Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH		0/5/	2/1/	4/0/
AIH/PBC/others		2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.
*$P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2-test; **$P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; ***$P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; ****$P < 0.05$ vs cirrhosis by χ^2-test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.[18] Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.[16]

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparafinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the α1 and α2 domains of MIC molecules shared by both MICA and MICB.[2] To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t-test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

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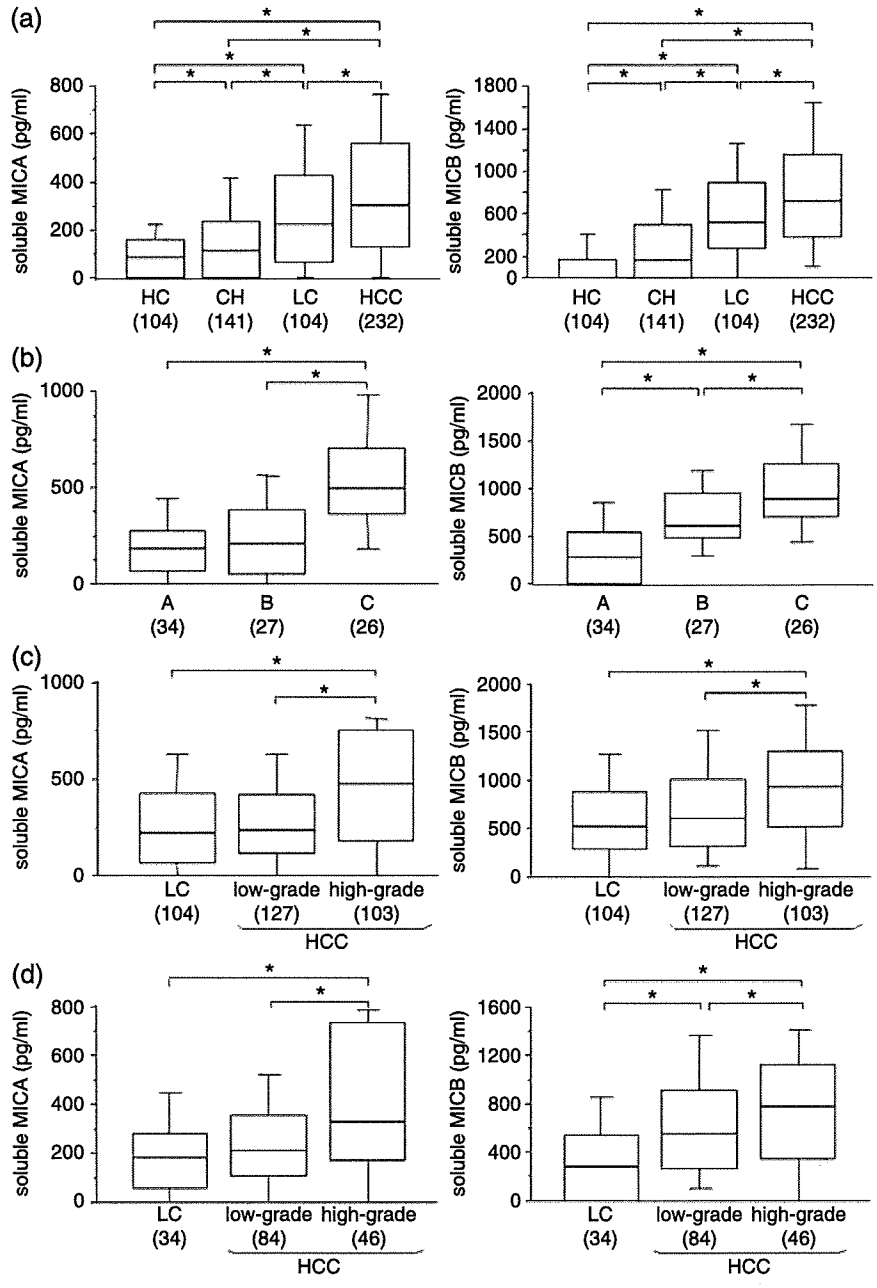

Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child–Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child–Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. $*P < 0.05$ by Kruskal–Wallis test and post hoc Steel–Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhosis patients were stratified based on Child–Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child–Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

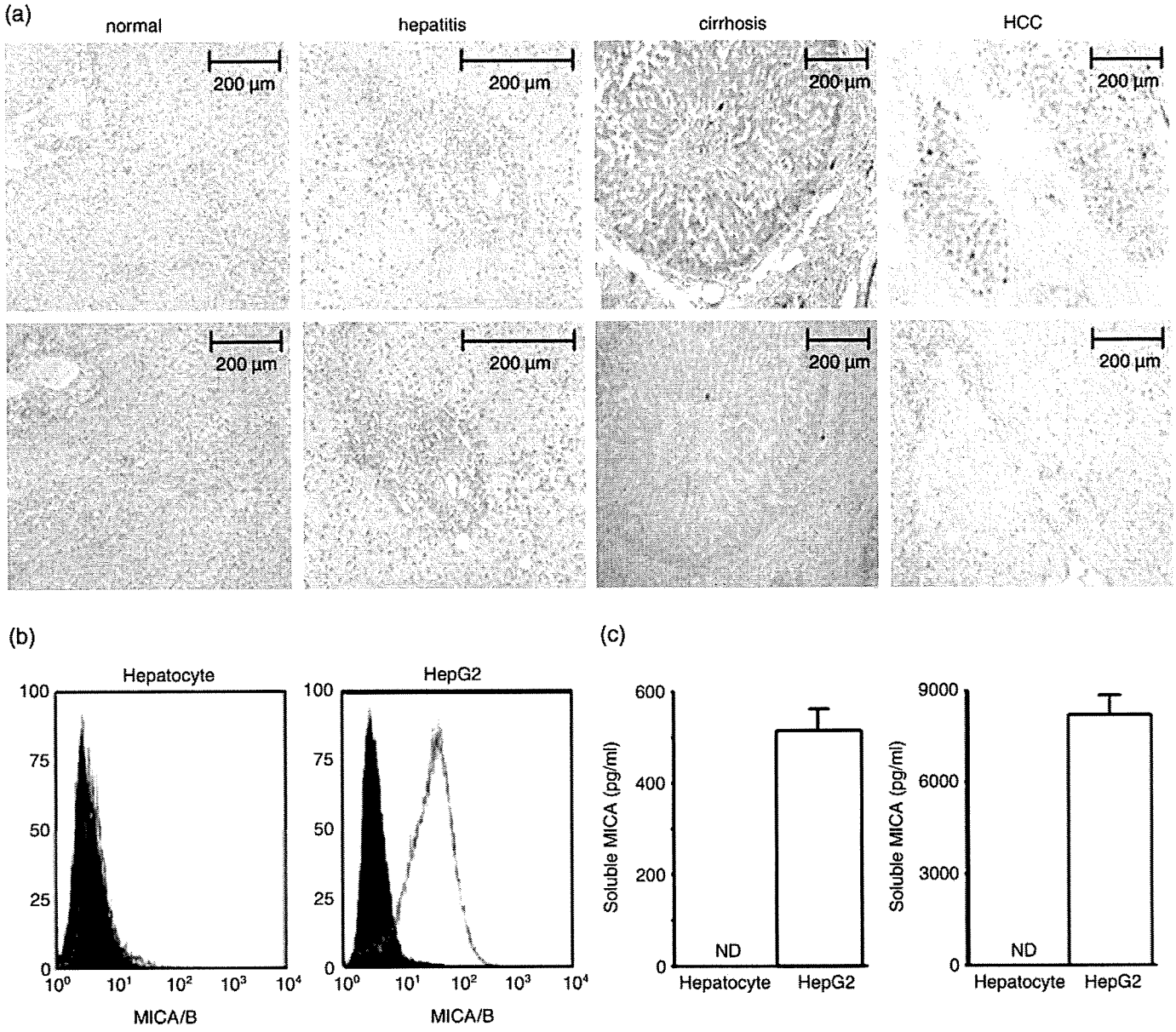

Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MICB released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.[3] Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

Downregulation of soluble MICA levels by TAE. The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

