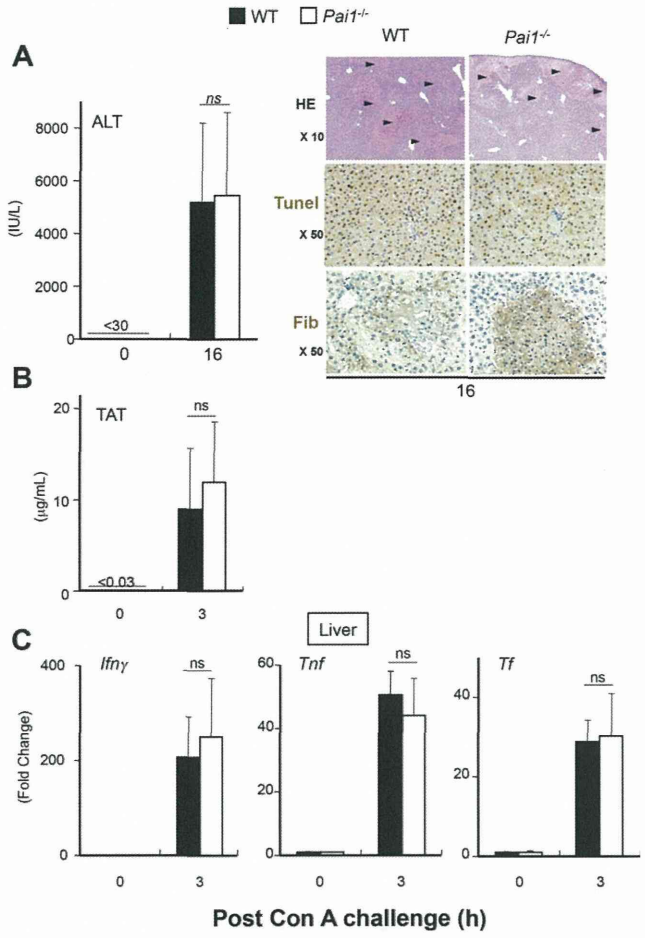

Fig. 4. Dispensability of PAI-1. WT mice (closed bars) or *Pai1*$^{-/-}$ mice (open bars) were challenged with Con A. At the indicated time points after Con A challenge, plasma and liver specimens were sampled, followed by the experiments, as shown in the legend to Fig. 3. Arrowheads indicated necrotic 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, *Ifnγ*$^{-/-}$, *Tnf*$^{-/-}$, and *Ifnγ*$^{-/-}$*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, *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice lacked fibrin deposition and were protected from Con A–induced hepatitis (Fig. 1D). *Ifnγ*$^{-/-}$ mice, like *Ifnγ*$^{-/-}$*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, *Ifnγ*$^{-/-}$ *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, *Ifnγ*$^{-/-}$ 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 *Ifnγ*$^{-/-}$*Tnf*$^{-/-}$ mice (Fig. 2B). *Tnf*$^{-/-}$ mice showed poor induction of *Tf* and *Pai1* as well, but their levels were significantly higher than those of *Ifnγ*$^{-/-}$ mice and *Ifnγ*$^{-/-}$ *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 *Ifnγ*$^{-/-}$, *Tnf*$^{-/-}$, and *Ifnγ*$^{-/-}$*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.

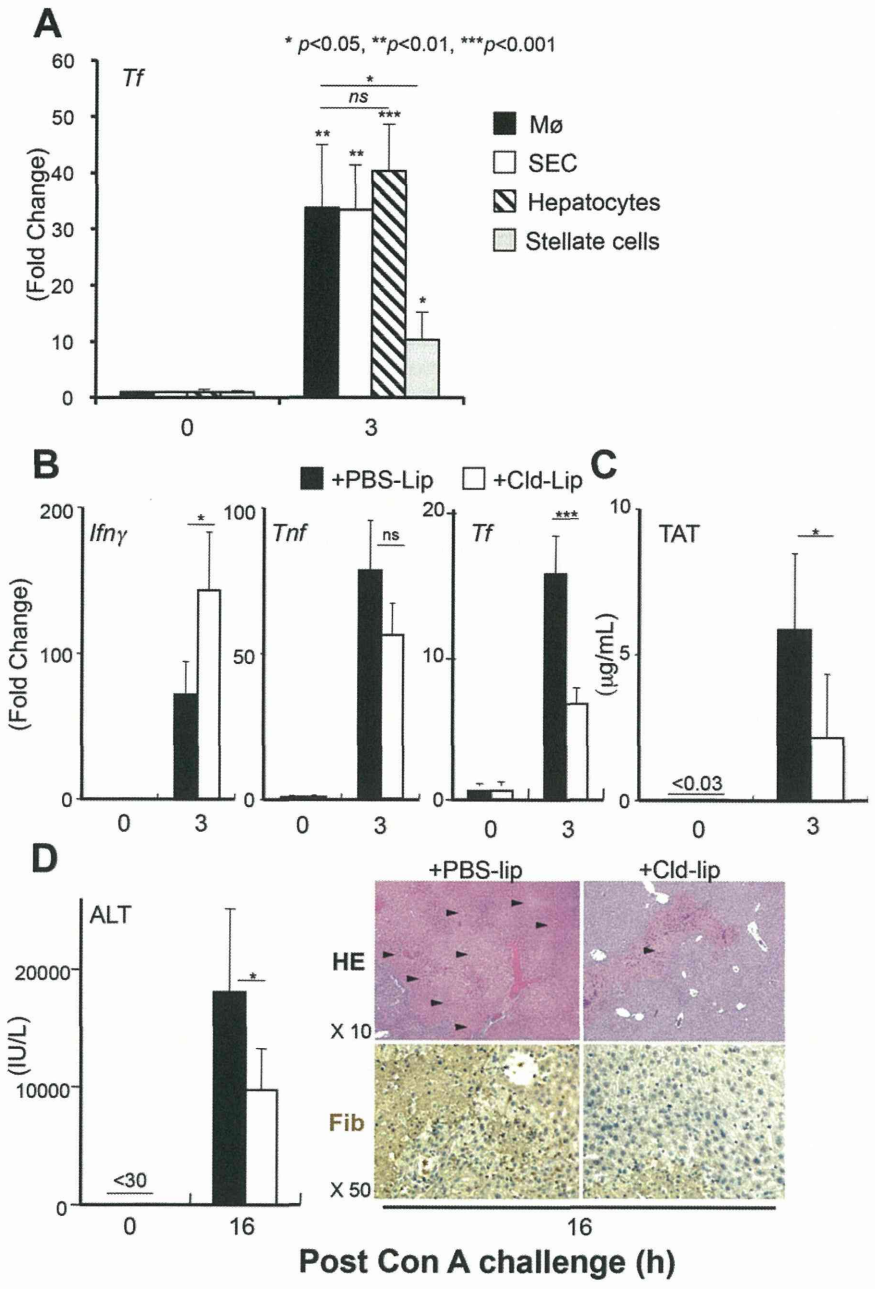

Fig. 5. Importance of macrophages for hepatic fibrin deposition. (A) Hepatic Mø (closed bars), SECs (open bars), hepatocytes (hatched bars), and stellate cells (gray bars) were isolated from WT mice at the indicated time points after Con A challenge. *Tf* was measured by qRT-PCR (A). (B-D) WT mice, having 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

