The rate of hepatocellular carcinoma (HCC) development increases with the progression of liver fibrosis, and the annual occurrence rate in HCV positive Japanese cirrhotic patients is high (~7–8%).[1] The incidence of HCC significantly decreases after viral eradication by IFN treatment (sustained viral response [SVR]) in patients with HCV-related CLD, even in patients with liver cirrhosis.[2–5] However, the SVR rate in cirrhotic patients with HCV genotype 1b is approximately 25%, that is significantly lower than that of chronic hepatitis patients (~50%). Hypersplenism due to portal hypertension is believed to be one of the causes of the low SVR rate of IFN treatment observed in cirrhotic patients.[6–9] In patients with hypersplenism, reducing the dose or discontinuing IFN is often required because of their thrombocytopenia and/or granulocytopenia. Furthermore, with the combined use of ribavirin (RBV), the adherence to treatment declines in association with the degree of anemia. Discontinuing or reducing the dose of antiviral agents (IFN and/or RBV) decreases the SVR rate,[10,11] and the presence of hypersplenism-related pancytopenia can be a major cause of this decrease. In particular, a low platelet count is the main factor that is linked to the discontinuation of IFN treatment. Therefore, in order to increase the platelet count and improve adherence to antiviral agents, splenectomy/partial splenic embolization (PSE) before the initiation of IFN therapy is considered a useful option for patients with hypersplenism-linked pancytopenia.[12–23]

In Japan, it is recommended that splenectomy/PSE be performed on patients with a low platelet count before IFN treatment, as specified in the Guidelines for Chronic Hepatitis C of 2008 and onwards.[24] However, neither splenectomy nor PSE is recommended in guidelines outside of Japan. Therefore, it is important and necessary to investigate whether these surgical or interventional treatments for anti-hypersplenism should be a standard precursor to IFN treatment for patients with a low platelet count. In addition, if splenectomy/PSE is indeed a valid therapeutic option, the patients that would most benefit from these treatments should be identified.[9,10,25–27]

In the present study, we investigated the current state of the treatment of splenectomy/PSE in HCV positive patients with low platelet count. We conducted a survey in the form of a questionnaire that probed the following topics with regard to splenectomy/PSE: the current status of implementation, associated complications, degree of increased platelets and its effect on IFN treatment.[15]

METHODS

Subjects

A SURVEY WAS conducted as a part of research by the "Standards and Clinical Research Aimed at Establishment of IFN Treatment Towards Cases with Low Platelet Counts" group from the scientific research grant from the Ministry of Health, Labor and Welfare in Japan. The sample included the 413 medical institutes to which the liver disease specialists (Japan Society of Hepatology, Board of Councilors of the Western and Eastern Association, Director of the Liver Cancer Study Group of Japan, and Councilor of The Japanese Society of Interventional Radiology) belong. Approval was obtained from the three associations (the Japan Society of Hepatology, the Liver Cancer Study Group of Japan and the Japanese Society of Interventional Radiology) prior to distributing this questionnaire. This study is a summary of the questionnaire responses, and ethical considerations toward patients were ensured by anonymizing personal information.

Questionnaires

Three types of questionnaires were prepared with internists, surgeons and radiologists as the subjects; each type of questionnaire was sent to 336, 46 and 31 institutes, respectively. As a general rule, one questionnaire was sent to one medical institute.

In this study, thrombocytopenia was defined as a platelet count of less than 100×10^9 platelets/L. A survey was conducted to determine whether splenectomy or PSE was performed to improve adherence to IFN treatment in patients with thrombocytopenia, and the selection criteria for splenectomy/PSE (including a platelet count and the liver function tests) were also queried in the first questionnaires (sent in September 2009 and collected on 22 December 2009). The state of the implementation of splenectomy/PSE was questioned again in the second questionnaire. The second questionnaire also focused on the appropriateness of performing splenectomy or PSE for IFN treatment in the patients, and investigated the aforementioned topics, including the efficacy of splenectomy/PSE, complications, and the prevalence of prophylactic administration of pneumococcal vaccine (sent in September 2010 and collected on 14 January 2011).[28] The third questionnaire (sent in November 2011 and collected on 6 December 2011) was performed as a detailed investigation of the 11 cases in which death was reported in the second questionnaire.

Statistical analysis

Data were expressed as mean ± standard deviation. A χ^2-test was used to compare splenectomy/PSE implementation cases, and Student's *t*-test and Mann–Whitney *U*-test were used for other comparisons. $P < 0.05$ indicated statistically significant difference.

RESULTS

FOR THE FIRST and second questionnaire, responses were obtained from internists, surgeons and radiologists (Table 1). For the third questionnaire, responses were obtained from all 10 institutes (11 patients died in 10 institutes: 100% recovery of the questionnaire sheets).

Standard platelet count required to initiate IFN treatment and the initial dose of IFN for patients with low platelet count

Eighty-nine percent (95/107) of institutes began IFN treatment even when the platelet count was less than 100×10^9 platelets/L. The adherence to IFN treatment of the patients was also answered. In the patients with a platelet count of 80×10^9 platelets/L or more before IFN treatment, 90% (72/80) of the institutions initiated therapy with a sufficient (≥80% of the normal dose) initial dose of IFN. However, among patients with a platelet count of less than 80×10^9 platelets/L, only 27% (25/93) started treatment with an insufficient (<80% of the normal dose) initial dose of IFN. Thus, many patients with a platelet count of less than 80×10^9 platelets/L prior to IFN introduction received a dose of IFN that was reduced to a level at which the IFN SVR rate was predicted to be low.

Implementation status of splenectomy/PSE prior to IFN treatment in patients with low platelet counts

The questionnaire results clarified that splenectomy and/or PSE were performed in 61% of the specialized institutes providing IFN therapy.

The platelet count that each institute considered when performing splenectomy/PSE before IFN treatment was $64 \times 10^9 \pm 18 \times 10^9$ platelets/L ($n = 25$) for splenectomy and $79 \times 10^9 \pm 14 \times 10^9$ platelets/L ($n = 24$) for PSE, with splenectomy having a significantly low value compared to PSE ($P = 0.002$).

Reasons for not performing splenectomy

In the questionnaire given to the internists (114 institutes), 60 institutes responded "splenectomy is not performed for IFN treatment".

In these 60 institutes, 28 described the possible severe complications as the reason for not carrying out splenectomy. Of these 28 institutes, four institutes that performed splenectomy for IFN treatment in the past experienced cases of portal thrombosis.

In the 26 institutes in which the surgeon-specific questionnaire was completed, 18 (69%) were performing splenectomy before IFN treatment; of these institutes, 59% experienced cases of portal thrombosis. In the questionnaire with internists and surgeons as subjects, the respondents strongly indicated complications as a reason for not performing splenectomy.

Reasons for not performing PSE

From the questionnaire that targeted internists (114 institutes), 70 institutes responded "PSE is not performed for IFN treatment".

In these 70 institutes, 36 described the possible severe complications as the reason for not performing splenectomy.

Of the 10 institutes in which questionnaires were completed by radiologists, one institute responded that PSE should not be performed for IFN treatment because of complication issues.

Platelet count transition and period before IFN treatment initiation following splenectomy/PSE

The changes in platelet count following splenectomy/PSE were investigated at each institute (Fig. 1), and a significantly increased platelet count was observed after carrying out splenectomy or PSE. However, this platelet count increase appeared to be higher and more sustained in patients who underwent splenectomy relative to those that underwent PSE. IFN administration was initiated within 1 – 3 months following PSE and within

Table 1 Questionnaire collection rates

	Internists	Surgeons	Radiologists
1st response	32% (107/336)	52% (24/46)	23% (7/31)
2nd response	34% (114/336)	57% (26/46)	32% (10/31)

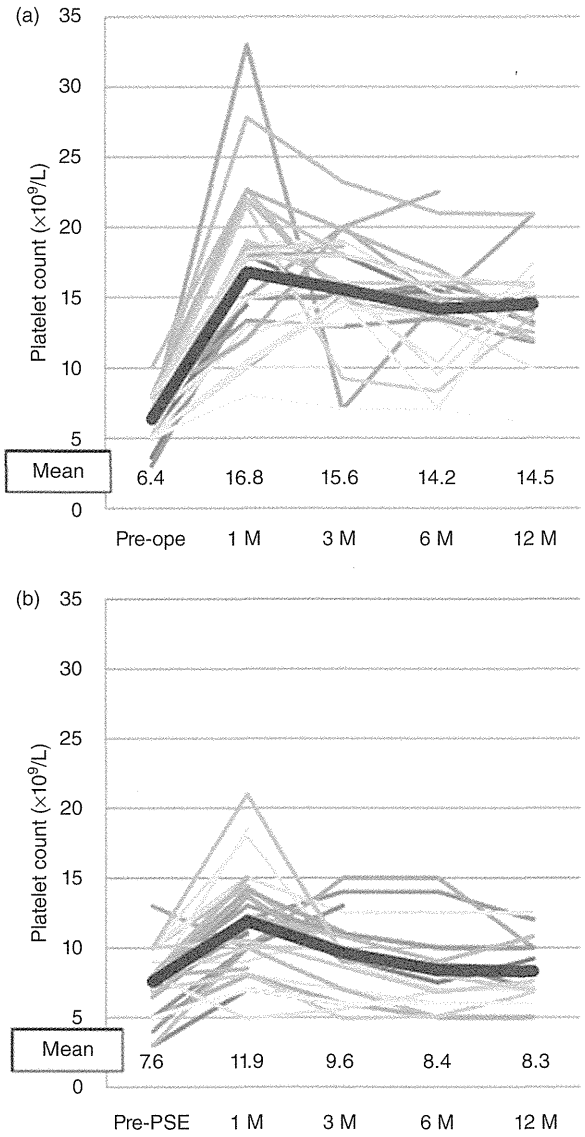

Figure 1 Platelet count transition of each institute following splenectomy/partial splenic embolization (PSE); average platelet count number of institutes. The bold line represents the transition of the mean platelet counts of each institute. (a) Splenectomy cases. (b) PSE cases.