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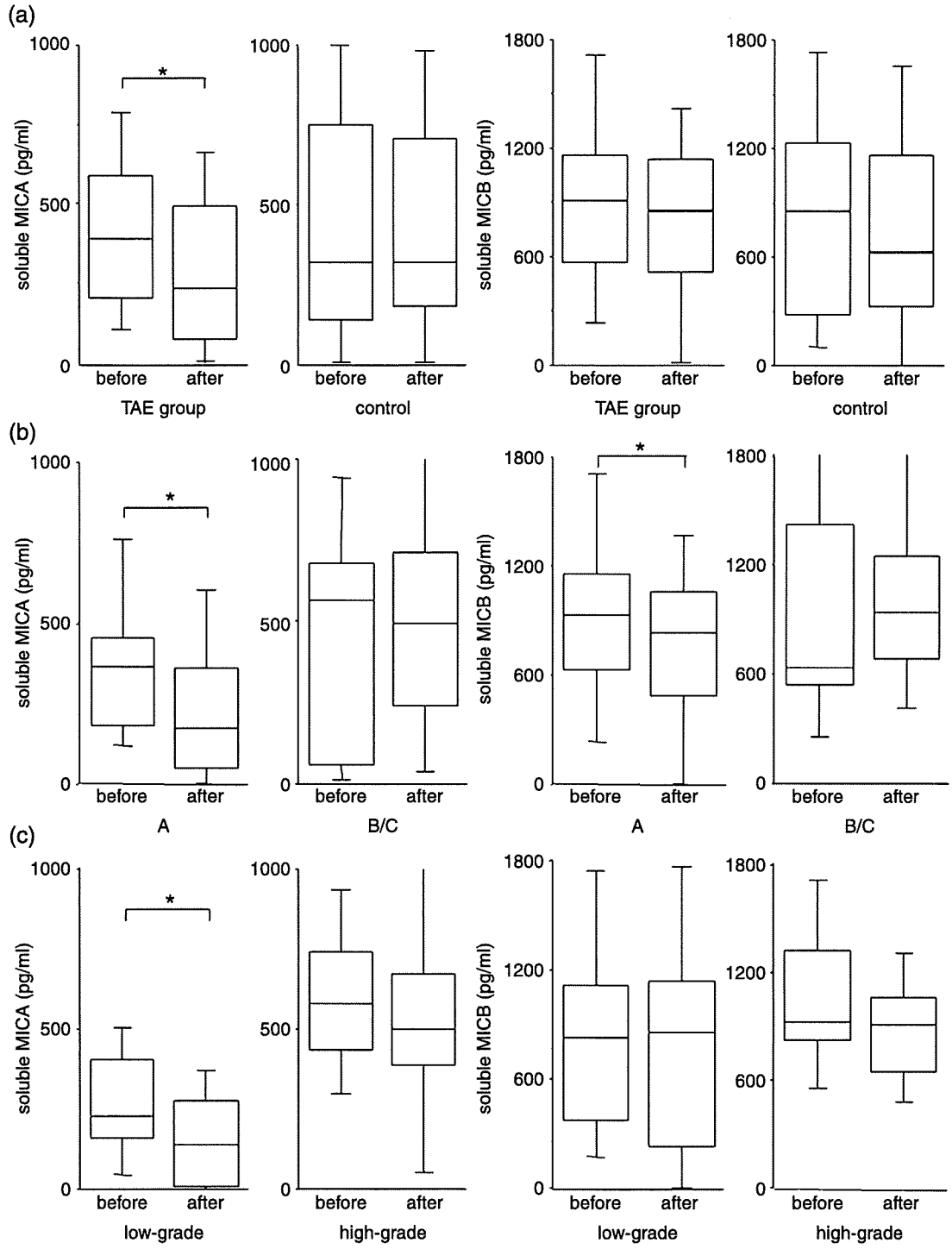

Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divide into two groups: Child–Pugh A ($n = 29$) and Child–Pugh B and C ($n = 9$). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ($n = 24$) and high-grade HCC ($n = 14$). *$P < 0.05$ by paired t-test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble MICA/B, TAE-treated patients were divided according to their Child–Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child–Pugh A patients but not in Child–Pugh B and C patients (Fig. 3b). Interestingly, Child–Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child–Pugh B and C patients did not. As for tumor stage, a significant decrease in

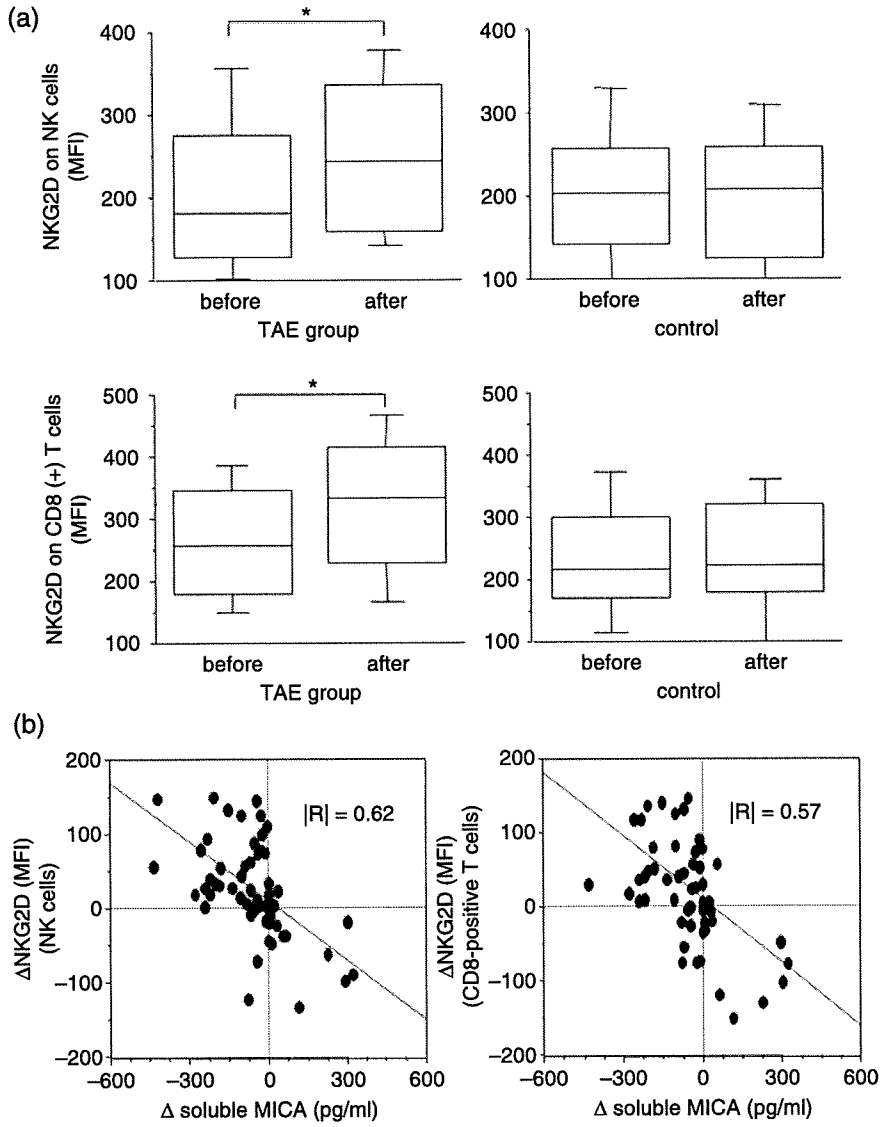

Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). $*P < 0.05$ by paired *t*-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

Upregulation of NKG2D expression by TAE. The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.[12] However, little is known about soluble MICA/B in the premalignant condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.[12,13] They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.[2] In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed

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hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.[19] It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.[20] It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.[16] To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child–Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child–Pugh A patients than Child–Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child–Pugh A patients in our cohort showed a larger decease in α-fetoprotein levels after TAE therapy than Child–Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

It is generally speculated that soluble MICA/B produced from tumors may deactivate NKG2D-mediated immune responses.[8,9] *In vitro* experiment indicates that soluble MICA could down-regulate NKG2D expression and effector cell function. However, the regulation by soluble forms of NKG2D ligands would be more complicated *in vivo*. First, soluble forms of NKG2D ligands could be produced not only from malignant tissues but also from non-malignant tissues, as shown in the present study. Second, MHC-encoded MICA/B may not be the sole family of proteins serving as NKG2D ligands. Non-MHC-encoded UL16-binding proteins also act as NKG2D ligands and were very recently found to be cleaved proteolytically from tumor cells.[21] The present study provides evidence that soluble MICA is derived, at least in part, from HCC and regulates NKG2D expression on NK and CD8-positive T cells. Although several species of soluble NKG2D ligands may exist in the circulation, the present study suggests that soluble MICA regulates NKG2D expression directly in cancer patients.

In conclusion, soluble MICA and MICB are significantly increased in the sera of patients not only with HCC but also with chronic liver disease. Soluble MICA/B increases together with the progression of liver disease as well as the tumor. Therapeutic intervention for HCC leads to reduction of soluble MICA levels in association with upregulation of NKG2D on immune cells, offering *in vivo* evidence of soluble MICA regulating NKG2D expression. Thus, cancer therapy may have a beneficial effect on the NKG2D-mediated immune response even if some of the soluble NKG2D ligands are produced from non-cancerous premalignant tissues.

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Original Article

Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C

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Aim: Ribavirin, used to treat chronic hepatitis C, can induce hemolytic anemia, forcing the discontinuance of treatment. To establish a predictive measure to help circumvent this, we evaluated the relationship of hemoglobin (Hb) decline with the discontinuance of treatment during the progression of ribavirin-induced anemia.

