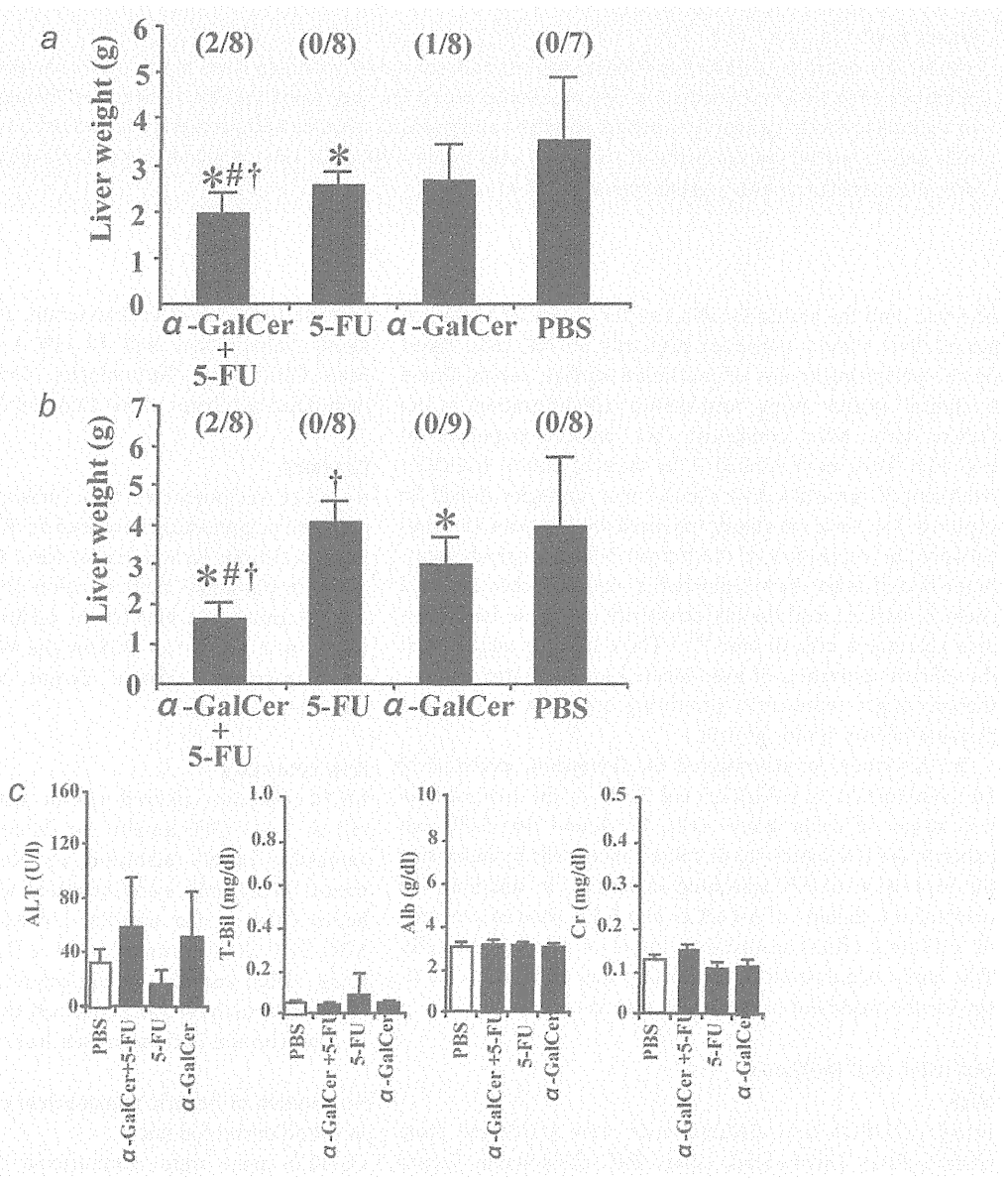

Figure 1. The antitumor effect of the α-GalCer and 5-FU combination therapy against MC38 liver tumors. (*a*, *b*) C57BL/6 mice or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0. To evaluate the efficacy of the α-GalCer and 5-FU combination therapy, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine intrahepatic tumor growth. $N = 7$–9 mice/group. Each data point represents the mean liver weight ± SD. The fraction of mice achieving tumor rejection in each treatment group is shown in parentheses. $^*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* 5-FU group, $\dagger p < 0.05$ *versus* α-GalCer group. (*c*) Blood samples from treated C57BL/6 mice were obtained 1 day after the final injection of each treatment. The serum levels of ALT, T-Bil, Alb, and Cr were examined. $N = 3$ /group. No significant differences were observed between any of the groups.

were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated or nontreated MC38 cells as previously described.[12] The assays were performed in triplicate, and the spontaneous release of all assays did not exceed 25% of the maximum release. In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 (R&D Systems) or H60 (R&D Systems).

Animal experiments

C57BL/6 or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0.

To evaluate the efficacy of the combination therapy of α-GalCer and 5-FU, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight respectively) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine the intrahepatic tumor growth. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-asialo GM-1 (ASGM1) Ab (WAKO, Osaka, Japan) on Days −1, 4, and 9 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometric analysis of splenocytes using PE-conjugated anti-DX5 mAbs (BD-Pharmingen) as previously described.[8] NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of treated mice were removed, and the liver weight was measured to examine the intrahepatic tumor growth.

NKG2D lignads and DNAM1 ligands expression in MC38 tumor tissues and nontumor tissues in 5-FU-treated mice

C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry.

Blood biochemistry test

Blood samples were obtained 24 hr after treatment. The levels of serum alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin (Alb), and creatinine (Cr) were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Statistics

All values are expressed as the mean and SD. Statistical analyses were performed by the unpaired Mann–Whitney *U* test or one-way ANOVA unless otherwise indicated. When ANOVA analyses were applied, differences in the mean values among groups were examined by the Scheffe post hoc correction. We defined statistical significance as $p < 0.05$.

Results

The combination therapy of α-GalCer and 5-FU showed a synergistic antitumor effect against MC38 liver tumors

We examined the antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 liver tumors. C57BL/6 mice were injected intrahepatically with MC38 cells. The mice were treated with α-GalCer on Day 0 and/or 5-FU for 5 consecutive days after tumor inoculation. As shown in Figure 1*a*, the liver weights of the mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with 5-FU were significantly lower than those of nontreated mice, but treatment with α-GalCer did not produce this effect. We also examined the antitumor effect of α-GalCer plus 5-FU in a Colon26 liver tumor model. The liver weights of mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with α-GalCer were significantly lower than those of nontreated and 5-FU-treated mice (Fig. 1*b*). Tumor rejection in the MC38 liver tumor model was observed in 2/8 of the α-GalCer plus 5-FU-treated

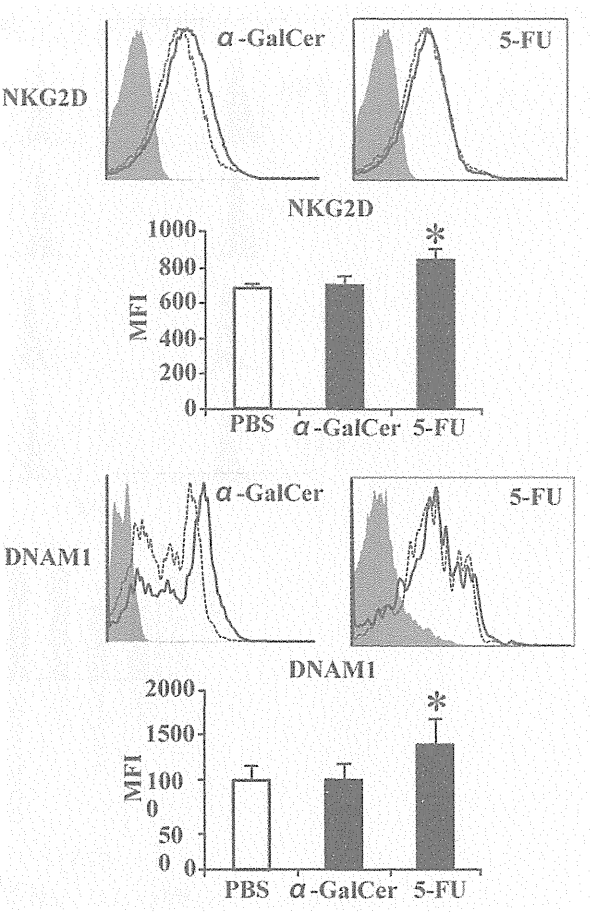

Figure 2. Expression of NKG2D and DNAM1 on liver NK cells isolated from α-GalCer- or 5-FU-treated mice. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or with 5-FU (10 mg/kg body weight) for 3 consecutive days. Liver NK cells were isolated from α-GalCer or 5-FU-treated mice, and the expression levels of NKG2D and DNAM1 were evaluated by flow cytometry. Black bold line histograms: NKG2D or DNAM1 staining of NK cells from α-GalCer or 5-FU-treated mice; dotted line histograms: NKG2D or DNAM1 staining of NK cells from PBS-treated mice; shaded/gray histograms: control staining. The data are represented as the average of the MFI obtained from 3 separate experiments. *$p < 0.05$ *versus* PBS-treated group.

