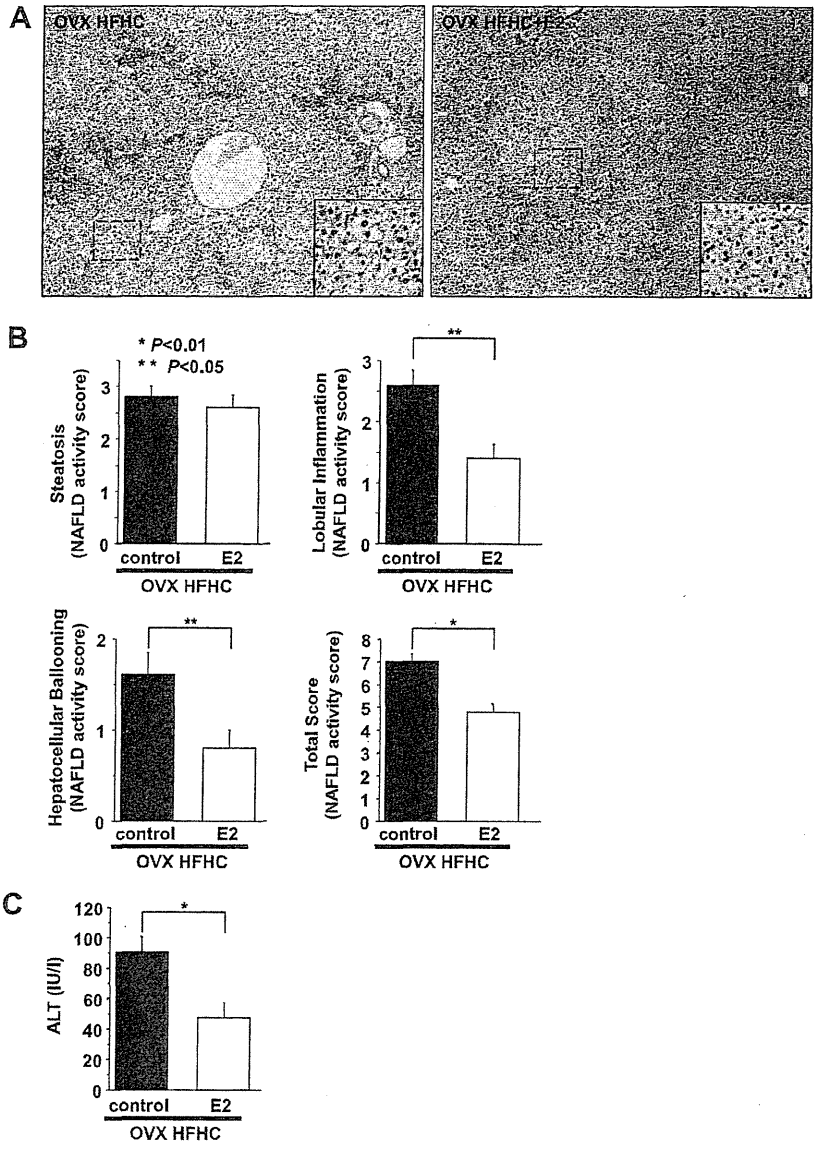

Fig. 5. Treatment with estrogen protects liver injury in OVX mice fed the HFHC diet. *A*: H&E staining of mice liver sections. OVX-HFHC+E2; estrogen pellet-implanted OVX-HFHC mice. *Insets*: magnified views of typical hepatocytes in mice (dashed squares indicate the magnified areas). Original magnification was ×100. *B*: NAFLD activity scores in mice. The graphs represent the scores for hepatic steatosis, lobular inflammation, hepatocellular ballooning, and total NAFLD activity. *C*: serum ALT levels in mice. Solid bars, OVX-HFHC mice (control), open bars: OVX-HFHC+E2. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$.

TNF or fatty acid synthase. In addition, dietary cholesterol induced hepatic MCP1 gene expression (48), and monocyte CCR2 expression was increased in hypercholesterolemic patients compared with normocholesterolemic controls (14). Cholesterol directly stimulates the CCR2 promoter activity through its cholesterol response element (7, 14), and, in this study, elevated serum cholesterol in OVX-HFHC mice increased, not only hepatocyte MCP1 expression, but also monocyte CCR2 expression in mice. Enhanced MCP1 expression in mice liver recruited CCR2-positive monocytes into the liver, and these recruited monocytes transformed into inflammatory macrophages.

The accumulation of macrophages at sites of inflammation and injury in the liver is thought to be mediated by chemokines such as MCP1. Accumulated inflammatory macrophages in mice liver produce various inflammatory cytokines, such as TNF-α and IFN-γ, leading to persistent liver injury. Estrogen at physiological concentrations inhibits the spontaneous secretion of these inflammatory cytokines in whole blood cultures (43). Moreover, hydrogen peroxide-induced TNF-α and MCP1 expressions were attenuated by estradiol in peritoneal macrophages from female mice (18), whereas estrogen inhibited MCP1 expression in human endometrial stromal cells, and tamoxifen, an estrogen antagonist, reversed the inhibition of MCP1 by estrogen (2). Finally, estrogen significantly decreased mice monocyte CCR2 expression and decreased chemotaxis of monocytes toward MCP1 (21). These findings suggest that estrogen itself may have hepatoprotection against inflammation by preventing macrophage accumulation and inhibiting proinflammatory cytokine production.

Estrogen has both anti-inflammatory and antifibrotic effects in the liver. During liver fibrogenesis, hepatic stellate cells (HSC) play a central role (11). HSC possess functional ER-β, but not ER-α, to respond directly to estrogen exposure. In addition, estrogen prevented reactive oxygen species and TGF-β production in cultured rat HSC by suppressing NADH/

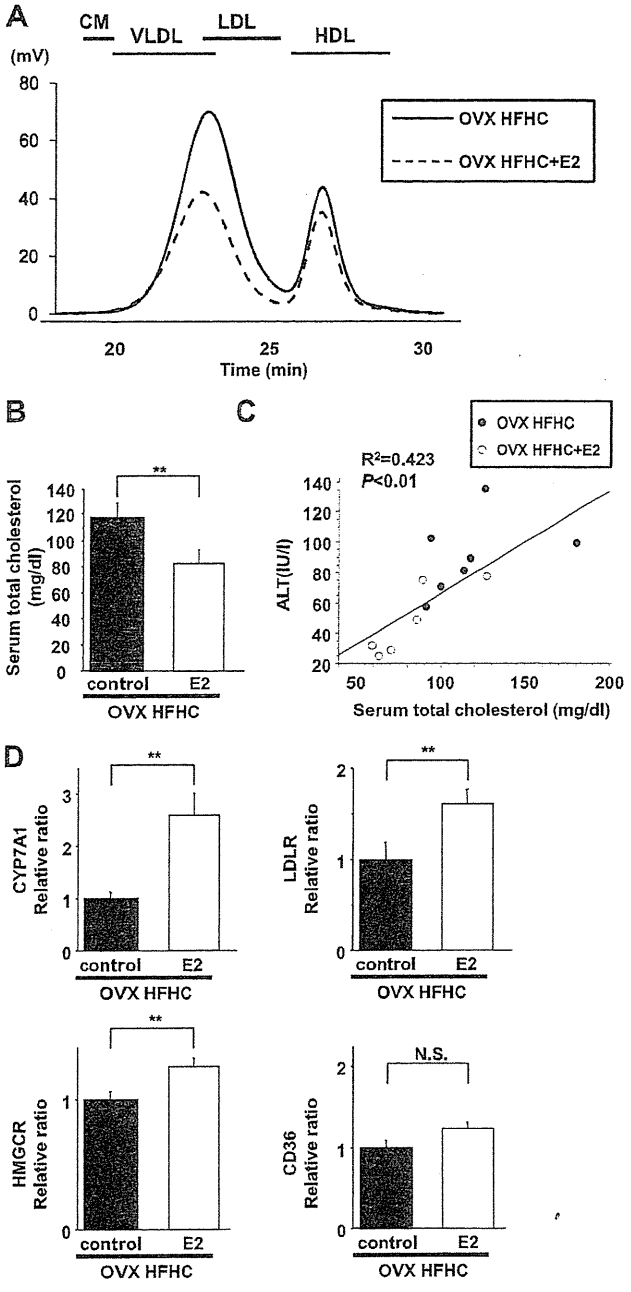

Fig. 6. Cholesterol lipoprotein distribution and serum cholesterol levels in mice treated with or without estrogen. *A*: serum cholesterol lipoprotein distribution analyzed by HPLC in mice. *B*: serum cholesterol levels in mice. Data are means ± SD. $^{**}P < 0.05$. *C*: correlations between serum ALT and cholesterol levels in mice. *D*: hepatic cholesterol metabolism-related gene expression changes in mice. Liver GAPDH-normalized CYP7A1, LDLR, HMGCR, and CD36 gene expressions. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$, N.S., not significant.

NADPH oxidase activity (20). In our study, fibrogenic genes, TGF-β1 and collagen Iα1, were upregulated in OVX-HFHC mice, and estrogen treatment improved the hepatic collagen Iα1 expression.