3 – 6 months following splenectomy in the majority of cases (Table 2).

Complications following splenectomy/PSE

The splenectomy-associated complications experienced in each institute included portal thrombosis (28 of 63 institutes), postoperative infectious diseases (11 of 63 institutes) and ascites (12 of 62 institutes). However, the incidence of these complications varied depending on the institute.

In 64 institutes, fever ($n = 28$), thrombosis ($n = 22$), abscess ($n = 4$) and ascites ($n = 12$) were reported as frequent complications after PSE. However, the incidence of these complications also varied depending on the institute.

From 2005 to 2010, patient deaths were observed in seven of 788 (0.89%) cases of splenectomy, and in four of 474 (0.84%) cases of PSE. In nine of the 11 death cases, there appeared to be causal relationship between death and splenectomy as well as PSE (a causal relationship was indicated to exist by five of seven institutes for splenectomy and four of four institutes for PSE).

The age at the time of death ranged 46–70 years, with many patients older than 60 years. The sex included six male cases and five female cases, and there were two cases of chronic hepatitis and nine cases of liver cirrhosis (Child–Pugh classification grade A, two cases; B, six cases; and unknown, one case). The cirrhotic patients that died tended to have higher Child–Pugh scores and poor residual hepatic function. Pneumococcal vaccine inoculation was only performed in one splenectomy patient, and the other 10 patients were not inoculated. The cause of death was related to infectious diseases in nine cases (there was one patient with an apparent pneumococcal infection who was not inoculated with a pneumococcal vaccine). In most cases, death occurred within 3 months after treatment (splenectomy or PSE), although it also occurred over 3 months after treatment. Three patients died during IFN treatment (two cases after splenectomy, one case after PSE). Two patients died within 3 months after IFN treatment (two cases after splenectomy) (Table 3).

SVR rate of cases in which IFN treatment was performed following splenectomy or PSE

Among patients with low platelet count, IFN treatment was introduced in 92% (236/257) of the cases in which

Table 2 Period from splenectomy/PSE to initiation of IFN treatment

	Splenectomy ($n = 64$)	PSE ($n = 56$)
Within 1 month	6 (9%)	23 (42%)
>1 to 3 months	26 (41%)	23 (42%)
>3 to 6 months	17 (27%)	6 (10%)
>6 to 12 months	8 (12%)	3 (5%)
>12 months	7 (11%)	1 (1%)

IFN, interferon; PSE, partial splenic embolization.

Table 3 Period from splenectomy/PSE to death

	Splenectomy (7 cases of death)	PSE (4 cases of death)
Within 3 months	3 §Postoperative bleeding (hemophilia) §Pancreatic fistula, local infection §Intra-abdominal abscess (MRSA)	3 §Thrombocytopenia, cerebral hemorrhage §Pneumonia, ARDS, sepsis (MRSA) §Peritonitis
Within 6 months	0	1 †Spondylodiscitis, sepsis
Within 1 year	2 †SAH, bacteremia (MRSA) ‡Sepsis	0
Within 2 years	1 ‡Liver failure, suspect of SBP	0
Over 2 years	1 †Pneumococcal infection	0

†Death occurred during IFN treatment.
‡Death occurred within 3 months after IFN treatment.
§Death except † and ‡.
ARDS, acute respiratory distress syndrome; MRSA, methicillin-resistant *Staphylococcus aureus*; PSE, partial splenic embolization; SAH, subarachnoid hemorrhage; SBP, spontaneous bacterial peritonitis.

splenectomy was performed for IFN, 94% (295/314) of the cases in which PSE was performed for IFN, and 84% (241/285) of cases in which such pretreatment (splenectomy or PSE) was not performed before the introduction of IFN. Discontinuation of IFN occurred in 22% of cases of splenectomy, 28% of cases of PSE and 33% of those without pretreatment. Due to the pretreatment, the IFN introduction rates were increased ($P < 0.001$) and discontinuation rate declined ($P = 0.02$).

The pretreatment platelet count was $64 \times 10^9 \pm 17 \times 10^9$ platelets/L in splenectomy cases and $76 \times 10^9 \pm 21 \times 10^9$ platelets/L in PSE cases, while that of cases without pretreatment was $85 \times 10^9 \pm 16 \times 10^9$ platelets/L. In patients with a platelet count of 80×10^9 platelets/L or more, the majority of IFN treatments were without pretreatment to increase the platelet count.

The tabulation of the IFN treatment effects of cases in each institute is shown in Table 4. The SVR rate of cases of HCV genotype 1b and high viral load was 42 of 228 (22%) for the PSE group and 63 of 228 (28%) for the splenectomy group, with an odds ratio of 0.78 ($P = 0.19$). The SVR rate of so-called "others" (patients other than those with genotype 1b and high viral load) was 62 of 110 (56%) for the PSE group and 84 of 119 (71%) for the splenectomy group, with an odds ratio of 0.54 ($P = 0.025$). Additionally, in the "others" group, the SVR rate following IFN treatment was higher in patients who underwent splenectomy compared to that of patients who underwent PSE.

DISCUSSION

THE PRESENT STUDY was conducted to clarify the current conditions of splenectomy/PSE performed for the purpose of IFN treatment. This was the first national questionnaire conducted in Japan, and no similar studies have been reported previously. The results of these questionnaires revealed that the lower limit of the platelet count achieved prior to IFN administration varied widely depending on the institute in

Table 4 SVR rate of IFN treatment following splenectomy/PSE

	Splenectomy	PSE	*P* (odds ratio)
1b-high	28% (63/228)	22% (42/190)	0.19 (0.74)
Others	71% (84/119)	56% (62/110)	0.025 (0.54)

A difference in SVR rate was observed between splenectomy and PSE groups. In patients with hepatitis C virus genotype 1b and a high viral load, there was no significant difference in the low SVR rate. The SVR rate was high in cases other than those of a 1b genotype/high viral load, with splenectomy having a significantly higher SVR rate compared to PSE.
IFN, interferon; PSE, partial splenic embolization; SVR, sustained virological response.

Japan. Currently, for pegylated (PEG) IFN-α-2a/RBV treatment of liver cirrhosis, Japanese insurance considers a platelet count of 75×10^9 platelets/L or more as the standard count for treatment initiation and less than 50×10^9 platelets/L as that for discontinuation. However, it is now clear that half of the specialized institutes surveyed were initiating IFN therapy even in patients with platelet counts below the recommended value, and 9% of institutes were administrating IFN even in patients with platelet counts below the standard discontinuation value. Moreover, there was disproportionate selection and application of splenectomy and/or PSE, perhaps because each institute tended to select its experienced method. Therefore, in order to obtain consensus in the future, it is necessary to investigate the actual situation of splenectomy/PSE treatment in HCV positive patients with a low platelet count through the collection of clinical data.

Beneficial information regarding the effects of splenectomy and/or PSE on IFN treatment for the patients was also obtained from this questionnaire. The platelet counts increased after splenectomy and/or PSE, thus resulting in the improvement of adherence to IFN treatment. However, an increased SVR rate was not prominent in patients with HCV genotype 1b and a high viral load. It was presumed that if the viral factor shows IFN-resistant characteristics and liver cirrhosis exists as an intractable factor on the patient side, only a small number of patients will achieve a SVR with PEG IFN/RBV treatment, despite increased platelet counts and IFN adherence following splenectomy and/or PSE. However, in the case of "others", a relatively high SVR rate was observed (Table 4). Interestingly, the SVR rate of the "others" splenectomy group was significantly higher than that of the "others" PSE group, despite the pretreatment platelet count being lower in the splenectomy group than that of the PSE group. If the platelet count is less than 80×10^9 platelets/L in cases of "others," anti-hypersplenism treatment should be performed to increase the platelet count, and we suggest that splenectomy should be selected because of its strong effect on the increase of the platelet count.

Many complications regarding patients' safety were observed in both splenectomy and PSE groups, and it should be noted that slightly less than 1% cases resulted in death. Most patients had liver cirrhosis. These patients typically have several medical problems that can become severe if they are not receiving anti-HCV therapy, including decompensated cirrhosis and/or the development of HCC. However, the high mortality rate reported in the present study should not be overlooked. We recommend that the application of splenectomy/PSE prior to IFN treatment should be chosen with careful consideration.