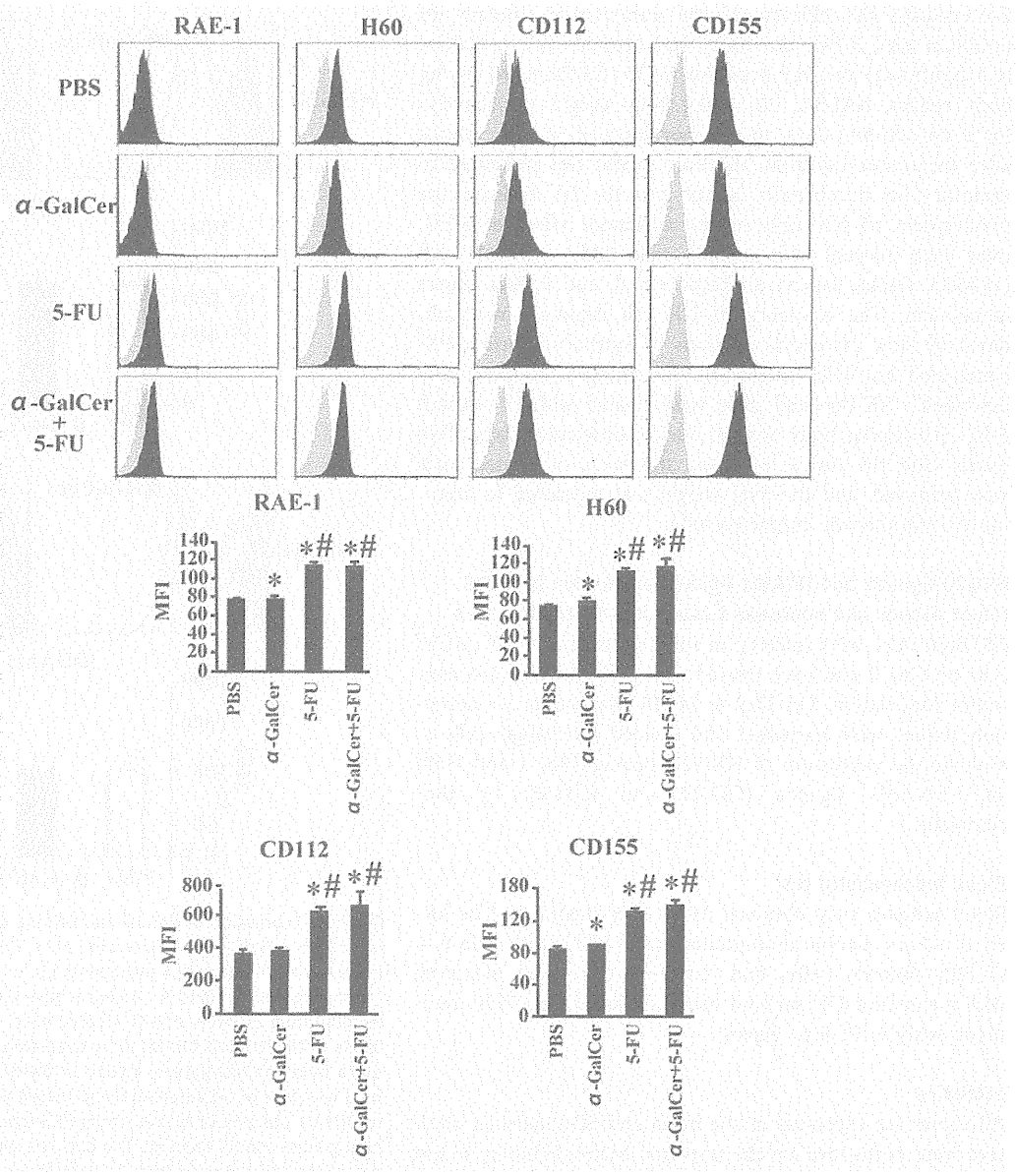

Figure 3. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells treated with α-GalCer and/or 5-FU. MC38 cells were cultured with or without α-GalCer (100 ng/ml) or 5-FU (500 nmol/l) for 24 hr. The treated cells were harvested and evaluated for the expression levels of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells by flow cytometry. Upper panel: representative data. Shaded/black histograms: NKG2D or DNAM1 ligand staining of α-GalCer or 5-FU-treated MC38 cells; shaded/gray histograms, control staining. Lower panel: data are represented as the average of MFI obtained from 3 separate experiments. $*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* α-GalCer group.

mice, 0/8 of the 5-FU-treated mice, 1/8 of the α-GalCer-treated mice, and 0/7 of the PBS-treated mice (Fig. 1*a*). These results were consistent with those of another Colon26 liver tumor model in BALB/c mice, where tumor rejection was observed in 2/8 of the α-GalCer plus 5-FU-treated mice, 0/8 of the 5-FU-treated mice, 0/9 of the α-GalCer-treated mice, and 0/8 of the PBS-treated mice (Fig. 1*b*). These results demonstrated that the combination therapy of α-GalCer and 5-FU produced a synergistic antitumor effect against liver tumors in both the MC38 and Colon26 models. To evaluate the safety of this combination therapy, serum levels of ALT, T-Bil, Alb, and Cr were evaluated in C57BL/6 mice immunized with α-GalCer plus 5-FU, 5-FU, α-GalCer, or PBS. There was no toxic effect upon the ALT, T-Bil, Alb, or Cr levels for any of the treatment groups (Fig. 1*c*). These results demonstrated that the combination therapy of α-GalCer and

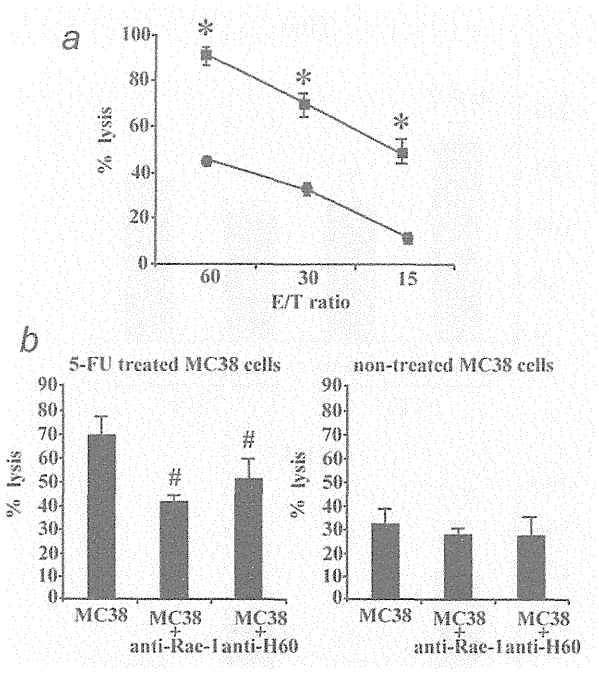

Figure 4. The cytolytic activity of α-GalCer-activated MNCs against 5-FU-treated MC38 cells. C57BL/6 mice were injected i.p. with α-GalCer (2 μg/mice) to activate NK cells. Liver MNCs were prepared on Day 3 after α-GalCer injection. (*a*) MC38 cells were cultured with or without 5-FU (500 nmol/l) for 24 hr. α-GalCer-activated liver MNCs were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated (■) or nontreated (●) MC38 cells. (*b*) In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 or H60 at an E/T ratio of 30:1. Similar results were obtained from 3 independent experiments. $*p < 0.05$ *versus* the cytolytic activity of activated NK cells against nontreated cells, $\#p < 0.05$ *versus* the cytolytic activity of activated NK cells against 5-FU-treated cells.

5-FU is not toxic to hepatocytes and does not harm the liver or kidney.

α-GalCer, but not 5-FU, treatment induced NK activating receptors on NK cells

We examined the expression levels of activating (NKG2D and DNAM1) receptors on liver NK cells. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or 5-FU (10 mg/kg body weight) for 3 consecutive days and liver NK cells were isolated from 5-FU- and α-GalCer-treated mice. As shown in Figure 2, the expression levels of NKG2D and DNAM1 on liver NK cells from α-GalCer-treated mice were significantly higher than those from PBS-treated mice. In contrast, the expression of NKG2D and DNAM1 on liver NK cells from 5-FU-treated mice was similar to that of PBS-treated mice. These results demonstrated that α-GalCer, but not 5-FU, could activate liver NK cells.