Methods: One hundred and sixteen patients (71% male) with genotype 1 chronic hepatitis C were treated with pegylated interferon (PegIFN) α-2b and ribavirin. The mean age was 50.6 years and 55% were IFN naïve. A decline of Hb concentration by 2 g/dL at two weeks from the start of the treatment ("2 by 2" standard) was adopted as the predictive factor for the progression of anemia.

Results: By applying the "2 by 2" standard, with $\Delta Hb \geq 2$ g/dL (34%, $n = 39$), treatment was discontinued in 12 cases (31%), three of which (8%) because of severe anemia. For $\Delta Hb < 2$ g/dL (64%, $n = 76$), treatment was discontinued in 11 (14%) cases; none due to severe anemia. Ten percent (4/39) of patients showed the minimum Hb ≤ 8.5 g/dL in the $\Delta Hb \geq 2$ g/dL group, with none in the $\Delta Hb < 2$ g/dL group ($P = 0.001$). Furthermore, the patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Conclusion: Monitoring the Hb decline using the "2 by 2" standard can identify patients who are prone to developing severe anemia. Further prospective studies are needed using ribavirin reduction based on the "2 by 2" standard.

Key words: "2 by 2" standard, chronic hepatitis C, pegylated interferon and ribavirin combination therapy, progression of anemia

INTRODUCTION

THE AIM OF antiviral therapy for hepatitis C virus (HCV) is to obtain a sustained viral response (SVR) and to reduce the occurrence rate of hepatocellular carcinoma or hepatic disease-related mortality.[1,2] The current optimal therapy for patients with chronic hepatitis C is a combination of pegylated interferon (PegIFN) and ribavirin. This combination can significantly improve the SVR rate and is recommended as a standard regimen worldwide.[3–8] However, the SVR rates for the combination therapy of ribavirin with PegIFN for naïve patients with HCV genotype 1 has been reported to be 42–52%,[6,9,10] which means that eradication of HCV is not complete in approximately half of these patients. Recently, long-term treatment[11] and a higher dosage

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of drugs[12,13] have been used to try to raise the SVR rate for patients with HCV genotype 1. However, it remains to be established what constitutes satisfactory efficacy. In this study we focused on a treatment strategy to enable the prediction of severe side-effects in order to avoid the need to discontinue treatment and raise the SVR rate by PegIFN and ribavirin combination therapy. It is important that ribavirin, the key drug for eradicating HCV, is continued until the end of treatment in order to attain the maximum SVR rate. Hemolytic anemia induced by ribavirin is known as one of the most important adverse effects in the combination therapy of PegIFN and ribavirin.[14–17] To decrease the discontinuance rate of ribavirin due to severe anemia, epoetin alfa has been used for patients with progressing anemia, which can maintain the dose level of ribavirin as well as the quality of life of the patients.[18–20] However, from a cost-effectiveness standpoint, it would be difficult for this treatment strategy to become standard. Also, side-effects other than anemia arising from an overload of ribavirin mainly due to renal dysfunction cannot be avoided by the additional administration of epoetin alfa.

Hemolysis induced by ribavirin has been suggested to be related to a high plasma concentration of ribavirin.[21] The apparent clearance of ribavirin (CL/F), which reflects its plasma concentration at four weeks after the start of combination therapy, has been used as a predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[22–24] However, the progression of hemolytic anemia occurs due not only to hemolysis, but also impaired hematogenous function. On the other hand, hemoglobin (Hb) dynamics directly reflect the degree of progression of anemia. We have reported that the early decline of Hb correlates with the progression of anemia during IFN and ribavirin combination therapy.[25] It is necessary to verify that a similar early predictor for the progression of anemia can be adopted in PegIFN and ribavirin combination therapy, since PegIFN is known to induce less depression of bone marrow function than usual IFN.

In this study, we evaluated the utility of the early decline of Hb in comparison with the CL/F to predict the progression of anemia in the combination therapy of PegIFN and ribavirin.

METHODS

Patients

THIS STUDY WAS conducted at 12 institutions in Japan. A total of 116 patients with chronic hepatitis C were enrolled and treated with a combination of PegIFN and ribavirin. All patients were anti-hepatitis C virus antibody positive, had HCV-RNA detectable in their serum by the polymerase chain reaction (PCR) method, and showed elevated serum alanine transaminase (ALT) (above the upper limit of the normal), serum Hb concentration ≥12 g/dL, neutrocytes ≥1500/mm^3 and platelets ≥10^5/mm^3 within six months before the treatment. Exclusion criteria were the presence of hepatitis B surface antigen, antihuman immunodeficiency virus antibody and other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis).

Table 1 Patient characteristics

Age (years)	50.6 ± 10.1 (24–70)
Gender (male/female)	82/34 (male 70.7%)
Body weight (kg)	64.5 ± 11.1
Previous IFN therapy (naïve/relapser/no responder)	64/38/14
HCV-RNA level (KIU/L) (<500/500–850/850<)	18/27/71
ALT (IU/L)	110 ± 60 (33–76)
Crnn (mg/dL)	0.9 ± 0.2
Liver histology	
Fibrosis (F1/F2/F3/unknown)	35/49/31/1
Activity (A1/A2/A3/A4)	15/33/56/12
WBC (/mm^3)	5317 ± 1207
Neutrocytes (/mm^3)	2778 ± 902
Platelets (×10^4/mm^3)	17.4 ± 4.0
RBC (×10^4/mm^3)	459 ± 41
Hemoglobin (g/dL)	14.5 ± 1.2

Data are given as the mean ± SD.
ALT, alanine transaminase; RBC, red blood cells; WBC, white blood cells.

The baseline characteristics of the patients are shown in Table 1. The mean age was 50.6 ± 10.1 years, and 71% (82 patients) were male. All patients had HCV-RNA with genotype 1 and high viral loads (more than 10^5 copies/mL serum by Amplicor-HCV monitor assay). The mean ALT level was 110 ± 60 IU/L. Sixty-four patients (55%) were IFN naïve and the others were undergoing retreatment.

Treatment schedule

All patients were treated with a combination of PegIFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (Rebetol; Schering-Plough) for 48 weeks. PegIFN was administered at a mean of 1.5 μg/kg body weight subcutaneously once a week. Ribavirin was given orally twice a day for the total dose. Dosages of both medications were decided based on the

body weight of the patients: those with a body weight of 40–60 kilograms (kg) were given PegIFN 75 μg/body and ribavirin 600 mg/day, those with a body weight of 60–80 kg were given PegIFN 105 μg/body and ribavirin 800 mg/day, and those with a body weight of 80–100 kg were given PegIFN 135 μg/body and ribavirin 1000 mg/day. The PegIFN dose was reduced by 50% if the neutrocyte count was below 750/mm^3 or the platelet (Plt) count was below 8×10^4/mm^3. The PegIFN was discontinued if the neutrocyte count was below 500/mm^3 or the Plt count was below 5.0×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dL and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dL, in accordance with the drug information for ribavirin. No ferric medicine or erythropoietin to prevent anemia was administered.

Patients with persistently undetectable HCV-RNA six-months after the end of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level, hematological and biochemical tests just before therapy, at the end of week 2 and every four weeks during the treatment. When the treatment was completed, the patients were assessed every four weeks up to 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar *et al.*, CL/F at the start of the treatment was calculated as follows: CL/F (L/h) = 32.3 × BW × (1 − 0.0094 × age) × (1 − 0.42 × sex)/Scr (BW, body weight; sex = 0 for male and 1 for female; Scr = serum creatinine).[17]

Definition of "severe anemia" leading to the discontinuance of ribavirin

In this study, the "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dL or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dL from the start of the combination therapy.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, white blood cell count, red blood cell count, Hb concentration, Plt, serum ALT levels and serum creatinine are expressed as mean ± SD. The SVR rate was evaluated using the intention-to-treat analysis (ITT analysis). The differences in proportions were tested by the χ^2-test and Mantel–Haenszel χ^2-test. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed by SAS program 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

Frequency and reasons for dose reduction or discontinuance of PegIFN and/or ribavirin

OF THE 116 patients, 92 completed 48 weeks of therapy, but 24 patients (21%) had to discontinue both PegIFN and ribavirin. Thirty-nine patients (34%) completed the entire treatment schedule without reduction or discontinuance of either drug. The ribavirin dose was decreased for 39 patients (34%) and the PegIFN dose was decreased for 33 patients (28%), including 19 patients for whom both drugs had to be reduced. The reasons for discontinuance of both drugs included anemia, thyroid dysfunction, skin eruption and neutropenia, with the major reasons being anemia (17%) and thyroid dysfunction (17%).

