCR-mediated signalling events just downstream of TCR.

SOCS-1 induced by flagellin negatively regulated TCR-mediated T cell activation

SOCS proteins are known to be negative regulators in a variety of signalling cascades including LPS or TCR-mediated signalling (Marine *et al.*, 1999; Matsuda *et al.*, 2000; Banerjee *et al.*, 2002; Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002; Egan *et al.*, 2003). We speculated that SOCS-1 was one of the candidates that negatively regulate TCR-mediated responses in cells pre-treated with flagellin, and examined the level of SOCS-1 expression

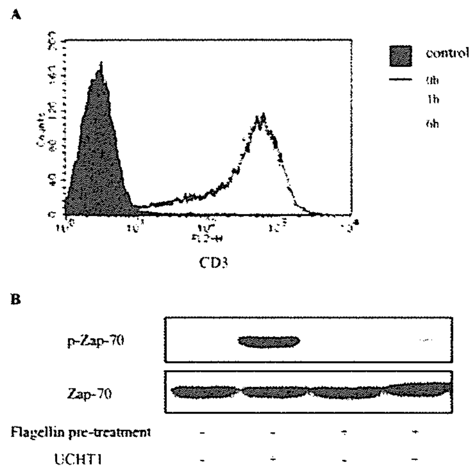

Fig. 4. Flagellin pre-treatment had no effect on the surface expression of TCRs, but inhibited TCR-induced tyrosine phosphorylation of Zap-70.
A. Jurkat T cells were stimulated with or without 100 ng ml⁻¹ flagellin for the indicated times, and stained with PE-conjugated anti-CD3 Ab. The surface expression of flagellin was analysed by FACS.
B. Jurkat T cells were pre-treated with or without 100 ng ml⁻¹ flagellin for 6 h prior to anti-CD3 Ab (UCHT1) stimulation. Then cells were stimulated with 1 μg ml⁻¹ anti-CD3 Ab for 1 min. Cell lysates were blotted with phospho-Zap-70 Ab (top) and Zap-70 Ab as a loading control (bottom).

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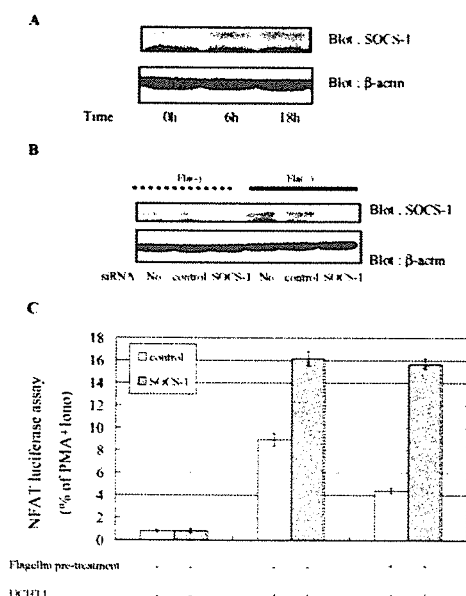

Fig. 5. Flagellin stimulation induced SOCS-1, and siRNA specific for SOCS-1 inhibited flagellin-induced SOCS-1 production and reinstated TCR-induced NFAT activation in cells pre-treated with flagellin.
A. Jurkat T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.
B. Jurkat T cells were transfected with 10 μg of siRNA specific for SOCS-1, control siRNA or vehicle by electroporation. After incubation for 24 h, cells were stimulated with or without 100 ng ml^{-1} flagellin for 6 h. Cells were lysed and subsequently subjected to SDS-PAGE for Western blotting. The blots were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and the blots were re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.
C. SOCS-1 siRNAs or non-specific control siRNAs were co-transfected with the NFAT luciferase reporter construct and the pRL-TK internal control into Jurkat T cells by electroporation. Then the cells were treated with or without 100 ng ml^{-1} flagellin for 6 h, and stimulated in the presence or absence of anti-CD3 Ab (UCHT1) for 6 h. The luciferase activities were calculated as described in Fig. 3A.

after flagellin stimulation in Jurkat T cells. As shown in Fig. 5A, expression levels of SOCS-1 were very low in unstimulated Jurkat cells, while they were augmented in cells treated with flagellin for 6 or 18 h, indicating that SOCS-1 was induced by flagellin stimulation in Jurkat T cells.