5-FU, but not α-GalCer, treatment induced NK activating molecules on colon cancer cells

We next examined the expression of the NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 colon cancer cells treated with α-GalCer and/or 5-FU. We first examined the cytotoxicity of 5-FU on MC38 cells by the WST-8 assay. The addition of more than 1 μmol/l of 5-FU resulted in a significant decrease in the growth of MC38 cells (data not shown). On the basis of these findings, we used 500 nmol/l of 5-FU to evaluate the biological effect on MC38 cells. MC38 cells were incubated with α-GalCer (100 ng/ml) and/or 5-FU (500 nmol/l) for 24 hr and the expression levels of NK activating molecules on MC38 cells were evaluated by flow cytometry. 5-FU induced the expression of Rae-1, H60, CD112, and CD155 on MC38 cells (Fig. 3). The expression of these molecules on 5-FU-treated MC 38 cells was significantly higher than that of nontreated MC38 cells. The induction of these NK activating molecules was dose-dependent (data not shown). In contrast, α-GalCer could not induce the expression of Rae-1, H60, CD112, or CD155 on MC38 cells. Even in the MC38 cells treated with this combination of α-GalCer and 5-FU, α-GalCer failed to induce additional expression of NK activating molecules. These results demonstrated that 5-FU, but not α-GalCer, could enhance the expression of NK activating molecules on colon cancer cells.

The cytolytic activity of α-GalCer activated liver MNCs against 5-FU-treated MC38 cells

We next examined the cytolytic activity of α-GalCer-activated liver MNCs against 5-FU-treated MC38 cells. We isolated liver MNCs from normal α-GalCer injected mice and the cytolytic activity of these α-GalCer-activated liver MNCs was measured. The cytolytic activity of liver MNCs against 5-FU-treated MC38 cells was significantly higher than that against non-treated cells (Fig. 4*a*). The cytolytic activity against 5-FU-treated MC38 cells decreased significantly following the addition of blocking Abs against Rae-1 or H60 (Fig. 4*b*).

5-FU treatment induced the expression of NK activating molecules in MC38 liver tumor tissues but not in MC38 nontumor tissues

We examined the induction of NKG2D ligand (Rae-1 and H60) and DNAM1 ligand (CD112 and CD155) expression in MC38 liver tumor or nontumor tissues of 5-FU-treated mice. As shown in Figure 5, the expression of Rae-1 in liver tumor tissues of 5-FU-treated mice was significantly higher than that of liver tumor and nontumor tissues of PBS-treated mice and that of nontumor tissues of 5-FU-treated mice. The expression of H60 and CD112 was similar to that of Rae-1. The expression of CD155 in liver tumor tissues of 5-FU-treated mice tended to be higher than that of PBS-treated mice, although the difference was not statistically significant. These results demonstrated that 5-FU treatment induced the expression of NK activating molecules in liver tumor tissues but not in nontumor tissues consistent with the *in vitro* results.

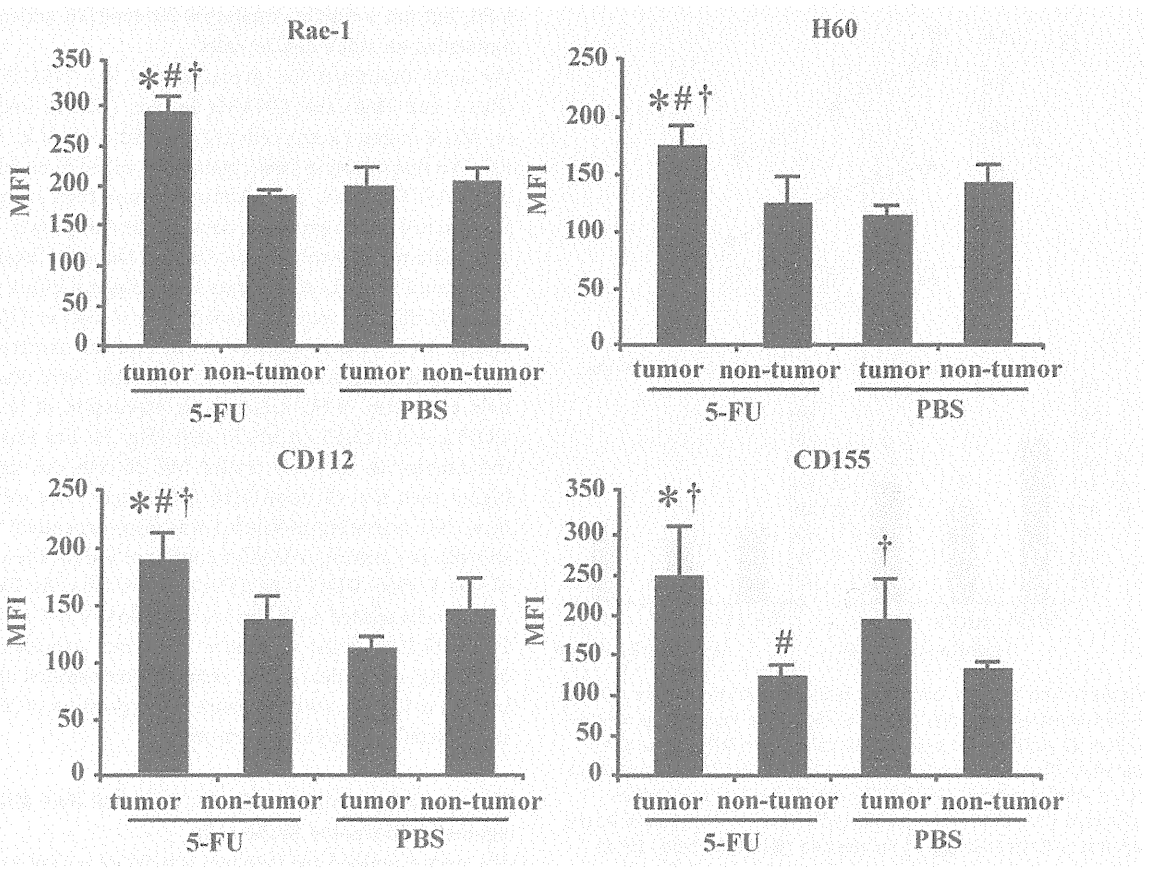

Figure 5. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 liver tumor tissues of mice treated with 5-FU. C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry. $N = 3$/group. *$p < 0.05$ *versus* nontumor tissues of PBS group, #$p < 0.05$ *versus* tumor tissues of PBS group, †$p < 0.05$ *versus* nontumor tissues of 5-FU group.

The antitumor effect of 5-FU depended on both direct cytotoxicity and the cytolytic activity of NK cells in mouse colon cancer

The above results suggested that 5-FU could enhance the NK sensitivity of MC38 cells. To confirm that NK activity played a role in the antitumor effect of 5-FU, we examined the antitumor effect of 5-FU against MC38 liver tumors in NK depleted mice. As shown in Figure 6, the liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. Depletion of NK cells significantly inhibited the antitumor efficacy of 5-FU against MC38 liver tumors. These results suggested that the antitumor effect of 5-FU depended on not only on the direct cytotoxic effect of 5-FU but also on the cytolytic activity of NK cells. Therefore, NK activity plays a role in the antitumor effect of 5-FU in the liver which contains abundant NK cells.

Discussion

The lymphocytes in the liver are typically enriched with a higher number of NK cells than that found in the peripheral blood in a normal mouse.[3,4] Efficient activation of the abundant NK cells in the liver might be important in antitumor defense against liver tumors. Interferon-α (IFN-α) could activate liver NK cells efficiently.[14] Bui *et al.*[15] reported that IFN-α reduced the expression of H60 on MCA sarcoma cells, suggesting that IFN-α treatment may reduce the NK sensitivity of cancer cells. We and others have previously demonstrated that the systemic administration of α-GalCer can lead to antitumor effects against metastatic liver tumors through the efficient activation of liver NK cells.[8,16] Although α-GalCer has not yet been officially accepted for clinical application in cancer treatment, these previous results encouraged us to evaluate the antitumor effect of the combination of α-GalCer and 5-FU against MC38 liver tumors. In most reports, high dose (2 μg/mouse) α-GalCer was applied for the treatment of liver tumors. However, administration of these high dose resulted in liver injury.[9,17,18] In the present study, we used low dose (0.4 μg/mouse) α-GalCer in the combination therapy. The administration of low dose α-GalCer is enough to activate liver NK cells and did not affect

the expression of NK activating molecules on MC38 cells. Importantly, the administration of this low dose α-GalCer did not cause liver injury. The antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 and Colon26 liver tumors was stronger than that of 5-FU alone or α-GalCer alone. The antitumor effect of the combination therapy of low dose (0.4 μg/mouse) α-GalCer and 5-FU was equal to that of the combination therapy of high dose (2 μg/mouse) α-GalCer and 5-FU (Aketa *et al.*, unpublished data). Our results might offer new chemo-immunotherapy strategies, especially for those patients with advanced stages of cancer.