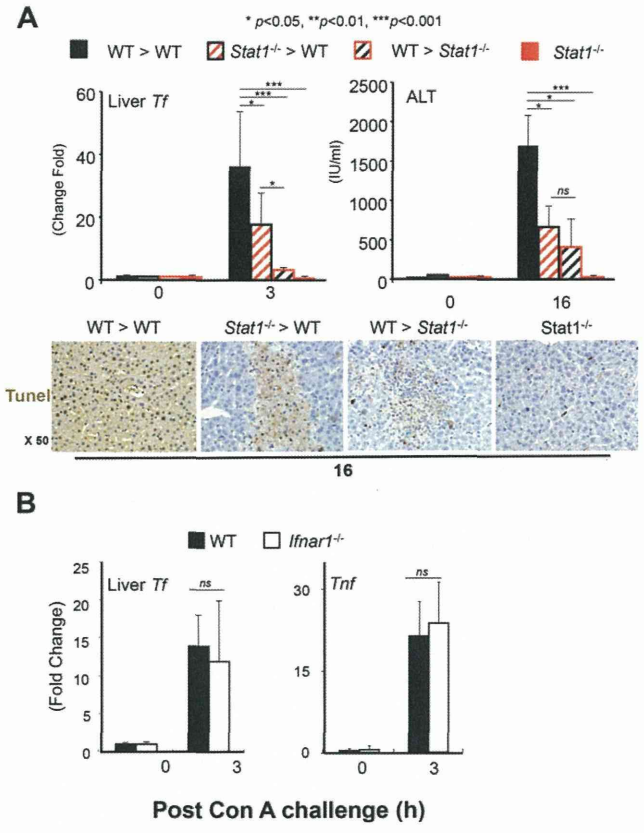

Fig. 6. A pivotal role of IFN-γ/STAT1 signaling in both hematopoietic and nonhematopoietic cells in Con A hepatitis. (A) Host mice were treated with clodronate liposome 2 days before reconstitution. After irradiation, host CD45.1 B6 WT received BM cells from congenic B6 CD45.2 WT (WT>WT) or *Stat1*$^{-/-}$ 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

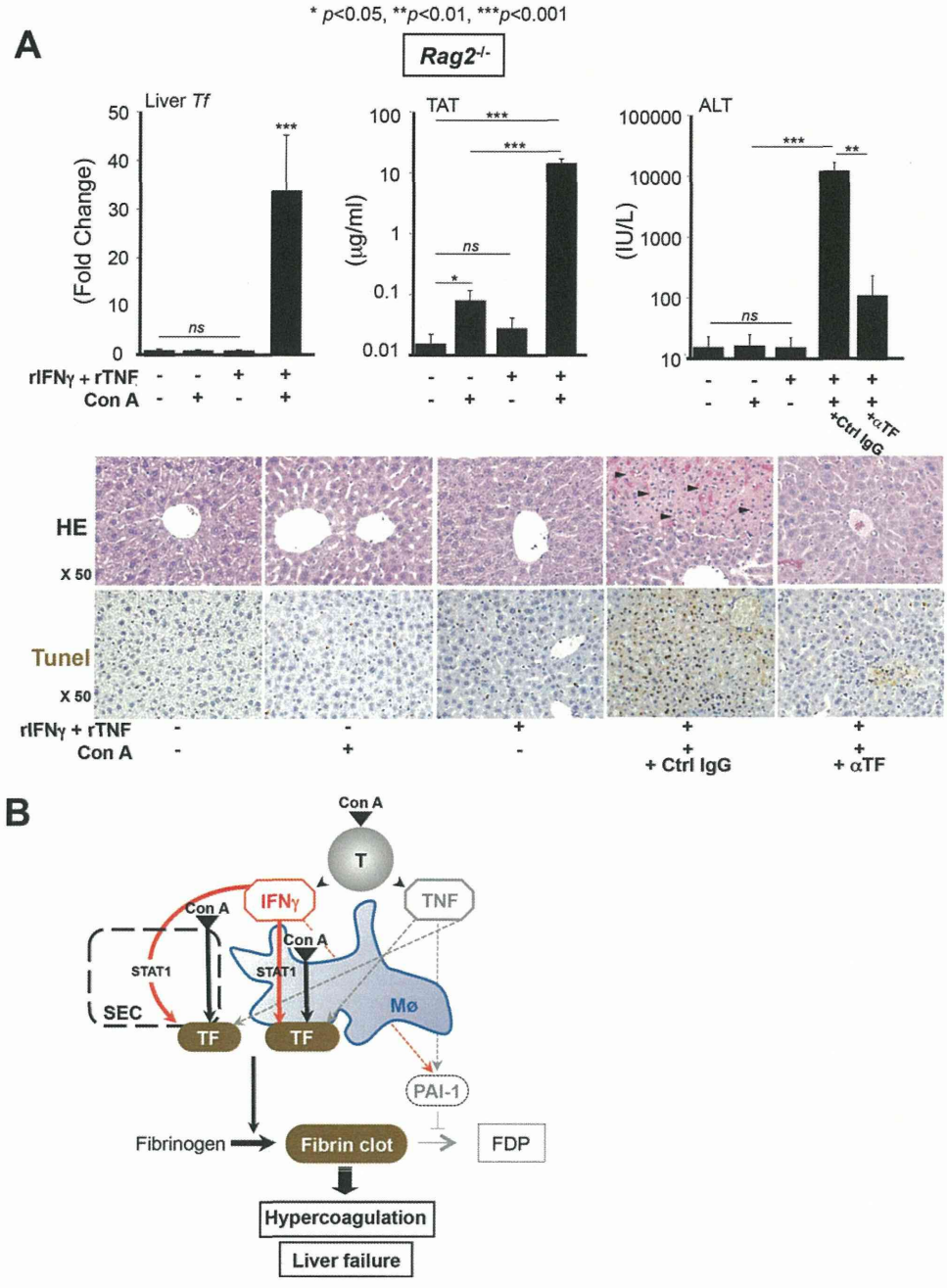

Fig. 7. IFNγ, TNF, and Con A signalings, likely in hepatic Mø and SECs, are necessary and sufficient for hepatitis involving hypercoagulation. (A) *Rag2*$^{-/-}$ mice were treated with rIFN-γ plus rTNF, Con A or Con A plus rIFNγ plus rTNF, or, additionally, with control rat IgG (Ctrl IgG) or neutralizing 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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BRIEF ARTICLE

Hyperglycemia is a significant prognostic factor of hepatocellular carcinoma after curative therapy

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Author contributions: Hosokawa T and Kurosaki M contributed equally to this work; Kurosaki M and Izumi N made substantial contributions to the conception and design of the study; Tsuchiya K, Matsuda S, Muraoka M, Suzuki Y, Tamaki N, Yasui Y, Nakata T, Nishimura T, Suzuki S, Ueda K, Nakanishi H, Itakura J, Takahashi Y and Izumi N collected the clinical data; Hosokawa T and Kurosaki M contributed to the analysis and interpretation of the data; Hosokawa T wrote the draft of the manuscript; Kurosaki M and Izumi N made critical revisions of the manuscript; and Izumi N obtained a research fund.

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Abstract

AIM: To evaluate whether metabolic factors are related to distant recurrence of hepatocellular carcinoma (HCC) and survival after curative treatment.

METHODS: This retrospective study included 344 patients whose HCC was treated curatively by radiofrequency ablation (RFA) therapy. The mean age was 67.6 years and the mean observation period was 4.04 years. The etiological background of liver disease was hepatitis B virus infection in 30, hepatitis C virus infection in 278, excessive alcohol drinking in 9, and other in 27 patients. The Child-Pugh classification grade was A (n = 307) or B (n = 37). The number of HCC nodules was one in 260, two in 61, and three in 23 patients. For surveillance of HCC recurrence after curative therapy with RFA, patients were radiologically evaluated every 3 mo. Factors associated with distant recurrence of HCC or survival were studied.