To confirm that flagellin-induced SOCS-1 protein inhibited TCR-mediated NFAT activity, we inhibited expression of SOCS-1 protein by transfection of small interfering RNA (siRNA) specific for SOCS-1. Cells transfected with non-specific siRNA induced the same levels of basal and flagellin-induced SOCS-1 protein as those in control cells (Fig. 5B). However, transfection of siRNA specific for SOCS-1 inhibited expression levels of SOCS-1 protein in both cells pre-treated with or without flagellin (Fig. 5B), indicating that the siRNA inhibited both basal and flagellin-induced SOCS-1 protein expression. We then examined whether inhibition of SOCS-1 protein by siRNA would alter TCR-mediated NFAT activity in cells treated with flagellin. We compared TCR-mediated NFAT luciferase activity between cells transfected with siRNAs specific or non-specific for SOCS-1. As shown in Fig. 5C, NFAT activities were inhibited by flagellin pre-treatment in cells transfected with non-specific SOCS-1 siRNA. However, pre-treatment of siRNA specific for SOCS-1 augmented TCR-mediated activation of NFAT, and flagellin pre-treatment did not inhibit TCR-mediated activation of NFAT (Fig. 5C). These results indicate that flagellin-induced SOCS-1 inhibited TCR-mediated activation of Jurkat T cells.

SOCS-1 forms a complex with Zap-70 after TCR stimulation

To explore the molecular mechanism by which SOCS-1 protein suppressed TCR-mediated NFAT activity, we established a stable cell line that expressed SOCS-1. As shown in Fig. 6A, SOCS-1 was expressed constitutively in these cells. We next compared NFAT luciferase activity and tyrosine phosphorylation of Zap-70 between cells stably transfected with or without SOCS-1. Both TCR-mediated NFAT activity and tyrosine phosphorylation of Zap-70 were inhibited in cells that expressed SOCS-1 constitutively compared with control cells (Fig. 6B and C). In addition, we examined whether SOCS-1 forms a complex with Zap-70 after TCR stimulation. As shown in Fig. 6D, Zap-70 interacted with SOCS-1, and the interaction between Zap-70 and SOCS-1 was dependent on TCR stimulation. These results collectively indicate that SOCS-1 was a negative regulator of TCR-mediated activation of T cells, inhibiting activation of Zap-70 and NFAT.

Effects of flagellin stimulation on human primary T cells

We also determined whether pre-treatment of flagellin inhibited TCR-mediated activation of primary T cells. First, we examined whether flagellin stimulation induced activation of human primary T cells, and analysed flagellin-induced phosphorylation of MAPKs as a representative. As shown in Fig. 7A, three MAPKs, ERK, JNK and p38, were all phosphorylated by flagellin stimulation. Next, we examined whether pre-treatment of flagellin suppressed TCR-mediated phosphorylation of Zap-70. As observed in Jurkat T cells, it was also inhibited in cells pre-treated with flagellin for 6 h before stimulation with anti-CD3 Ab (Fig. 7B). Finally, we showed that SOCS-1 was induced