In this study, we demonstrated that 5-FU treatment enhanced the expression of both NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells. In contrast, 5-FU treatment did not affect the activating molecules on NK cells. Both pathways involving NKG2D and DNAM1 play critical roles in the activation of NK cells and have been implicated in tumor surveillance.[19] The expression of NKG2D ligands has been associated with a good prognosis in patients with colon cancer.[20] Thus, these results suggest that the upregulation of NKG2D ligand expression might improve the prognosis of patients with colon cancer. Gasser *et al.*[21] previously reported that DNA-damaging agents and DNA-synthesis inhibitors including 5-FU could induce the expression of NKG2D ligands on tumor cells. We also demonstrated that 5-FU treatment could induce the expression of NK activating molecules in MC38 liver tumor tissues but not in nontumor tissues, which was consistent with the *in vitro* results. Our present results suggest that 5-FU treatment might have strong immune-editing potential to enhance the NK sensitivity of colon cancer cells by regulating DNAM1 and NKG2D ligands.

In this study, we demonstrated that 5-FU treatment enhanced the susceptibility of MC38 cells to the cytolytic activity of liver MNCs *via* the NKG2D-NKG2D ligand pathway. Because the blocking antibody of the DNAM1-DNAM1 ligand is not commercially available, we could not evaluate the involvement of this pathway. We have previously demonstrated that membrane-bound MICA, an activating molecule of NK cells, on HCC cells is essential in the NK sensitivity of HCC cells.[12,13] The addition of both epirubicin and sorafenib enhanced the NK sensitivity of HCC cells by increasing the membrane-bound MICA.[12,13] This finding is consistent with this study of a colon cancer model. Interestingly, the expression of death receptors, such as FAS and TRAIL receptors, on MC38 cells was significantly increased by 5-FU treatment (Aketa *et al.*, unpublished data). This result may also explain the enhancement of the susceptibility of MC38 cells to the cytolytic activity of liver MNCs. We previously demonstrated that α-GalCer administration resulted in rapid and strong activation of liver NK cells and that the cytolytic activity of liver MNCs early after α-GalCer administration mainly depended primarily on liver NK cells and not on NKT or T cells.[8,10] Taken together, these results suggest that the addition of 5-FU enhanced the NK sensitivity of MC38 cells by increasing the

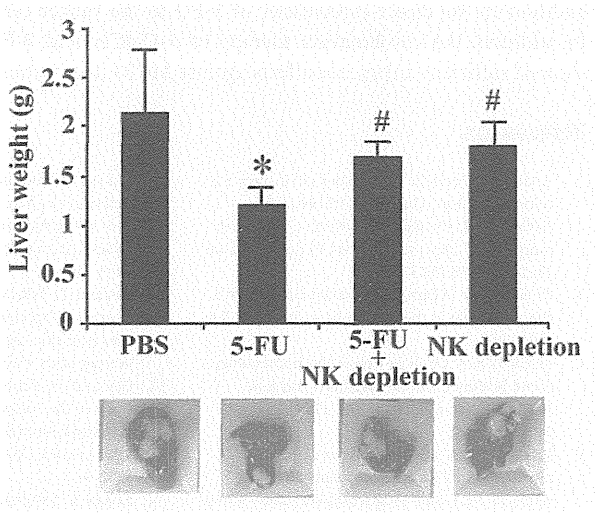

Figure 6. The antitumor effect of 5-FU against MC38 liver tumors in NK-depleted mice. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-ASGM1 Ab. NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine the intrahepatic tumor growth. $*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* 5-FU group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of Rae-1 or H60 on MC38 cells. Therefore, 5-FU treatment might be expected to enhance the susceptibility of MC38 cells to the cytolytic activity of NK cells by modifying the expression of NKG2D and DNAM1 ligands.

NK depletion decreased the antitumor effect of 5-FU against MC38 liver tumors, demonstrating that the antitumor effect of 5-FU depends on NK activity in addition to direct cytotoxicity. We also examined the antitumor effect of 5-FU against the Colon26 liver tumor model, derived from BALB/c colon cancer. The liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. The depletion of NK cells also significantly inhibited the antitumor efficacy of 5-FU against Colon26 liver tumors in BALB/c mice. A significant upregulation of Rae-1, H60, CD112, and CD155 could also be observed in 5-FU-treated Colon26 cells derived from BALB/c mice (Aketa *et al.*, unpublished data). These results were consistent with the results of C57BL/6 mice and suggest that the antitumor effect of 5-FU may always depend on NK activity in the liver. The liver contains abundant NK cells. In cancer tissues that are rich in NK cells, the combination therapy of α-GalCer and 5-FU might have a potential as a new chemo-immunotherapeutic strategy.

The liver is the most common site of metastasis of gastrointestinal cancers (*i.e.*, colorectal, gastric, and pancreatic cancers). Thus, new therapeutic approaches of cancer immunotherapy for metastatic liver cancer need to be developed. We have shown here that 5-FU can enhance the NK sensitivity of cancer cells by inducing the expression of NK activating molecules in

addition to the direct cytotoxicity of 5-FU to the cancer cells. In addition, the combination therapy of α-GalCer and 5-FU showed sufficient antitumor effects against MC38 liver tumors. These findings indicate that this new combination chemo-immunotherapy might represent a particularly promising approach for patients with metastatic liver cancer.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Incidence of hepatocellular carcinoma in HCV-infected patients with normal alanine aminotransferase levels categorized by Japanese treatment guidelines

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Abstract

Background This study was conducted to evaluate Japanese treatment guidelines for patients with chronic hepatitis C virus (HCV) infection and normal alanine aminotransferase (N-ALT) levels from the viewpoint of the incidence of hepatocellular carcinoma (HCC).

Methods Four groups of patients with chronic HCV infection treated with pegylated interferon (Peg-IFN) plus ribavirin, and classified according to the N-ALT guidelines, were examined for HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and platelet (PLT) $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$; group C ($n = 233$), 30 < ALT $\leq$ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), 30 < ALT $\leq$ 40 IU/L and PLT $<15 \times 10^4/mm^3$. The mean observation period was 36.2 ± 16.5 months

Results In groups A and C, the HCC incidence was low even in patients with non-response (NR) (cumulative rates at 3 years, 0.0 and 2.9 %, respectively). In groups B and D, 14.5 and 5.3 % of NR patients had developed HCC at 3 years, but none of the patients with sustained virologic response (SVR) or relapse had developed HCC. In group B, no patients with mild fibrosis developed HCC irrespective of the antiviral effect of the treatment. Among patients with PLT $<15 \times 10^4/mm^3$ (group B plus group D), the HCC incidence was significantly lower in patients with SVR and relapse than in NR patients ($p < 0.001$, $p = 0.021$, respectively).

Conclusion These results suggest that N-ALT patients with PLT $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy.

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Introduction

Continuous hepatitis C virus (HCV) infection causes liver inflammation and can lead to liver fibrosis, which may progress to cirrhosis and hepatocellular carcinoma (HCC) [1–4]. Because HCV carriers with persistent normal alanine aminotransferase (PNALT) levels have minimal liver inflammation and the progression of liver fibrosis in such patients is slow, they are generally considered to be at low risk for carcinogenesis [5–7]. Moreover, patients with PNALT had not been considered as candidates for antiviral therapy in the era of interferon (IFN) monotherapy because of reports of ALT flare-up owing to antiviral therapy in some cases (47–67 %) [8–10].

However, in recent years, the antiviral efficacy of pegylated IFN (Peg-IFN) plus ribavirin combination therapy for patients with chronic HCV infection has been reported to be equivalent for patients with normal alanine aminotransferase (N-ALT) levels and those with elevated ALT levels [11–15]. In addition, for patients with PNALT, there have been fewer cases of ALT flare-up caused by Peg-IFN plus ribavirin combination therapy than with IFN monotherapy [12, 15]. Thus, patients with chronic HCV infection and N-ALT have come to be treated with Peg-IFN plus ribavirin combination therapy.

Treatment guidelines for patients with chronic HCV infection and N-ALT levels have been prepared by a Japanese group conducting "Research on Hepatitis" supported by Health and Labour Sciences Research Grants from the Japanese Government. In these guidelines, HCV carriers with N-ALT (≤40 IU/L) are categorized into four groups according to their ALT levels (≤30 or ≥31 IU/L) and platelet (PLT) counts (≥15 or $<15 \times 10^4/mm^3$). Briefly, the therapeutic strategies are as follows: patients with ALT levels of more than 31 IU/L are candidates for antiviral treatment, but observation is recommended for patients with ALT levels of <30 IU/L. However, the goal of antiviral treatment is to improve the long-term prognosis, including inhibition of HCC. Therefore, the indication of antiviral therapy for patients with chronic HCV infection and N-ALT should be decided based on whether or not Peg-IFN plus ribavirin combination therapy can suppress the cumulative rate of HCC incidence and improve prognosis. It is thus very important to examine the effect of inhibition of HCC induced by antiviral therapy in patients with chronic HCV infection and N-ALT.