NASH is reported to be correlated with atherosclerotic cardiovascular diseases independently of Mets (47). In this study, we used HFHC diet (containing 15% cocoa butter, 1.25% cholesterol, and 0.5% cholate) to induce steatohepatitis in mice. This diet is known to be an atherogenic diet. This HFHC diet is also known to induce liver steatosis, inflammation, and fibrosis (34). This model seems to be a more physiological dietary model of NASH than existing animal models, which require genetic defects or the depletion of nutrients, such as methionine and choline deficiency diet-induced model. Moreover, the liver pathology induced by HFHC diet feeding involves steatohepatitis with hepatocellular ballooning, a necessary histological feature defining human NASH. These results indicate that the HFHC diet-induced NASH model in mice is one of the better experimental models of human NASH.

It was reported that mice fed HFHC diet were smaller than control mice, and insulin sensitivity and glucose tolerance were similar between HFHC-fed mice and NC diet-fed mice (29, 34). However, hepatic insulin resistance, as assessed by the liver insulin receptor substrate-2 (IRS-2) expression, was increased in HFHC-fed mice (34). In this study, both body weight and insulin resistance, as assessed by HOMA-IR, in HFHC diet-fed mice did not increase compared with in NC diet-fed mice, and IRS-2 gene expression was decreased in HFHC diet-fed mice liver (Table 2). These results indicated that hepatic insulin resistance would exist in HFHC-fed mice in our study.

In conclusion, our study demonstrated that estrogen deficiency promoted NASH progression and that estrogen treatment improved NASH progression in mice fed HFHC diet. Extrapolation of our results into the clinical setting suggests that estrogen deficiency and estrogen deficiency-induced hypercholesterolemia in postmenopausal women could potentially exacerbate NASH progression through enhanced liver inflammatory macrophage infiltration.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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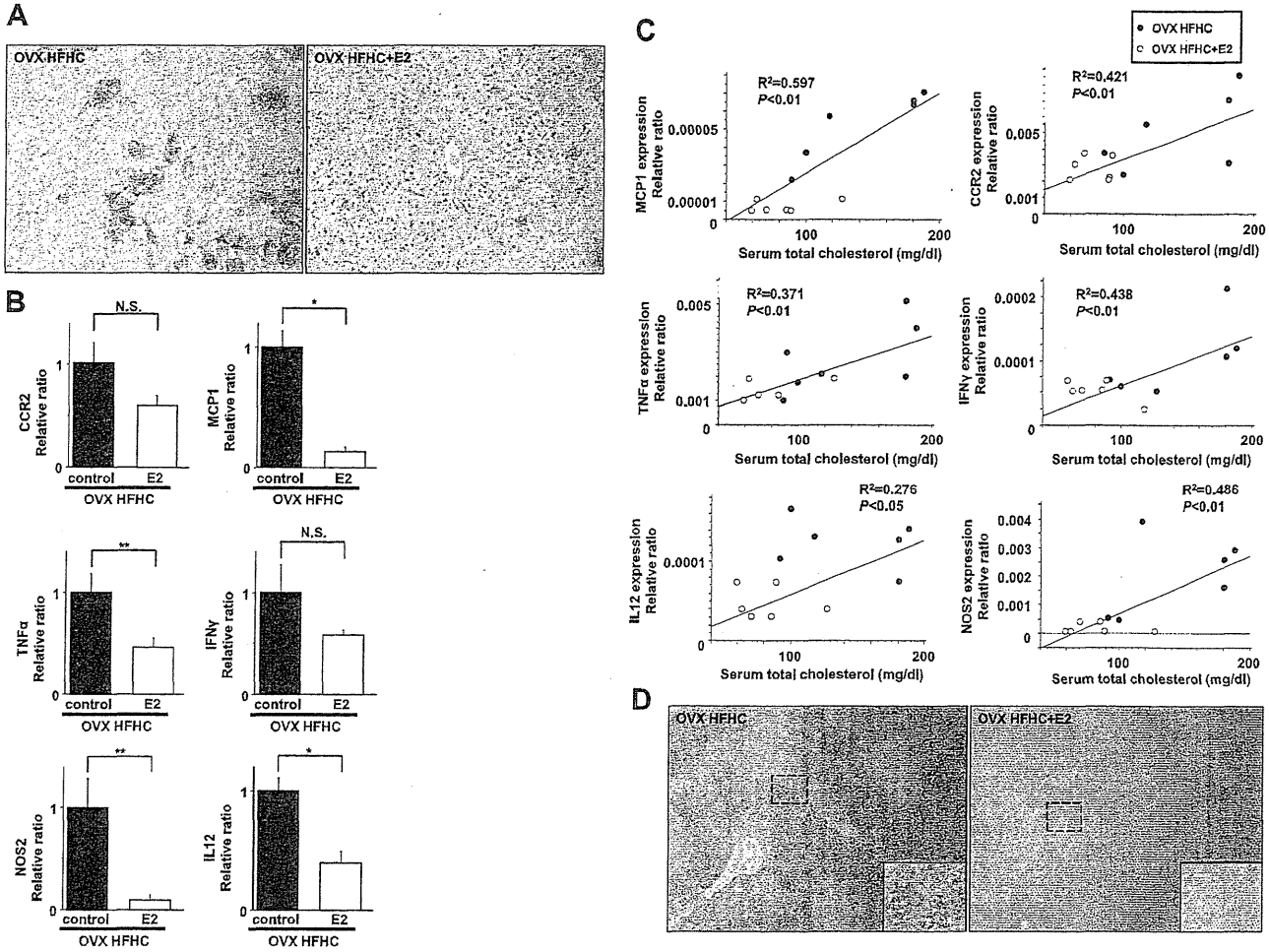

Fig. 7. OVX-exacerbated liver macrophage infiltration in mice fed HFHC diet was attenuated by estrogen treatment. *A*: F4/80 immunohistochemistry of mice livers. Original magnification, ×200. *B*: hepatic inflammation-related gene expression changes in mice livers. Liver GAPDH-normalized CCR2, MCP1, TNF-α, IFN-γ, NOS2, and IL12 gene expression determined by real-time RT-PCR. Data are means ± SD. $*P < 0.01$, $**P < 0.05$. N.S., not significant. *C*: correlations between serum cholesterol levels and liver inflammation-related gene expressions. *D*: MCP1 immunohistochemistry of mice livers. *Insets*: magnified views of mice livers (dashed squares indicate the magnified areas). Original magnification, ×100.

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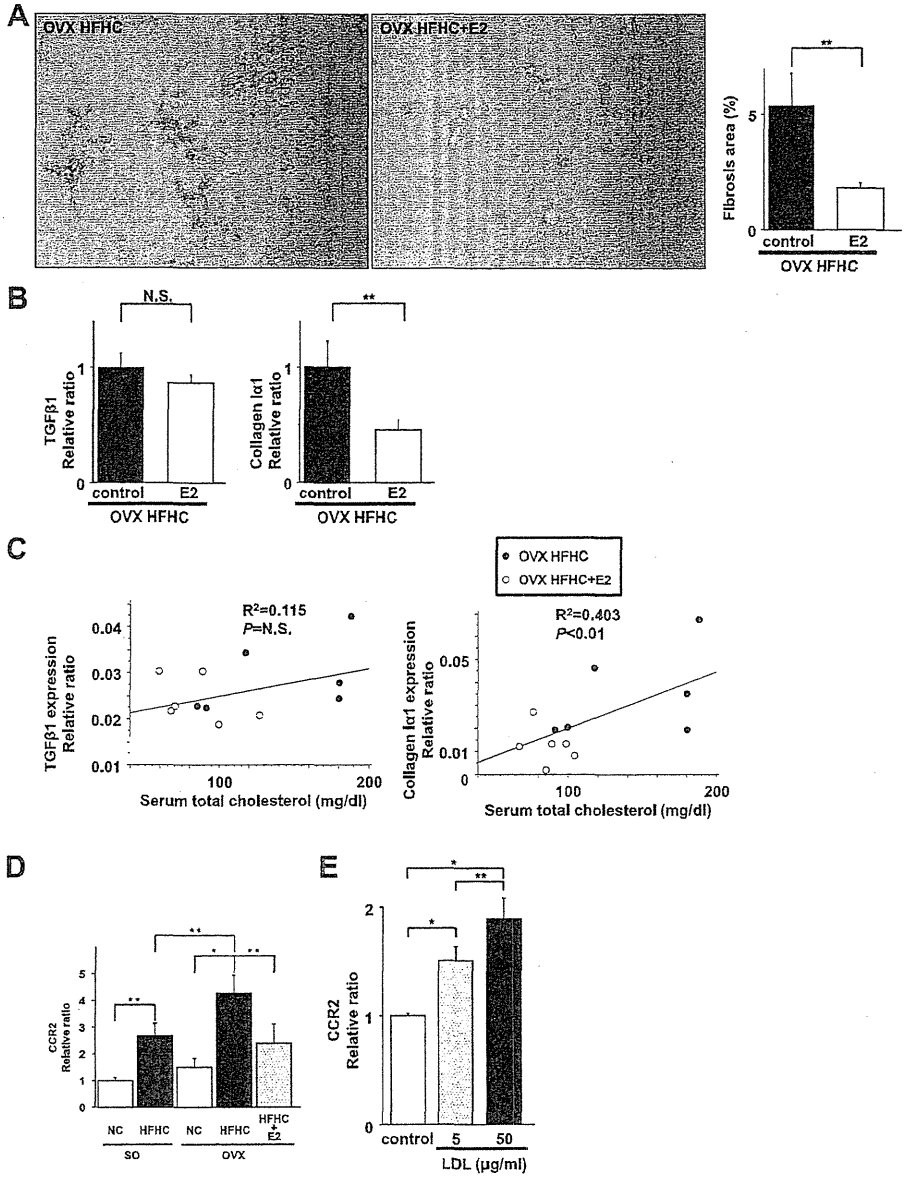