Efficacy of the combination therapy with dose reduction or discontinuance of PegIFN and/or ribavirin

The SVR rate was 57% (66/116) for all according to ITT analysis. According to the category of response to previous IFN therapy, the SVR rates were 43% (6/14) in

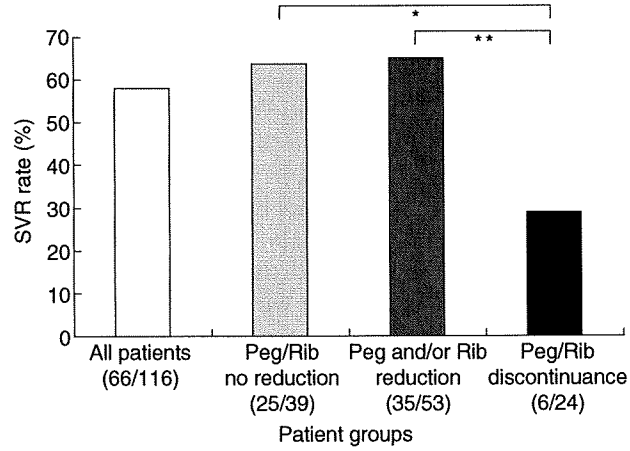

Figure 1 SVR rate due to PegIFN/ribavirin dose reduction or discontinuance. (□), All patients; (▨), patients without dose reduction; (▩), patients with dose reduction; (■), patients with drug discontinuance. Significant levels: *$P = 0.003$; **$P = 0.001$.

Table 2 Rate of the ribavirin reduction or discontinuance due to adverse effects according to CL/F level

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
20 ≤ CL/F (n = 12)	67% (8/12)	25% (3/12)	8% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	57% (13/23)	30% (7/23)	13% (3/23)	0
10 ≤ CL/F < 15 (n = 39)	46% (18/39)	31% (12/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	33% (14/42)	40% (17/42)	26% (11/42)	5% (2/42)

P = 0.031 (Mantel–Haenszel χ^2-test).

Table 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy according to CL/F level

	10 g/dL < Hb	8.5 < Hb ≤ 10 g/dL	Hb ≤ 8.5 g/dL
20 ≤ CL/F (n = 12)	92% (11/12)	12% (1/12)	0
15 ≤ CL/F < 20 (n = 23)	83% (19/23)	17% (4/23)	0
10 ≤ CL/F < 15 (n = 39)	72% (28/39)	23% (9/39)	5% (2/39)
CL/F < 10 (n = 42)	50% (21/42)	43% (18/42)	7% (3/42)

P = 0.009 (Mantel–Haenszel χ^2-test).

non-responders, 61% (23/38) in relapsers, and 58% (37/64) in naïve patients. The relationship between dose reduction or discontinuance of PegIFN and ribavirin and the SVR rate on ITT analysis is shown in Figure 1. Similar SVR rates were obtained in the groups without dose reduction of PegIFN and ribavirin (64%, 25/39) and with reduction of PegIFN and/or ribavirin (66%, 35/53); in detail, the SVR rate was 79% (11/14) in the group with reduction of only PegIFN, 55% (11/20) with reduction of only ribavirin, and 63% (12/19) with reduction of both PegIFN and ribavirin. In the group where both drugs were discontinued, the SVR rate was 25% (6/24), significantly lower than the group without reduction of both drugs (P = 0.003), and the group with reduction of PegIFN and/or ribavirin (P = 0.001).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients showed a median of 12.6 L/h (range 4.5–27.9). At the start of the treatment, 36% (42/116) were under 10 L/h, 34% (39/116) were 10–15 L/h, 20% (23/116) were 15–20 L/h and 10% (12/116) were 20 L/h or more.

The rate of dose reduction or discontinuance of ribavirin is shown in Table 2 for different levels of CL/F. The rate of discontinuance of ribavirin in all cases was 8% (1/12) for the CL/F ≥ 20, 13% (3/23) for the 15 ≤ CL/F < 20, 23% (9/39) for the 10 ≤ CL/F < 15, and 26% (11/42) for the CL/F < 10 group. Ribavirin did not have to be discontinued due to severe anemia among patients with 15 ≤ CL/F, but did for the 18% (2/11) of those with CL/F < 10 and 22% (2/9) of those with 10 ≤ CL/F < 15. The rate of reduction and discontinuance of ribavirin correlated significantly with the CL/F level.

CL/F and minimum hemoglobin level during treatment

To examine the relationship between anemia and the cessation of ribavirin in further detail, we evaluated the minimum hemoglobin level during treatment. Table 3 presents the different levels in relation to CL/F. The patients with minimum Hb ≤ 8.5 g/dL, the criterion for discontinuance of ribavirin, accounted for 7% (3/42) of the group of CL/F < 10, and 5% (2/39) of the group of 10 ≤ CL/F < 15. No patients of the group of CL/F ≥15 showed minimum Hb ≤ 8.5 g/dL.

Early decline of Hb and progression of anemia during combination therapy

Following the initiation of combination therapy, the Hb concentration decreased rapidly until the end of four weeks. At the end of two weeks, Hb had decreased by 1.1 ± 1.0 g/dL among the patients without dose reduction of ribavirin (n = 53), 1.6 ± 1.2 g/dL among those with dose reduction (n = 39), and 1.8 ± 1.0 g/dL among

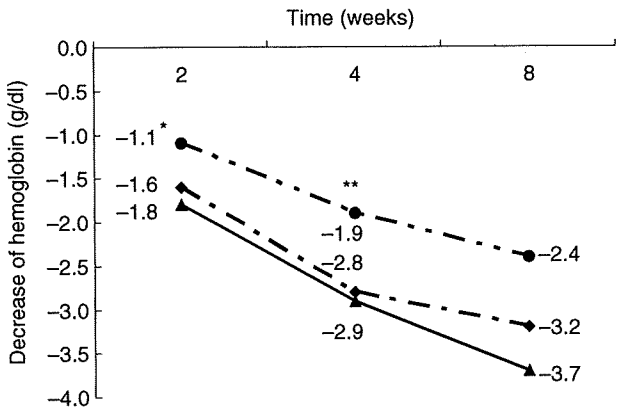

Figure 2 Course of ΔHb in the initial phase. (—··—), No reduction; (—·—·), reduction; (——), discontinuance. *Significantly different between patients with discontinuance and patients with no reduction ($P = 0.04$). **Significantly different between patients with discontinuance and patients with no reduction ($P = 0.008$), and between patients with discontinuance and patients with reduction ($P = 0.003$).

those who had discontinued ribavirin ($n = 24$). It was significantly different between the patients with no reduction and those with discontinuance of therapy ($P = 0.04$). At the end of four weeks, Hb had decreased by 1.9 ± 1.2 g/dL among the patients without dose reduction of ribavirin, 2.8 ± 1.2 g/dL among those with dose reduction, and 2.9 ± 1.2 g/dL among those who had discontinued ribavirin. Hb decline at the end of four weeks was significantly greater in the patients who had discontinued treatment and those who had reduced it, than in those with no reduction ($P = 0.008$, $P = 0.003$, respectively) (Fig. 2).

In this study, we selected the Hb decrease at the end of two weeks as the predictive factor for anemia progression. This is because the judgment of Hb decrease at the end of four weeks is too late to prevent progression of anemia or to perform appropriate counter-measures, such as the administration of epoetin or reduction of ribavirin. Next, we tried to use two borderlines of ΔHb: ΔHb 2.0 indicates a 2 g/dL Hb decrease at the end of two weeks and ΔHb 1.5 indicates a 1.5 g/dL Hb decrease. When ΔHb 2.0 was adopted, the rate of discontinuance of drugs was 31% (12/39) in the ΔHb ≥ 2.0 and 14% (11/76) in the ΔHb < 2.0. When ΔHb 1.5 was adopted, it was 23% (14/60) in the ΔHb ≥ 1.5 and 16% (9/55) in the ΔHb < 1.5. Comparison of the ΔHb 2.0 and ΔHb 1.5 standards showed the sensitivity to be 52% (12/23) and 61% (14/23), and the specificity to be 71% (65/92) and 50% (46/92), respectively. With respect to discontinuance due to anemia, both ΔHb 2.0 and ΔHb 1.5 gave 100% sensitivity (3/3), and the specificities were 68% (76/112) using ΔHb 2.0 and 49% (55/112) using ΔHb 1.5. We decided to adopt the standard of ΔHb 2 g/dL at the end of two weeks from the start of the pegylated IFN and ribavirin combination therapy as the predictive factor for anemia progression ("2 by 2" standard), which has been taken as a predictive factor for anemia in the IFN and ribavirin combination therapy.[25]

Applying the "2 by 2" standard to PegIFN plus ribavirin combination therapy, the rate of reduction or discontinuance of the ribavirin dose was examined with respect to the Hb decrease level (Table 4). Only one patient was excluded from this study, because the treatment was discontinued on the 11th day. In the group of ΔHb (the decrease in Hb concentration at two weeks from the baseline) ≥ 2 g/dL ($n = 39$), the doses were reduced for 18 patients (46%) and discontinued for 12 (31%), three of whom (8%) had severe anemia. For the group of ΔHb < 2 g/dL (76 patients), the doses were reduced for 21 patients (28%) and discontinued for 11 (14%); none due to severe anemia.