RESULTS: Inadequate maintenance of blood glucose in diabetic patients was associated with higher incidence of distant recurrence. The 1-, 2-, and 3-year recurrence rates were significantly higher in diabetic patients with inadequate maintenance of blood glucose compared with the others: 50.6% *vs* 26.8%, 83.5% *vs* 54.4%, and 93.8% *vs* 73.0%, respectively (P = 0.0001). Inadequate maintenance of blood glucose was an independent predictor of distant recurrence [adjusted relative risk 1.97 (95%CI, 1.33-2.91), (P = 0.0007)] after adjustment for other risk factors, such as number of HCC nodules [2.03 (95%CI, 1.51-2.73), P < 0.0001] and initial level of serum alpha fetoprotein (AFP) [1.43 (95%CI, 1.04-1.97), P = 0.028]. Obesity was not an independent predictor of recurrence. The incidence of distant recurrence did not differ between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients. Among 232 patients who had HCC recurrence, 138 had a second recurrence. The 1-, 2-, and 3-year rates of second recurrence were significantly higher in diabetic patients with inadequate maintenance of blood glucose than in the others: 9.0% *vs* 5.9%, 53.1% *vs* 24.3%, and 69.6% *vs* 42.3%, respectively (P = 0.0021). Inadequate maintenance of blood glucose in diabetic patients [1.99 (95%CI, 1.23-3.22), P = 0.0049] and presence of multiple HCC nodules [1.53 (95%CI, 1.06-2.22), P = 0.024] were again significantly associated with second HCC recurrence. Inadequate maintenance of blood glucose in diabetic

patients was also a significant predictor of poor survival [2.77 (95%CI, 1.38-5.57), P = 0.0046] independent of excessive alcohol drinking [6.34 (95%CI, 1.35-29.7), P = 0.019], initial level of serum AFP [3.40 (95%CI, 1.88-6.18), P < 0.0001] and Child-Pugh classification grade B [2.24 (95%CI, 1.12-4.46), P = 0.022]. Comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the 1-, 2-, and 3-year survival rates were significantly lower in diabetic patients with inadequate maintenance of blood glucose: 92% *vs* 99%, 85% *vs* 96%, and 70% *vs* 92%, respectively (P = 0.0003).

CONCLUSION: Inadequate maintenance of blood glucose in diabetic patients is a significant risk factor for recurrence of HCC and for poor survival after curative RFA therapy.

Key words: Hyperglycemia; Hepatocellular carcinoma; Recurrence; Radio frequency ablation; Survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1] and its incidence has been increasing in many countries[2]. Surgical resection, liver transplantation, and local ablation therapy, such as radiofrequency ablation (RFA) therapy, have been considered as efficient curative therapies for HCC. RFA therapy is now widely performed in patients with small HCC[3] and a randomized controlled study demonstrated that the survival rates were similar in patients with small HCC receiving RFA or surgical resection[4]. A characteristic of HCC is its high rate of recurrence after curative resection or local ablation therapy, reaching approximately 80% within 5 years[5-7]. Identification of factors related to recurrence of HCC and therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival.

Tumor factors, such as the number of HCC nodules and their size, are associated with the recurrence of HCC and survival prognosis[8-10]. Another factor that is associated with the recurrence of HCC and survival is the hepatic reserve function at the time of HCC therapy[8,10,11]. Hepatitis C virus (HCV) and hepatitis B virus (HBV) infection are the major causes responsible for 80% of HCC cases[2] and antiviral therapy targeting HCV[12,13] or HBV[14] has been shown to decrease HCC recurrence, and improve hepatic reserve function and survival. Non-alcoholic steatohepatitis (NASH) has also received attention as a cause of HCC[15]. Metabolic factors, such as obesity and diabetes, are closely linked to the etiology of NASH. These metabolic factors have also been identified as risk factors for several other types of cancer. Obesity is associated with increased mortality rates of several cancers[16,17] and diabetes is also reported as a risk factor for liver, pancreatic, renal, and colon cancers[18,19]. If these metabolic factors are related to the recurrence of HCC, therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival. The impact of diabetes on the recurrence of HCC after treatment has been discussed, but with conflicting results[20-23].

In this study, factors contributing to the recurrence and prognosis of HCC after curative treatment were analyzed. We found that inadequate maintenance of blood glucose was related to the high rate of HCC recurrence and poor survival.

MATERIALS AND METHODS

Patients whose HCC was treated by RFA at the Musashino Red Cross Hospital were studied retrospectively for factors associated with recurrence of HCC and survival. The inclusion criteria were as follows: (1) HCC treated curatively with RFA at the Musashino Red Cross Hospital between 1999 and 2007; (2) maximum diameter of HCC nodule $\leqslant$ 3 cm; (3) number of HCC nodules $\leqslant$ 3; (4) no previous history of treatment for HCC; and (5) follow-up observation for at least 6 mo after RFA therapy. 344 patients met these criteria, including 140 women and 204 men, with a mean age of 67.6 years and mean observation time of 4.04 years. The clinical characteristics of the patients are summarized in Table 1. The etiological background of liver disease was HBV infection in 30, HCV infection in 278, excessive alcohol drinking (intake of ethanol $\geqslant$ 60 g/d for $\geqslant$ 5 years continuously) in 9, and non-B non-C non-alcoholic etiology in 27 patients. The Child-Pugh classification grade was either A (n = 307) or B (n = 37). The number of HCC nodules was one in 260, two in 61, and three in 23 patients. Thus, 260 patients had a single lesion, and 84 had multiple lesions. The maximum diameter of HCC nodules was 19.9 ± 0.3 mm.

Obesity was defined as a body mass index > 25 kg/m^2 according to the definition of the Japan Society for the Study of Obesity[24]. Blood glucose was measured monthly for 6 mo after HCC treatment and the average value was determined. Inadequate maintenance of blood glucose was defined as an average value of blood glucose $\geqslant$ 200 mg/dL. The level of hemoglobin A1c (HbA1c) was not used in the present study because the lifespan of erythrocytes is shortened due to hypersplenism in patients with chronic hepatitis or cirrhosis, leading to lower HbA1c levels relative to the blood glucose level[25]. Diagnosis of type 2 diabetes was made according to the

Table 1 Characteristics of patients undergoing curative radiofrequency ablation for hepatocellular carcinoma *n* (%)

Variable	Value
Sex (male/female)	204/140
Age(yr)	67.6 ± 8.4
Etiology of liver disease: HBV/HCV/NBNC	30/278/36
AST (IU/L)	84.0 ± 34.5
ALT (IU/L)	73.2 ± 36.5
GGT (IU/L)	82.9 ± 96.8
T-Chol (mg/dL)	157.8 ± 32.0
TG (mg/dL)	112.3 ± 55.7
Mean blood sugar (mg/dL)	139.3 ± 44.0
Diabetes mellitus	159 (48)
BMI > 25 kg/m^2	86 (25)
Maximum diameter of HCC nodule (mm)	19.9 ± 0.3
Number of HCC nodules: single/2 or 3	260/84
AFP (ng/mL)	214 ± 1025
Alcohol drinking > 60 g/d	9 (2.6)
Child-Pugh grade: A/B	307/37

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNV: Neither HBV nor HCV; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltransferase; T-Chol: Total cholesterol; TG: Triglyceride; BMI: Body mass index; AFP: α-fetoprotein; HCC: Hepatocellular carcinoma.

American Diabetes Association criteria of a fasting blood glucose level ≥ 126 mg/dL (≥ 7.0 mmol/L) and/or HbA1c level ≥ 6.5[26]. After initial treatment of HCC by RFA, the ablated area was confirmed by contrast-enhanced computed tomography (CT) within one week. If the ablated area was not sufficient, then RFA therapy was repeated until the HCC nodule was completely ablated.

HCC surveillance and diagnosis of recurrence

Diagnosis of HCC was based on abdominal ultrasonography, contrast-enhanced CT, magnetic resonance imaging (MRI), or angiography. Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

For surveillance of HCC recurrence after curative therapy with RFA, patients were evaluated by abdominal ultrasonography, contrast-enhanced CT, or contrast-enhanced MRI every three months. Recurrence of HCC was diagnosed based on a new lesion detected by ultrasonography showing vascular enhancement with washout on CT or MRI. If the tumor was not hypervascular, a tumor biopsy was performed to confirm the diagnosis.

Statistical analysis

For analysis of survival and recurrence, the time of initial RFA treatment was defined as day zero. Survival rate and recurrence rate were analyzed by the Kaplan-Meier method and log rank test. Multivariate analysis was performed using a Cox proportional hazard model. Data were analyzed using StatView Version 5.0 (SAS Institute Inc, Cary, North Carolina, United States) and IBM-SPSS statistics version 18 (IBM SPSS Inc, Chicago, IL, United States). Statistical significance was set at $P < 0.05$.