At present, splenectomy/PSE is also mentioned in the Guidelines for IFN Treatments in Liver Cirrhosis C in Japan as treatments for patients with low platelet counts, although the risk of death due to splenectomy/PSE for the purpose of IFN treatment has not been studied previously. Death was related to infections in many cases, but the rate of pneumococcal vaccine inoculation was low in cases of death. According to the questionnaire results, within institutes administrating pneumococcal vaccines when performing splenectomy, the vaccination rate was 80% or more in the department of internal medicine and 60% or more in the surgical department; however, this rate was only approximately 20% for PSE. The pneumococcal vaccine is inoculated at a high rate for splenectomy based on the recommendations from the insurance guidelines[29] and infection prevention guidelines.[30–32] In contrast, there is no evidence indicating the usefulness of pneumococcal vaccine inoculation when performing PSE, in which splenic function is preserved. Therefore, this vaccine was not given to patients undergoing PSE in many cases. However, considering the fact that most causes of death were related to infections, pneumococcal vaccine inoculation should also be necessary when carrying out PSE. Moreover, vaccinations against bacteria other than *Streptococcus pneumoniae* such as *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis*, which mainly exhibit immune reactions in the spleen, may be important to administrate before splenectomy and/or PSE.[33–36]

Deaths were primarily observed in Child–Pugh B or C cirrhotic patients and those aged above 60 years. Therefore, splenectomy/PSE must be applied with care in patients with poor residual hepatic function and elderly patients.[37,38]

As a result of this questionnaire, we determined that adherence to IFN treatment was increased by splenectomy and/or PSE; however, in patients with HCV genotype 1b and a high viral load, the rate of SVR was not improved. Therefore, splenectomy and/or PSE must be limited to the cases in which IFN is likely to be effective. In addition, it is essential to predict the sensitivity of IFN treatment by evaluating the IFN sensitivity-determining region of HCV, core domain amino acid 70 of HCV and interleukin-28B, as well as hepatic functional reserves and age before splenectomy and/or PSE. In the future, treatment by various oral therapeutic agents (direct antiviral agents) may be selected without administrating IFN for patients with low platelet counts.[39,40]

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Interferon-Gamma–Mediated Tissue Factor Expression Contributes to T-Cell-Mediated Hepatitis Through Induction of Hypercoagulation in Mice

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Concanavalin A (Con A) treatment induces severe hepatitis in mice in a manner dependent on T cells, interferon (IFN)-gamma, and tumor necrosis factor (TNF). Treatment with the anticoagulant heparin protects against hepatitis, despite healthy production of IFN-γ and TNF. Here, we investigated molecular and cellular mechanisms for hypercoagulation-mediated hepatitis. After Con A challenge, liver of wild-type (WT) mice showed prompt induction of *Ifnγ* and *Tnf*, followed by messenger RNA expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1), which initiate blood coagulation and inhibit clot lysis, respectively. Mice developed dense intrahepatic fibrin deposition and massive liver necrosis. In contrast, *Ifnγ*$^{-/-}$ mice and *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice neither induced *Pai1* or *Tf* nor developed hepatitis. In WT mice TF blockade with an anti-TF monoclonal antibody protected against Con A–induced hepatitis, whereas *Pai1*$^{-/-}$ mice were not protected. Both hepatic macrophages and sinusoidal endothelial cells (ECs) expressed *Tf* after Con A challenge. Macrophage-depleted WT mice reconstituted with hematopoietic cells, including macrophages deficient in signal transducer and activator of transcription-1 (STAT1) essential for IFN-γ signaling, exhibited substantial reduction of hepatic *Tf* and of liver injuries. This was also true for macrophage-depleted *Stat1*$^{-/-}$ mice reconstituted with WT macrophages. Exogenous IFN-γ and TNF rendered T-cell-null, Con A–resistant mice deficient in recombination-activating gene 2, highly susceptible to Con A–induced liver injury involving TF. *Conclusions*: Collectively, these results strongly suggest that proinflammatory signals elicited by IFN-γ, TNF, and Con A in both hepatic macrophages and sinusoidal ECs are necessary and sufficient for the development of hypercoagulation-mediated hepatitis. (HEPATOLOGY 2013;57:362-372)

Concanavalin A (Con A)-induced hepatitis is a well-characterized, representative mouse model of T-cell-mediated acute liver failure.[1] After Con A challenge, mice show elevation of circulating proinflammatory cytokine levels, subsequently resulting in massive liver necrosis with dense infiltration of leukocytes. Because interferon (IFN)-γ or tumor necrosis factor (TNF) blockade and gene depletion of *Ifnγ* or

Abbreviations: Abs, antibodies; ALI, acute liver injury; ALT, alanine aminotransferase; B6, C57BL/6; BM, bone marrow; Ccl2, *CC chemokine ligand 2 gene clodronate liposome, liposome-encapsulated dichloromethylene bis-phosphonate; Con A, concanavalin A; ECs, endothelial cells; H&E, hematoxylin and eosin; IFN, interferon; IFNAR1, IFN-α receptor 1; IgG, immunoglobulin G; IHC, immunohistochemistry;* Il6, *interleukin-6 gene;* Il1β, *interleukin-1β gene; IP, intraperitoneal; IV, intravenously; KO, knockout; Mø, macrophages; mAb, monoclonal antibody; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time reverse-transcriptase polymerase chain reaction; RAG2, recombination-activating gene 2; rIFN-γ, recombinant IFN-γ; rRNA, ribosomal RNA; rTNF, recombinant TNF; SC, subcutaneously; SEC, sinusoidal endothelial cells; STAT1, signal transducer and activator of transcription 1; TAT, thrombin antithrombin III complex; TF, tissue factor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling; WT, wild type.*

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**These authors equally contributed to the present study.*

Tnf rescues mice from Con A–induced hepatitis,[2,3] IFN-γ and TNF are convincingly regarded as the cytokines necessary for the development of this type of liver injury. Thus, one may assume that endogenous IFN-γ and TNF initiate both the hepatic inflammatory responses and the liver parenchymal cell death.[4] However, as previously reported, massive liver necrosis is accompanied by severe thrombocytopenia and intrahepatic hemostasis, and heparin pretreatment substantially protects against liver injury without down-regulating the production of IFN-γ and TNF.[2] This may imply that microcirculatory disturbances resulting from hepatic thrombosis contribute to liver injury independent of IFN-γ/TNF-mediated hepatic inflammation and hepatocytotoxicity. Alternatively, IFN-γ and/or TNF might be causative for hepatic thrombosis, perhaps by inducing procoagulant activity within the liver. Thus, it is important to elucidate whether and how IFN-γ and/or TNF contribute to the hepatic hypercoagulation and whether IFN-γ and/or TNF are sufficient to trigger these pathological changes.

Tissue factor (TF) is a transmembrane cofactor for the coagulation factor, VIIa, and is constitutively expressed in the blood vessel wall and its expression is induced by various mediators in several cell types including macrophage (Mø) and endothelial cells (ECs).[5-7] Endothelial damage or TF expression on circulating monocytes/macrophages brings TF in contact with circulating factor VIIa to initiate the blood coagulation cascade, which eventually results in the activation of prothrombin, leading to fibrin formation and platelet activation. The coagulation system is tightly regulated by the fibrinolytic system, which comprises plasminogen, the tissue-type plasminogen activator (tPA), and its inhibitor, plasminogen activator inhibitor-1 (PAI-1).[8,9] *Pai1*$^{-/-}$ mice have been reported to be resistant to alcohol-induced or cholestatic liver injuries.[10-12] Therefore, it is possible that PAI-1 as well as TF may play a role in coagulation-mediated liver injuries.

In this study, we investigated the mechanisms by which Con A treatment induces the prothrombotic state. We found strong induction of hepatic *Tf* and *Pai1* expressions, dense hepatic fibrin deposits, and massive liver necrosis in Con A–treated wild-type (WT) mice, but not in *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice. TF blockade protected WT mice from the intrahepatic fibrin deposition and resultant hepatitis. Both hepatic macrophages (Mø) and sinusoidal ECs (SECs) expressed *Tf* in Con A–challenged WT mice. IFN-γ signaling was crucial for *Tf* induction in both these cell types. Con A–resistant mice that have Mø and SECs, but not T cells, became highly susceptible to Con A when treated simultaneously with IFN-γ and TNF. Collectively, these results indicate that IFN-γ-, TNF-, and Con A–activated signaling pathways in hepatic Mø and SECs are necessary and sufficient for the development of intrahepatic hemostasis-mediated massive liver injuries.

Materials and Methods

Reagents. Con A was purchased from J-Oil Mills (Tokyo, Japan). Neutralizing rat antimouse TF monoclonal antibody (mAb) (1H1) was described elsewhere.[13] Purified rat immunoglobulin G (IgG) was purchased from Beckman Coulter (Fullerton, CA). Recombinant murine IFN-γ and TNF were from PeproTech (Rocky Hill, NJ). Liposome-encapsulated dichloromethylene bis-phosphonate (clodronate liposome) and phosphate-buffered saline (PBS) liposomes were prepared as described previously.[14,15]

Induction of Acute Hepatitis. Con A was administered to mice (20 mg/kg) through a tail vein.[2] In some experiments, mice received Con A intravenously (IV), promptly followed by intraperitoneal (IP) treatment with recombinant IFN-γ (rIFN-γ; 500 ng) and recombinant TNF (rTNF; 500 ng). In some experiments, mice were treated IP with neutralizing anti-TF mAb, 1H1, or subcutaneously (SC) with heparin (5,000 U/kg) 30 minutes before Con A challenge. At various time points after challenge, plasma and liver specimens were sampled.[16] Plasma alanine aminotransferase (ALT) and aspartate aminotransferase levels were measured (SRL, Osaka, Japan).

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction. We performed quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR), as shown in the Supporting Materials. RNA content was normalized based on amplification of 18S ribosomal RNA (rRNA) (18S).[17] Change folds = normalized data of experimental sample/normalized data of control.