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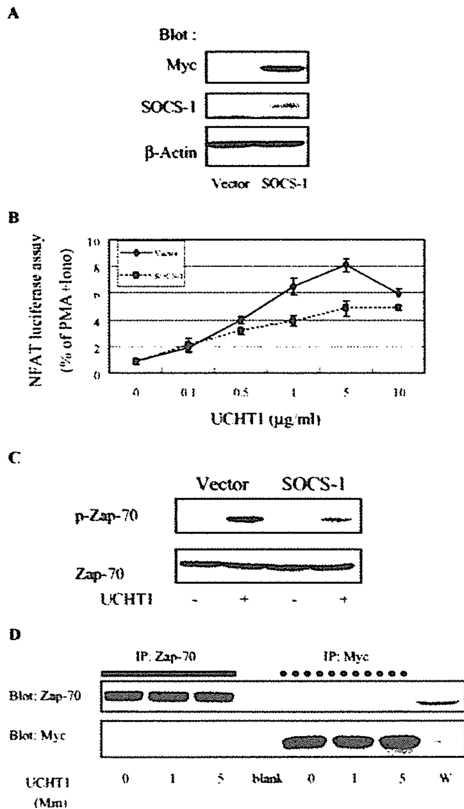

Fig. 6. TCR-induced activation of NFAT and tyrosine phosphorylation of Zap-70 was suppressed in cells stably expressed with SOCS-1.
A. To establish a cell line that stably expresses SOCS-1, Jurkat T cells were transfected with pcDNA3-SOCS1-Myc or pcDNA3 (control), and selected in the medium with 600 μg ml^{-1} G418. Protein expression of SOCS-1 in cells stably expressing SOCS-1 or control cells was examined by Western blotting with anti-Myc Ab.
B. Cells stably expressing SOCS-1 or control cells were transfected with the NFAT luciferase reporter construct and pRL-TK by electroporation. Twenty-four hours after transfection cells were stimulated with anti-CD3 Ab (UCHT1). Six hours after stimulation luciferase activities were measured as described in Fig. 3A.
C. Cells stably expressing SOCS-1 or control cells were stimulated with anti-CD3 Ab for 1 min. Then the cell lysates were blotted with a phosphospecific Ab for Zap-70 or Zap-70 Ab as a loading control.
D. Jurkat cells stably expressing SOCS-1 or control cells (E6.1) were stimulated with anti-CD3 Ab for indicated times. Then the cell lysates were immunoprecipitated with anti-Myc or anti-Zap-70 Ab and blotted with anti-Zap-70 or anti-Myc Ab. Whole-cell lysates of Jurkat T cells stably expressing SOCS-1 (W) were used as a control.

by flagellin stimulation in primary T cells (Fig. 7C). These results collectively indicated that pre-treatment of flagellin inhibited the TCR-mediated activation of primary T cells and that flagellin stimulation induced SOCS-1.

Discussion

We demonstrated for the first time that stimulation by flagellin itself induced tyrosine phosphorylation of TLR5 (Fig. 1B). TLR2 has a YXXM motif whose phosphorylation is important for phosphatidylinositol 3-kinase (PI3-K)

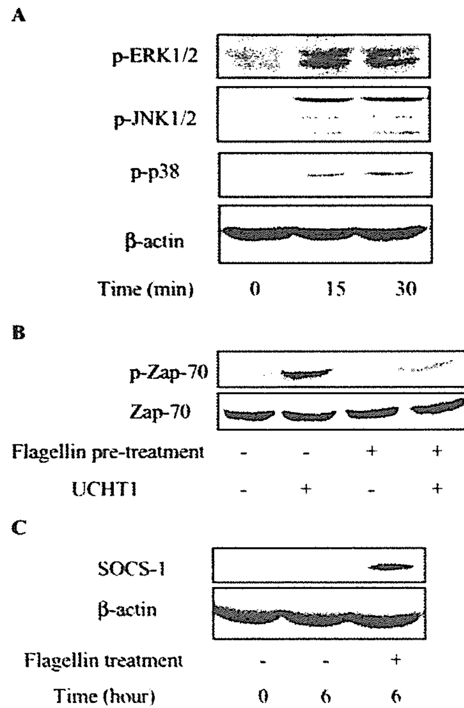

Fig. 7. Flagellin stimulation led to phosphorylation of MAPKs, inhibited TCR-mediated activation and induced SOCS-1 in human primary T cells.
A. Human primary T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with a phosphospecific Ab for ERK1/2, JNK1/2 and p38, as indicated. Cell lysates were also blotted with β-actin Ab as a loading control.
B. Primary T cells were pre-treated with or without 100 ng ml^{-1} flagellin for 6 h prior to anti-CD3 Ab (UCHT1) stimulation. Then cells were stimulated with 1 μg ml^{-1} anti-CD3 Ab for 1 min. Cell lysates were blotted with phospho-Zap-70 (top) or Zap-70 Ab as a loading control (bottom).
C. Primary T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.