RESULTS

Factors associated with HCC recurrence

Of the 344 patients whose HCC was curatively treated by RFA, 232 had HCC recurrence. The 1-, 2-, and 3-year recurrence rates were 29.3%, 57.5%, and 75.2%, respectively. On univariate analysis, inadequate maintenance of blood glucose, higher initial level of serum AFP and multiple HCC nodules were significantly associated with HCC recurrence. Obesity ($P = 0.06$) and diabetes ($P = 0.65$) were not significantly associated with HCC recurrence.

Thirty-seven patients had diabetes with inadequate maintenance of blood glucose, 122 patients had diabetes with adequate maintenance of blood glucose, and 185 patients did not have diabetes. The HCC recurrence rate was significantly higher in diabetic patients with inadequate maintenance of blood glucose than in the others ($P = 0.0001$) (Figure 1A).

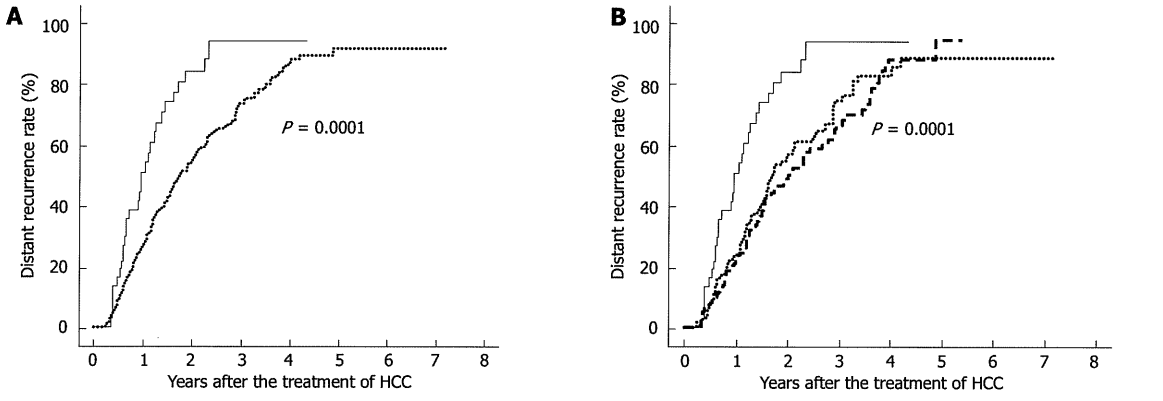
Comparing patients with diabetes ($n = 159$) and patients who did not have diabetes ($n = 185$), there was no significant difference in the recurrence rate ($P = 0.65$). Upon comparison of the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group, the recurrence rate was significantly higher in the diabetes with inadequate maintenance of blood glucose group than in the other two groups ($P = 0.0001$) (Figure 1B). On the other hand, there was no significant difference in the HCC recurrence rate between the diabetes patients with adequate maintenance of blood glucose group and the non-diabetes group.

In terms of the number of HCC nodules, namely, single ($n = 260$) *vs* multiple ($n = 84$), the recurrence rate was significantly higher in patients with multiple HCC nodules ($P = 0.0001$). Within each subgroup of patients with single and multiple HCC nodules, diabetes with inadequate maintenance of blood glucose was significantly associated with recurrence of HCC (single, $P = 0.006$; multiple, $P = 0.025$) (Figure 2A, B). In terms of the initial level of serum AFP ≥100 ng/mL ($n = 70$) *vs* < 100 ng/mL ($n = 274$), the recurrence rate was significantly higher in patients with AFP ≥ 100 g/mL ($P = 0.018$). Within each subgroup of patients with AFP ≥ 100 ng/mL and < 100 ng/mL, diabetes with inadequate maintenance of blood glucose was associated with a higher rate of recurrence (AFP ≥ 100 ng/mL, $P = 0.005$; AFP < 100 ng/mL, $P = 0.017$) (Figure 2C, D).

Independent risk factors for distant recurrence of HCC on multivariate analysis were inadequate maintenance of blood glucose in diabetic patients [adjusted relative risk, 1.97 (95%CI, 1.33-2.91), $P = 0.0007$], multiple HCC nodules [2.03 (1.51-2.73), $P < 0.0001$], and AFP ≥ 100 ng/mL [1.43 (1.04-1.97), $P = 0.028$] (Table 2).

Factors associated with second recurrence

Among the 232 patients who had HCC recurrence, 138 had a second recurrence. Regarding second recurrence, inadequate maintenance of blood glucose in diabetic pa-



Recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	50.6%	83.5%	93.8%
Others	26.8%	54.4%	73.0%

Recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	50.6%	83.5%	93.8%
Diabetes with adequate maintenance of blood glucose	21.9%	48.7%	66.5%
Non-diabetes	23.6%	55.5%	74.4%

Figure 1 Kaplan-Meier curves showing a higher rate of hepatocellular carcinoma recurrence in diabetic patients with hyperglycemia. A: The cumulative incidence of the recurrence of hepatocellular carcinoma (HCC) was significantly higher in diabetic patients with inadequate maintenance of blood glucose (blood glucose ≥ 200 mg/dL solid line) than in the others (dotted line) (P = 0.0001); B: The HCC recurrence rate was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.0001). There was no significant difference in HCC recurrence rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

Table 2 Multivariate analysis of factors associated with recurrence of hepatocellular carcinoma

Factors	Odds ratio (95%CI)	*P*-value
First recurrence		
Inadequate maintenance of blood glucose	1.97 (1.33-2.91)	0.0007
Multiple HCC nodules	2.03 (1.51-2.73)	< 0.0001
AFP ≥ 100 ng/mL	1.43 (1.04-1.97)	0.028
Second recurrence		
Inadequate maintenance of blood glucose (mg/dL)	1.99 (1.23-3.22)	0.0049
Multiple HCC nodules	1.53 (1.06-2.22)	0.024

Inadequate maintenance of blood glucose was defined as an average of casual blood glucose of ≥ 200 mg/dL. HCC: Hepatocellular carcinoma; AFP: α-fetoprotein.

tients and multiple HCC nodules were again significantly associated with HCC recurrence. Obesity (P = 0.18), diabetes (P = 0.31) and initial level of serum AFP (P = 0.08) were not associated with second recurrence. In terms of the number of HCC nodules, namely, single *vs* multiple, the 1-, 2-, and 3-year recurrence rates were significantly higher in patients with multiple lesions (6.4% *vs* 6.1%, 39.3% *vs* 23.1%, and 52.5% *vs* 42.3%, respectively, P = 0.013). Upon comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the rate of second recurrence was significantly higher in diabetic patients with inadequate maintenance of blood glucose (P = 0.0021) (Figure 3A). Upon comparing patients with diabetes *vs* patients who did not have diabetes, the rates of second recurrence were not significantly different (P = 0.31). Upon comparison of the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group, the second recurrence rate was again significantly higher in the diabetes with inadequate maintenance of blood glucose group than in the other two groups (P = 0.0035) (Figure 3B). On the other hand, there was no significant difference in the second recurrence rate between the diabetes with adequate maintenance of blood glucose group and the non-diabetes group.

Independent risk factors for second recurrence of HCC on multivariate analysis were inadequate maintenance of blood glucose [1.99 (95%CI, 1.23-3.22), P = 0.0049] and multiple HCC nodules [1.53 (95%CI, 1.06-2.22), P = 0.024] (Table 2).

Factors associated with survival

There were 52 HCC-related or hepatic failure deaths. On univariate analysis, inadequate maintenance of blood glucose, excessive alcohol drinking, higher initial level of serum AFP and Child-Pugh classification grade B were significantly associated with survival. Obesity (P = 0.81) and diabetes (P = 0.11) were not significantly associated with survival.