Early decline of Hb and minimum hemoglobin level during treatment

As in the case of ΔHb, we evaluated the minimum hemoglobin level during treatment, as shown in Figure 3. The patients with minimum Hb ≤ 8.5 g/dL accounted for 10% (4/39) of the group of ΔHb ≥ 2 g/dL, and there was no patient with minimum Hb ≤ 8.5 g/dL

Table 4 Rate of the ribavirin reduction or discontinuance due to adverse effects according to Hb decrease levels

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb < 2 g/dL ($n = 76$)	58% (44/76)	28% (21/76)	14% (11/76)	0
ΔHb ≥ 2 g/dL ($n = 39$)	23% (9/39)	46% (18/39)	31% (12/39)	8% (3/39)

$P = 0.004$ (Mantel–Haenszel χ^2-test).

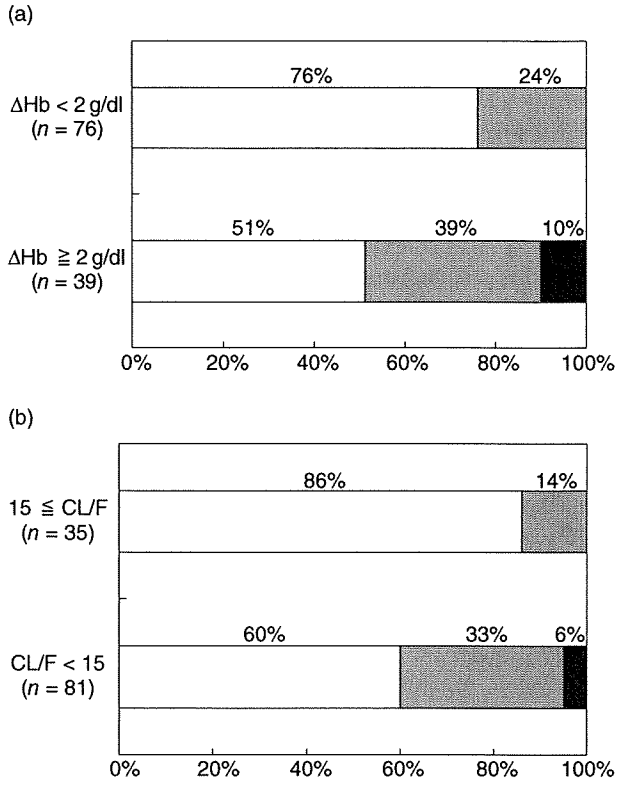

Figure 3 Minimum hemoglobin levels during PegIFN/ribavirin combination therapy. (□), 10 g/dL < minimum Hb; (▩), 8.5 < minimum Hb ≤ 10 g/dL; (■), minimum Hb ≤ 8.5 g/dL. (a) According to the "2 by 2" standard (Hb 2 g/dL decrease at two weeks from the baseline). $P = 0.009$ (Mantel–Haenszel χ^2-test). (b) according to CL/F levels. $P = 0.001$ (Mantel–Haenszel χ^2-test).

in the ΔHb < 2 g/dL group (Fig. 3a). The patients with minimum Hb ≤ 8.5 g/dL accounted for 6% (5/81) of the group of CL/F < 15, and there was no patient with minimum Hb ≤ 8.5 g/dL in the 15 ≤ CL/F group (Fig. 3b). The number of patients with minimum Hb ≤ 8.5 g/dL during PegIFN and ribavirin combination therapy according to "2 by 2" standard and CL/F levels is shown in Table 5. The patients with minimum Hb ≤ 8.5 g/dL were found only in the "2 by 2" standard-positive and low CL/F (<15) group (4/29, 14%).

Table 5 The number of patients with minimum hemoglobin ≤8.5 g/dL during PegIFN/ribavirin combination therapy according to "2 by 2" standard and CL/F levels

	ΔHb < 2 g/dL ($n = 76$)	ΔHb ≥ 2 g/dL ($n = 39$)
CL/F ≥ 15 ($n = 35$)	0/25	0/10
CL/F < 15 ($n = 80$)	0/51	4/29 (14%)

DISCUSSION

PREDICTION OF THE progression of anemia is necessary to decide whether drugs can be continued, with minimization of the disadvantages induced by anemia. Recently, CL/F has been used as a marker of progressing anemia that necessitates discontinuance of treatment. For example, if the patients have a low CL/F level, they should start treatment with a low ribavirin dose. In this study, we attempted to use the CL/F level measurement for our patients. To predict which patients might have to discontinue the treatment, the target range had to be CL/F < 15 because 6% of patients ($n = 5$) in this range showed minimum Hb ≤ 8.5 g/dL, which is the level at which ribavirin should be discontinued. No patients of the CL/F ≥15 group showed minimum Hb ≤ 8.5 g/dL. Our findings showed that 70% of the patients (81/116) with CL/F < 15 should be discriminated from the others (Table 3). In the same manner, using ΔHb as the marker, 34% of the target patients in the ΔHb ≥ 2 g/dL group were identified because 10% in this range showed minimum Hb ≤ 8.5 g/dL. No patients in the ΔHb < 2 g/dL group showed minimum Hb ≤ 8.5 g/dL. Compared to CL/F, ΔHb is considered to be more sensitive and convenient for identifying the high risk patients for whom treatment would need to be discontinued. Furthermore, the application of "2 by 2" standard in the group with low level of CL/F < 15 can be the most sensitive method for this (Table 5), since no patients with progression of anemia were found in the "2 by 2" standard-negative group with CL/F < 15.

In Japan, ribavirin doses are set at 600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for ≥80 kg, which are lower doses than those used in Europe and the USA. In this study, the mean ribavirin level at the start of treatment was 743 mg per day, while the AASLD practice guideline for genotype 1 hepatitis C is a daily dose of 1000 mg for body weight ≤ 75 kg and 1200 mg if >75 kg[26] In Japan, the use of lower doses is why fewer patients treated with PegIFN and ribavirin combination therapy are forced to discontinue the treatment due to severe anemia. Since the "2 by 2" model and/or CL/F can identify the patients who are prone to develop severe anemia, the other patients could be candidates for ribavirin dose-up strategies to raise SVR rates.

A considerable number of patients with chronic hepatitis C are over 60 years old in Japan (mean age is

around 55 years old),[27] although the mean age of this study was 50.6 years old. The number of aged patients with chronic hepatitis C is expected to increase in Europe and the USA, as well as in Japan. In IFN and ribavirin combination therapy, the discontinuance rate due to anemia was significantly higher in aged patients (≥60 years old, 21%) than in younger patients (<60 years old, 9%) ($P < 0.001$).[25] Earlier prediction of anemia is necessary to reduce the ribavirin dose in order to prevent the progression of severe anemia or to start epoetin alfa administration as needed, especially with aged patients. The "2 by 2" standard in PegIFN and ribavirin combination therapy should be a useful and convenient device for predicting the progress of anemia and treatment discontinuance in Europe and the USA, as well as in Japan.

CONCLUSION

IN CONCLUSION, THIS paper has shown that the SVR rate can be raised by preventing the discontinuance of ribavirin in PegIFN and ribavirin combination therapy. What is now needed is a prospective study of whether the early reduction of ribavirin in "2 by 2" standard-positive patients can improve the SVR rates, to ascertain the utility of the "2 by 2" standard in PegIFN and ribavirin combination therapy.

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Signal Transducer and Activator of Transcription 3 Signaling Within Hepatocytes Attenuates Systemic Inflammatory Response and Lethality in Septic Mice

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Sepsis is an infection-induced syndrome with systemic inflammatory response leading to multiorgan failure and occasionally death. During this process, signal transducer and activator of transcription 3 (STAT3) is activated in the liver, but the significance of this molecule has not been established. We generated hepatocyte-specific STAT3-deficient mice (L-STAT3 KO) and examined the susceptibility of these mice to cecal ligation and puncture–induced peritonitis, a well-established septic model. L-STAT3 KO mice showed significantly higher mortality and produced lesser amounts of various acute phase proteins than control littermates. Although blood bacterial infection did not differ between L-STAT3 KO mice and control mice, the former showed deterioration of the systemic inflammatory response as evidenced by a significant increase in various cytokines such as tumor necrosis factor α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1β. A similar hyperinflammatory response was observed in another septic model caused by lipopolysaccharide (LPS) injection. In vitro analysis revealed that soluble substances derived from hepatocytes and dependent on STAT3 were critical for suppression of cytokine production from LPS-stimulated macrophage and splenocytes. *Conclusion:* STAT3 activation in hepatocytes can attenuate a systemic hyperinflammatory response and lethality in sepsis, in part by suppressing immune cell overactivation, implying a critical role of hepatocyte STAT3 signaling in maintaining host homeostasis. (HEPATOLOGY 2007;46: 1564-1573.)