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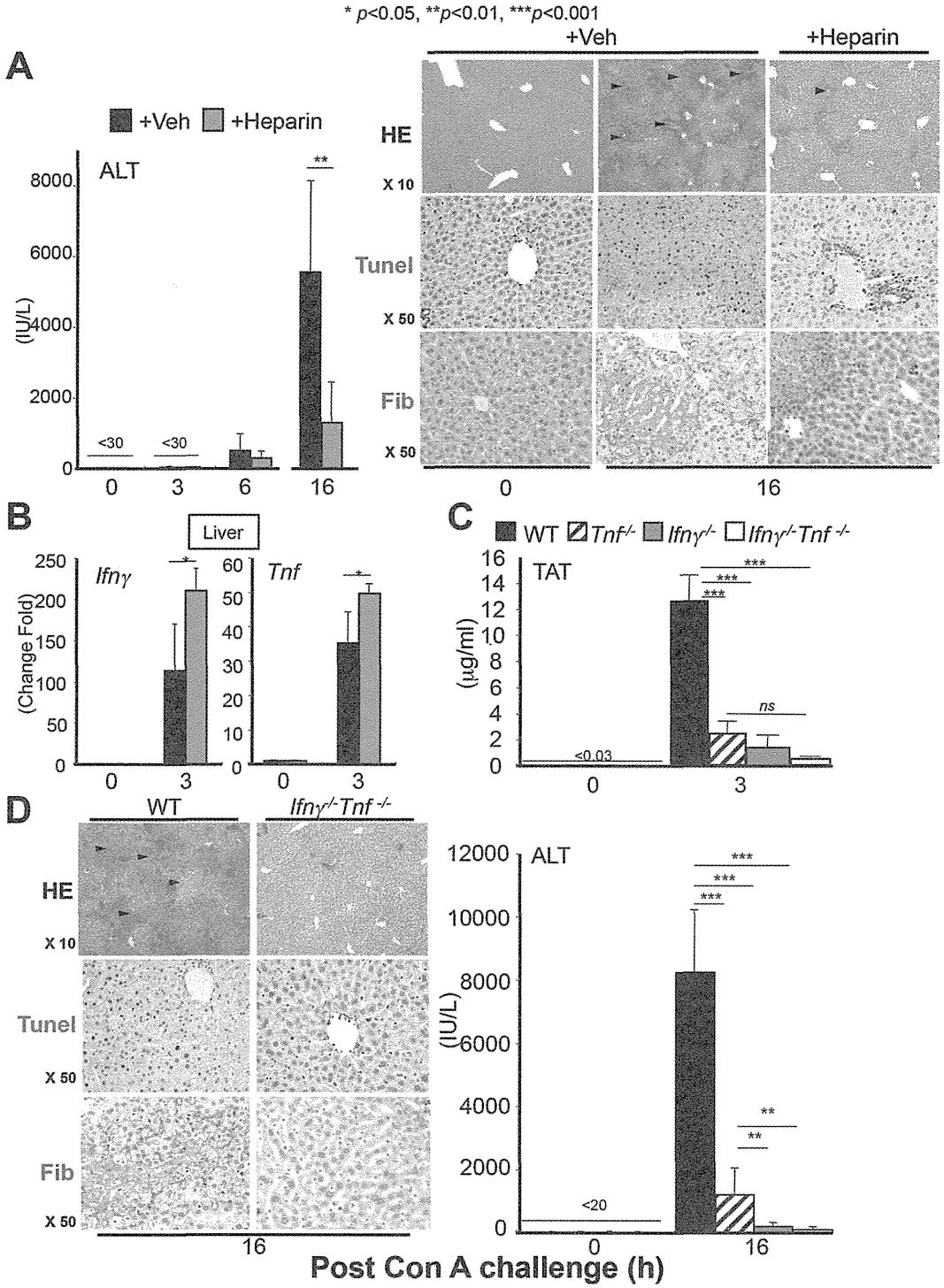

Fig. 1. Both IFN-γ and TNF are necessary for the development of thrombus-associated ALI. (A and B) WT mice were treated SC with heparin (red columns) or vehicle (Veh, closed columns), then with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of ALT (A) and hepatic *Ifnγ* (B) and *Tnf* (B), respectively. Fold increase of mRNA expression was calculated after normalization to 18S (B). Histological (H&E) and immunohistological study for TUNEL and fibrin deposition (A) were also performed. (C and D) WT (closed bars), $Tnf^{-/-}$ (hatched bars), $Ifn\gamma^{-/-}$ (gray bars), and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (open bars) were treated with Con A. At the indicated time point, plasma and liver specimens were sampled for measurement of TAT (C) and histological/immunohistological study, as shown in (B), respectively. Representative data are shown (A and D left panels). Original magnification, ×10 (A and D, left upper panels) and ×50 (A and D, left lower panels). Arrowheads indicated necrotic area.

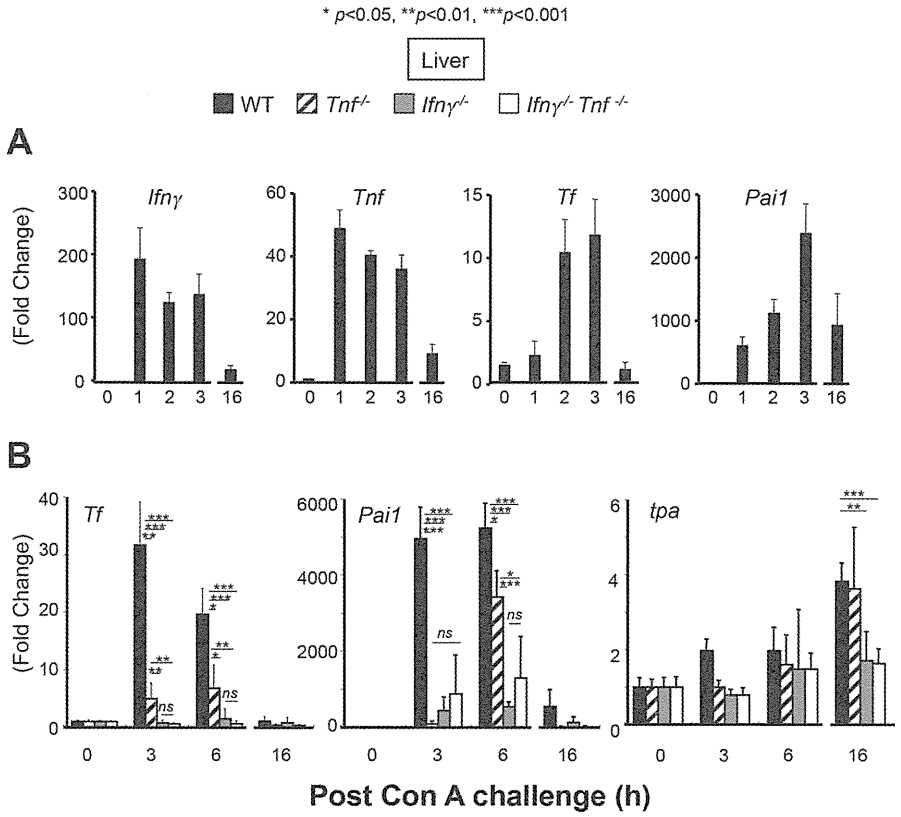

Fig. 2. Requirement of IFN-γ and TNF for the induction of hepatic *Tf* and *Pai1*. WT (closed bars), $Tnf^{-/-}$ (hatched bars), $Ifn\gamma^{-/-}$ (gray bars), or $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (open bars) were challenged IV with Con A, and their liver specimens were sampled for measurement of mRNA expression levels of IFN-γ (A), TNF (B), TF (A and B), PAI-1 (A and B), and tPA (B) by real-time qRT-PCR. Fold increase of mRNA expression was calculated after normalization to 18S.

Assay for Thrombin Antithrombin III Complex. Plasma levels of thrombin antithrombin III complex (TAT) were measured by commercially available kits for TAT (Enzyme Research Laboratories, South Bend, IN).[16]

Preparation of Liver Cells. Hepatic nonparenchymal cells from 3 mice were pooled.[15] $CD11b^+$ hepatic Mø and $CD146^+$ SECs were then enriched by magnetic-activated cell sorting (Miltenyi Biotec GmbH, Cologne, Germany) using anti-CD11b and anti-CD146 microbeads (Miltenyi Biotec), according to the manufacture's instruction, respectively. Stellate cells and liver parenchymal cells were prepared as described.[18,19]

Histological and Immunochemical Analyses. Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E).[2]

For detection of fibrin deposition, livers were perfused through a portal vein with PBS,[2] and liver specimens were rapidly sampled, fixed in 10% zinc fixative (Becton Dickinson, San Diego CA), and embedded in paraffin. Tissue sections were incubated overnight with rabbit antimouse fibrinogen antiserum (1:5,000) (Molecular Innovations, Inc., Novi, MI), followed by treatment with the rabbit Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA). Antigen-antibody (Ab) complexes were detected by using a DAB Substrate Kit (Vector Laboratories). Formalin-fixed liver sections were analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphage nick-end labeling (TUNEL) assay.[20]

In Vivo *Depletion of Mø.* Mø were depleted by the IV injection of clodronate liposome, as described previously.[14]

Mouse Reconstitution. To abolish irradiation-resistant Mø, we injected IV clodronate liposome into host mice and, 2 days later, irradiated them, followed by transfer of donor bone marrow (BM) cells.[21,22] CD45.1 WT mice were transferred with CD45.2 WT or CD45.2 $Stat1^{-/-}$ BM cells, and CD45.2 $Stat1^{-/-}$ mice were transferred with CD45.1 WT BM cells.[22] Two months later, the reconstituted mice were used.