Fig. 8. Amelioration of hepatic fibrosis in OVX-HFHC mice treated with estrogen. *A*: picrosirius red staining of mice livers. *Original magnification*, ×100. *Histographic* representation of quantified data. Areas of fibrosis were measured as described in MATERIALS AND METHODS. The degree of fibrosis was expressed as the percentage of the total area measured. *B*: gene expression changes related to fibrosis. Liver GAPDH-normalized TGF-β1 and collagen Iα1 gene expression was determined by real-time RT-PCR. Data are means ± SD. $^{**}P < 0.05$; N.S., not significant. *C*: correlation between serum cholesterol level and fibrosis-related genes (TGF-β1, collagen Iα1). *D*: CCR2 gene expression changes in mice spleen monocytes from 5 groups in this study. *E*: CCR2 gene expression changes in THP1 monocytes treated with LDL. CCR2 gene expression changes were determined by real-time RT-PCR. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$.

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Fibroblast growth factor-2 enhances NK sensitivity of hepatocellular carcinoma cells

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The roles of fibroblast growth factor-2 (FGF-2) in the hepatocellular carcinoma (HCC) development are still controversial. In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. The FGF-2 expressions were detected in the liver tissues of patients, but not in normal liver. The serum FGF-2 levels of the patients with CH, liver cirrhosis (LC) or HCC were significantly higher than those of healthy volunteers. The serum FGF-2 levels of patients decreased with the progression of chronic liver disease. HCC occurrence of LC patients with high levels of serum FGF-2 was significantly lower than that with low levels of serum FGF-2. Proinflammatory cytokines, such as IL-1β and IL-6, induced FGF-2 expressions in HCC cells and normal hepatocytes. FGF-2 stimulation resulted in increasing the expression of the membrane-bound major histocompatibility complex class I-related chain A (MICA), an NK activating molecule, and decreasing that of human leukocyte antigen (HLA) class I, an NK inhibitory molecule, on HCC cells. This did not occur with normal hepatocytes. Adding anti-FGF receptor-2 neutralizing antibody resulted in inhibiting the change of MICA and HLA class I expressions on FGF-2 stimulated HCC cells. FGF-2 stimulation on HCC cells resulted in increasing NK sensitivity against HCC cells. These findings indicate that FGF-2 produced by HCC cells or normal hepatocytes of chronic liver disease may play critical roles in eliminating HCC cells by innate immunity.

Fibroblast growth factor (FGF)-2 is one of a family of FGFs that includes 22 structurally related members.[1] FGF-2 has been shown to exert a potent angiogenic effect by interacting with tyrosine kinase receptors, FGFR1, FGFR2 and FGFR3, in various cancers including hepatocellular carcinoma (HCC).[2–4] Aside from its angiogenic effect, FGF-2 has also been shown to act as a mitogen for HCC cell proliferation *via* an autocrine mechanism.[5] Uematsu *et al.* reported that the serum FGF-2 of chronic liver disease patients without HCC tended to be higher than that of those with HCC.[6] Decrease of serum FGF-2 could be observed prior to the emergence of HCC, and this suggests that FGF-2 may play a critical role in the surveillance of HCC. However, the immunological significance of elevating the FGF-2 levels in chronic liver disease patients remains unclear.

HCC is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and nonalcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis (LC), in particular, being considered a premalignant condition.[7,8] The liver contains a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells),[9,10] but the activation process of these immune cells in HCC development remains unclear. A recent study has demonstrated that the innate immune system may play a critical role in tumor surveillance *via* an NKG2D signal.[11] Knowing the details of how to activate the abundant NK cells in the liver could lead to the establishment of attractive new strategies for HCC treatment.

In this study, we investigated the expression of FGF-2 in chronic hepatitis (CH) type C patients with or without HCC and the immunoregulation of FGF-2 in NK sensitivity of HCC cells. Of importance are the findings that serum FGF-2 levels in patients with CH and LC without HCC were significantly higher than that in those with HCC and that FGF-2 enhanced the NK sensitivity of HCC cells. The present study

Key words: FGF-2, hepatocellular carcinoma, NK cells, MICA, HLA class I
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Table 1. Clinical backgrounds

	Normal	Hepatitis	Cirrhosis	HCC
Number	24	80	84	112
				Stage I/II 51
				Stage III/IV 61
Sex (M/F)	12/12	45/35	44/40	67/45
Age	64 ± 15	56 ± 13	62 ± 13	66 ± 11
Etiology		HCV	HCV	HCV

Abbreviations: Stage: TNM stage; M: male; F: female; HCV: hepatitis C virus.

sheds light on previously unrecognized immunological effects of FGF-2 on HCC cells and thus suggests a role of FGF-2 in HCC development in patients with CH type C.

Material and Methods

Liver tissues and immunohistochemistry

Human HCC tissues (n = 6) and normal liver tissues (n = 2) were obtained at surgical resection. CH tissues (n = 4) and LC tissues (n = 4) were obtained as liver biopsy samples. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. Liver sections were subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA). The primary antibody (Ab) was antihuman FGF-2 Ab (Abcam, Cambridge, MA). To confirm the specificity of the staining, the primary antibody was incubated with recombinant human FGF-2 protein (R&D Systems, Minneapolis, MN) for 3 hr and then applied onto liver sections in parallel with staining of the primary antibody as the absorption test.

HCC cell lines

HepG2 and PLC/PRF/5, human hepatoma cell lines, were purchased from American Type Culture Collection (Rockville, MD) and were cultured with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C.

ELISA

The sera from CH patients (n = 80), LC patients (n = 84), HCC patients (n = 112, StageI/II n = 51 and StageIII/IV n = 61) and age-matched healthy volunteers (HVs) (n = 24) were subjected to analysis of the FGF-2 level. Clinical backgrounds of patients were summarized in Table 1. Informed consent, under an Institutional Review Board-approved protocol, was obtained from all patients before sample acquisition. The level of FGF-2 and soluble major histocompatibility complex class I-related chain A (MICA) were determined using Quantikine Human FGF basic (R&D Systems) and DuoSet MICA eELISA kit (R&D Systems), respectively.

HCC cells and normal hepatocyts cultures

Both HepG2 and PLC/PRF/5 cells or normal hepatocytes (ScienCell Research Laboratories, Carlsbad, CA) were cultured for 72 hr in the presence or absence of human interleukin-1β (IL-1β) (50 ng/ml, Peprotech, Rocky Hill, NJ), human IL-6 (300 ng/ml, Peprotech), human transforming growth factor-β1 (TGF-β1) (50 ng/ml, R&D Systems) and human tumor necrosis factor-α (TNF-α) (100 ng/ml, Peprotech), and the treated cells were harvested and evaluated for expression of FGF-2. In some experiments, HepG2 and PLC/PRF/5 cells were cultured in the presence or absence of recombinant human FGF-2 protein (250 ng/ml, R&D Systems) with or without antihuman FGFR2 neutralizing Ab (10 μg/ml, R&D Systems) for 48 hr, and the hepatoma cells were harvested and evaluated for the immunological regulation of the NK cells.

Flow cytometry

For the detection of membrane-bound MICA, cells were incubated with anti-MICA specific Ab (2C10, Santa Cruz Biotechnology, Santa Cruz, CA) and stained with Goat F(ab')2 fragment anti-Mouse IgG(H+L)-PE (Beckman Coulter, Fullerton, CA) as a secondary reagent and then subjected to flow cytometric analysis. For the detection of human leukocyte antigen (HLA) class I, cells were incubated with PE-conjugated antihuman HLA-A,B,C Ab (w6/32, BD Biosciences, San Jose, CA). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Western blotting

The total cellular protein was electrophoretically separated using sodium dodecyl sulfate-12% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked in Tris-buffered saline-Tween20 containing 5% skim milk for 1 hr and then probed with rabbit polyclonal Ab to FGF-2 (Abcam) at room temperature overnight. Horseradish peroxidase-conjugated anti-rabbit IgG and SuperSignal West Pico System (Pierce, Rockford, IL) were used for the detection of blots.

Real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assay (Applied Biosystems) was used for the quantification of FGF-2 (ID: Hs00960934_m1), MICA (Hs00792195_m1) and β-actin (Hs:99999903_m1) mRNAs according to the manufacturer's instructions. β-actin mRNA from each sample was quantified as endogenous control of internal RNA.