Upon comparing diabetic patients with inadequate maintenance of blood glucose *vs* the others, the survival rate was significantly lower in patients with inadequate maintenance of blood glucose (P = 0.0003) (Figure 4A). Upon comparing diabetic patients *vs* non-diabetic patients, the survival rates were not significantly different

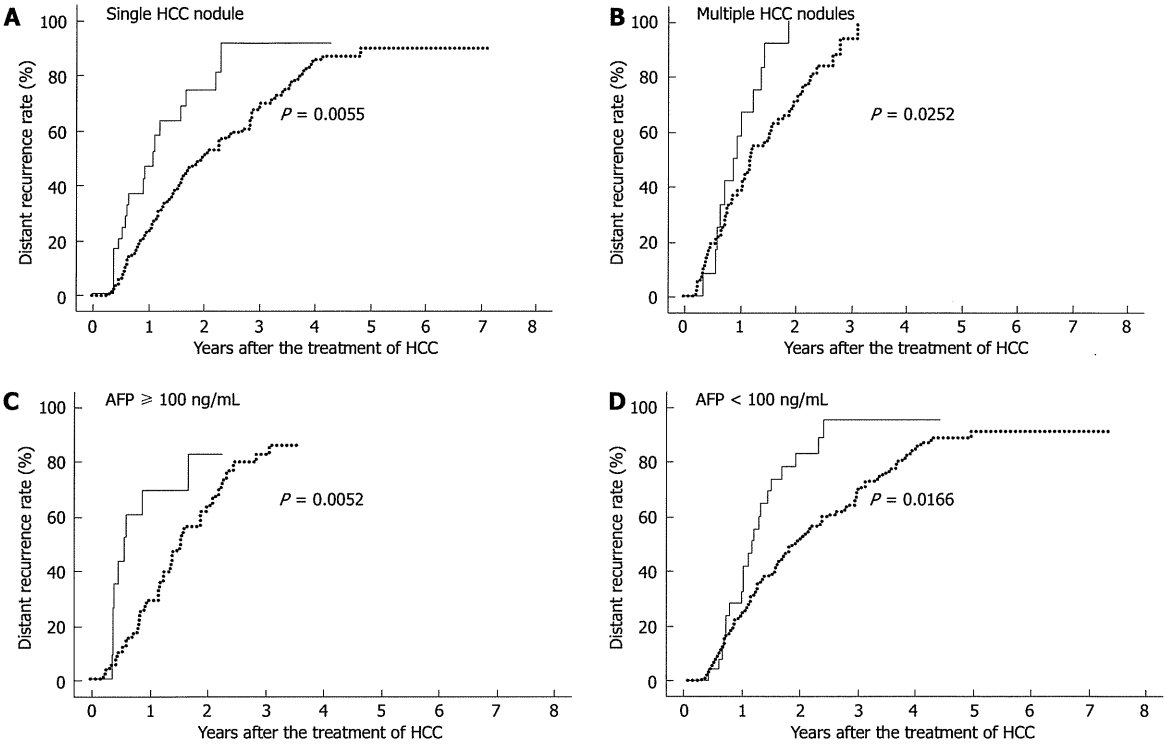


Figure 2 Diabetic patients with inadequate maintenance of blood glucose have higher rate of hepatocellular carcinoma recurrence after stratification by other risk factors. A: $P = 0.006$ for single hepatocellular carcinoma (HCC) nodule; B: $P = 0.025$ for multiple HCC nodules; C: $P = 0.005$ for AFP ≥ 100 ng/mL; D: $P = 0.017$ for α-fetoprotein (AFP) < 100 ng/mL. The cumulative incidence of the recurrence of HCC was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in the others (dotted line). after stratification by number of HCC nodules and by initial level of AFP.

Table 3 Multivariable analysis of factors associated with survival

Factors	Odds ratio (95%CI)	*P*-value
Inadequate maintenance of blood glucose	2.77 (1.38-5.57)	0.0046
Alcohol drinking ≥ 60 g/d	6.34 (1.35-29.7)	0.019
Child Pugh grade B	2.24 (1.12-4.46)	0.022
AFP ≥ 100 ng/mL	3.40 (1.88-6.18)	< 0.0001

Inadequate maintenance of blood glucose was defined as an average of casual blood glucose of ≥ 200 mg/dL. AFP: α-fetoprotein.

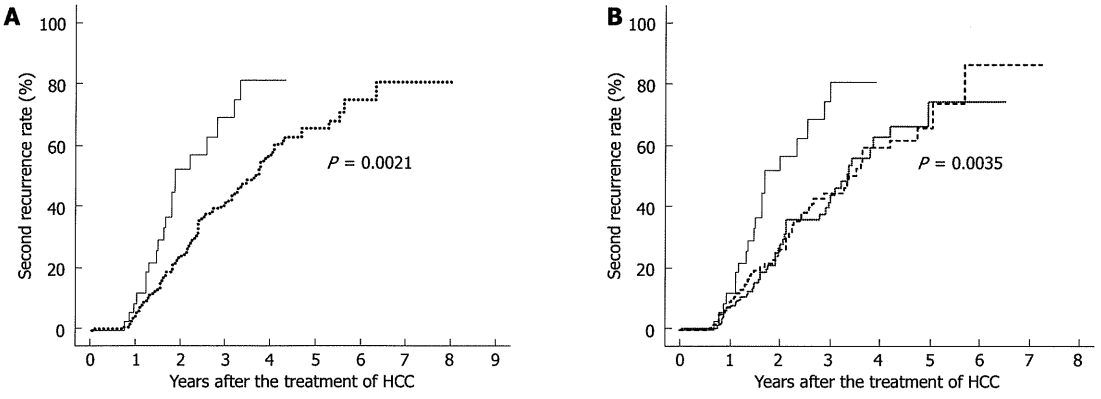
($P = 0.11$). of the survival rate was compared among the three groups, i.e., the diabetes with inadequate maintenance of blood glucose group, the diabetes with adequate maintenance of blood glucose group, and the non-diabetes group. The survival rate was significantly poorer in the diabetes with inadequate maintenance of blood glucose group than in the other two groups ($P = 0.0003$) (Figure 4B), while it did not differ between the diabetes with adequate maintenance of blood glucose group and the non-diabetes group.

The number of HCC nodules, which was a significant factor for HCC recurrence, was not related to survival ($P = 0.34$). Patients with excessive alcohol drinking had poor survival prognosis compared to those with non-excessive or no alcohol drinking ($P = 0.046$). Survival was better in patients in Child-Pugh A class than in patients in Child-Pugh B class ($P = 0.0082$). AFP ≥ 100 ng/mL was associated with poor survival compared with AFP < 100 ng/mL ($P < 0.0001$).

On multivariate analysis, inadequate maintenance of blood glucose was a significant predictor of poor survival [2.77 (95%CI, 1.38-5.57), $P = 0.0046$] independent of excessive alcohol drinking [6.34 (95%CI, 1.35-29.7), $P = 0.019$], initial level of serum AFP ≥ 100 ng/mL [3.40 (95%CI, 1.88-6.18), $P < 0.0001$] and Child-Pugh classification grade B [2.24 (95%CI, 1.12-4.46), $P = 0.022$] (Table 3).

DISCUSSION

The impact of metabolic factors, such as hyperglycemia, diabetes and obesity, on distant recurrence and survival after curative RFA therapy for HCC was analyzed retrospectively. We identified that inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and a risk factor for poor survival, whereas obesity and diabetes were not. Diabetic patients with inadequate maintenance of blood glucose had a higher rate of HCC recurrence and poorer survival compared with diabetic patients with adequate maintenance of blood glucose and non-diabetic patients. In other words, even in patients with diabetes, if



Second recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	9.0%	53.1%	69.6%
Others	5.9%	24.3%	42.3%

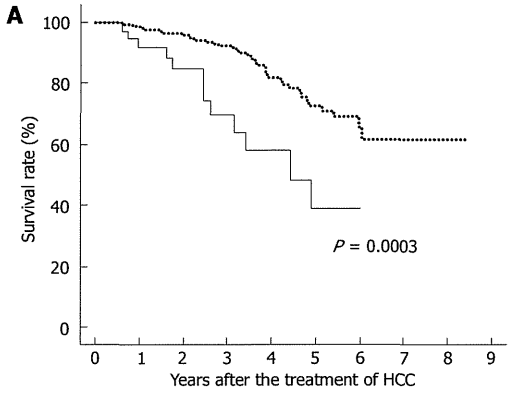
Second recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	9.0%	53.1%	69.6%
Diabetes with adequate maintenance of blood glucose	5.9%	22.1%	43.7%
Non-diabetes	5.6%	21.5%	36.5%