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association and activation of the TLR2-mediated signal transduction cascade (Arbibe *et al.*, 2000), while phosphorylation of TLR4 is important for activation of NF-κB and IL-8 production (Chen *et al.*, 2003). We speculated that phosphorylation of TLR5 is also important for the activation of the TLR5-mediated signal transduction cascade, although which tyrosine is phosphorylated in TLR5 remains to be determined. In our studies, tyrosine phosphorylation of TLR5 indicates that flagellin activated the cells through TLR5. As Jurkat T cells do not express TLR4 and were not activated by LPS in our study (data not shown), activation was carried out by flagellin via TLR5, not by impure ingredients such as LPS. Flagellin stimulation induced activation of both MAPKs (Figs 2A and 7A) and NF-κB (Fig. 2B), which are important for the TLR-mediated signal transduction cascade in T cells. Although stimulation by flagellin is involved in cytokine production in macrophages or dendritic cells, we did not detect flagellin-induced production of cytokines, such as TNF-α, IL-1β, IL-6 and IL-2, in Jurkat T cells (data not shown), indicating that the role of flagellin-induced activation of T cells may be different from that of macrophages or dendritic cells.

Stimulation by flagellin itself did not induce NFAT activation in T cells, while stimulation via TCR lead to the activation of NFAT (Fig. 3C). However, TCR-mediated NFAT activation was inhibited in cells pre-stimulated by flagellin (Fig. 3A). We speculated that flagellin stimulation might downregulate cell surface expression of TCR or inhibit molecules in the signal transduction cascade downstream of TCR. However, pre-treatment of flagellin had no effect on the expression of TCR on the cell surface (Fig. 4A). Interestingly, pre-treatment of flagellin inhibited tyrosine phosphorylation of Zap-70 which is a crucial tyrosine kinase in TCR-mediated activation of T cells (Figs 4B and 7B), thus indicating that pre-treatment of flagellin interfered with activation of the molecules just downstream of TCR in TCR-mediated signal transduction cascade. The inhibitory effect of flagellin on Zap-70 phosphorylation appears to be greater than that on NFAT-mediated gene expression. One possibility is that NFAT gene expression might be regulated in part by the molecules that is not located downstream of Zap-70 in the TCR-mediated signal cascade and that is not affected by flagellin pre-stimulation. Another possibility is that the difference might be due to using different assay systems, Western blotting and luciferase assay.