Statistical Analyses. All data are shown as the mean ± standard deviation of samples in each experimental group. Five to seven mice were used for each experimental group. Significance between the experimental and control groups was examined by the unpaired Student *t* test. *P* values less than 0.05 were considered significant. Two to three experiments were

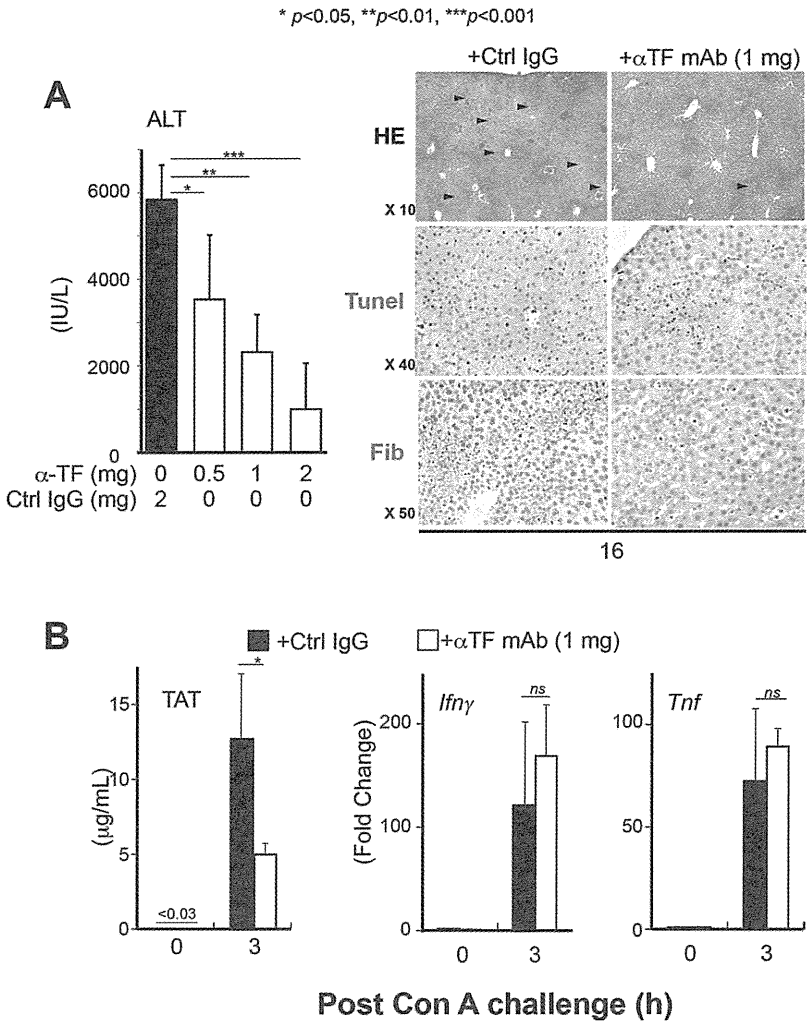

Fig. 3. TF is necessary for the development of Con A hepatitis. We administered various doses of neutralizing anti-TF mAb (open bars) or control rat IgG (Ctrl IgG) (closed bars) into WT mice 30 minutes before Con A challenge. At the indicated time points after Con A challenge, plasma and liver specimens were sampled for measurement of ALT (A) and TAT (B) as well as measurement of *Ifnγ* and *Tnf* expressions (B) and histological/immunohistological studies (A), respectively. Arrowheads indicated necrotic area.

separately performed, and representative data were shown.

Results

IFN-γ- and TNF-Dependent Hepatic Hypercoagulation Underlies Con A–Induced Hepatitis. We previously reported that by use of electron microscopy, many microthrombi, consisting of platelets, red blood cells, and fibrin deposits were observed in hepatic sinusoids of Con A–treated mice.[2] Immunohistochemistry (IHC) with antifibrinogen Abs further substantiated the dense fibrin deposition in the hepatic sinusoids (Fig. 1A, right middle lower panel). Pretreatment with the anticoagulant, heparin, protected against Con A–induced liver injuries with abundant TUNEL-positive hepatocytes and resulted in greatly reduced fibrin deposition (Fig. 1A). Consistent with our previous report,[2] heparin pretreatment did not down-regulate hepatic *Ifnγ* or *Tnf* (Fig. 1B). This is also true for interleukin-1β (*Il1β*), interleukin-6 (*Il6*), and CC chemokine ligand 2 (*Ccl2*) genes (Supporting Fig. 1). These results clearly indicated the importance of intrahepatic fibrin deposition for liver injury, and suggested that induction of these proinflammatory cytokines/chemokine was insufficient for the development of liver injuries in the absence of hepatic thrombosis.

Because plasma TAT is an excellent indicator of thrombin formation in the circulation,[16] we measured plasma TAT levels of Con A–challenged mice. Concomitant with dense fibrin deposition in the liver (Fig. 1A), plasma TAT levels were strongly elevated after challenge of WT mice with Con A, indicating that Con A treatment induced a systemic coagulation response along with hepatic hypercoagulation. Our previous report revealed that blockade of IFN-γ and

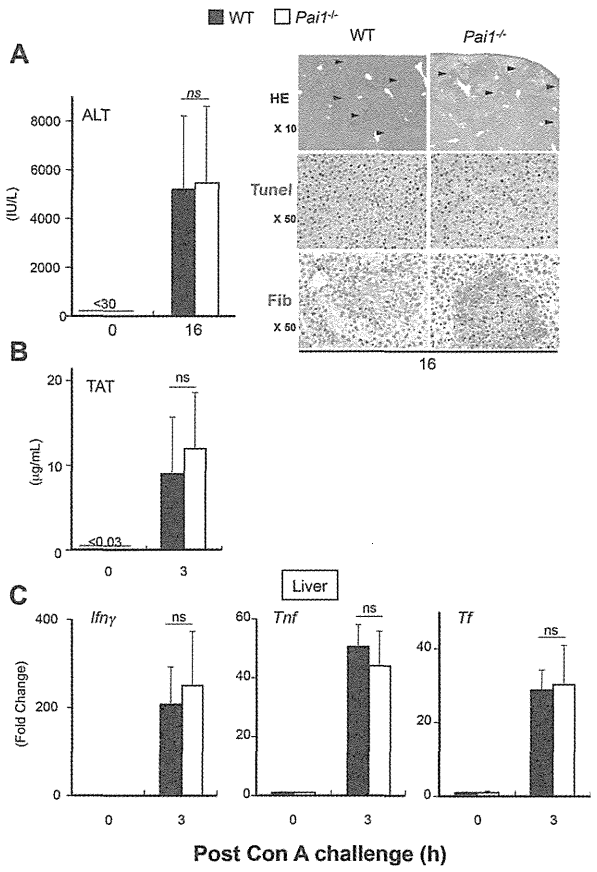

Fig. 4. Dispensability of PAI-1. WT mice (closed bars) or $Pai1^{-/-}$ mice (open bars) were challenged with Con A. At the indicated time points after Con A challenge, plasma and liver specimens were sampled, followed by the experiments, as shown in the legend to Fig. 3. Arrowheads indicated necrotic area.

TNF reduced hepatic coagulation response.[2] The involvement of IFN-γ and TNF was examined in more detail by using knockout (KO) mice, including single- and double-KO mice. Expectedly, $Ifn\gamma^{-/-}$, $Tnf^{-/-}$, and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice had lower TAT elevation than WT mice (Fig. 1C). This clearly indicated the requirement of IFN-γ and TNF for the Con A–induced hypercoagulation response. In agreement, $Ifn\gamma^{-/-}Tnf^{-/-}$ mice lacked fibrin deposition and were protected from Con A–induced hepatitis (Fig. 1D). $Ifn\gamma^{-/-}$ mice, like $Ifn\gamma^{-/-}Tnf^{-/-}$ mice, were free from liver injury, whereas $Tnf^{-/-}$ mice showed only partial reduction of liver injury (Fig. 1D, right panel), suggesting that endogenous IFN-γ is more important than TNF for promoting liver injury. In contrast, $Ifn\gamma^{-/-}Tnf^{-/-}$ mice showed significantly reduced, but still substantial induction of, *Il1β*, *Il6*, and *Ccl2* (Supporting Fig. 2). Taken together, these results demonstrated that both IFN-γ and TNF are important initiators in the development of massive liver necrosis, which is mediated by the induction of intrahepatic hypercoagulation.

***Requirement of IFN-γ and TNF for Hepatic Induction of* Tf *and* Pai1.** Next, we investigated how IFN-γ and/or TNF contributed to hepatic thrombosis. Because TF and PAI-1 were reported to induce the prothrombotic state,[5,9] we measured both *Tf* and *Pai1* levels in livers of Con A–challenged WT mice. Hepatic *Tf* levels started to increase at 2 hours, with a peak at 3 hours after Con A challenge (Fig. 2A,B). Hepatic *Pai1* levels began to increase at 1 hour and peaked at approximately 3-6 hours (Fig. 2A,B). Intriguingly, both *Ifnγ* and *Tnf* levels increased immediately after Con A challenge, and the peaks of *Ifnγ* and *Tnf* preceded those of *Tf* and *Pai1* (Fig. 2A). In sharp contrast to WT mice, $Ifn\gamma^{-/-}$ mice showed no increase in *Tf* and only little increase in *Pai1* levels (Fig. 2B), indicating the importance of IFN-γ for the induction of both *Tf* and *Pai1*. This was also the case for $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (Fig. 2B). $Tnf^{-/-}$ mice showed poor induction of *Tf* and *Pai1* as well, but their levels were significantly higher than those of $Ifn\gamma^{-/-}$ mice and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (Fig. 2B). Compared to *Tf* and *Pai1*, the Con A–mediated increase of messenger RNA (mRNA) levels of tPA, a target protease of PAI-1, were much less pronounced and peaked at a much later time point in WT mice (Fig. 2B). In addition, *tpa* levels were only slightly reduced in $Ifn\gamma^{-/-}$, $Tnf^{-/-}$, and $Ifn\gamma^{-/-}Tnf^{-/-}$ mice (Fig. 2B). These results strongly suggested that Con A stimulates hepatic T cells to produce both IFN-γ and TNF, which then induce the expression of hepatic *Tf* and *Pai1*.