Figure 3 Kaplan-Meier curves showing a higher rate of second recurrence of hepatocellular carcinoma in diabetic patients with inadequate maintenance of blood glucose. A: The cumulative incidence of the second recurrence of hepatocellular carcinoma (HCC) was significantly higher in diabetic patients with inadequate maintenance of blood glucose (blood glucose ≥ 200 mg/dL solid line) than in the others (dotted line) (P = 0.002); B: The rate of second recurrence of HCC was significantly higher in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.004). There was no significant difference in the rate of second recurrence of HCC between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

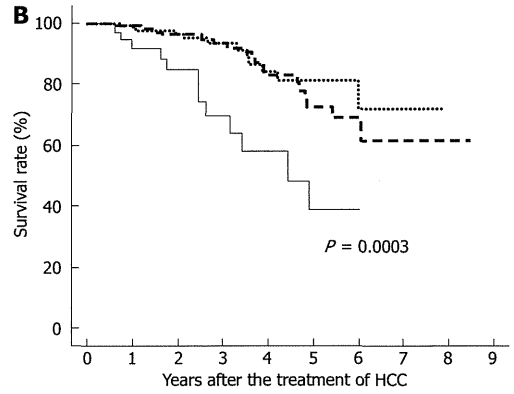
the blood glucose was adequately maintained, the HCC recurrence rate and survival did not differ significantly compared with those in non-diabetic patients. These results indicate the possibility that adequate management of hyperglycemia may lead to reduction in the risk of HCC recurrence and improvement of overall survival.

The contribution of diabetes to the development of HCC has been confirmed in several reports[27-30]. The impact of diabetes on the recurrence of HCC after treatment has also been discussed, but with conflicting results[20-22]. A recent study from Taiwan demonstrated that diabetes may not affect the intra-hepatic HCC recurrence and survival after RFA[23]. The results of the present study also indicated that diabetes itself is not a significant risk factor if the level of blood glucose is adequately managed. Rather, hyperglycemia was a significant risk factor for the recurrence of HCC. There may be several mechanisms involved in the relationship between hyperglycemia and HCC recurrence. Hyperglycemia promotes cancer cell proliferation in pancreatic cancer cells and breast cancer cells[31-33] through accelerated cell cycle progression or through the production of reactive oxygen species, leading to activation of protein kinase C and increased DNA synthesis in cancer cells[34]. A previous study in hepatitis C patients indicated that hyperglycemia after challenge with 75-g oral glucose tolerance test was associated with the risk for HCC while hyperglycemia at fasting was not[35]. A possible reason for this result may be that patients with post-challenge hyperglycemia may have higher fluctuations in daily glucose levels that lead to oxidative stress[35], because it was reported that acute fluctuations in blood glucose levels cause greater oxidative stress than sustained chronic hyperglycemia[36]. Taken together, a possible mechanism for the relationship between higher level of casual blood glucose and development of HCC in the present study may be that daily fluctuations in serum glucose levels caused greater oxidative stress. Alternatively, hyper-insulinemia or increased level of insulin-like growth factor, which are caused by hyperglycemia, may be related to carcinogenesis[37-39]. Insulin levels were not measured in our study; therefore, the effects of insulin could not be identified.

Discussions are now taking place on methods of treating diabetes from the standpoint of cancer prevention. Control of hyperglycemia could reduce cancer incidence, which means that hyperglycemia could directly contribute to the development of cancer[39]. The results of our study also showed that adequate management of hyperglycemia may lead to reduction in the risk of HCC recurrence and improvement of overall survival. Improvement in insulin resistance is undoubtedly the most important factor for the treatment of diabetes, but glycemic control is often difficult to achieve with dietary therapy, exercise, or insulin resistance-improving drugs alone. It was reported that metformin may be associated with a lower risk of cancer[38] and there is a theoretical concern that exogenous insulin may be associated with an increased risk of cancer[40]. In fact, a recent study reported that insulin therapy in patients with HCV infection is linked with the development of HCC[41]. On the other hand, with insulin treatment, concomitant use of metformin has been reported to offset the carcinogenic risk of insulin[42]. Whether glycemic control should be a priority, or whether avoiding hyper-insulinemia because



Second recurrence rate at	1-year	2-year	3-year
Inadequate maintenance of blood glucose	91.7%	84.6%	69.7%
Others	98.7%	95.9%	92.4%



Second recurrence rate at	1-year	2-year	3-year
Diabetes with inadequate maintenance of blood glucose	91.7%	84.6%	69.7%
Diabetes with adequate maintenance of blood glucose	99.2%	96.3%	93.7%
Non-diabetes	99.2%	96.4%	93.3%

Figure 4 Patients with inadequate maintenance of blood glucose have a lower survival rate. A: The survival rate after curative local ablation therapy for hepatocellular carcinoma (HCC) was significantly lower in diabetic patients with inadequate maintenance of blood glucose (blood glucose ≥ 200 mg/dL solid line) than in the others (dotted line) (P = 0.0003); B: The survival rate was significantly lower in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) (P = 0.0003). There was no significant difference in survival rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

of therapy should be a priority, is an issue for future investigation.

In terms of survival of HCC patients, associations with liver function and tumor factors have been reported[10], but conflicting results have been reported for the relationship with diabetes[20,21]. These two studies involved heterogeneous groups of HCC patients treated with various therapies, including surgery, local ablation therapy and transcatheter arterial embolization. This heterogeneity may have led to the conflicting results, because the survival of HCC patients may be strongly affected by the initial treatment. Our study involved a homogeneous patient population, i.e., all patients were initially treated curatively by RFA. The results of our study suggest that glycemic control in diabetic patients, more so than diabetes itself, plays a role in survival. The mechanism by which glycemic control and survival are related is unknown, but frequent recurrence of HCC in hyperglycemic patients and the accumulation of damage in liver function because of repeated treatment intervention for HCC may lead to worsening survival.

In conclusion, inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and for poor survival. Adequate management of hyperglycemia in diabetic patients may lead to reduction in the risk of HCC recurrence and improvement in overall survival.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Radiofrequency ablation (RFA) therapy is an efficient curative therapy for HCC, but long-term survival is limited because of the high rate of distant recurrence of approximately 80% within 5 years. Identification of factors related to recurrence of HCC and therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved survival.

Research frontiers

Metabolic factors, such as obesity and diabetes, have been identified as risk factors for several types of cancer, such as cancer of the liver, pancreas, kidney, and colon. These metabolic factors may be related to recurrence of HCC. The impact of diabetes on the recurrence of HCC after treatment has been discussed, but with conflicting results.

Innovations and breakthroughs

The authors identified that inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC and a risk factor for poor survival, whereas diabetes was not. In other words, even in patients with diabetes, if the blood glucose was adequately maintained, then the HCC recurrence rate and survival did not differ significantly from those in non-diabetic patients.

Applications

The results of the present study indicate the possibility that adequate management of hyperglycemia in diabetic patients may lead to reduction in the risk of HCC recurrence and improvement of overall survival.

Peer review

This is an important study in which the effect of inadequate maintenance of blood glucose in diabetes has been shown as a significant risk factor for distant recurrence of hepatocellular carcinoma and poor survival after curative radiofrequency ablation therapy.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Inhibition of hepatocellular carcinoma by PegIFNα-2a in patients with chronic hepatitis C: a nationwide multicenter cooperative study

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Abstract
Background We investigated whether the administration of maintenance doses of interferon prevented hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.
Methods Study 1: A multicenter, retrospective, cooperative study was carried out to determine whether long-term administration of low-dose peginterferon alpha-2a (PegIFNα-2a) prevented HCC development in patients with chronic hepatitis C. In total, 594 chronic hepatitis C patients without a history of HCC were enrolled and treated with 90 μg PegIFNα-2a administered weekly or bi-weekly for at least 1 year. Study 2: HCC developed in 16 of 99 additional patients without PegIFNα-2a treatment during 3.8 years of observation. A propensity-matched control study was then carried out to compare the incidence of

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HCC between the 59 patients who received low-dose PegIFNα-2a (PegIFNα-2a group) and 59 patients who did not receive PegIFNα-2a treatment (control group), matched for sex, age, platelet count, and total bilirubin levels.