Proteins in SOCS family inhibit signal transduction cascades mainly through cytokine receptors (Elliott and Johnston, 2004), and negatively regulate TCR-mediated activation of T cells (Marine *et al.*, 1999; Matsuda *et al.*, 2000; Egan *et al.*, 2003). For example, SOCS-3 inhibits TCR-mediated NFAT activation by binding to calcineurin (Banerjee *et al.*, 2002). In addition, SOCS-1 is induced by LPS stimulation (Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002). As T cells over-respond to stimulation through TCR in SOCS-1-deficient mice (Egan *et al.*, 2003), and activation of NFAT is suppressed by binding Splenic Tyrosine Kinase (Syk) to SOCS-1 in HEK293 cells that express Syk and SOCS-1 (Matsuda *et al.*, 2000), we speculated that SOCS-1 might be involved in suppression of TCR-mediated activation of NFAT in cells pre-stimulated with flagellin. We showed that SOCS-1 is induced by flagellin stimulation (Figs 5A and 7C). Furthermore, inhibiting SOCS-1 expression by SOCS-1-specific siRNA reinstates TCR-mediated activation of NFAT in cells pre-stimulated with flagellin (Fig. 5C). These results collectively indicated that SOCS-1 inhibited TCR-mediated NFAT activation directly or indirectly in cells pre-treated with flagellin. In addition, TCR-mediated activation of NFAT was also inhibited in cells stably expressing SOCS-1 and pre-stimulated with flagellin (Fig. 6B). However, the level of inhibition of NFAT-mediated gene expression was lower in cells stably expressing SOCS-1 than that in control cells stimulated with flagellin. We speculated that NFAT-mediated gene expression might be inhibited in part by molecules other than SOCS-1. SOCS-1 is associated with activated Jak family kinases through its SH2 domain in interferon or cytokine receptor signalling cascades, and negatively regulates their signal transduction cascades (Elliott and Johnston, 2004). As co-stimulation by interferons or cytokines also augments TCR-mediated T cell activation (Romano *et al.*, 1996; Zella *et al.*, 2000), we cannot deny the possibility that SOCS-1 might inhibit action of interferons or cytokines, and thus negatively regulate T cell activation via TCR. However, we demonstrated that activation via TCR leads to a complex forming between SOCS-1 and Zap-70 upon induction of the TCR-mediated signalling cascade in Jurkat T cells (Fig. 6D). We also observed that the degrees of TCR-mediated NFAT activation in Jurkat T cells stably expressing SOCS-1 were significantly lower than those in control Jurkat T cells when they were cultured in the absence of serum overnight (data not shown). Thus, we speculated that SOCS-1 induced by flagellin stimulation directly inhibited TCR-mediated NFAT activation, probably by interfering with activation of Zap-70.

In this article, we report that pre-stimulation by flagellin inhibited TCR-mediated activation in T cells. Our study is important for understanding the molecular mechanisms of host defences mediated by T cells. Why are T cells that are stimulated with flagellin made refractory to TCR-mediated activation? We speculate that inhibition of TCR-mediated activation by flagellin might represent a host mechanism aimed at limiting inflammatory damage on activation of the immune system by flagellated bacteria because excessive inflammatory responses is potentially harmful to the host and may lead to microcirculatory dysfunction, causing tissue damage and septic shock. Thus,

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our study may lead to the development of improved treatments for inflammatory responses to flagellated bacteria that may cause sepsis.

Experimental procedures

Purification of human primary T cells

Peripheral blood mononuclear cells (PBMC) were isolated from human healthy volunteers by a standard density gradient centrifugation on Ficoll-Paque (MP Biomedicals, OH), and T cells were subsequently purified by negative selection with magnetic beads according to the manufacturer's instructions (StemCell Technologies, Canada). Purity of T cells were about 95–97% measured by flow cytometry using Phycoerythrin (PE)-conjugated anti-CD3 mouse monoclonal Ab.

Cell line, reagents and antibodies

The human acute leukaemia T cell line, Jurkat E6.1 (American Type Culture Collection, Manassas, VA) and purified T cells were maintained in RPMI 1640 supplemented with 2 mM glutamine (Sigma, St Louis, MO), 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (ICN, Aurora, OH) and 10% fetal bovine serum (Sanko, Japan). *Salmonella muenchen* flagellin was purchased from Calbiochem (San Diego, CA). *Salmonella typhosa* LPS, phorbol myristate acetate (PMA), ionomycin calcium salt were purchased from Sigma. The following Abs were used in the experiments: anti-TLR5 goat Ab, c-Myc mouse monoclonal Ab (9E10), fluorescein isothiocyanate (FITC)-conjugated anti-goat donkey Ab, control goat IgG Ab and PE-conjugated mouse IgG Ab (Santa Cruz Biotechnology), anti-phosphotyrosine mouse monoclonal Ab (4G10; Upstate, Lake Placid, NY), anti-β-actin mouse monoclonal Ab (Abcam, Cambridge, UK), anti-phosphorylated JNK1/2 Ab and anti-phosphorylated p38 Ab (Cell Signalling Berverly, MA), anti-CD3 mouse monoclonal Ab and PE-conjugated anti-CD3 mouse monoclonal Ab (Pharmingen, San Diego, CA), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Dako, Denmark), anti-SOCS-1 Ab (Zymed Laboratories, South San Francisco, CA).