NK cell analysis

NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyi Biotech, Auburn, CA).[12] The cytolytic ability of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells was assessed by 4-hr ^{51}Cr-release assay with or without antihuman MICA/B Ab (BD Biosciences) as previously described.[12] The expressions of NKG2D and NKG2A on NK cells were analyzed by flow cytometry with PE-conjugated antihuman NKG2D Ab (BD Biosciences) and PE-conjugated IgG antihuman NKG2A Ab (R&D Systems).

Statistics

For human sample data, values were expressed as the median and interquartile range using box plots and the 10th and 90th percentiles as horizontal bars. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel-Dwass procedure. Differences between retreatment and post-treatment values were tested by the paired t-test. FGF-2 mRNA values were expressed as the mean and SD, and the statistical significance of differences between the groups was determined by applying Student's t test after each group had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $p < 0.05$.

Results

FGF-2 is expressed in the liver and serum of patients with chronic liver diseases

We first examined the FGF-2 expressions in the livers of normal volunteers and the patients with chronic liver diseases. Immunohistochemical analysis revealed that FGF-2 was not expressed in normal liver tissues. In contrast, the expressions of FGF-2 were detected in chronic liver tissues (Fig. 1*a*). We evaluated the serum FGF-2 levels by specific ELISA. All of the chronic liver disease patients were hepatitis C virus (HCV)-RNA positive. As shown in Figure 1*b*, the serum FGF-2 levels in CH and LC patients were significantly higher than those of HV, but those in HCC patients were not. Those in CH patients were also significantly higher than those in LC or HCC patients. Those in LC patients tended to be higher than those in HCC patients, although this was not significant. The serum FGF-2 levels in HCC patients were low and significant difference between StageI/II patients and III/IV patients was not observed (data not shown). We compared the serum FGF-2 levels before and after the

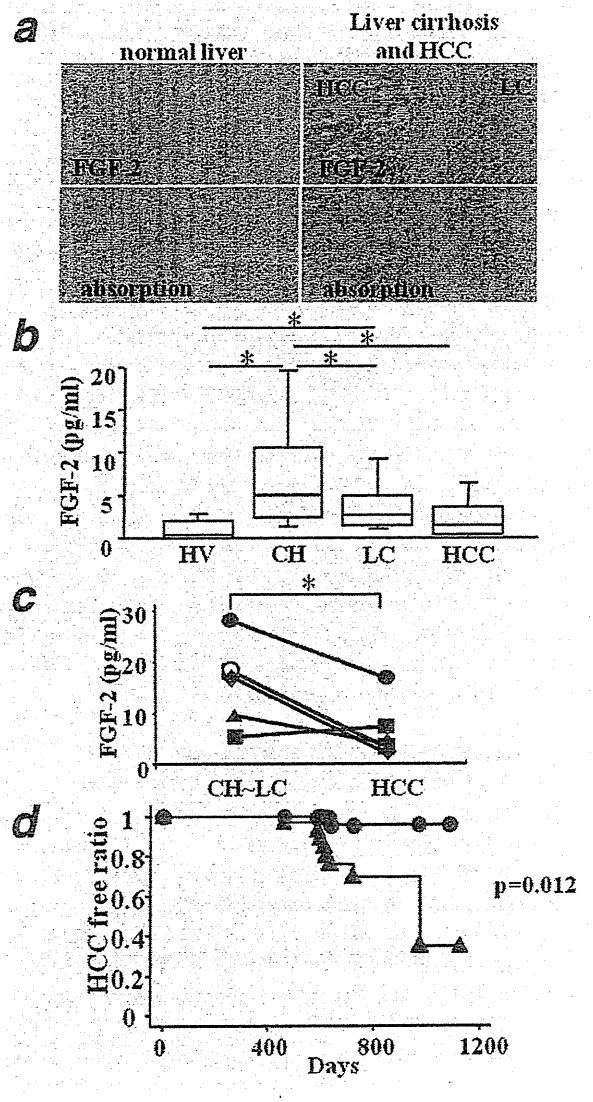

Figure 1. Expressions of FGF-2 in the liver of patients with chronic liver diseases and serum FGF-2 levels in chronic liver disease patients were associated with HCC incidence. (*a*) Immunohistochemical analysis of FGF-2 in normal liver tissues ($N = 2$), chronic hepatitis tissues ($N = 4$), liver cirrhosis (LC) tissues ($N = 4$) and hepatocellular carcinoma (HCC) tissues ($N = 6$). Liver sections were stained with the FGF-2 Ab (*upper panels*). The primary Ab was incubated with recombinant FGF-2 protein and then applied to liver sections in parallel as the absorption test (*lower panels*). Representative pictures are shown. (*b*) Serum FGF-2 levels in chronic hepatitis patients (CH, $N = 80$), liver cirrhosis patients (LC, $N = 84$) and HCC patients ($N = 112$) were evaluated by specific ELISA. All patients were HCV-RNA positive. Comparison of serum FGF-2 levels of each group. * $p < 0.05$. (*c*) Serum FGF-2 levels were compared between before and after HCC development in six chronic liver disease patients. The mean follow-up period was nine years. * $p < 0.05$. (*d*) The correlation of the FGF-2 level and HCC incidence was evaluated. 84 LC patients were divided into two groups according to serum FGF-2 levels; high (serum FGF-2 concentration > 1.8 pg/ml; 40 patients, ●) and low (≦1.8 pg/ml; 44 patients, ▲). We followed these LC patients for three years and compared the rate of HCC-free survival in these groups.

development of HCC in six chronic liver disease patients. The mean follow-up period was nine years. The serum FGF-2 levels of the patients before the occurrence of HCC were significantly higher than those of the same patients after the occurrence of HCC (Fig. 1*c*). These results demonstrated that the serum FGF-2 levels were highest in CH patients and significantly decreased as the liver disease progressed.

FGF-2 levels were associated with the incidence of HCC in chronic liver disease patients

The earlier results suggested that increased FGF-2 levels might prevent HCC tumor development. We investigated the correlation of the serum FGF-2 level and HCC incidence. The 84 LC patients were divided into two groups according to serum FGF-2 levels, high (serum FGF-2 concentration > 1.8 pg/ml; 40 patients) and low ($\leqq$1.8 pg/ml; 44 patients), because the median of FGF-2 levels in these patients was 1.8 pg/ml. We followed these LC patients for three years and compared the rates of HCC-free survival. As shown in Figure 1*d*, the HCC free ratio of the high FGF-2 patients was significantly higher than that of the low FGF-2 patients. These results suggested that FGF-2 production from chronically diseased liver tissues might be associated with the occurrence of HCC.

Inflammatory cytokines increased FGF-2 expression in HCC cells and normal hepatocytes

Previous reports demonstrated that FGF-2 expressions were detected in both tumor cells and normal hepatocytes in addition to sinusoidal endothelial cells in HCC tissues.[5] Some inflammatory cytokines, such as IL-1β, IL-6, TGF-β and TNF-α, are known to increase in CH patients.[13–15] To examine the effect of such inflammatory cytokines on FGF-2 expression in liver cells, we cultured HepG2 and PLC/PRF/5 HCC cells for 72 hr in the presence or absence of these cytokines. As shown in Figure 2*a*, IL-1β and IL-6 increased FGF-2 protein levels in both HepG2 and PLC/PRF/5 cells. FGF-2 mRNA levels in HepG2 and PLC/PRF/5 cells treated with IL-1β and IL-6 were significantly higher than those in nontreated control HCC cells (Fig. 2*b*). We also examined FGF-2 levels in the supernatants of the HCC cells cocultured with inflammatory cytokines. FGF-2 levels of IL-1β- or IL-6-treated HepG2 cells or PLC/PRF/5 cells tended to increase compared with those of nontreated HCC cells (data not shown). FGF-2 mRNA levels in normal hepatocytes treated with IL-1β, but not IL-6, were also significantly higher than those in nontreated control cells (Fig. 2*c*). These results suggested that both IL-1β and IL-6 were capable of inducing FGF-2 expression in HCC cells and normal hepatocytes. We also examined whether TGF-β1 and TNF-α could induce FGF-2 expressions on HCC cells. We found that FGF-2 expression levels in treated HCC cells did not change in Western blotting or real-time RT-PCR analysis (data not shown).