Results Study 1: HCC developed in 49 patients. The risk of HCC was lower in patients with undetectable hepatitis C virus RNA, ≤40 IU/L alanine aminotransferase (ALT), or ≤10 ng/L alpha-fetoprotein (AFP) 24 weeks after the start of therapy. Study 2: The incidence of HCC was significantly lower in the PegIFNα-2a group than in the control group.

Conclusions Low-dose and long-term maintenance administration of PegIFNα-2a decreased the incidence of HCC in patients with normalized ALT and AFP levels at 24 weeks compared with patients without normal ALT and AFP levels.

Keywords Chronic hepatitis C · Hepatocellular carcinoma · Peginterferon

Introduction

Hepatocellular carcinoma (HCC), the sixth most common cancer worldwide, often develops because of long-term hepatitis B or C virus infection [1, 2]. In particular, chronic hepatitis C and hepatic cirrhosis increase the risk of HCC; the annual incidence of tumor development in such patients may be as high as 2–4 % [3–5]. The incidence of HCC decreases in patients who achieve a sustained virological response (SVR) to interferon (IFN) treatment, although the incidence remains high in non-SVR patients [6–9]. A detailed analysis of HCC development revealed that chronic hepatitis C patients aged 65 years or more, especially those with advanced fibrosis of the liver, were at an increased risk of developing HCC [10]. For patients 65 years or older with advanced liver fibrosis, the dose of ribavirin is often reduced or the agent is discontinued, resulting in lower SVR rates in those with discontinuation of ribavirin. Establishing an effective treatment strategy for preventing the development of HCC is important for these high-risk patients.

Factors related to the development of HCC have been analyzed in patients who did not achieve an SVR even after IFN treatment; advanced fibrosis of the liver and high levels of serum alanine aminotransferase (ALT), and alpha-fetoprotein (AFP) are risk factors for HCC development [11, 12]. A randomized controlled trial was conducted in Western countries to determine whether combined peginterferon and ribavirin treatment with weekly administration of 90 μg peginterferon alpha-2a (PegIFNα-2a) could prevent HCC in non-responders. A 3.5-year follow up showed that administration of a maintenance dose of PegIFNα-2a did not reduce tumor incidence in these patients [13]. However, after 8.5 years of observation, the incidence of HCC was decreased among those in the PegIFNα-2a group with cirrhosis [14]. Meanwhile, Bruix et al. [15] reported that maintenance therapy with PegIFNα-2b did not prevent HCC in chronic hepatitis C patients with cirrhosis. In Japan, long-term low-dose administration of natural IFN has been reported to decrease the incidence of HCC [16]. In light of these conflicting results, investigations should be carried out in a large number of patients with chronic hepatitis C to resolve the question of whether IFN treatment prevents the development of HCC.

We carried out a multicenter retrospective cooperative study of patients with chronic hepatitis C to determine whether those treated with 90 μg PegIFNα-2a without ribavirin had a reduced incidence of HCC compared with those not treated with IFN.

Patients and methods

Study 1: analysis of risk factors for HCC in patients treated with long-term low-dose-PegIFNα-2a

In total, at 21 hepatitis centers throughout Japan, 743 patients with hepatitis C who had received 90 μg of PegIFNα-2a therapy weekly or bi-weekly for 1 year or more without having received the full dose (180 μg) since December 2003 were examined retrospectively for the development of HCC. The end of enrollment in this study was the end of December 2008 and the end of follow up was the end of December 2010. Patients with a history of HCC before the start of therapy and those with a therapy period of less than 48 weeks were excluded, leaving 594 patients who had undergone long-term administration of PegIFNα-2a for analysis. At the 21 centers involved in this

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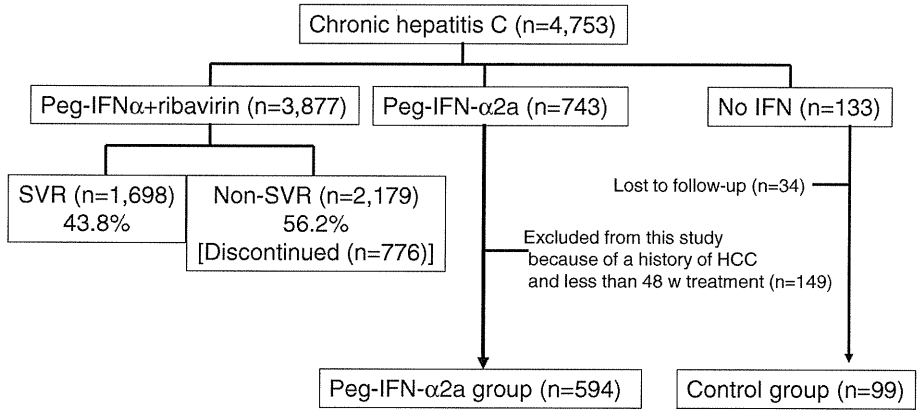

Fig. 1 Flow diagram of the patients' enrollment in the study. *Peg-IFNα* pegylated interferon α, *SVR* sustained viral response, *HCC* hepatocellular carcinoma, *w* week

study, 4,753 patients with chronic hepatitis C had been treated; Peg-IFN and ribavirin combination treatment had been administered to 3,877 patients, 743 patients had received Peg-IFN alone, and 133 patients had not agreed to receive IFN (a flow diagram of the enrollment of patients in this study is shown in Fig. 1). In the patients with Peg-IFN and ribavirin combination treatment, the SVR rate was 43.8 %; SVR was not achieved in 2,179 patients, and in 776 of these patients, the combination therapy was discontinued owing to adverse events or the patient's choice. Patients who failed to achieve an SVR were not included in this study, because the incidence of HCC is known to be reduced even in non-responders to IFN [17].

The backgrounds of the 594 patients studied are shown in Table 1. Findings from the liver biopsies of the patients were classified according to international standards [18]. Long-term PegIFNα-2a treatment is approved by the Japanese Medical Insurance system. Written informed consent was obtained from all patients prior to participation in this study. The study design was approved by the regional ethics committees of the 21 centers involved in this study, including the Musashino Red Cross Hospital, in accordance with the Helsinki Declaration. The 743 patients treated with PegIFNα-2a alone were not indicated for Peg-IFNα and ribavirin combination therapy because of anemia or heart disease. The 133 patients who did not agree to receive IFN served as the control group (see Fig. 1). A large proportion of the 594 study patients had advanced fibrosis of the liver and active inflammation. A dose of 90 μg PegIFNα-2a was administered to 512 and 82 patients weekly and biweekly, respectively, according to the patients' wishes. There were no significant differences between the weekly and biweekly groups in the patients' background data (data not shown).

The median duration of follow up in the PegIFNα-2a group was 1,273 days (range 228–2,768 days) and HCC was observed in 49 of the 594 patients (Table 1). Pre-treatment and on-treatment factors associated with the development of HCC were analyzed by Student's *t*-test, the Mann–Whitney *U*-test, and the χ^2 test (Table 2). Independent factors for the development of HCC were assessed by multivariate analysis using logistic regression. The

Table 1 Background data of patients treated with PegIFNα-2a ($n = 594$)

	$n = 594$
Age (years)	61.7 ± 11.7
Sex (male/female)	258/336
BMI	23.2 ± 3.3
Genotype (1/2)	443/151
Diagnosis (ASC/CH/LC)	4/460/130
History of excess alcohol consumption (≥60 g/day; yes/no)	118/376
Fibrosis (F0, 1, 2/F3, 4)	443/151
Inflammatory activity (A0, 1/A2, 3)	469/125
Diabetes mellitus (no/yes)	499/95
LDL cholesterol (mg/dL)	94.2 ± 31.1
Fasting blood sugar (mg/dL)	106.3 ± 28.5
White blood cell count (/mm^3)	4,360 ± 1,470
Red blood cell count (×10^6/μL)	423.8 ± 56.4
Hemoglobin (g/dL)	13.3 ± 1.8
Platelet count (×10^3/μL)	137 ± 56
Albumin (g/dL)	4.0 ± 0.5
Total bilirubin (mg/dL)	0.8 ± 0.6
AST (IU/L)	65.8 ± 47.8
ALT (IU/L)	72.1 ± 68.0
Gamma-GTP (IU/L)	55.2 ± 51.3
Esophageal varices (no/yes)	344/31
Alpha fetoprotein (ng/L)	6.9 (4.2–13.8)
Once weekly or biweekly PegIFNα-2a	512:82
Baseline HCV RNA (KIU/mL)	1,024 (73–2,130)
Development of HCC (no/yes)	545/49