Flow cytometric analysis

Flow cytometric analysis was performed essentially as described previously (Nagase *et al.*, 2000). Briefly, cells were incubated with anti-TLR5, followed by staining with FITC-conjugated anti-goat donkey Ab, or cells were stained with PE-conjugated anti-CD3 mouse monoclonal Ab. Expression of TLR5 or CD3 was analysed by using a BD LSR (BD biosciences, Franklin Lakes, NJ).

Establishing a stable cell line that expresses SOCS-1

To establish a cell line that stably expresses SOCS-1, 1×10^7 Jurkat T cells were transfected by electroporation with 50 μg of pcDNA3MycSOCS-1 construct (kindly provided by Dr A. Yoshimura), using a Gene Pulser II (Bio-Rad) set at 290 V, 970 μF. Cells that expressed SOCS-1 were selected by incubation in media containing 600 μg ml^{-1} G418 (Life Technologies, Gaithersburg, MD) and cloned by a standard limiting dilution method. Protein expression of SOCS-1 in cells was examined by Western blotting.

Transfection and dual luciferase reporter assay

Transfection and luciferase reporter assays were performed essentially as previously reported (Nakayama *et al.*, 2003). Briefly, 1×10^7 Jurkat T cells were co-transfected by electroporation with 30 μg of NFAT luciferase reporter plasmid (kindly provided by Drs B. Barbara and R.L. Wange) or 30 μg of pNF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA), and 0.03 μg of *Renilla* luciferase reporter vector pRL-TK (Promega, Madison, WI). Twenty-four hours after transfection, cells were either left untreated or were incubated with anti-CD3 monoclonal Ab UCHT1, flagellin, LPS or PMA (30 ng ml^{-1}) plus ionomycin (1.5 μM). Six hours after stimulation, cells were lysed, and luciferase activities were measured by a dual luciferase reporter assay system (Promega, Madison, WI). Data were obtained by calculating the ratio of *Firefly* luciferase activity (experimental) and *Renilla* luciferase activity (control) and were expressed as the relative luciferase activities representing the mean ± SD of triplicate experiments. NFAT luciferase activities were reported as the percentage of those produced by stimulation with PMA and ionomycin.

Immunoprecipitation and immunoblotting

Immunoprecipitation and Western blotting were performed as previously described (Okugawa *et al.*, 2003). Briefly, cells were lysed in ice-cold NP-40 lysis buffer containing 1% NP-40, 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin and 1 mM phenylmethylsulphonyl fluoride. For immunoprecipitation studies, cell lysates were mixed with the indicated Abs for 1 h. Cell lysates were then mixed with protein G-coupled Sepharose beads and rotated for 1 h at 4°C. After the beads were washed three times with ice-cold NP-40 lysis buffer, the precipitated proteins were boiled for 5 min and eluted with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For the precipitation of total-cell lysates, cells were lysed directly by the addition of SDS-PAGE sample buffer containing 2-mercaptoethanol.

Immunoprecipitated proteins and cell lysates were separated by SDS-PAGE under reducing conditions and were electrically transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature with 1% bovine serum albumin in TBS (Tris-buffered saline) buffer. The membrane was then incubated with the indicated Ab and the reactive bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia Biotech, UK).

Constructions and transfection of siRNA

The constructions of siRNA molecules specific for SOCS-1 were evaluated by B-Bridge International (Sunnyvale, CA). The oligonucleotide sequences used in the experiments were as follows: 5′-CggAACTgCTTTTTCgCCCTT-3′. SOCS-1-specific and negative control RNAs were obtained from Dharmacon (CO). siRNAs specific for SOCS-1 or control RNAs were transfected into Jurkat T cells by electroporation.