Signal transducer and activator of transcription 3 (STAT3) mediates a signal from the IL-6 family of cytokines such as IL-6, oncostatin M, leukemia inhibitory factor, and ciliary neutrophic factor, and activates transcription of various target genes.[1] Although a STAT3 is now known to be ubiquitously expressed in variety of cells and has pleiotropic functions, it was formerly termed *acute phase response factor* and was first identified in the liver as an inducible DNA binding protein binding to type 2 IL-6–responsive elements within the promoter of hepatic acute phase protein (APP) genes.[2,3] Because deletion of STAT3 leads to embryonic lethality in mice, the significance of STAT3 in adult organs has been investigated using conditional knockout animals generated by the Cre/loxP recombination system.[4] Research has shown that STAT3 signaling within hepatocytes controls a variety of physiological or pathological processes, including hepatocyte proliferation after partial hepatectomy,[5] apoptosis resistance of hepatocytes during Fas-mediated liver injury,[6] and regulation of hepatic gluconeogenic genes.[7] Although STAT3 is activated in response to a rise of circulating cytokines, the significance of hepatic STAT3 has not been elucidated under systemic inflammatory conditions.

Sepsis is an infection-induced systemic syndrome, the incidence of which is estimated at 750,000 cases annually in North America with overall mortality being approxi-

Abbreviations: APP, acute phase protein; CLP, cecal ligation and puncture; LPS, lipopolysaccharide; L-STAT3 KO, hepatocyte-specific STAT3-deficient mice; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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mately 30%, but rising to 40% in the elderly.[8] Sepsis develops when the initial, appropriate host response to an infection becomes amplified and then dysregulated.[9] Among those harmful or damaging responses is the rise of a variety of circulating cytokines such as IL-6, tumor necrosis factor α (TNF-α), IL-10, and IFN-γ. These cytokines lead directly to the development of systemic inflammatory response syndrome. During this process, an increasing proportion of patients will develop adult respiratory distress syndrome, disseminated intravascular coagulation, and/or acute renal failure, leading to the multiple organ dysfunction syndrome.[10] The liver is also one of the target organs of multiple organ dysfunction syndrome, although liver dysfunction may cause patient death less frequently than cardiovascular dysfunction.[11] Conversely, sepsis is a serious complication of severe liver diseases such as fulminant hepatitis[12] and decompensated cirrhosis.[13] Thus, research on the relevance of signal transduction in liver cells in the septic condition would not only satisfy basic scientific interest but would also have clinical implications.

In the present study, we used hepatocyte-specific STAT3-deficient (L-STAT3 KO) mice and examined the significance of STAT3 signaling within hepatocytes in a well-established murine model of sepsis. We found that STAT3 deficiency in hepatocytes causes exacerbation of the hyperinflammatory response by attenuating hepatic production of soluble substances that can suppress immune cell activation and also increases mortality in septic mice. This study identified an anti-inflammatory function of hepatic STAT3 signaling and its protective role against systemic inflammation, providing genetic evidence for a close link between hepatocytes and the immune system.

Materials and Methods

Animals. Mice carrying a STAT3 gene with 2 *loxP* sequences flanking exon 22 and a STAT3 null allele (*STAT3 fl/−*) have been described previously.[14] To generate mice with hepatocyte-specific STAT3 deficiency, we crossed STAT3 fl/− mice and Alb-Cre transgenic mice,[15] which express the Cre recombinase gene under the regulation of the albumin gene promoter. We crossed Alb-Cre STAT3 fl/fl mice and STAT3 fl/− mice. The resulting Alb-Cre STAT3 fl/− mice were used as L-STAT3 KO mice. Sex-matched STAT3 fl/− mice obtained from the same litter were used as control mice. All mice were used at the age of 12-15 weeks. All animals were housed under specific pathogen-free conditions and were treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Cecal Ligation and Puncture and Lipopolysaccharide Injection. Cecal ligation and puncture (CLP) is a well-established murine model of septic shock. The mice underwent CLP surgery as described previously.[16] In brief, the mice were anesthetized via intraperitoneal injection of sodium pentobarbital. Under sterile condition, the cecum was assessed via a 1-cm midline incision of the lower abdomen, ligated with a suture below the ileocecal valve, and punctured once with a 23-gauge needle. The cecum was replaced in the peritoneum, and the abdomen was closed with sutures. The mice were injected with 1 mL of lactate Ringer's solution subcutaneously for fluid resuscitation. As another septic model, lipopolysaccharide (LPS) (form *Escherichia coli* 055: B5; Sigma, St. Louis, MO) was injected intraperitoneally at a dose of 4 mg/kg body weight.

Preparation of Peritoneal Macrophage. To isolate peritoneal macrophages, we injected mice intraperitoneally with 2 mL of 4% thioglycollate. Peritoneal exudates cells were isolated from the peritoneal cavity 4 days after injection. The cells were incubated for 4 hours in 96-well plates and washed 3 times with phosphate-buffered saline. We used the adherent cells as peritoneal macrophages for further experiments.

Determination of the Bacterial Load. Mice were sacrificed 24 hours after CLP surgery. Samples of blood were obtained in sterile condition. Fifty microliters of the blood were then plated on heart-infusion plates. The heart-infusion plates were incubated at 37°C overnight, and the number of bacteria colonies was counted. Results were expressed as $\log_{10}$ of CFU.

Blood Biochemistry. Blood samples were obtained 24 hours after CLP or LPS injection. Acute phase proteins, cytokines, and chemokines in plasma were determined via MultiAnalyte Profile testing (Rules Based Medicine, Austin, TX). Levels of serum ALT and creatinine were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Measurement of Culture Supernatant. Levels of cytokines (TNF-α, IL-6, IL-10, and IFN-γ) in the culture supernatants were measured using commercially available ELISA kits in accordance with the manufacturer's instructions (BD Biosciences-Pharmingen, San Diego, CA). Haptoglobin was determined in cell-free supernatants by using a commercially available ELISA kit (Immunology Constants Laboratory, Newberg, OR).

Western Blot Analysis. The total cellular protein was extracted with the RIPA buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4). Twenty micrograms of protein were separated

via 7.5% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline 0.1% Tween 20 containing 5% skim milk or Blocking One-P (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, the membrane was incubated overnight at 4°C with antibodies to STAT3 or tyrosine^{705}-phosphorylated STAT3 (Cell Signaling Technology, Danvers, MA), respectively. After washing with Tris-buffered saline 0.1% Tween 20, the membrane was incubated with anti–horseradish peroxidase–linked antibody for 1 hour at room temperature. The immune complex was detected by an enhanced chemiluminescent assay. In some experiments, tyrosine^{701}-phosphorylated STAT1 antibody (Cell Signaling Technology) was also used. This antibody recognizes the phosphorylated form of both STAT1α and STAT1β.

Histology and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling. The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed using an ApopTag kit according to the manufacturer's instructions (Serological Corporation, Norcross, GA).

Primary Culture of Hepatocytes. Livers were digested using a standard in situ 2-step collagenase perfusion procedure (Gibco BRL, Rockville, MD). Hepatocytes were isolated from nonparenchymal cells via subsequent centrifugation at 50*g* for 1 minute. In a selected experiment, nonparenchymal cells in the supernatants were pelleted at 1,500 rpm for 5 minutes and subjected to western blot analysis. Isolated hepatocytes with >90% viability were cultured in Williams' medium E containing 10% fetal bovine serum overnight. On the next day, the cells were stimulated with recombinant IL-6 (PeproTech, London, UK). The cells were harvested after 2 hours for the analysis of STAT3 activation. In another experiment, supernatants were harvested after 48 hours.

Cytokine Production by Macrophage and Splenocytes. The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). RAW cells were plated at a density of 5×10^5/well in a 96-well plate and were incubated at 37°C in culture supernatants of hepatocyte from L-STAT3 KO mice or control mice. As a control, RAW cells were also cultured in Williams' medium E. After 24 hours, LPS was added to achieve a final concentration of 100 ng/mL. After 24 hours of incubation at 37°C in an atmosphere of 5% CO_2, the supernatant was collected and stored at −80°C for measurement of TNF-α, IL-6, and IL-10. Splenocytes were isolated by way of a standard procedure for wild-type mice[17] and incubated with hepatocyte culture supernatant. Twenty-four hours after incubation, the cells were stimulated with LPS (1,000 ng/mL) for 24 hours. The resultant culture supernatant was subjected to IFN-γ ELISA.

Statistics. Kaplan-Meier curves were used to show survival over time. Data are expressed as interquartile range and median and compared using the Mann-Whitney *U* test. Statistical significance was set at $P < 0.05$.

Results

Mice with hepatocyte-specific STAT3 deficiency were produced by crossing floxed STAT3 mice and Alb-Cre transgenic mice carrying the Cre recombinase gene under the regulation of the albumin gene promoter. L-STAT3 KO mice were born and grew without any gross abnormality. Western blot analysis revealed that STAT3 expression was substantially decreased in the liver but not in other organs (Fig. 1A). Isolation of hepatocytes from nonparenchymal cells by liver perfusion followed by centrifugation confirmed that STAT3 deficiency is specific in hepatocytes (Fig. 1B). In addition, STAT3 expression did not differ in peritoneal macrophages between L-STAT3 KO mice and control littermates (Fig. 1C). Those cells isolated from L-STAT3 KO mice produced similar levels of TNF-α in response to LPS compared with those from control littermates (Fig. 1D).