Importance of TF, but Not PAI-1, for Liver Injuries. To examine the respective roles of TF and PAI-1 for the hypercoagulation response, we determined the effects of Con A treatment in WT mice pretreated with an anti-TF mAb and in $Pai1^{-/-}$ mice. Compared to mice receiving control rat IgG, treatment with the neutralizing anti-TF mAb, 1H1, just before Con A challenge reduced ALT plasma levels and fibrin deposition in a concentration-dependent manner (Fig. 3A). This indicated the importance of TF in mediating liver injury. Notably, TF blockade protected against plasma elevation of TAT without affecting hepatic *Ifnγ*, *Tnf*, *Il1β*, *Il6*, and *Ccl2* inductions (Fig. 3B and Supporting Fig. 3). In contrast, $Pai1^{-/-}$ mice underwent massive liver injuries similar to WT mice in respect to hepatic fibrin deposition, plasma TAT elevation and induction of hepatic *Ifnγ*, *Tnf*, and *Tf* (Fig. 4). These results demonstrated a pivotal role for TF, but not PAI-1, in hypercoagulation response and the development of liver injuries.

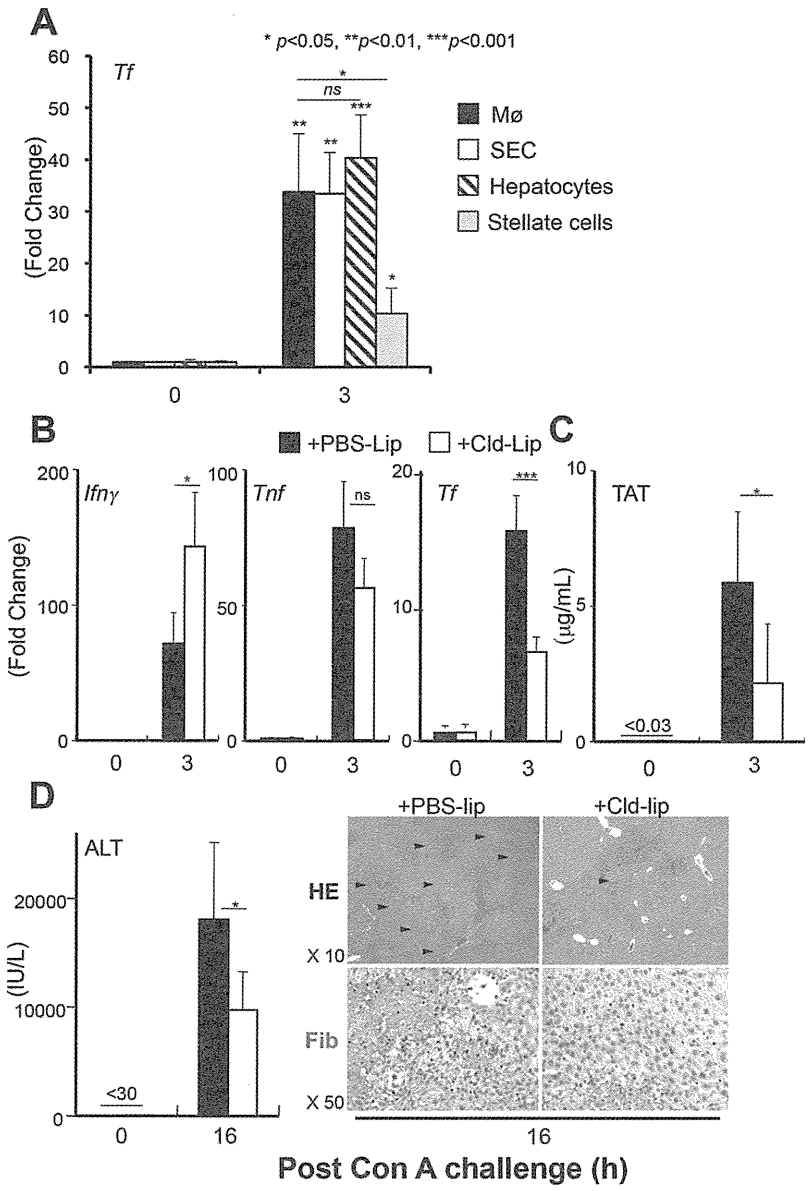

Fig. 5. Importance of macrophages for hepatic fibrin deposition. (A) Hepatic Mø (closed bars), SECs (open bars), hepatocytes (hatched bars), and stellate cells (gray bars) were isolated from WT mice at the indicated time points after Con A challenge. *Tf* was measured by qRT-PCR (A). (B-D) WT mice, having received clodronate liposome (Cld-lip) or control PBS liposome (PBS-lip), were challenged with Con A. At the indicated time points after Con A challenge, plasma and liver specimens were sampled, followed by the method shown in the legend to Fig. 1. Arrowheads indicated necrotic area.

***Liver Cells Both Inside and Outside of the Sinusoid Expressed* Tf.** Various cell types are localized within the hepatic sinusoid, such as SECs and liver Mø, including Kupffer cells. To identify the cell types that expressed TF mRNA upon Con A challenge, we isolated hepatic $CD11b^+$ Mø and $CD146^+$ SECs from Con A–treated mice and measured *Tf* expression. Both Mø and SECs prepared from livers of mice at 3 hours after Con A challenge showed a remarkable increase in *Tf* expression levels, as compared to naïve mice (Fig. 5A). Furthermore, cells outside of the sinusoid, such as hepatocytes and stellate cells, also increased the expression of *Tf* after Con A challenge (Fig. 5A). These results suggested that TF on Mø and SECs directly triggered the coagulation cascade within the hepatic sinusoid.

To analyze the roles of Mø in liver thrombosis, we generated Mø-depleted mice by injection of clodronate liposome.[15] Upon Con A challenge, Mø-depleted WT mice displayed significant diminution in hepatic *Tf* induction without reduction in hepatic *Ifnγ* and *Tnf* induction, as compared to PBS liposome-pretreated control mice (Fig. 5B). This suggested that the impaired TF induction was not attributed to the impaired induction of the upstream cytokines, but was rather the result of a decrease in the number of

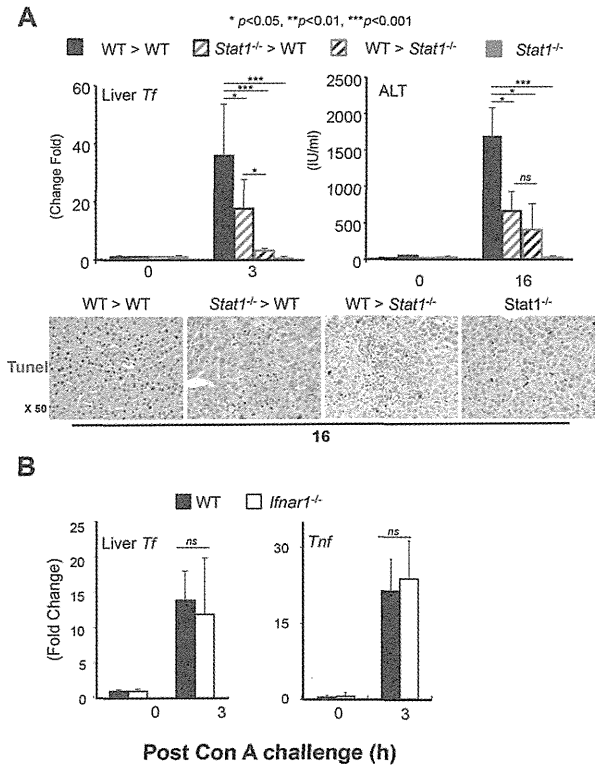

Fig. 6. A pivotal role of IFN-γ/STAT1 signaling in both hematopoietic and nonhematopoietic cells in Con A hepatitis. (A) Host mice were treated with clodronate liposome 2 days before reconstitution. After irradiation, host CD45.1 B6 WT received BM cells from congenic B6 CD45.2 WT (WT>WT) or *Stat1*$^{-/-}$ CD45.2 B6 mice (*Stat1*$^{-/-}$>WT). Irradiated *Stat1*$^{-/-}$ B6 CD45.2 mice received BM cells from CD45.1 B6 WT (WT>*Stat1*$^{-/-}$). *Stat1*$^{-/-}$ mice were used as well. The reconstituted mice were challenged with Con A. At the indicated time points, plasma and liver specimens were sampled for measurement of hepatic *Tf*, TUNEL assay, and plasma ALT levels. (B) WT mice (closed bars) or *Ifnar1*$^{-/-}$ mice (open bars) were challenged with Con A. *ns*, not significant.

TF-expressing cells. Furthermore, Mø-depleted mice showed impairment in plasma TAT increase, hepatic fibrin deposition, and liver injuries (Fig. 5C,D). The findings suggested that Mø were an important cellular source of functional TF.