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CASE REPORT

Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report

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Abstract

We present a case of fetal liver failure caused by the activation of lamivudine-resistant hepatitis B virus (HBV) nine months after lamivudine treatment. A 57-year old man visited our hospital for the treatment of decompensated chronic hepatitis B. Lamivudine was started in December 2001. Subsequently, serum HBV was negative for HBV DNA with seroconversion from HBeAg to anti-HBe and improvement of liver function. However, HBV DNA and HBeAg were again detected in September 2002. He was complicated by breakthrough hepatitis and admitted to our hospital in November for severely impaired liver function. Vidarabine treatment was started and serum HBV DNA and alanine aminotransferase (ALT) decreased transiently. However, after the start of α-interferon treatment, HBV DNA level increased and liver function deteriorated. He died 1 mo after admission. An analysis of amino acid sequences in the polymerase region revealed that rtM204I/V with rtL80I/V occurred at the time of viral breakthrough. After the start of antiviral treatment, rtL180M was detected in addition to rtM204I/V and rtL80I/V, and became predominant in the terminal stage of the disease. HBV clone with a high replication capacity may be produced by antiviral treatment leading to the worsening of liver function. Antiviral therapy for patients with breakthrough hepatitis in advanced liver disease should be carefully performed.

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Key words: **Hepatitis B virus; Lamivudine; Polymerase; Interferon; Tyrosine-methionine-aspartate-aspartate**

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INTRODUCTION

Lamivudine is a nucleoside analogue that interrupts the reverse transcription of hepatitis B viral (HBV) pregenomic RNA. Lamivudine is effective for controlling chronic hepatitis B and currently recommended as the first line of treatment for chronic active hepatitis B[1,2]. Even for patients with decompensated liver cirrhosis, lamivudine improves liver function and extends transplantation free intervals[3-10]. Since more than 10% of patients with chronic HBV infection are estimated to develop liver cirrhosis and may eventually suffer from decompensated liver cirrhosis or hepatocellular carcinoma, the role of lamivudine in the treatment of advanced liver disease caused by chronic HBV infection is large[11-14].

The major problems concerning lamivudine treatment are the viral and biochemical breakthroughs caused by drug resistance. Amino acid mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif can occur six months after treatment and often increases alanine aminotransferase (ALT) level. Although the increase is usually mild, a marked increase in ALT level leading to fatal hepatic failure has been reported[15-17]. Factors other than the YMDD motif mutation that are associated with the worsening of liver function remain to be clarified.

Here, we report a case of fatal hepatic failure caused by lamivudine-resistant HBV. A serial analysis of viral amino acid sequences indicated that the acquisition of mutations outside the YMDD motif might be related to the deteriolation of the patient's condition.

CASE REPORT

A 57-year old man visited our hospital in September 2001

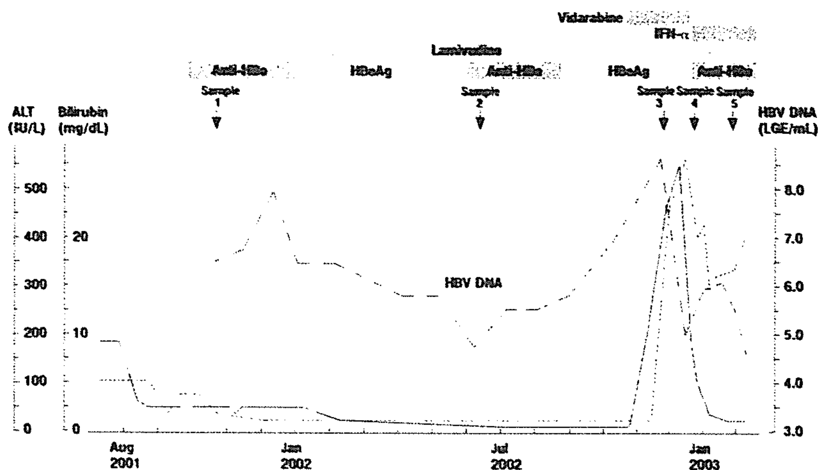

Figure 1 Clinical course of our patient. HBV DNA level was quantified by transcription-mediated amplification assay. The levels of HBV DNA started to increase 8 mo after treatment with reappearance of HBeAg. Breakthrough hepatitis developed 12 mo after treatment. The timing of serum sample analysis for mutations is shown by the arrowhead.