In the present study, we evaluated the treatment guidelines for patients with chronic HCV infection and N-ALT from the viewpoint of HCC inhibition by analyzing the differences in the cumulative rates of HCC incidence among the above four groups. The treatment guidelines also recommend that if patients with ALT ≤30 IU/L and PLT $<15 \times 10^4/mm^3$ have moderate to severe liver fibrosis (F2–4), they should receive antiviral therapy. We also evaluated the effect of Peg-IFN plus ribavirin on HCC incidence according to the degree of fibrosis in this group.

Patients and methods

This retrospective study was conducted by Osaka University and institutions participating in the Osaka Liver Forum. Among patients with chronic HCV infection who had received Peg-IFN plus ribavirin combination therapy from December 2004 to December 2009, four groups of patients, classified according to the N-ALT guidelines, who had not suffered from HCC, were examined for their HCC incidence: group A ($n = 353$), ALT ≤30 IU/L and PLT $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT ≤30 IU/L and PLT $<15 \times 10^4/mm^3$; group C ($n = 233$), $30 <$ ALT ≤ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), $30 <$ ALT ≤ 40 IU/L and PLT $<15 \times 10^4/mm^3$. The Kaplan–Meier method was used to examine the cumulative rates of HCC incidence in the four groups. Excluded from this study were patients who developed HCC within 12 months from the start of Peg-IFN plus ribavirin combination therapy, patients with co-infection with hepatitis B or human immunodeficiency virus, patients with drug-induced or alcoholic liver disorders, and patients with autoimmune hepatitis. The protocol was performed after obtaining informed consent from each patient before treatment in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008. This study was approved by the Institutional Review Board and registered in the Universal Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN unique trial number, C000000197).

Treatment protocol

All patients received Peg-IFN alpha-2b (PEGINTRON; Merck & Co., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD) for the duration of the study. Peg-IFN alpha-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight ≤60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients. Dose modification according to the intensity of the hematological adverse effects followed, as a rule, the manufacturer's drug information. The dose of Peg-IFN alpha-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the PLT count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the PLT count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced, from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <8.5 g/dL. Both Peg-IFN alpha-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no medicine containing iron or hematopoetic growth factors, such as erythropoietin alpha, or granulocyte–macrophage colony-stimulating factor, was administered. The serum HCV RNA levels were qualitatively analyzed using the COBAS AMPLICOR HCV Test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics, Branchburg, NJ, USA), and the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml). In the patients with HCV genotype 1, as a rule, treatment duration was 48 weeks, but the patients with detectable HCV RNA (≥50 IU/mL) at week 12 and undetectable HCV RNA (<50 IU/mL) at week 24 were treated for 72 weeks. Patients with HCV genotype 2 were treated for 24 weeks.

Definition of virologic response

A sustained virologic response (SVR) was defined as undetectable HCV RNA at the end of treatment and at 24 weeks after completion of treatment. A relapse was defined as undetectable HCV RNA at the end of treatment but detectable HCV RNA at 24 weeks after completion of treatment. A non-response (NR) was defined as detectable HCV RNA at the end of treatment.

Histological evaluation

Liver biopsy was performed immediately before initiation of the Peg-IFN plus ribavirin combination therapy. Liver biopsy specimens were scored using the METAVIR system, and the grade of activity and stage of fibrosis were evaluated [16].

HCC surveillance

Ultrasonography or computed tomography (CT) was carried out before the initiation of the Peg-IFN plus ribavirin combination therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by CT or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out, with the patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

End point

The observation period was defined as the period from the start of Peg-IFN plus ribavirin combination therapy. Patients who developed HCC and patients whose treatments were switched to other types of IFN therapy were defined as censored cases at that point in time.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD. To analyze differences between baseline data among the four groups, analysis of variance or the χ^2 test was performed. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with the log-rank test. A value of $p < 0.05$ (two-tailed) was considered to indicate significance. The statistical software used for this analysis was IBM SPSS for Windows v. 19.0.0 (SPSS, Armonk, NY, USA).

Results

Baseline characteristics of patients categorized by the treatment guidelines

The baseline clinical features of the patients are shown in Table 1. There were significant differences in age; sex; body mass index (BMI); HCV genotype; past history of IFN therapy; grade and stage of liver histology; WBC, neutrophil, and PLT counts; Hb levels; and virologic response among the four groups. The mean ages of the patients in groups B and D were significantly higher than those of the patients in groups A and C. The proportion of males was lowest in group A (26 %) and highest in group C (41 %). The proportion of patients with progression of liver fibrosis (F3–4) diagnosed by the METAVIR score was 7.8 % among all patients tested and highest in group D (22.5 %). In groups B and D, peripheral blood cell counts (WBC, neutrophils, Hb, PLT) were significantly lower and the

Table 1 Baseline characteristics of the patients with chronic HCV infection and normal ALT levels

	Group A ALT ≤30 IU/L PLT count ≥15 × 10^4/mm^3	Group B ALT ≤30 IU/L PLT count <15 × 10^4/mm^3	Group C 30 < ALT ≤ 40 IU/L PLT count ≥15 × 10^4/mm^3	Group D 30 < ALT ≤ 40 IU/L PLT count <15 × 10^4/mm^3	*p* value
Number of patients	353	123	233	100	
Age (years)	55.6 ± 11.3	60.3 ± 8.4	54.6 ± 11.8	60.7 ± 8.6	<0.001
Sex: male/female	95/258	44/79	95/138	35/65	0.005
BMI (kg/m^2)	22.6 ± 3.3	22.1 ± 3.0	23.2 ± 3.4	22.3 ± 2.6	0.029
HCV genotype: 1/2	203/144	86/35	180/52	81/16	<0.001
HCV RNA (KIU/mL), mean ± SD	2333 ± 1664	2276 ± 1478	2261 ± 1599	2354 ± 1644	0.998
Past IFN therapy: naïve/experienced[a]	266/81	79/41	173/52	63/33	0.018
Histology[b]: activity: A0/A1/A2/A3	32/179/48/1	6/64/23/0	20/105/36/1	0/46/24/1	0.026
Fibrosis: F0/F1/F2/F3/F4	41/169/40/9/1	4/49/29/7/5	16/107/31/7/1	0/34/21/13/3	<0.001
White blood cell count (/mm^3)	5543 ± 1606	4405 ± 1211	5601 ± 1638	4677 ± 1337	<0.001
Neutrophil count (/mm^3)	3008 ± 1213	2332 ± 948	2999 ± 1243	2578 ± 1026	<0.001
Hemoglobin (g/dL)	13.3 ± 1.3	13.3 ± 1.4	13.9 ± 1.4	13.3 ± 1.3	<0.001
Platelet count (×10^4/mm^3)	21.1 ± 4.7	12.2 ± 2.1	21.3 ± 4.8	12.1 ± 2.2	<0.001
ALT (IU/L)	22.8 ± 5.2	23.5 ± 5.4	35.4 ± 2.9	35.8 ± 2.9	<0.001
Virologic response: SVR/relapse/NR	218/82/53	59/32/32	133/51/49	44/26/30	0.005

BMI body mass index, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *SVR* sustained virologic response, *NR* non-response, PLT platelet

[a] Virologic response to previous treatment was unknown for 22 patients

[b] Fibrosis stages are evaluated on a scale of 0–4 and activity grades are evaluated on a scale of 0–3 according to the METAVIR histological score. Fibrosis data were not available for 222 patients. Activity data were not available for 223 patients

numbers of patients with progression of liver fibrosis were significantly higher than in groups A and C. The mean duration of the observation period was 36.2 ± 16.5 months.

Antiviral efficacy of Peg-IFN plus ribavirin combination therapy

In genotype 1 patients, the rates of SVR, relapse, and NR were 50.7, 25.1, and 24.1 %, respectively, in group A; 39.5, 24.4, and 36.0 % in group B; 52.2, 23.9, and 23.9 % in group C; and 39.5, 25.1, and 35.2 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate was significantly higher in groups A and C than that in groups B and D (groups A and C: SVR 51.4 %, relapse 24.5 %, NR 24.0 %; groups B and D: SVR 39.5 %, relapse 25.1 %, NR 35.2 %, $p = 0.012$). In genotype 2 patients, the rates of SVR, relapse, and NR were 77.8, 20.1, and 2.1 %, respectively, in group A; 65.7, 31.4, and 2.9 % in group B; 75.0, 15.4, and 9.6 % in group C; and 62.5, 31.3, and 6.3 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate tended to be higher in groups A and C than that in groups B and D (groups A and C: SVR 77.0 %, relapse 18.9 %, NR 4.1 %; groups B and D: SVR 64.7 %, relapse 31.4 %, NR 8.9 %, $p = 0.152$).