FGF-2 induced the expression of membrane-bound MICA and suppressed the expression of HLA class I on HCC cells, but FGF-2 did not change the expressions of NKG2D and NKG2A on NK cell

The above findings suggested that decreasing FGF-2 might affect the HCC development in the patients with chronic liver disease. To investigate whether or not FGF-2 protein directly activates NK cells, we examined whether FGF-2 affected the expression of NKG2D (activating receptor) or NKG2A (inhibitory receptor) on NK cells. We cultured CD56+ NK cells obtained from HVs with FGF-2 for 24 hr and then subjected them to flow cytometric analysis. The expressions of both NKG2D and NKG2A on NK cells did not change by adding FGF-2 protein (Fig. 3*a*), suggesting that FGF-2 did not have a direct effect on NK cells. We next examined the immunological modification of human HCC cells by adding human FGF-2 protein. We evaluated the expressions of membrane-bound MICA (NK activating molecule) and HLA class I (NK inhibitory molecule) in HepG2 and PLC/PRF/5 cells by flow cytometry. The expressions of MICA on FGF-2-treated cells were higher than those on nontreated cells in both HepG2 and PLC/PRF/5 cells (Fig. 3*b*). In contrast, those of HLA class I on FGF-2-treated cells were lower than those on nontreated cells in both types of HCC cells (Fig. 3*b*). FGF-2-treatment could modify the expressions of MICA and HLA class I on HCC cells in a dose-dependent manner (data not shown). The mRNA level of MICA in FGF-2-treated HepG2 cells was also significantly higher than that in nontreated HepG2 cells. The mRNA level of MICA in FGF-2-treated PLC/PRF/5 tended to be higher than that in nontreated cells, although the difference was not statistically significant (Fig. 3*b*). We examined the expressions of MICA and HLA class I on FGF-2-treated normal hepatocytes. The expressions of both molecules did not change in FGF-2-treated normal hepatocytes (Fig. 3*c*). We also evaluated FGF-2-dependent MICA regulation on a gastric cancer cell line (KATOIII), colon cancer cell lines (HCT116, HT29) and a cervical cancer cell line (IIela). The MICA expression was induced in FGF-2-treated HCT116 cells and weakly in FGF-2-treated Hela cells, but not in the other two cell lines (data not shown). These results suggested that FGF-2 could modify the MICA expressions in several types of cancers.

The signal via FGF-2/FGF-receptor2 is essential for the induction of MICA and HLA class I expressions on HCC cells

We examined the FGF receptors (FGFR1, FGFR2, and FGFR3) on both types of HCC cells by flow cytometry. The expressions of FGFR2 were high for both cell types. While FGF-2 has cross-reactivity with FGFR1 and FGFR3, the expressions of FGFR1 and FGFR3 were very low on both types of HCC cells (Fig. 4*a*). To examine whether the interaction between FGF-2 and FGFR2 could induce the expressions of MICA and HLA class I on both types of HCC cells, we evaluated the expressions of both molecules on FGF-2-treated

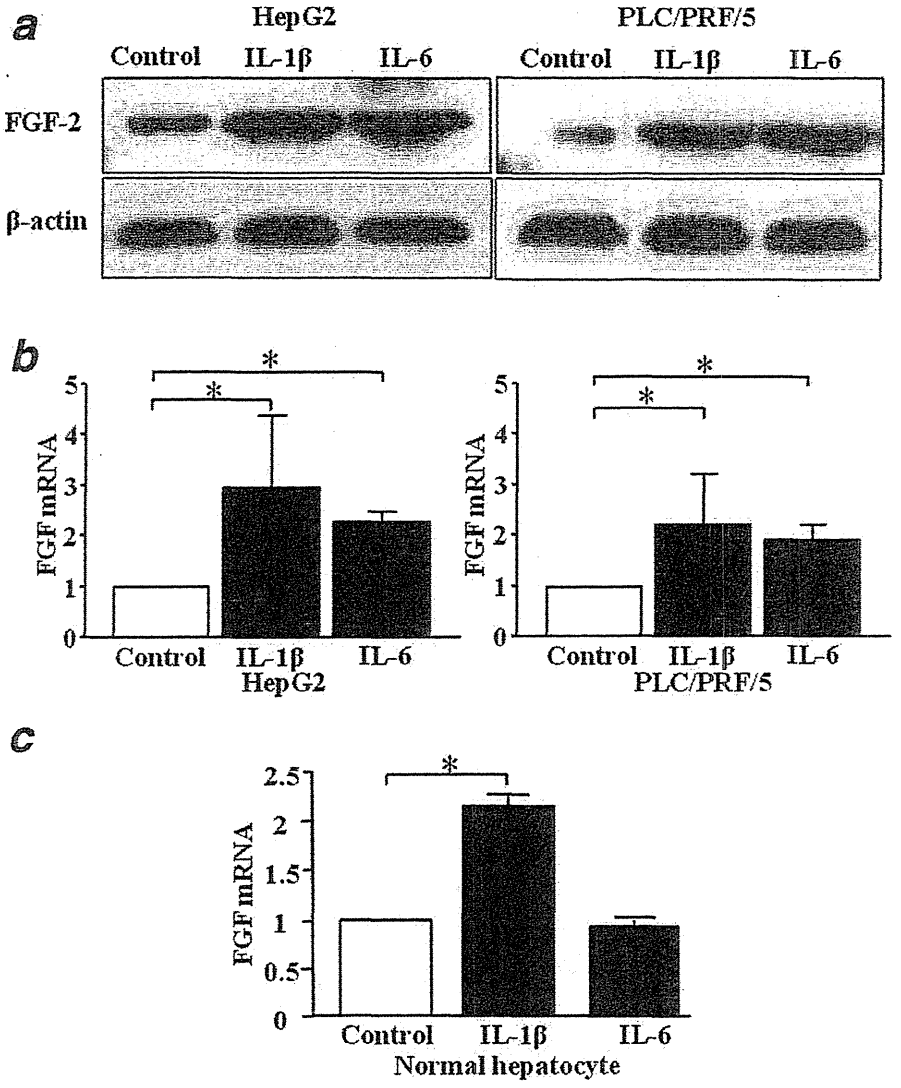

Figure 2. IL-1β and IL-6 increased FGF-2 expressions on human HCC cells and normal hepatocytes. To examine the effect of IL-1β and IL-6 on FGF-2 expression, HepG2 and PLC/PRF/5 cells (*a,b*) or normal hepatocytes (*c*) were cultured for 72 hr in the presence or absence of IL-1β (50 ng/ml) and IL-6 (300 ng/ml). FGF-2 expression in these cells was evaluated by Western blotting analysis (*a*) and real-time RT-PCR analysis (*b,c*). (*a*) The proteins were subjected to Western blot assay using each specific Ab. *Upper panel* is FGF-2 and *lower panel* is β-actin. (*b,c*) Total RNA was extracted and reverse transcribed. Relative copy numbers of FGF-2 were determined by real-time PCR analysis and normalized with β-actin expression. Results are expressed as mean ±SD. Similar results were obtained in two independent experiments. * $p < 0.05$.

HCC cells with anti-FGFR2 neutralizing Ab. The anti-FGFR2 Ab blocks the ability of FGF-2 to modulate MICA and HLA class I on both HepG2 and PLC/PRF/5 cells (Fig. 4*b*).

FGF-2 enhanced susceptibility to NK cells of HCC cells and the correlation of serum FGF-2 and soluble MICA levels in patients with chronic liver disease

The earlier results suggested that FGF-2 might enhance the susceptibility to NK cells of HCC cells. We next examined whether FGF-2 could modify the NK sensitivity of human HCC cells. The cytolytic activities of NK cells against FGF-2-treated HepG2 and FGF-2-treated PLC/PRF/5 cells were higher than those against nontreated HCC cells (Fig. 5*a*). The cytolytic activity against FGF-2-treated HCC cells decreased to the control levels on addition of anti-MICA/B blocking antibody (Fig. 5*a*) but not on addition of isotype IgG antibody (Fig. 5*b*). These results demonstrated that adding FGF-2 enhanced the NK sensitivity of HCC cells *via*