for the treatment of decompensated chronic hepatitis B. In 1978, He was found to be positive for serum HBs antigen (HBsAg). In July 2001, he was admitted to a nearby hospital for ascites where he was diagnosed as having decompensated cirrhosis with exacerbated chronic hepatitis B. The symptomatic control of his ascites improved his general condition. For further treatment, he was referred to our hospital.

On his first visit, he showed no symptoms or signs of worsening hepatic failure or encephalopathy. No ascites or leg edema was observed. His bulbar conjunctiva was slightly jaundiced. Dilated vasculature was observed in his neck and chest. His ALT, total bilirubin and albumin were 50 IU/L, 3.1 mg/dL and 3.7 g/dL, and his prothrombin time was 76%. He was diagnosed as having liver cirrhosis with a Child-Pugh score of 8. He was negative for HBe antigen (HBeAg) and his HBV DNA level measured by transcription-mediated amplification and hybridization protection assay[18] was $10^{6.5}$ genome copies/mL.

In November 2001, he was found to be positive for HBeAg and showed an increase in HBV DNA level. Because he had a history of decompensated chronic hepatitis B, lamivudine treatment (100 mg/d) was started in December. Figure 1 shows the clinical course. The high serum levels of bilirubin and ALT decreased and normalized within 6 mo after lamivudine treatment was started. The patient became negative for HBV DNA and HBeAg.

However, in September 2002, he was found to be positive for HBeAg again and showed an increase in HBV DNA level. In November 2002, he observed jaundice of his bulbar conjunctiva and was admitted to our hospital. Although he was alert, his bulbar conjunctiva and skin were jaundiced. His ALT, total bilirubin, were 474 IU/L, 11.4 mg/dL and 4.3 g/dL. His HBV DNA level was $10^{8.6}$ genome copies/mL. He was diagnosed as having breakthrough hepatitis caused by lamivudine-resistant mutants of HBV. HBV with an amino acid substitution in the YMDD motif in the domain C of polymerase region was detected.

Because interferon is not indicated in patients with decompensated cirrhosis, vidarabine, which is effective for the control of active HBV infection[19-21], was administered together with lamivudine under informed consent. Liver function improved transiently with a decrease in HBV DNA within 2 wk. As prolonged vidarabine administration may induce several complications[22], vidarabine was switched to interferon-α. After the start of interferon-α treatment, HBV DNA level increased and liver function worsened. He died of hepatic failure and rupture of esophageal varices 1 mo after his admission.

The histolopathology of the patient's liver after necropsy showed cirrhosis with zonal necrosis. Hepatocyte regeneration was scarce (Figure 2).

To elucidate the viral factors affecting early viral breakthrough and fatal outcome, amino acid sequences of the upstream polymerase region (aa 1-250) of HBV DNA in serum were examined at 5 points as shown in Figure 1. The methods were as follows.

First, DNA was extracted from 100 μL of a serum sample using the QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). Three fragments spanning the upper polymerase region of HBV DNA were amplified by nested PCR with the primers shown in Table 1. The first stage of amplification was carried out using a thermal cycler for 40 cycles (94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min) in 100 μL of reaction mixture containing 200 mmol/L dNTPs, 1.0 mmol/L each of the primers and 1 × PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase gold (Perkin Elmer Cetus Corp., CT). Two microliters of the PCR products was subjected to the second stage of amplification under the same conditions as the first stage.

Second, PCR products were purified using Wizard PCR preps DNA purification resin (Promega, WI) and cloned into a plasmid vector using the TA cloning kit (PCR cloning kit Qiagen, CA). Four clones were selected from