Cumulative rate of HCC incidence according to the treatment effect of Peg-IFN plus ribavirin combination therapy

Eleven patients developed HCC during the observation period, and all were infected with HCV genotype 1. Figure 1 shows the cumulative rates of HCC incidence according to the treatment effect in the four groups.

In group A, no patients developed HCC during the 3 years of observation, regardless of the effect of Peg-IFN plus ribavirin combination therapy. Moreover, among those with SVR and relapse, no patients developed HCC during the 3-year observation period, while in NR patients the cumulative rate of HCC incidence at 5 years was 4.0 %. No significant difference in HCC incidence was found among the patients with SVR, relapse, and NR ($p = 0.071$) (Fig. 1a). In group C, no significant difference in HCC incidence was found among the patients with SVR, relapse, and NR (cumulative rates of HCC at 3 years, 2.2, 0.0, and 2.9 %, respectively; at 5 years, 3.7, 0.0, and 2.9 %, respectively, $p = 0.631$) (Fig. 1c). In group B, a marginally significant difference was found in HCC incidence among patients with SVR, relapse, and NR ($p = 0.054$), and patients with SVR had a significantly lower rate of HCC incidence than that of patients with NR (SVR vs. relapse, $p = 0.346$, SVR vs. NR, $p = 0.013$, relapse vs.

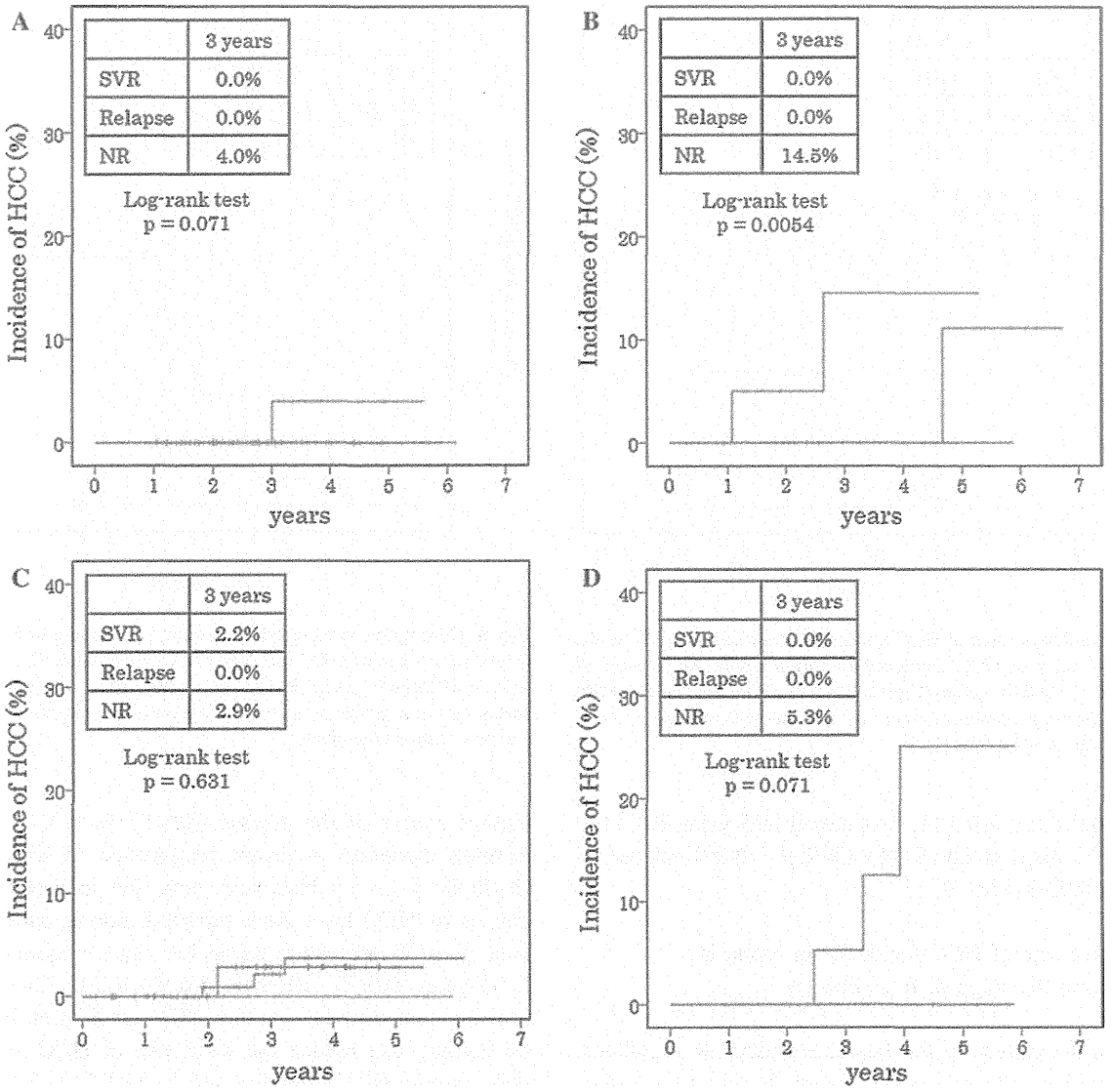

Fig. 1 Cumulative rates of hepatocellular carcinoma (*HCC*) incidence in groups A, B, C, and D, categorized according to the treatment effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy. **a** Group A (patients with alanine aminotransferase [ALT] level ≤30 IU/L and platelet [PLT] count ≥15 × 10^4/mm^3), **b** group B (patients with ALT ≤30 IU/L and PLT <15 × 10^4/mm^3), **c** group C (patients with 30 < ALT ≤ 40 IU/L and PLT ≥15 × 10^4/mm^3), **d** group D (patients with 30 < ALT ≤ 40 IU/L and PLT < 15 × 10^4/mm^3). *Blue line* patients with sustained virologic response (*SVR*), *green line* patients with relapse, *red line* patients with non-response (*NR*)

NR, $p = 0.250$). Of the NR patients, 14.5 % had developed HCC at 3 years, while none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1b). In group D, there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.006$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (SVR vs. NR, $p = 0.012$, relapse vs. NR, $p = 0.047$). In the NR patients, 5.3 % had developed HCC at 3 years and 25.0 % had developed HCC at 5 years, but none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1d).

In the analysis of the differences in the cumulative rates of HCC incidence in the patients with 30 < ALT ≤ 40 IU/L (group C plus group D), the p value for a significant difference was 0.059 among the patients with SVR, relapse, and NR (Fig. 2). In the analysis of the differences in the cumulative rates of HCC incidence among the patients with PLT counts of less than 15 × 10^4/mm^3 (group B plus group D), there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p < 0.001$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (cumulative rates of HCC incidence at

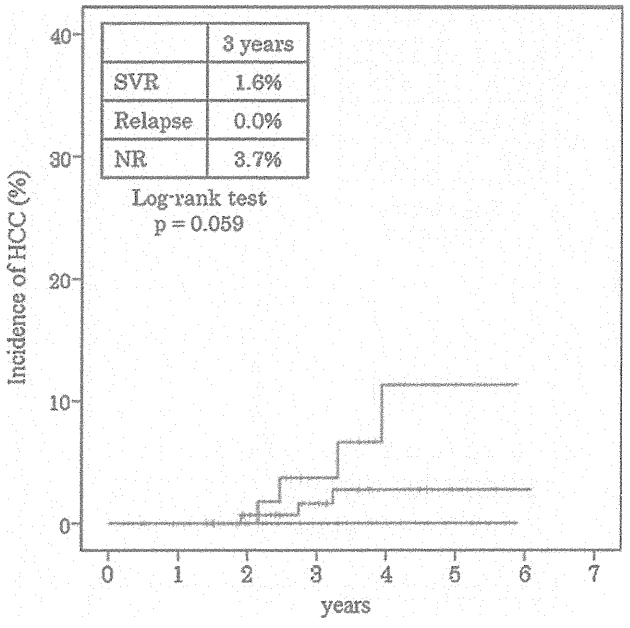

Fig. 2 Cumulative rates of HCC incidence according to ALT levels. Cumulative rates of HCC incidence in patients with ALT levels of 30 < ALT ≤ 40 IU/L (group C plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

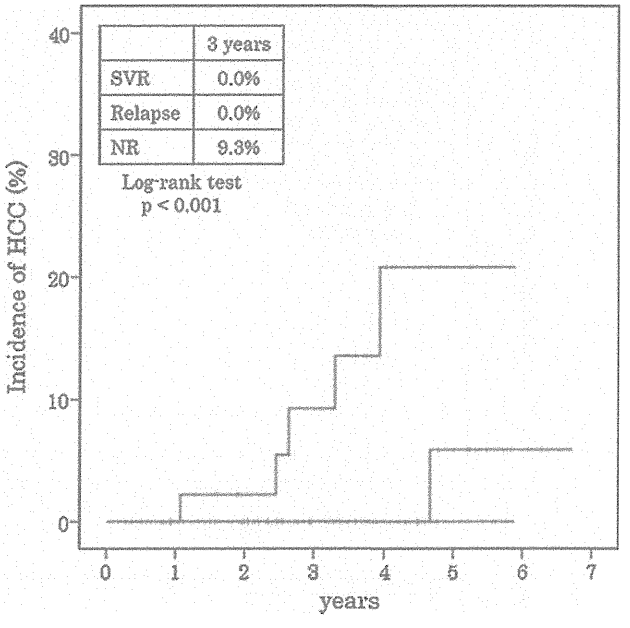

Fig. 3 Cumulative rates of HCC incidence according to PLT counts. Cumulative rates of HCC incidence in patients with PLT counts of $<15 \times 10^4/mm^3$ (group B plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

3 years, 0.0, 0.0, and 9.3 %, respectively; at 5 years, 0.0, 11.1, and 20.8 %, respectively; SVR vs. NR, $p < 0.001$, relapse vs. NR, $p = 0.021$) (Fig. 3).