Requirement of IFN-γ/STAT1 Signaling in Both Mø and SECs for the Hepatic* Tf *Induction. Because endogenous IFN-γ appeared more important than TNF for hepatic *Tf* induction (Fig. 2B), we further investigated the IFN-γ signaling pathway in liver cells (Fig. 6A). *Stat1*$^{-/-}$ mice, like *Ifnγ*$^{-/-}$ mice (Fig. 1D), showed a strongly impaired hepatic *Tf* induction and completely evaded Con A hepatitis (Fig. 6A), indicating the importance of the IFN-γ/STAT1-signaling pathway for these events. To exclude the possible involvement of type I IFN-mediated STAT1 signaling, we carried out experiments with mice deficient in the receptor for type I IFN, IFNAR. *Ifnar*$^{-/-}$ mice displayed healthy hepatic induction of *Tf* and *Tnf* (Fig. 6B), indicating that STAT1-mediated *Tf* up-regulation is not dependent on type I IFN. Next, we examined whether hepatic Mø or nonhematopoietic liver cells, including SECs, hepatocytes, and stellate cells, were responsible for STAT1-dependent *Tf* expression. We generated reciprocal BM chimeric mice by using WT and *Stat1*$^{-/-}$ mice. Mø are somewhat irradiation resistant. To improve depletion of host Mø, we pretreated host mice with clodronate liposome before reconstitution.[22] WT mice reconstituted with WT hematopoietic cells (control mice) showed *Tf* induction in their livers after Con A challenge (Fig. 6A). WT mice transferred with *Stat1*$^{-/-}$ BM cells exhibited partly impaired induction of *Tf*, as compared to the control mice (Fig. 6A). *Stat1*$^{-/-}$ mice reconstituted with WT hematopoietic cells showed further reduction in *Tf* induction, as compared to *Stat1*$^{-/-}$ mice receiving WT BM cells (Fig. 6A). IHC with antiphosphorylated STAT1 mAb revealed its nuclear localization in the corresponding WT Mø and nonhematopoietic liver cells of the chimeric mice (Supporting Fig. 4). Thus, the *Tf* inductions in Mø and nonhematopoietic liver cells were largely dependent on STAT1. WT mice transferred with *Stat1*$^{-/-}$ hematopoietic cells and *Stat1*$^{-/-}$ mice with WT BM cells both developed significantly mild liver injuries, compared to control mice (Fig. 6A). Intriguingly, severities of the liver injuries were comparable between these two types of chimeric mice (Fig. 6A). *Stat1*$^{-/-}$ mice reconstituted with *Stat1*$^{-/-}$ BM cells exhibited the phenotypes equivalent to *Stat1*$^{-/-}$ mice (data not shown). Collectively, these results strongly indicated that the IFN-γ/STAT1 signalings in both hematopoietic Mø and nonhematopoietic liver cells are equally important for the development of Con A hepatitis.

Con A Signaling in Non-T Non-B Cells Collaborates With IFN-γ and TNF Signaling in Thrombosis-Mediated Liver Injury. T cells have been documented to be essential for Con A hepatitis.[1] In agreement, *Rag2*$^{-/-}$ mice lacking T and B cells did not show hepatic *Tf* induction, elevation of plasma TAT concentrations, or liver damage after Con A challenge (Fig. 7A). T cells, including natural killer T cells, are necessary for the production of IFN-γ and TNF.[2,23] Both *Ifnγ* and *Tnf* inductions were absent in the liver of Con A–challenged *Rag2*$^{-/-}$ mice (Supporting Fig. 5). Because both IFN-γ and TNF mediate hypercoagulation and liver injury (Fig. 1C,D), we hypothesized that T cells may contribute to liver damage by producing IFN-γ and TNF. To test this possibility, we

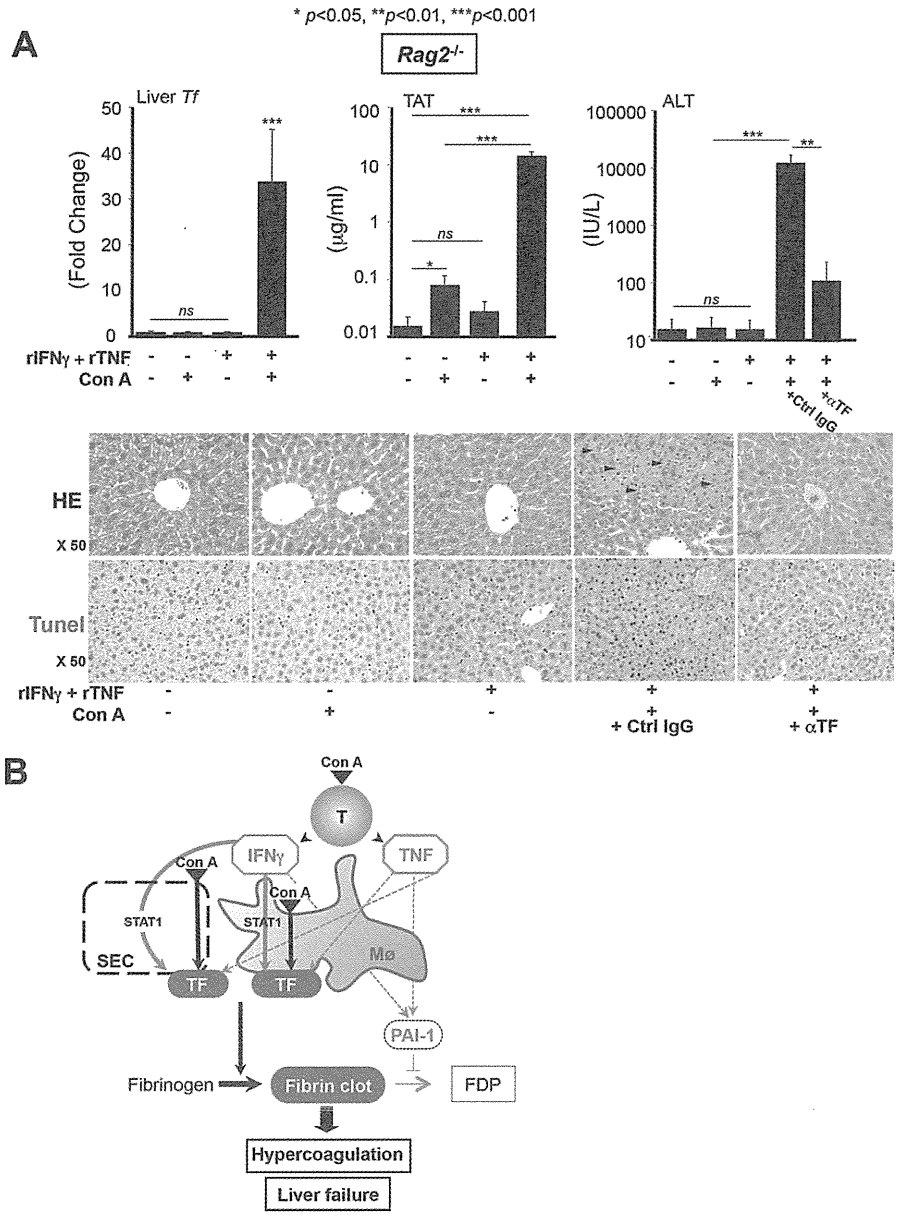

Fig. 7. IFNγ, TNF, and Con A signalings, likely in hepatic Mø and SECs, are necessary and sufficient for hepatitis involving hypercoagulation. (A) *Rag2*$^{-/-}$ mice were treated with rIFN-γ plus rTNF, Con A or Con A plus rIFNγ plus rTNF, or, additionally, with control rat IgG (Ctrl IgG) or neutralizing anti-TF mAb (αTF). At 3 hours, plasma and liver specimens were sampled for measurement of TAT and *Tf* expression, respectively. At 9 hours, plasma and liver specimens were sampled for measurement of ALT and histological study, respectively. *ns*, not significant. Arrowheads indicated necrotic area. (B) A proposal model for Con A–induced liver injury. Upon Con A challenge, T cells produce IFN-γ and TNF, which, together with Con A, cooperatively stimulate sinusoidal Mø and SECs to produce TF in a STAT1-dependent manner. TF then starts to rapidly and aberrantly activate the coagulation cascade to generate hepatic sinusoidal thrombus, eventually leading to massive liver injuries.

administered rIFN-γ plus rTNF into *Rag2*$^{-/-}$ mice and challenged them with Con A. This treatment resulted in hepatitis accompanied by considerable hepatic *Tf* induction, along with *Il1β*, *Il6*, and *Ccl2* inductions (Supporting Fig. 6) and a strong increase in plasma TAT levels (Fig. 7A). Notably, TF blockade protected against the elevation of plasma TAT levels and the liver injuries in Con A plus rIFN-γ/rTNF-treated *Rag2*$^{-/-}$ mice (Fig. 7A and Supporting Fig. 7). However, in the absence of Con A, treatment with IFN-γ and TNF alone could not induce any of those alterations (Fig. 7A). Thus, in addition to signals elicited by IFN-γ and TNF, Con A signaling in the cells of *Rag2*$^{-/-}$ mice, likely mediated by hepatic Mø and SECs, was required for the development of thrombosis-mediated liver injuries.

Discussion

Results presented here demonstrate that both endogenous IFN-γ and TNF are essential for the development of Con A–induced liver injuries through the induction of TF-dependent coagulation. In particular, the IFN-γ/STAT1-signaling pathway, in both hepatic

Mø and SECs, was directly critical for the development of hypercoagulation and resultant acute liver injuries (ALIs). However, exogenous or endogenous IFN-γ and TNF were not sufficient to induce the hypercoagulation response or liver injuries. However, exogenous IFN-γ and TNF rendered *Rag2*$^{-/-}$ mice highly susceptible to Con A treatment, suggesting that Con A, IFN-γ, and TNF act in concert on hepatic Mø and SECs to elicit a procoagulant response. Based on these results, we propose a model of Con A–induced acute liver damage (illustrated in Fig. 7B). After stimulation with Con A, T cells produce IFN-γ and TNF. In hepatic Mø and SECs within the sinusoid, the cellular signaling pathways initiated by Con A, TNF, and IFN-γ through STAT1 activation synergize to elicit a robust expression of TF. TF then activates the coagulation system, leading to hepatic fibrin deposition and liver injury.