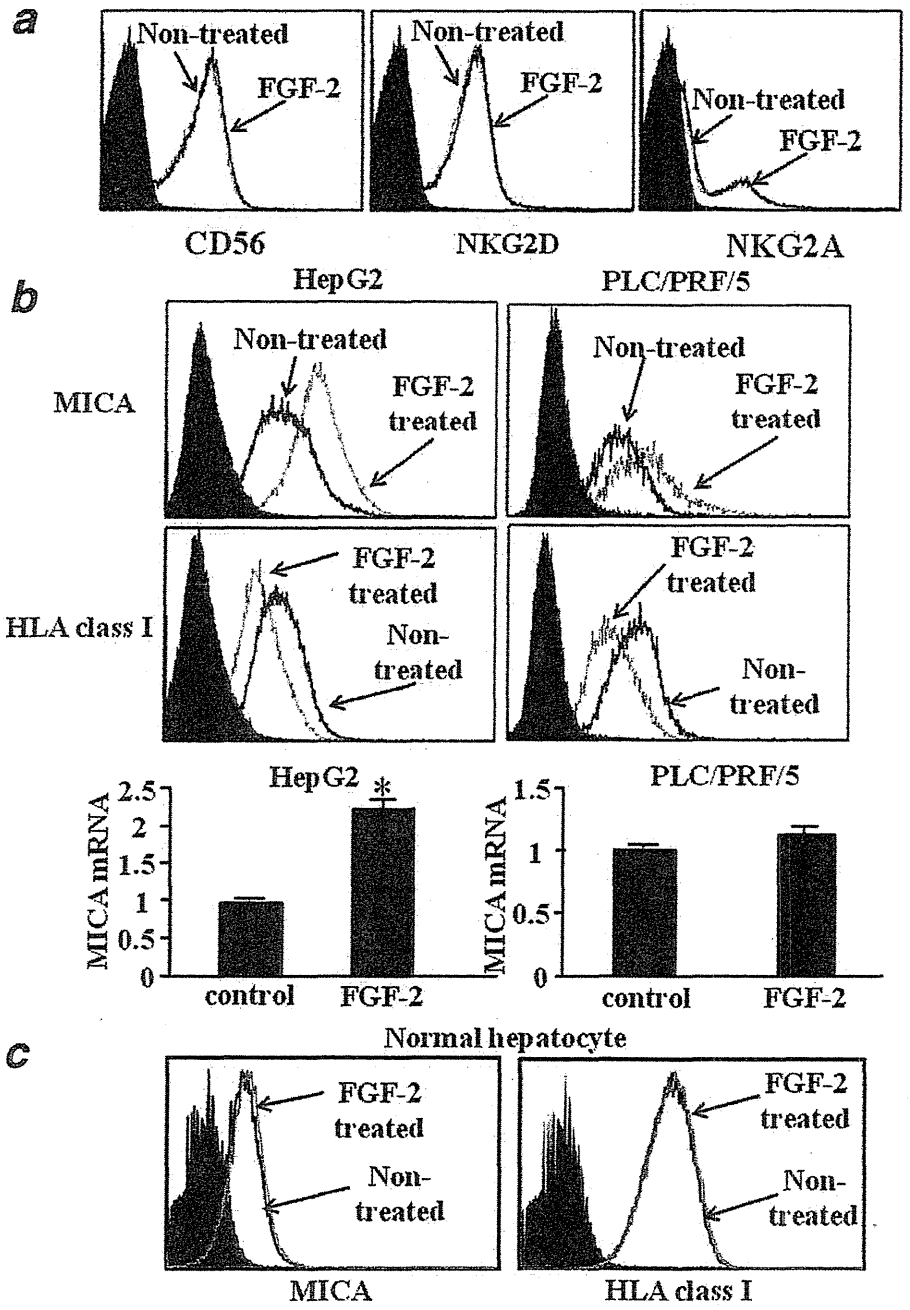

Figure 3. The expressions of NKG2D and NKG2A on FGF-2-treated NK cells and the expressions of MICA and HLA class I on FGF-2-treated hepatoma cells. (*a*) The expressions of NKG2D or NKG2A on FGF-2-treated or nontreated NK cells were evaluated. NK cells obtained from healthy volunteers (2×10^6 cells/well) were cultured with or without FGF-2 protein (250 ng/ml) for 24 hr, and the expressions of NKG2D and NKG2A on NK cells were evaluated by flow cytometry. Representative results were shown. (*b,c*) HCC cells (B: HepG2 and PLC/PRF/5) or normal hepatocytes (*c*) were treated with 250 ng/ml FGF-2 or control medium for 48 hr and subjected to flow cytometric analysis of MICA and HLA class I surface expression. Black line histograms: MICA or HLA class I staining of nontreated cells; gray line histograms: MICA or HLA class I staining of FGF-2-treated cells; shaded/black histograms: control IgG isotype Ab staining of each molecule. (*b*) *Lower panel*, mRNA levels of MICA in FGF-2-treated or nontreated HCC cells were examined by real-time PCR. Representative data are shown. Similar results were obtained from two independent experiments. * $p < 0.05$.

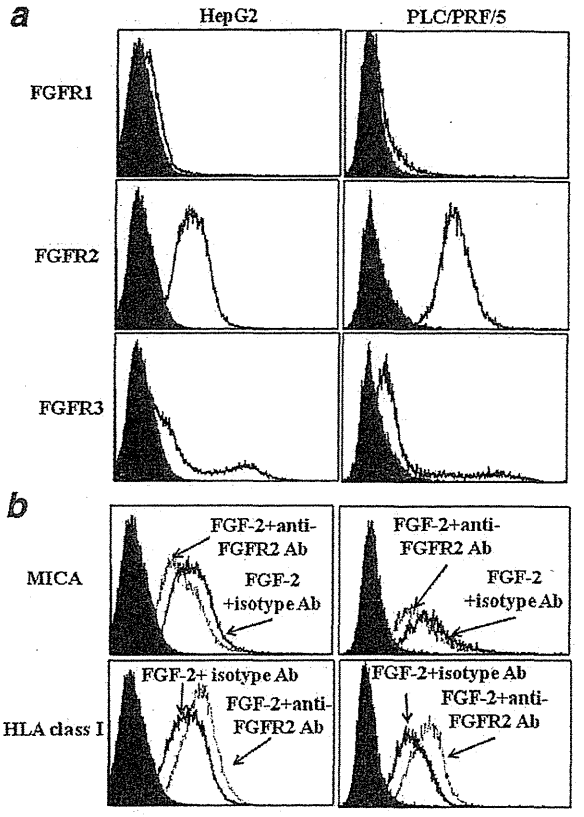

Figure 4. The expressions of FGF receptors on hepatoma cells. (*a*) The expressions of FGF receptors (FGFR1, FGFR2, and FGFR3) on both HepG2 and PLC/PRF/5 cells were evaluated by flow cytometry. Black line histograms: staining of each FGF receptors (FGFR1, FGFR2, FGFR3), shaded/black histograms: control isotype Ab staining of each molecule. (*b*) To confirm that adding of FGF-2 protein resulted in modifying the expressions of MICA and HLA class I on both HCC cells, the expressions of both molecules on FGF-2- (250 ng/ml) treated HCC cells with anti-FGFR2 neutralizing Ab (10 μg/ml) or isotype control Ab (murine isotype control IgG 10 μg/ml) were evaluated by flow cytometry. FGF-2+anti-FGFR2 Ab, the expression of MICA or HLA class I on FGF-2-treated HCC cells with anti-FGFR2 neutralizing Ab. FGF-2+isotype Ab, the expression of MICA or HLA class I on FGF-2-treated HCC cells with isotype control Ab. shaded/black histograms: control isotype Ab staining of each molecule. Representative results were shown. Similar results were obtained in three independent experiments.

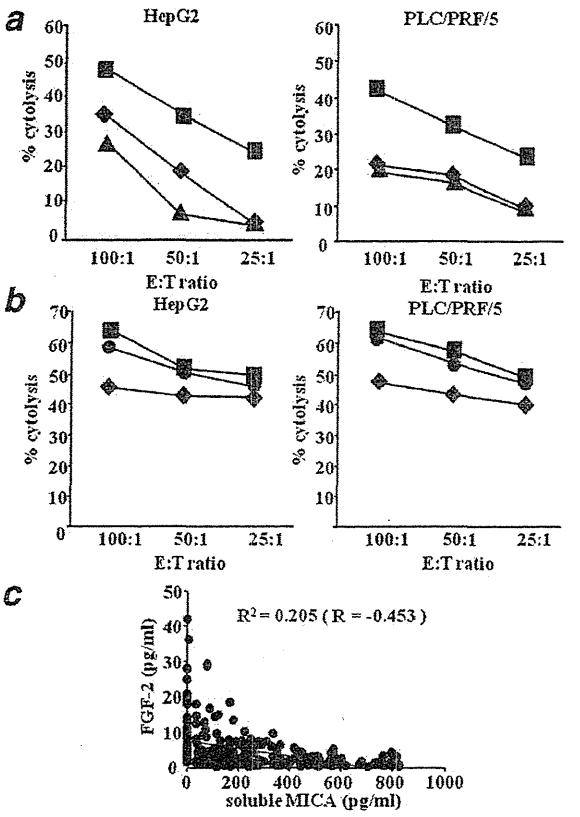

Figure 5. The cytolytic activity against FGF-2-treated HCC cells and the correlation between serum FGF-2 and soluble MICA in patients with chronic liver disease. (*a,b*) Both HepG2 and PLC/PRF/5 cells were cultured with or without FGF-2 protein (250 ng/ml) for 48 hr, and the cytolytic activities of NK cells against FGF-2-treated HepG2 and PLC/PRF/5 cells or nontreated HCC cells were evaluated by ^{51}Cr-release assay. Nontreated HCC cells (◆) or FGF-2-treated HCC cells without (■) or with blocking Ab of MICA/B (6D4) (*a*, ▲) or isotype IgG Ab (*b*, ●). Representative results are shown. Similar results were obtained from three independent experiments. (*c*) Correlation of serum FGF-2 levels and soluble MICA levels in patients with chronic liver disease (chronic hepatitis patients, N = 80, liver cirrhosis patients, N = 84 and HCC patients, N = 112). The serum FGF-2 and soluble MICA were evaluated by specific ELISA respectively.

increased expression of membrane-bound MICA. We next examined the correlation of serum FGF-2 and soluble MICA in patients with chronic liver disease. Serum FGF-2 levels in patients with chronic liver disease correlated with soluble MICA levels (Fig. 5*c*). These results suggested that high FGF-2 levels in patients with chronic liver disease may prevent the shedding of MICA in liver tissues.