Cumulative rate of HCC incidence in group B according to the stage of liver fibrosis

Based on the pattern of the Japanese treatment guidelines, we categorized the patients in group B into two groups according to the stage of liver fibrosis (F0–1 or F2–4) and compared the cumulative rates of HCC incidence. Patients with no fibrosis or mild fibrosis (F0–1) showed no HCC development regardless of the virologic response (SVR, relapse, or NR). Of note, in those with moderate to severe fibrosis (F2–4) in group B, there was no significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.174$), although SVR patients tended have a lower rate of HCC incidence than NR patients (SVR vs. relapse, $p = 0.414$, SVR vs. NR, $p = 0.071$, relapse vs. NR, $p = 0.383$). No patient in the SVR or relapse groups developed HCC, while the cumulative rate of HCC incidence at 3 years for the NR group was 25.0 % (Fig. 4).

Discussion

Patients with chronic HCV infection and N-ALT have been reported to show the possibility of ALT flare-up during the natural course of the disease (22–27 %) [17, 18] and to develop moderate to severe progression of liver fibrosis (5–30 %) [18–21]. However, very low cumulative incidences of HCC have been reported among patients with average ALT integration values less than or equal to 20 IU/L (5-year, 0.0 %, 10-year, 3.6 %) [22]. Therefore, it remains controversial whether HCV eradication by antiviral therapy can reduce the incidence of HCC in patients with chronic HCV infection and N-ALT [23–26].

The definition of N-ALT remains unclear because its cutoff value is still under consideration [22, 27, 28]. In Japan, treatment guidelines for patients with chronic HCV infection and N-ALT define N-ALT as serum ALT levels of ≤40 U/L, and the therapeutic strategy is decided after categorizing patients into four groups according to ALT levels and PLT counts. However, the indication of antiviral therapy should be based on whether or not HCC incidence can be suppressed by the antiviral therapy. Therefore, we examined the treatment guidelines from the viewpoint of inhibiting HCC in patients with chronic HCV infection and N-ALT.

In the present study, the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with chronic HCV infection and N-ALT was almost equivalent to the efficacy in those with elevated ALT levels, as previously reported [11–15]. The SVR rate was significantly higher in groups A and C than in groups B and D for patients with genotype 1, and the same tendency was found

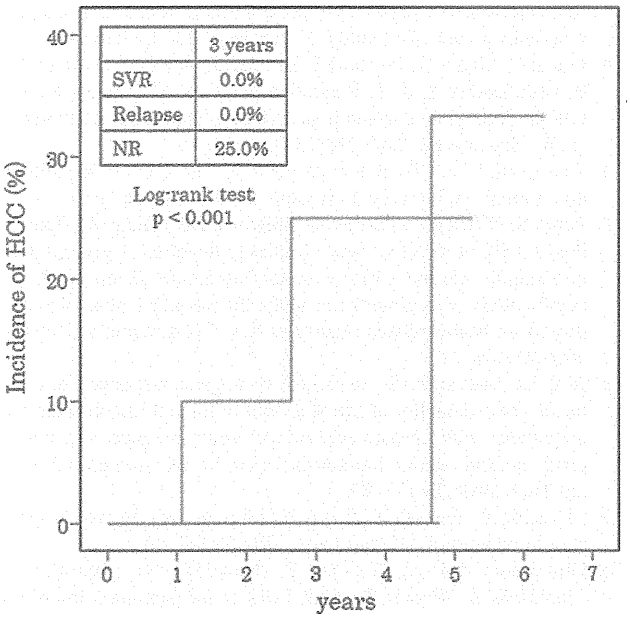

Fig. 4 Cumulative rates of HCC incidence in group B patients (ALT $\leq$ 30 IU/L and PLT $< 15 \times 10^4/mm^3$) with moderate to severe liver fibrosis (F2–4), according to the treatment effect of Peg-IFN plus ribavirin combination therapy. *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

for those with genotype 2. The reason for this was considered to be that groups B and D included many patients with moderate to severe liver fibrosis (F3–4, 17.0 %), which can lead to a lower SVR rate [23, 29, 30].

The present study revealed the cumulative rates of HCC incidence according to the treatment effect in the four groups. In group D, the cumulative rate of HCC incidence in the SVR and relapse patients was significantly lower than that for the NR patients. This result supports the recommendation by the treatment guidelines that patients in group D be managed in the same way as patients with chronic hepatitis C (CH-C) and elevated ALT levels.

In group B patients, the treatment guidelines recommend antiviral therapy for those who have moderate to severe liver fibrosis (F2–4). In our present study, patients with no fibrosis to mild fibrosis (F0–1) did not develop HCC, and in the patients with moderate to severe fibrosis (F2–4), the cumulative rate of HCC incidence tended to be lower in the SVR group than that in the NR group ($p = 0.071$). These results also indicate the appropriateness of the Japanese treatment guidelines. However, further study is needed because of the small number of cases studied here.

It appears that group A patients have time to wait for therapy with the next generation of direct antiviral agents (DAAs), such as Peg-IFN plus ribavirin plus a second-generation protease inhibitor, because none of the patients had developed HCC at 3 years. Even in group C, for which the treatment guidelines recommend antiviral therapy, there was no significant difference in the cumulative rate of HCC incidence among the SVR, relapse, and NR patients, with the incidence being below 5 % at 3 years. Accordingly, patients with PLT counts of more than $15 \times 10^4/mm^3$ (groups A or C) have time to wait until the next generation of DAAs becomes available, because patients with PLT counts of more than $15 \times 10^4/mm^3$ have a low 3-year carcinogenesis rate.

The Japanese treatment guidelines recommend antiviral therapy for patients with 30 < ALT $\leq$ 40 IU/L levels. However, in the present study, in the patients with 30 < ALT $\leq$ 40 IU/L levels, the p value for a significant difference in the cumulative rate of HCC incidence among the patients with SVR, relapse, and NR was 0.059. This result indicates that the patients with 30 < ALT $\leq$ 40 IU/L levels have the potential to be candidates for antiviral therapy, and further study is needed to clarify this. However, these patients may not be candidates for immediate antiviral therapy because the cumulative rates of HCC incidence at 3 years in the patients with SVR, relapse, and NR were low (cumulative rates of HCC at 3 years: 1.6, 0.0, and 3.7 %). On the other hand, as mentioned above, in the patients with PLT counts of $<15 \times 10^4/mm^3$, the cumulative rate of HCC incidence was significantly lower in the SVR and relapse patients than that in the NR patients (cumulative rates of HCC at 3 years: 0.0, 0.0, and 9.3 %; at 5 years: 0.0, 11.1, and 20.8 %; $p < 0.001$). This result suggests that patients with PLT counts of $<15 \times 10^4/mm^3$ may be candidates for antiviral therapy.

A limitation of this study was that the incidence of HCC was not compared between a treatment group and a non-treatment group. This study showed the suppressive effect of antiviral therapy on HCC incidence by comparing patients according to the treatment's antiviral effect. Peg-IFN plus ribavirin combination therapy has become acceptable for patients with chronic HCV infection and N-ALT levels. However, if there were no difference in HCC incidence between patients with SVR and non-SVR in the group receiving Peg-IFN plus ribavirin combination therapy, it would not be necessary for patients with chronic HCV infection and N-ALT to receive this therapy. In this study, we compared the incidence of HCC according to the treatment effect in HCV-infected patients with N-ALT levels categorized by the Japanese treatment guidelines. Indeed, although our results did not demonstrate that N-ALT patients should be treated, they indicated that it could be appropriate to treat N-ALT patients, because the incidence of HCC in these patients with SVR was suppressed compared with that in the NR patients.

In conclusion, in patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ who received Peg-IFN plus ribavirin

combination therapy, the cumulative rate of HCC incidence was significantly lower in those with SVR or relapse than in those with NR. Therefore, HCV-infected patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy for the purpose of reducing the risk of developing HCC.