The IFN-γ/STAT1-mediated signaling in SECs is important for Con A–induced liver injury. Mø-depleted *Stat1*$^{-/-}$ mice reconstituted with WT Mø showed reduction in hepatic *Tf* induction and evaded Con A–induced liver injury. This suggested that IFN-γ/STAT1 induction of TF in SECs might evoke the hypercoagulation response relevant to the liver injury. A recent report verified a crucial role of endogenous IFN-γ in SEC damage of Con A–treated mice.[24] SEC damage has been believed to be a potent inducer of intrahepatic coagulation.[25] Therefore, IFN-γ/STAT1-mediated induction of TF in SECs may contribute to intrahepatic coagulation within the context of IFN-γ/STAT1-mediated cellular damage.

Under normal conditions, hepatocytes and stellate cells are anatomically segregated from the sinusoid. However, IFN-γ induction of SEC damage allows them to be exposed to the sinusoidal circulation, which might facilitate thrombosis. Thus, IFN-γ/STAT1-mediated induction of TF in hepatocytes and stellate cells might amplify procoagulant response.

Con A is a well-known T-cell mitogen, suggesting an important role of T cells in Con A–induced hypercoagulation. However, our present results verified the replacement of T cells by IFN-γ/TNF and the importance of Con A signaling in non-T cells, presumably exemplified by hepatic Mø and SECs. We are currently investigating the signaling pathway of Con A in Mø and SECs.

Thrombin-cleaved osteopontin was shown to be involved in this type of hepatitis.[23,26] These reports are consistent with the view that hepatic thrombosis is an essential contributor to Con A–induced liver injuries, at least through the induction of the thrombin-cleaved form of osteopontin and, perhaps, hepatic microcirculatory disturbance.

Mouse hepatitis virus infection is associated with intrahepatic thrombosis.[27,28] Patients with chronic hepatitis C show increase in plasma TF levels, whereas those with viral clearance by IFN-α therapy, such as healthy controls, do not.[29] Furthermore, there is a growing recognition of the role of hypercoagulation in chronic liver injury and fibrosis.[30] Although our current study was focused on ALI, the similar mechanism likely underlies acute and chronic viral hepatitis and fibrosis.

In summary, our present study demonstrates that IFN-γ/STAT1-mediated signaling in hepatic various cells, including Mø and SECs, is the underlying mechanism of Con A–induced aberrant activation of coagulation resulting in massive liver necrosis. In hepatic Mø and liver sinusoid, signaling through the IFN-γ/STAT1 pathway induced expression of *Tf*, which, in conjunction with IFN-γ/STAT1-mediated damage to the endothelium, triggered the coagulation reactions. The resulting formation of extensive microthrombi induced microcirculatory disturbances and hepatic inflammation involving thrombin-mediated conversion of precursor proteins, eventually leading to massive liver necrosis. It is conceivable that similar mechanisms are driving the progression of lethal fulminant hepatitis. Although our study did not formally address this question, the findings presented here may incite future studies to investigate this possibility and, perhaps, lead to novel therapeutic approaches.

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LecT-Hepa, a Glyco-Marker Derived from Multiple Lectins, as a Predictor of Liver Fibrosis in Chronic Hepatitis C Patients

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Assessment of liver fibrosis in patients with chronic hepatitis C (CHC) is critical for predicting disease progression and determining future antiviral therapy. LecT-Hepa, a new glyco-marker derived from fibrosis-related glyco-alteration of serum alpha 1-acid glycoprotein, was used to differentiate cirrhosis from chronic hepatitis in a single-center study. Herein, we aimed to validate this new glyco-marker for estimating liver fibrosis in a multicenter study. Overall, 183 CHC patients were recruited from 5 liver centers. The parameters *Aspergillus oryzae* lectin (AOL) / *Dature stramonium* lectin (DSA) and *Maackia amurensis* lectin (MAL)/DSA were measured using a bedside clinical chemistry analyzer in order to calculate LecT-Hepa levels. The data were compared with those of seven other noninvasive biochemical markers and tests (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, aspartate aminotransferase-to-platelet ratio index [APRI], Forns index, Fib-4 index, and Zeng's score) for assessing liver fibrosis using the receiver-operating characteristic curve. LecT-Hepa correlated well with the fibrosis stage as determined by liver biopsy. The area under the curve (AUC), sensitivity, and specificity of LecT-Hepa were 0.802, 59.6%, and 89.9%, respectively, for significant fibrosis; 0.882, 83.3%, and 80.0%, respectively, for severe fibrosis; and 0.929, 84.6%, and 88.5%, respectively, for cirrhosis. AUC scores of LecT-Hepa at each fibrosis stage were greater than those of the seven aforementioned noninvasive tests and markers. *Conclusion*: The efficacy of LecT-Hepa, a glyco-marker developed using glycoproteomics, for estimating liver fibrosis was demonstrated in a multicenter study. LecT-Hepa given by a combination of the two glyco-parameters is a reliable method for determining the fibrosis stage and is a potential substitute for liver biopsy. (HEPATOLOGY 2012;56:1448-1456)

Accurate staging of hepatic fibrosis in patients with chronic hepatitis C (CHC) is most important for predicting disease progression and determining the need for initiating antiviral therapy, such as interferon (IFN) therapy.[1,2] Liver biopsy has been considered the gold standard for fibrosis staging for many years.[3] However, liver biopsy is invasive and painful,[4,5] with rare but potentially life-threatening complications.[6] In addition, this method may suffer from sampling errors since only 1/50,000 of the organ is examined.[7] Furthermore, inter- and intraobserver discrepancies reaching levels of 10% to 20% have been

Abbreviations: α2-MG, α2-macroglobulin; AFP, alpha-fetoprotein; AGP, alpha-1 acid glycoprotein; ALT, alanine aminotransferase; AOL, Aspergillus oryzae *lectin; CHC, chronic hepatitis C; DSA,* Datura stramonium *lectin; GGT, gamma-glutamyltransferase; HA, hyaluronic acid; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MAL,* Maackia amurensis *lectin; TIMP1, tissue inhibitors of metalloproteinases 1.*

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reported using this method, leading to misdiagnosis of cirrhosis.[8] Therefore, finding a noninvasive method for diagnosing liver fibrosis is an emerging issue in the care of patients with CHC.

Several methods have been studied for the noninvasive diagnosis of hepatic fibrosis or cirrhosis, including clinical[9] or blood markers,[10,11] and signal analysis (ultrasonography, magnetic resonance imaging, and elastography).[12,13] Although each method can play a substantial role in the diagnosis of cirrhosis, it is evident that the best way of monitoring hepatitis progression employs an accurate serological method for the quantitative evaluation of fibrosis. We developed a new glyco-marker using multiple lectins that performed well in estimating liver fibrosis in a single-center study.[14,15]

Recent progress in glycoproteomics has had a great influence on work toward ideal, disease-specific biomarkers for a number of conditions. Glycoproteins that exhibit disease-associated glyco-alteration and are present in serum or other fluids have the potential to act as biomarkers for the diagnosis of a target disease,[16] because the features of glycosylation depend on the extent of cell differentiation and the stage of the cell. Detecting hepatic disease-associated glyco-markers for clinical applications has been a continuous challenge since the early 1990s, because increased fucosylation on complex-type *N*-glycans has been frequently detected in glycoproteins from patients with hepatocellular carcinoma (HCC) and cirrhosis.[17,18] Of all the alpha-fetoprotein (AFP) glycoforms, more than 30% have been found to react to a fucose-binding lectin, *Lens culinaris* agglutinin. This fraction, designated AFP-L3, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the diagnosis and prognosis of HCC.[19] We have found that two fibrosis-indicator lectins (*Aspergillus oryzae* lectin [AOL] and *Maackia amurensis* lectin [MAL]) together with an internal, standard lectin (*Datura stramonium* lectin [DSA]) on an alpha 1-acid glycoprotein (AGP) could, using lectin microarray, clearly distinguish between cirrhosis and chronic hepatitis patients.[14] We have further simplified this quantitative method so that it could be performed using bedside, clinical chemistry analyzers.[15]

The aim of the current study was to evaluate this new glyco-marker (LecT-Hepa) using multiple lectins and bedside clinical chemistry analyzers for use in the assessment of liver fibrosis. In this multicenter study we compared the method's efficiency in estimating liver fibrosis with other noninvasive fibrosis markers and tests.

Materials and Methods

Study Population. This study included 183 consecutive adult patients with CHC who had undergone percutaneous liver biopsy at one of the following institutions: Hokkaido University Hospital, Musashino Red Cross Hospital, National Center for Global Health and Medicine, Hyogo College of Medicine Hospital, or Nagoya City University Hospital in Japan. A diagnosis of CHC was defined as detectable serum anti-hepatitis C virus (HCV) antibody and HCV-RNA, found using polymerase chain reaction assays, of at least 2 points. Exclusion criteria were coinfection with hepatitis B virus or human immunodeficiency virus (HIV), and other disorders that commonly cause liver diseases. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by our Institutional Review Board.

Histological Staging. Ultrasonography-guided liver biopsy was performed according to a standardized protocol. Specimens were fixed, paraffin-embedded, and stained with hematoxylin-eosin and Masson's trichrome. A minimum of six portal tracts in the specimen were required for diagnosis. All liver biopsy samples were independently evaluated by two senior pathologists who were blinded to the clinical data. Liver fibrosis stages were assessed using METAVIR fibrosis (F) staging.[20] Significant fibrosis was defined as METAVIR F $\geq$2, severe fibrosis as METAVIR F $\geq$3, and cirrhosis as METAVIR F4. Two patients were excluded from the study because of inadequate histological samples.

Clinical and Biological Data. The age and sex of the patients were recorded. Serum samples were collected immediately before or no more than 2 months

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