L-STAT3 KO Mice Are More Vulnerable to Septic Shock. To examine the role of hepatic STAT3 during septic shock, we used a well-examined clinically relevant murine model of sepsis performed by CLP.[16] CLP clearly activated liver STAT3, which was determined via phosphorylation of STAT3 in control mice (Fig. 2A), in agreement with a previous report.[18] Liver STAT3 activation during sepsis is mostly due to the activation of STAT3 in hepatocytes, because liver STAT3 was only marginally activated in L-STAT3 KO mice. CLP activated liver STAT1 both in L-STAT3 KO mice and wild-type mice, suggesting that the absence of STAT3 does not affect the activation of other STATs. Given that STAT3 is a well-known mediator for APP,[19] we measured APPs such as fibrinogen and haptoglobin in plasma after CLP (Fig. 2B). The levels of fibrinogen and haptoglobin clearly increased after CLP in wild-type mice. In contrast, induction of fibrinogen was completely diminished in L-STAT3 KO mice, whereas that of haptoglobin was partially inhibited. This is consistent with the previous notion that fibrinogen is a class 2 gene and haptoglobin is a class 1 gene; the class 2 gene is predominantly regulated by type 2 IL-6 responsive elements binding to STAT[20] and the class 1 gene by both type 1 IL-6 responsive elements

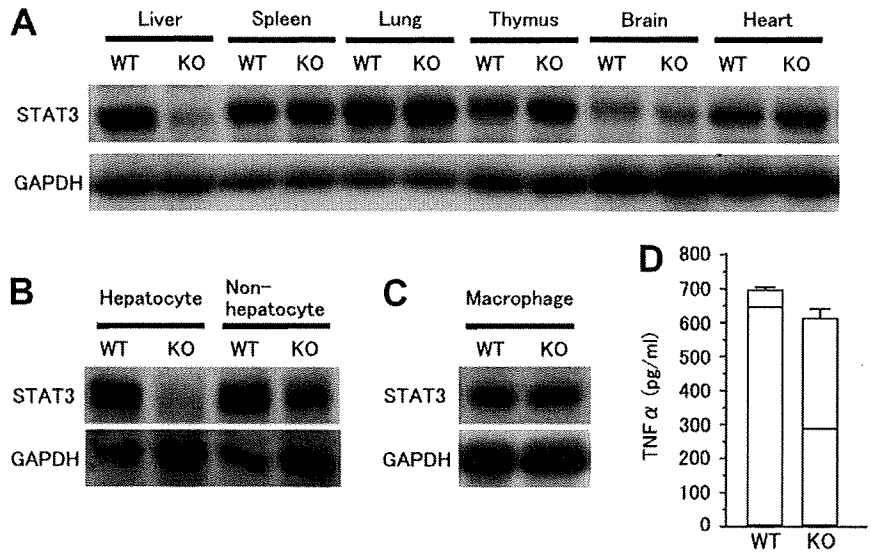

Fig. 1. Hepatocyte-specific STAT3 deficiency in mice. Floxed STAT3 mice were crossed with Alb-Cre transgenic mice. Floxed STAT3 mice having the Alb-Cre transgene were regarded as L-STAT3 KO mice (KO); those not having the Alb-Cre gene were used as a wild-type control (WT). (A) STAT3 expression in a variety of organs from L-STAT3 KO mice and wild-type mice via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (B) Expression of STAT3 of isolated hepatocytes and nonhepatocytes. Liver of L-STAT3 KO mice or wild-type mice was collagenase-perfused and separated into hepatocyte and nonhepatocyte fractions. STAT3 expression was determined via western blot analysis. Expression of GAPDH was served as a loading control. Representative blots are shown. (C) Expression of STAT3 in isolated macrophage. Peritoneal macrophage was isolated from L-STAT3 KO mice or wild-type mice and subjected to western blot analysis of STAT3 expression. Representative blots are shown. (D) LPS-stimulated TNF-α production of peritoneal macrophages. Peritoneal macrophages were isolated from L-STAT3 KO mice or wild-type mice ($n = 6$ for each group) and stimulated with LPS (100 ng/mL) for 24 hours. TNF-α production was determined via ELISA in culture supernatants.

binding to CCAAT enhancer-binding protein (C/EBP) and type 2 IL-6 responsive elements.[21]

To address the issue of whether hepatic STAT3 is involved in the outcome of CLP-induced lethality, we performed CLP blinded to the genetic background and checked the survival of the mice every 6 hours. L-STAT3 KO mice were significantly more vulnerable to CLP-induced lethality than wild-type littermates (Fig. 2C). To examine the possible difference in bacterial infection after CLP, we measured colony forming unit of blood bacteria 24 hours after CLP. Because there was no significant difference in bacterial amount between L-STAT3 KO mice and wild-type mice (Fig. 2D), we considered hepatic STAT3 to have had a beneficial effect on the outcome of septic shock without affecting bacterial infection.

Hepatic STAT3-Deficient Mice Show Exacerbated Liver Injury. To examine liver injury and renal dysfunction in CLP-induced sepsis, we measured ALT and creatinine levels. L-STAT3 KO mice showed increased levels of serum ALT and creatinine compared with wild-type littermates, although the difference in creatinine did not reach a significant level (Fig. 3A). TUNEL of the liver revealed that the number of apoptotic hepatocytes was significantly higher in L-STAT3 KO mice than in wild-type littermates (Fig. 3B,C). However, the liver injury itself presumably is not a direct cause of animal death, because histologic abnormality was modest. Furthermore, LPS injection, which is another model of septic shock, induced more hepatocyte apoptosis than CLP but did not kill any mice tested (Fig. 3A-C and data not shown), supporting the idea that increased liver injury could not explain the increased lethality in L-STAT3 KO mice.

Exacerbated Systemic Inflammatory Response in L-STAT3 KO Mice. Hypercytokinemia underlying systemic inflammatory response syndrome may play an important role in the development of multiple organ dysfunction syndrome and lethality.[9] We measured several circulating cytokines and chemokines in septic mice and found that TNF-α, IFN-γ, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) had clearly increased 24 hours after CLP in L-STAT3 KO mice. Of importance is the finding that the plasma levels of these cytokines and chemokines were significantly higher in L-STAT3 KO mice than in wild-type mice, although they did not differ before CLP. This result indicates that the increased lethality found in L-STAT3 KO mice is associated with hypercytokinemia (Fig. 4A). Although plasma insulin levels significantly increased 24 hours after CLP, there was no significant difference between L-STAT3 KO mice and wild-type mice, suggesting that insulin levels do not affect the difference in animal lethality (Supplementary Fig. 1).

Given that bacterial infection did not differ between L-STAT3 KO mice and wild-type mice, we examined the