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Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection

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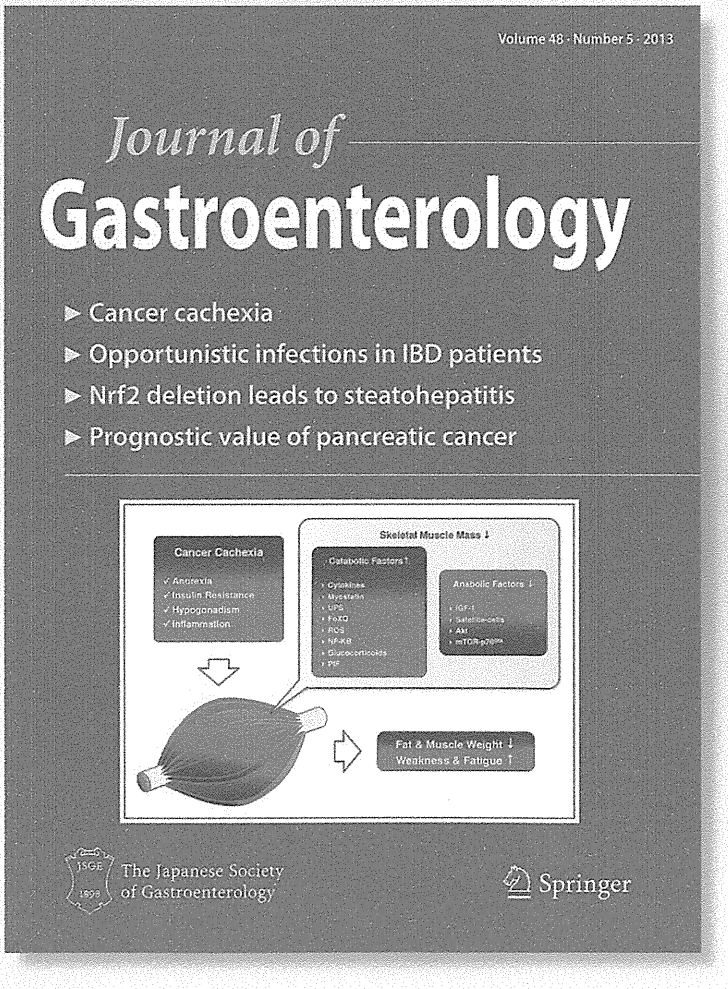

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association of enhanced activity of indoleamine 2,3-dioxygenase in dendritic cells with the induction of regulatory T cells in chronic hepatitis C infection

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Abstract

Background Altered functions of dendritic cells (DCs) and/or increases of regulatory T cells (Tregs) are involved in the pathogenesis of chronic hepatitis C virus (HCV) infection. A tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO), is reported to be an inducer of immune tolerance. Our aim was to clarify whether or not IDO is activated in chronic hepatitis C patients and its role in immune responses.

Methods This study enrolled 176 patients with chronic HCV infection and 37 healthy volunteers. Serum kynurenine concentration was evaluated by high-performance liquid chromatography, and its correlation with clinical parameters was examined. Monocyte-derived DCs were prepared from the subjects and subsequently stimulated with a combination of lipopolysaccharide and interferon-gamma to induce functional IDO (defined as IDO-DCs). The phenotypes, kynurenine or cytokine production, and T-cell responses with IDO-DCs were compared between the patients and healthy volunteers.

Results The serum kynurenine level in the patients was significantly higher than that in the healthy volunteers, and the level of serum kynurenine was positively correlated with the histological activity or fibrosis score. IDO activity in IDO-DCs from the patients was significantly higher than that in IDO-DCs from the volunteers. Furthermore, IDO-DCs from the patients induced more Tregs in vitro compared with those from the volunteers, and the frequency of induced Tregs by IDO-DCs was decreased with an IDO-specific inhibitor.

Conclusions Systemic IDO activity is enhanced in chronic hepatitis C patients in correlation with the degree of liver inflammation and fibrosis. In response to inflammatory stimuli, DCs from the patients tend to induce Tregs, with some of this action being dependent on IDO.

Keywords Hepatitis C virus · Dendritic cell · Regulatory T cell · Indoleamine 2,3-dioxygenase

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. It is estimated that 170 million people

are chronically infected with HCV and are at risk of developing liver cirrhosis and/or hepatocellular carcinoma [1]. Approximately 70 % of those exposed to HCV progress to a chronically infected state [2]. The mechanisms of HCV leading to persistent infection have been ascribed to escape mutations of the HCV genome and insufficient immune responses to HCV in hosts, but the precise mechanisms are still largely unknown.

Dendritic cells (DCs) are key regulators of the immune system and are capable of promoting or suppressing T-cell responses depending on their environment [3, 4]. One of the crucial machineries of HCV-induced immune dysfunction is impaired abilities of DCs. Several research groups, including ours [5, 6] have demonstrated that DCs from chronically HCV-infected patients have lower ability to stimulate T cells and to drive T-helper 1 (Th1) polarization than those from healthy controls [7, 8]. Regulatory T cells (Tregs) are specialized suppressor cells that maintain immune tolerance against auto-reactive T cells or against pathogens [9]. In patients with chronic HCV infection, the frequency of Tregs in peripheral blood mononuclear cells (PBMCs) is higher than that in healthy individuals, suggesting the active roles of Tregs in immune alteration or alleviation of inflammation [10, 11]. However, the mechanisms of DC dysfunction or Treg expansion in chronic HCV infection have not been completely elucidated.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyzes the initial and rate-limiting steps in the catabolism of the essential amino acid tryptophan (Trp), resulting in the generation of kynurenine (Kyn). IDO is widely expressed in human tissues [12] and cell subsets [13] and is induced during inflammation by interferon-gamma (IFN-γ) and/or other inflammatory cytokines [14–16]. Recent studies have demonstrated a crucial role of IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and cancers [17–21]. IDO expressed by DCs promotes immune tolerance by inhibiting T-cell activation and proliferation or by inducing Tregs through Trp starvation and/or the accumulation of Trp catabolites, such as Kyn, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid [22–25]. With respect to chronic HCV infection, a small-sized study showed that IDO expression was up-regulated in the liver and was associated with increased serum IDO activity [26]. However, the functions of IDO in immune cells in HCV infection still remain obscure.

In this study, we aimed to clarify whether or not IDO in DCs has a role in chronic HCV infection. We found that systemic IDO activity was enhanced in chronic hepatitis C patients. By comprehensively comparing the function of IDO-expressing DCs between the patients and healthy volunteers, we showed that IDO in DCs may be related to the induction of Tregs.

Subjects, materials, and methods

Subjects

This study enrolled 176 patients chronically infected with HCV serotype 1 (CHC group) who had been followed at Osaka University Hospital (Suita, Japan), National Hospital Organization Osaka National Hospital (Osaka, Japan), or Ikeda Municipal Hospital (Ikeda, Japan). All of them were confirmed to be positive for both serum anti-HCV antibody and HCV-RNA but were negative for other viral infections, including hepatitis B virus (HBV) and human immunodeficiency virus. The presence of other liver diseases, such as alcoholic, metabolic, or autoimmune hepatitis was ruled out, and the presence of liver cirrhosis and hepatocellular carcinoma was excluded by the use of laboratory and imaging analyses. As controls, we examined 37 healthy volunteers (HV group), working as medical staff at Osaka University Hospital, who were negative for HCV and HBV markers. As disease controls, 13 patients with chronic HBV infection followed at National Hospital Organization Osaka National Hospital were also enrolled. They were positive for hepatitis B surface (HBs) antigen and had abnormal levels of alanine aminotransferase (ALT). The characteristics of the group were: male/female 10/3, hepatitis B envelope (HBe) antigen-positive/HBe antigen-negative 6/7, mean age 43.9 ± 15.0 years, mean serum ALT level 218.7 ± 282.5 IU/L, and mean HBV-DNA level [assayed by the COBAS AmpliPrep™/COBAS TaqMan™ HBV test (Roche, Branchburg, NJ, USA)] 6.1 ± 2.3 Log copies/mL. At enrollment, written informed consent was obtained from each subject. The study protocol was approved by the ethics committee of each institution.

In this study, because of the limitations of sampling from multiple centers, the conditions for blood collection and preservation differed among the facilities. Thus, for the precise comparison of IDO activity between the patients and healthy volunteers, firstly, we examined the samples collected and preserved under the same conditions at Osaka University Hospital (Cohort I, Table 1). Secondly, because liver biopsy was not carried out in Cohort I patients, we used another cohort (Cohort II, Table 1) for our analysis of the correlation between IDO activity and clinical parameters. Cohort II consisted of the remaining 127 patients, whose samples were collected at National Hospital Organization Osaka National Hospital or Ikeda Municipal Hospital. Histological examination was performed according to the METAVIR scoring system. The clinical backgrounds of the patients in Cohorts I and II, except for HCV-RNA quantity, were not different.