Discussion

The FGF-2 levels in chronic liver disease, a premalignant condition, have not been well studied. Uematsu *et al.* reported that the serum FGF-2 levels of patients with LC or HCC were significantly higher than those of HVs, and serum FGF-2 levels of HCC patients tended to be lower than those of LC patients without HCC.[6] In contrast, Jinno *et al.* reported that the circulating FGF-2 levels in HCC patients were significantly higher than those in CH and LC patients.[16] In the present study, we analyzed the serum FGF-2 levels on

a larger scale for patients with chronic liver disease. Consistent with Uematsu's report, the serum FGF-2 levels significantly decreased along the progression of chronic liver disease and those in HCC patients were significantly lower than those in CH or LC patients. These results suggested that decreasing FGF-2 levels might be associated with the occurrence of HCC during the progression of chronic liver disease. FGF-2 has been shown to act as a potent angiogenic factor in a number of cell lines and solid tumors.[1,2] As for HCC development, FGF-2 has been reported to augment vascular endothelial growth factor (VEGF)-mediated angiogenesis in HCC development.[17] However, at present, in contrast to the clear roles of VEGF in the angiogenesis of HCC, the roles of FGF-2 in the HCC development are still controversial and should be elucidated.

Immunohistochemical analysis revealed that hepatocytes in patients with chronic liver diseases seemed to produce FGF-2, but those in healthy donors did not. This suggested that inflammatory responses in liver tissues might have roles in the production of FGF-2. Some inflammatory cytokines, such as IL-1β and IL-6, increased in CH or LC patients.[13–15] Aside from liver cells, IL-6 could induce FGF-2 expressions in basal cell carcinoma cell line[18] or Kaposi's sarcoma cell and human umbilical vein endothelial cells.[19] On the basis of these reports, we examined the effect of such inflammatory cytokines on FGF-2 expression in HCC cells and normal hepatocytes. The FGF-2 expression could be, at least in part, induced by IL-1β and IL-6. Both IL-1β and IL-6 are produced mainly by local immune cells, including activated Kupffer cells.[20] Although the detail mechanism of the induction of FGF-2 expression in HCC cells and normal hepatocytes is little known, the production of these cytokines might contribute to preventing HCC development *via* promoting FGF-2 expression in the liver.

Guerra *et al.* reported that NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy,[11] suggesting that NK-dependent immune-surveillance might play a critical role in tumor development. However, the mechanism of tumor surveillance of NK cells remains unclear in HCC development. We previously demonstrated that membrane-bound MICA on HCC cells plays essential roles in the NK sensitivity of HCC cells.[21] We therefore evaluated the MICA (activating molecule of NK cells) and HLA class I (inhibitory molecule of NK cells) on HCC cells treated with FGF-2. This treatment resulted in increasing MICA expression and decreasing HLA class I on HCC cells. Consistent with these results, the cytolytic activity of NK cells against FGF-2-treated HCC cells was higher than that against nontreated HCC cells. These results suggested that FGF-2 enhanced the NK sensitivity of HCC cells by upregulating MICA expression and downregulating HLA class I on the cellular surface. Interestingly, adding FGF-2 did not change the expressions of MICA and HLA class I on normal hepatocytes. These demonstrated that FGF-2 could enhance the NK sensitivity of HCC cells but not that of normal hepatocytes. We also evaluated the expressions of MICA and HLA class I on other growth factors (such as VEGF or PDGF)-treated HCC cells. The expressions of MICA and HLA class I on VEGF- or PDGF-treated HCC cells were similar to those on nontreated HCC cells (Tsunematsu H, unpublished data). In this study, we demonstrated that FGF-2 production from liver tissues decreased along the progression of chronic liver disease. FGF-2 production from liver tissues might prevent the occurrence of HCC by eliminating HCC cell by enhancing NK sensitivity. If the innate immunity of the liver can be efficiently activated, preventing the occurrence of HCC could be expected. We previously demonstrated that anti-HCC chemotherapy and molecular targeted therapy using sorafenib resulted in enhancing NK sensitivity of HCC cells *via* upregulation of membrane-bound MICA on HCC cells.[12,22] These results suggested the possibility of new routes for chemoprevention of HCC, which could improve the prognosis of chronic liver disease patients. Also, on the basis of our results, FGF-2 supplementation therapy may be a rational approach for eliminating HCC cells in the chronic liver disease.

The concentration of FGF-2 in our *in vitro* study was high compared with the serum FGF-2 concentration level. Previous reports demonstrated that FGF-2 produced in the liver tissues acts in an autocrine or paracrine fashion.[2,5] We demonstrated that serum FGF-2 levels in chronic liver disease were significantly higher than those in HVs and that serum FGF-2 levels decrease with the progression of liver disease. These results suggested that FGF-2 production from liver tissues might also decrease with the progression of liver disease. Although the local FGF-2 concentration in the liver tissues still remains unknown and may differ from the serum FGF-2 concentration, our results have at least demonstrated that FGF-2 could enhance NK sensitivity of HCC cells *via* modification of the activating and inhibitory molecules on HCC cells.

The expression of NKG2D has been reported in all NK cells. However, this has also been reported in most NKT cells, subsets of γδ T cells and all human CD8+ T cells and a subset of CD4+ T cells.[23] In addition to NK cells, the MICA-NKG2D pathway plays roles in the costimulation or recognition of each cell. Our results demonstrated that FGF-2 might increase the membrane-bound MICA on HCC cells. It might be possible that the increased expression of MICA may also activate other lymphocytes expressing NKG2D and that these cells may also contribute to the elimination of HCC cells.

The earlier results suggested that FGF-2 levels might contribute to the eradication of HCC cells in liver tissues, which would prevent the incidence of HCC in chronic liver disease. Our patients' data demonstrated that HCC occurrence of the patients with high levels of FGF-2 was significantly lower than that with low levels of FGF-2, which is consistent with the results of NK sensitivity of FGF-2-treated HCC cells. Moreover, the FGF-2 levels in patients before HCC occurrence were significantly higher than those in the same

patients after HCC occurrence. The decreasing levels of serum FGF-2 may be a prediction factor for the occurrence of HCC in chronic liver disease.

Despite recent progress in understanding HCC development, unknown mechanisms remain. We have shown here that FGF-2 levels in chronic liver disease were significantly higher than those in HVs, and serum FGF-2 levels decreases along the progression of liver disease. Importantly, FGF-2 enhances NK sensitivity of HCC cells *via* modification of the activating and inhibitory molecules on HCC cells. These findings suggested that FGF-2 might play roles in eliminating occurring HCC cells by innate immunity.

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ORIGINAL ARTICLE

Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice

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Abstract Although the anti-tumor effect of IL-12 is mediated mostly by IFNγ, which cell types most efficiently produce IFNγ and therefore initiate or promote the anti-tumor effect of IL-12 has not been clearly determined. In the present study, we demonstrated hydrodynamic injection of the IL-12 gene led to prolonged IFNγ production, NK-cell activation and complete inhibition of liver metastasis of CT-26 colon cancer cells in wild-type mice, but not in IFNγ knockout mice. NK cells expressed higher levels of STAT4 and upon IL-12 administration displayed stronger STAT4 phosphorylation and IFNγ production than non-NK cells. Adoptive transfer of wild-type NK cells into IFNγ knockout mice restored IL-12-induced IFNγ production, NK-cell activation and anti-tumor effect, whereas transfer of the same number of wild-type non-NK cells did not. In conclusion, NK cells are predominant producers of IFNγ that is critical for IL-12 anti-tumor therapy.

Keywords IFNγ · Innate immunity · Liver tumor · IL-12 · NK

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Introduction

IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits, mainly produced by antigen-presenting cells. IL-12 was originally found as a "natural killer-stimulating factor" and a "cytotoxic lymphocyte maturation factor" [1, 2]. IL-12 has multi-potent effects, inducing a Th1 response, enhancing the CD8 T-cell response, activating natural killer cells and inducing production of IFNγ [3, 4]. Therapeutic use of IL-12, either using its recombinant protein or gene, can induce an efficient anti-tumor effect on primary or metastatic tumors in various murine models and humans [5, 6].

Research has shown that IL-12 mediates anti-tumor effects in a variety of ways. They include anti-proliferative effects, anti-angiogenic effects [7, 8] and cytotoxic effects of effector lymphocytes. A variety of effector cells has been reported to be required for IL-12-mediated anti-tumor effects: they include CD8 T cells [9], NKT cells [10], CD4 T cells [11] and NK cells [12]. The relative contribution of these cells may differ among IL-12 doses and types of tumor models [13]. Endogenous IFNγ production is required for most, if not all, of the anti-tumor effects of IL-12 administration [14, 15]. IL-12 stimulates a variety of immune cells, such as T cells [16], B cells [17] and NK cells [18], to produce IFNγ. However, which cell types are most critical for producing IFNγ during IL-12 therapy is not clearly known.

In the present study, we used a murine model of liver metastasis of CT-26 colon cancer cells and found that NK cells highly expressed the IL-12 signaling molecule STAT4 and most efficiently produced IFNγ. IFNγ was essential for the anti-tumor effect of IL-12, and NK-cell production of IFNγ sufficed to produce the full-blown anti-tumor effects. These results demonstrated that NK cells

serve not only as an effector but also as an important mediator producing IFNγ that is critical for the anti-tumor effects of IL-12.