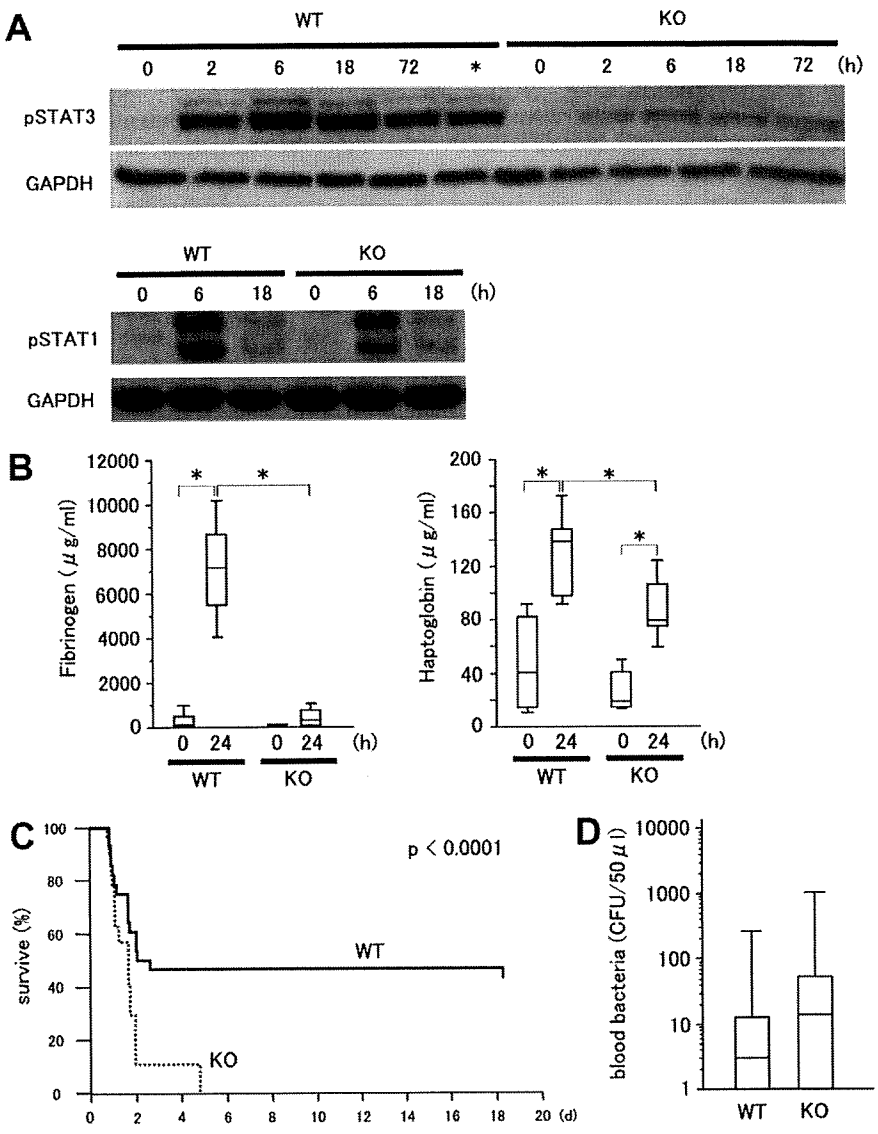

Fig. 2. STAT3 activation, APP production, and survival in CLP mice. (A) Western blot analysis of phosphorylated STAT3 and STAT1 in CLP mice. L-STAT3 KO mice (KO) and wild-type mice (WT) were treated with CLP and sacrificed at the indicated time points. Their liver tissues were subjected to analysis of Tyrosine 705 phosphorylation of STAT3 or Tyrosine 701 phosphorylation of STAT1 via western blot analysis. GAPDH expression served as a control. Representative blots are shown. *7 days. (B) Levels of circulating haptoglobin and fibrinogen before and 24 hours after CLP (n = 8 for each group). $*P < 0.05$. (C) Comparison of survival after CLP between L-STAT3 KO (n = 27) mice and wild-type littermates (n =28). (D) Colony-forming units of blood bacteria after CLP. L-STAT3 KO or wild-type mice were sacrificed 24 hours after CLP. Blood samples were subjected to analysis of bacterial growth (n = 10 for knockout mice and n = 9 for wild-type mice).

response of cytokine production upon endotoxin stimulation. To this end, we injected the same amount of LPS to L-STAT3 KO mice and control mice and measured circulating cytokines. LPS injection into L-STAT3 KO mice upregulated those cytokines to a lesser extent than CLP. In agreement with the finding on the CLP model, the levels of TNF-α, IL-10, MCP-1, and MIP-1β were significantly higher in L-STAT3 KO mice than in wild-type mice after LPS injection (Fig. 4B), indicating that L-STAT3 KO mice were highly sensitive to endotoxin and prone to show hypercytokinemia.

STAT3-Regulated Soluble Factors Produced by Hepatocytes Suppress Cytokine Production From Immune Cells. To examine the underlying mechanisms of the hyperimmune response in L-STAT3 KO mice, we hypothesized that STAT3-mediated soluble factors from hepatocytes repress cytokine production from immune cells. We isolated hepatocytes from L-STAT3 KO mice and control mice and stimulated them with or without IL-6, collecting the conditional medium of hepatocytes. Wild-type hepatocytes displayed STAT3 activation in primary culture without stimulation, but the levels increased upon IL-6 exposure, whereas KO hepatocytes did not show any STAT3 activation (Fig. 5A). Consistent with this was the finding that the wild-type hepatocytes produced more haptoglobin than KO hepatocytes, even in the absence of IL-6 (Fig. 5B).

Next, we cultured RAW cells, a murine macrophage cell line, in the presence or absence of culture supernatant of hepatocytes. RAW cells produced TNF-α, IL-6, and IL-10 but not IFN-γ upon stimulation of LPS, and hepatocyte culture supernatant suppressed the production of these cytokines (Fig. 5C). Importantly, the suppression was significantly weaker in the presence of conditional medium of KO hepatocytes than in the presence of conditional medium of wild-type hepato-

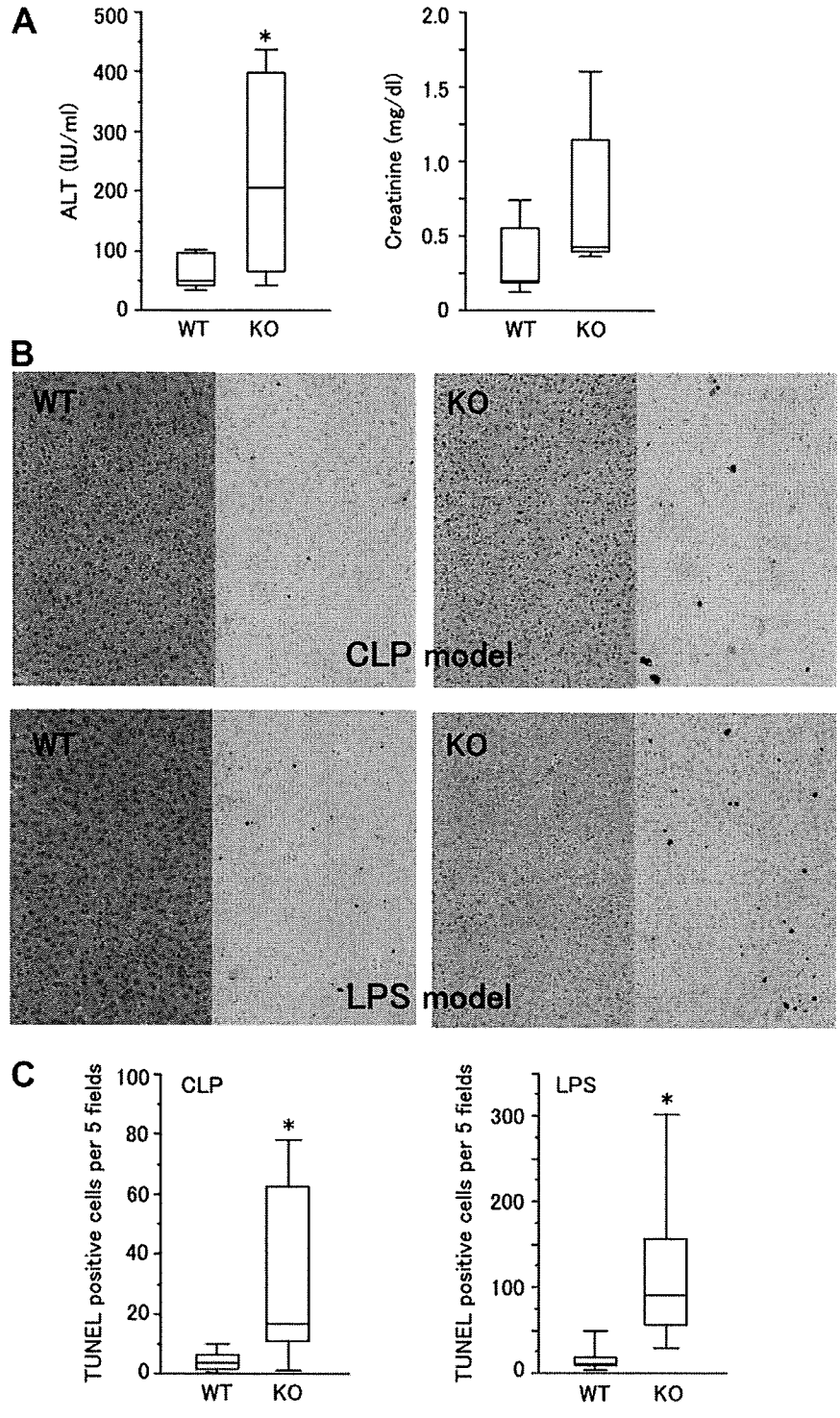

Fig. 3. Organ injury in septic mice. (A) Serum ALT and creatinine levels in L-STAT3 KO mice (KO) and control mice (WT) 24 hours after CLP. $*P < 0.05$. (B) Representative histology (left part of each panel) and TUNEL (right part of each panel) of liver sections 24 hours after CLP or LPS injection. (C) Comparison of TUNEL-positive hepatocytes for at least 9 mice in each group. $*P < 0.05$.

cytes. Furthermore, murine primary splenocytes produced IFN-γ upon LPS stimulation, and the production was also suppressed in the presence of conditional medium of hepatocytes. Again, IFN-γ production was significantly higher in splenocytes cultured with KO hepatocyte supernatant than in those with wild-type hepatocyte supernatant (Fig. 5D). These data indicate that soluble substances from hepatocytes suppressed activation of immune cells, which was critically dependent on STAT3.

Discussion

The present study clearly demonstrated that the absence of STAT3 in hepatocytes leads to high levels of circulating cytokines and increased mortality of CLP-in-