PegIFN pegylated interferon, *BMI* body mass index, *ASC* asymtomatic carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *LDL* low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GTP* guanosine triphosphate, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma

Values are means ± SD, with ranges in parentheses

incidence of HCC was analyzed according to the ALT, AFP, and hepatitis C virus (HCV) RNA levels 24 weeks after the start of PegIFNα-2a administration by using the Kaplan–Meier method. The risk of HCC was analyzed, using the Kaplan–Meier method, only in the non-responders with detectable HCV RNA during PegIFNα-2a administration by dividing them according to the ALT and AFP levels 24 weeks after the start of therapy. The incidence of HCC was compared between the patients with ALT levels of <41 IU/L and those with levels of ≥41 IU/L, and between patients with serum AFP levels of <10 ng/L and those with levels of ≥10 ng/mL at 24 weeks after starting treatment, because at most of the centers participating in the this study, the upper normal range of serum ALT is set at 40 IU/L, and the most significant difference in the incidence of HCC was observed between the PegIFNα-2a and control group with the cut-off serum ALT set at 41 IU/L and cutoff serum AFP set at 10 ng/mL, 24 weeks after starting treatment. The HCV RNA level was measured using the Amplicor Monitor method with a lower detection limit of 50 IU/L (Roche Diagnostics, Tokyo, Japan). A history of excess alcohol consumption was determined as >60 g alcohol per day in order to exclude alcoholic liver disease.

An asymptomatic carrier was defined as a patient with a serum ALT level within the normal range and minimal inflammation or fibrosis in the biopsied tissues of the liver. Chronic hepatitis was defined as mild-to-severe fibrosis of the liver according to liver biopsy [18]. The diagnosis of liver cirrhosis was based on the results of histological examination of the biopsied liver tissues.

Study 2: incidence of HCC in the PegIFNα-2a therapy and non-administration (control) groups in comparison with propensity-matched controls

Ninety-nine of the 133 chronic hepatitis C patients who had not received IFN were examined as controls; patients in this group received liver-protective agents such as glycyrrhizin or were untreated, and the group was observed for more than 1 year. None of the individuals in the control groups had received IFN alone or PegIFNα and ribavirin combination treatment. They were treated for a median of 1,395 days (range 75–6,556 days). Fifty-nine of these patients underwent liver biopsy before the treatment and were considered the control group for the propensity-matched study. For the propensity-matched study, 59 patients were selected from the PegIFNα-2a group according to their age, sex, platelet count, and total bilirubin levels, which had been identified as independent pretreatment risk factors for the development of HCC in Study 1. The rates of HCC were analyzed using the Kaplan–Meier method, and the risk of HCC was analyzed particularly in patients with advanced fibrosis of the liver (F3 and F4).

Table 2 Comparison of HCC and non-HCC patients with long-term PegIFNα-2a administration ($n = 594$)

	Patients with or without development of HCC		p value
	With HCC ($n = 49$)	Without HCC ($n = 545$)	
Pretreatment parameters			
Age (years)	63.8 ± 1.7	61.3 ± 0.5	<0.05
Sex (male/female)	32/17	226/319	<0.01
BMI	24.0 ± 0.5	23.1 ± 0.2	n.s.
Genotype (1/2)	47/6	397/148	n.s.
History of excess alcohol consumption (≥60 g/day; yes/no)	11/38	107/338	n.s.
Fibrosis (F0, 1, 2/F3, 4)	25/24	418/127	<0.001
Inflammatory activity (A0, 1/A2, 3)	7/42	462/83	<0.001
Diabetes mellitus (no/yes)	38/11	461/84	n.s.
LDL cholesterol (mg/dL)	88.2 ± 9.0	94.7 ± 2.6	n.s.
White blood cell count (/mm^3)	4,355 ± 210	4,360 ± 64	n.s.
Red blood cell count (×10^6/μL)	420.8 ± 8.1	424.1 ± 2.6	n.s.
Hemoglobin (g/dL)	13.6 ± 0.3	13.3 ± 0.1	n.s.
Platelet count (×10^3/μL)	106 ± 8	140 ± 2	<0.001
Albumin (g/dL)	3.8 ± 0.1	4.0 ± 0.1	<0.001
Total bilirubin (mg/dL)	1.2 ± 0.1	0.8 ± 0.1	<0.001
AST (IU/L)	78.1 ± 6.8	64.6 ± 2.1	n.s.
ALT (IU/L)	72.8 ± 9.7	72.0 ± 2.9	n.s.
Gamma-GTP (IU/L)	68.7 ± 7.5	53.9 ± 2.3	n.s.
Alpha fetoprotein (ng/L)	17.1 (4.4–36.8)	16.7 (4.1–23.1)	n.s.
Esophageal varices	29.0 % (9/31)	6.4 % (22/344)	<0.01
On-treatment parameters			
ALT (IU/L)	59.4 ± 5.7	44.6 ± 1.8	<0.05
Alpha fetoprotein (ng/L)	9.8 (4.6–17.4)	5.5 (3.7–11.1)	<0.01
HCV RNA level (KIU/mL)	236 (<0.5–2,210)	21 (<0.5–1,780)	<0.05

n.s. not significant

Statistical analysis

Categorical data were compared using the χ^2 test or Fisher's exact test. The distributions of continuous variables were analyzed using Student's t-test and the Mann–Whitney U-test for two groups. Multivariate analysis was

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at $p < 0.05$. Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFNα-2a group were included.

Results

Study 1

We analyzed the factors involved in the development of HCC in patients who received 90 μg PegIFNα-2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFNα-2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pretreatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFNα-2a maintenance treatment (Table 2).

Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of ≥41 IU/L and AFP levels of ≥10 ng/L 24 weeks after the start of the PegIFNα-2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of ≤40 IU/L than in those with ALT levels of ≥41 IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of <10 ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of ≥10 ng/mL (Fig. 3). The dose of PegIFNα-2a was reduced to 45 μg in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFNα-2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

Table 3 Independent risk factors for HCC development in patients treated with 90 μg PegIFNα-2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	*p*
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count (<120 × 10^3/μL vs. ≥120 × 10^3/μL)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) (≥41 vs. <40 IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) (≥10 vs. <10 ng/L)	3.78	1.92–11.8	<0.01

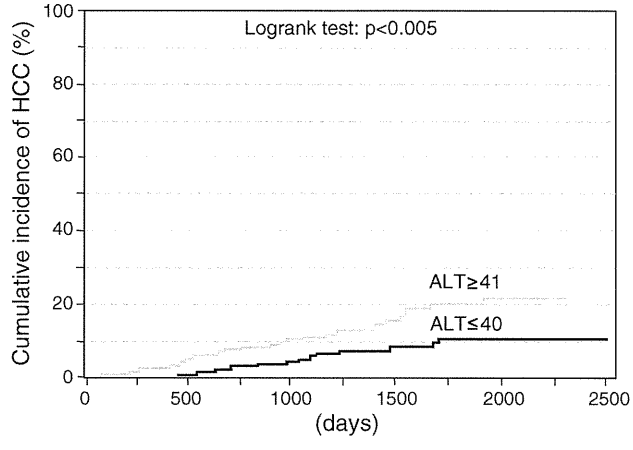

Fig. 2 Comparison of HCC rates in patients administered with PegIFNα-2a ($n = 594$) with respect to alanine aminotransferase (*ALT*) levels 24 weeks after the start of therapy. *Black line* patients with ALT ≥41 IU/L in the first 24 weeks, *gray line* patients with ALT ≤40 IU/L in the first 24 weeks