Materials and methods

Mice

Specific pathogen-free female Balb/c mice were purchased from Clea Japan, Inc (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice with a Balb/c background were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/c background were kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo). All mice used were at the age of 6 to 10 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intra-splenic injection of tumor cells was used to establish micro-disseminated liver tumors in mice [19]. CT-26 colon cancer cells originating from Balb/c mice were maintained in RPMI1620 supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 200 μl of PBS and injected into the spleen.

Injection of naked plasmid DNA

A plasmid coding the murine IL-12 gene, pCMV-IL-12, was generously provided by Dr. M Watanabe (Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center) [20]. Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany,) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described [21]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 26-gauge needle. DNA injection was completed within 5 to 8 s.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IL-12 p70, IFNγ (BD Biosciences-Pharmingen, San Diego, CA), IFNγ-inducible protein 10 (IP-10) and monokine induced by IFNγ (MIG) (R&D Systems, Inc, Minneapolis, MN) were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described. The NK activity of mononuclear cells was assessed by a standard 4-h ^{51}Cr-releasing assay using Yac1 cells as targets. In some experiments, mononuclear cells were separated into $DX5^+$ cells (NK cells) and $DX5^-$ cells (non-NK cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated NK-cell population was found to be greater than 90% by FACS analysis.

Flow cytometric analysis

Liver mononuclear cells were isolated 2 days after pCMV-IL-12 injection. Cytokine secretion was then blocked by the addition of brefeldin A for 4 h. Next, liver mononuclear cells were stained with FITC-conjugated anti-TCRβ antibody and biotin-conjugated anti-CD49b antibody (DX5), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with PE-conjugated anti-INFγ antibody or corresponding isotype controls. Analysis was performed using a FACSCalibur (Becton Dickinson), with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as $DX5^+/TCR\beta^-$ lymphocytes, NKT cells as $DX5^+/TCR\beta^+$ lymphocytes and T cells as $DX5^-/TCR\beta^+$ lymphocytes.

Adoptive transfer

For adoptive transfer experiments, GKO mice were injected intravenously 1 day before plasmid DNA injection with 2.0×10^8 whole mononuclear cells or 4.0×10^6 NK cells, or non-NK cells or whole mononuclear cells, all of which had been harvested from wild-type mice that can produce IFNγ.

Western blotting

Mouse recombinant IL-12 was purchased from R&D Systems, Inc (Minneapolis, MN). Mononuclear cells were treated with or without IL-12. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was stained with anti-STAT4 antibody (BD biosciences),

anti-phospho-specific STAT4 (pY693) antibody (BD biosciences), anti-STAT1 antibody (Cell Signaling), anti-phospho-specific STAT1 antibody (Cell Signaling) and visualized by chemiluminescence.

NK-cell depletion

For depletion of NK cells in vivo, anti-asialoGM1 antibody (WAKO, Osaka, Japan) was intraperitoneally administered. We determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. The percentage of $DX5^+/TCR\beta^-$ cells (NK cells) is 12.6 ± 2.4% in IgG-injected liver, whereas it decreased to 0.76 ± 0.04% one day after anti-asialo GM1 antibody injection ($N = 3$/group). This effect remained at least 3 days after anti-asialo GM1 antibody injection. NKT cells were less affected than NK cells, because 90% of $DX5^+/TCR\beta^+$ cells (NKT cells) still remained in the liver after the treatment. Anti-asialoGM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Histology

The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Acetone-fixed fresh frozen liver sections were immunostained with anti-mouse CD4 (H123.19), anti-mouse CD8α (53-6.7) or anti-CD31 (390) monoclonal antibody (all from BD Biosciences), using a VECSTAIN ABC kit (Vector Laboratories, Burlingame, California, USA).

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired t-test with Welch's correction. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of IL-12-expressing plasmid led to prolonged production of IFNγ

Hydrodynamics-based gene delivery into mice establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes. Serial measurement of serum IL-12 demonstrated that pCMV-IL-12 injection led to substantial IL-12 production on day 1. The levels of serum IL-12 then rapidly declined (Fig. 1a). We also measured IFNγ production in serum, since IL-12 is known to activate IFNγ production. pCMV-IL-12 and, to a lesser extent, pCMV injection increased serum IFNγ on day 1. In contrast to the pCMV injection group, high levels of serum IFNγ were maintained at later time points in the pCMV-IL-12 injection group (Fig. 1a). Thus, hydrodynamic injection of pCMV-IL-12 led to prolonged production of IFNγ. Transient IFNγ production followed by control plasmid may be an indirect effect of liver injury caused by bolus injection of saline or DNA injection.

IL-12 therapy induced NK activation and anti-metastatic effects, both of which are critically dependent on IFNγ

To examine the biological effects of the produced IL-12, we evaluated the NK activity of mononuclear cells from the liver. pCMV-IL-12 injection, but not control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells (Fig. 1b). When GKO mice were injected with pCMV-IL-12 or pCMV, the hepatic mononuclear cells did not display any lytic ability to Yac1 cells, suggesting that IL-12-mediated NK-cell activation required IFNγ.

To examine the anti-metastatic effect of IL-12, pCMV-IL-12 or pCMV was injected into wild-type mice 2 days after intrasplenic injection of CT-26 cells. At 14 days after tumor injection, the mice were killed for evaluation of liver tumor (Fig. 1c). While pCMV-injected mice displayed huge liver tumors, pCMV-IL-12-injected mice did not show any macroscopic or microscopic tumor (Fig. 1d). Liver weight was significantly higher in pCMV-injected mice than pCMV-IL-12-injected mice, reflecting liver tumor formation. To examine the involvement of IFNγ in the IL-12-induced anti-tumor effect, we injected pCMV or pCMV-IL-12 into GKO mice 2 days after CT-26 injection. At 14 days after CT-26 injection, both groups showed similar degrees of tumor formation and there was no significant difference in liver weight between the two. This indicated that IL-12-induced anti-metastatic effect was strictly dependent on IFNγ.

NK cells were the most potent producer of IFNγ during IL-12 therapy

To evaluate which cell types most efficiently produced IFNγ, we isolated hepatic mononuclear cells from mice 2 days after plasmid injection and then stained cell surface TCRβ and DX5 as well as intracellular IFNγ (Fig. 2). $TCR\beta^-/DX5^+$ NK cells, $TCR\beta^+/DX5^+$ NKT cells and $TCR\beta^+/DX5^-$ T cells from pCMV-IL-12-injected mice showed significant levels of IFNγ production compared

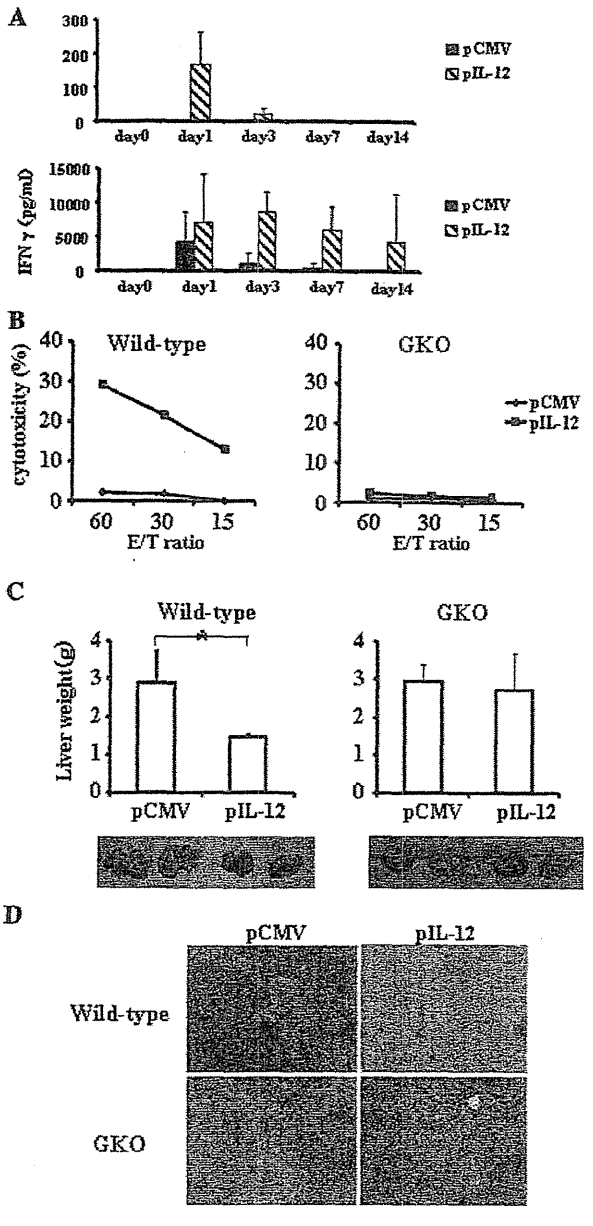

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and SD ($n = 6$/group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (*left*) or GKO mice (*right*) which had been injected with pCMV-IL-12 (*closed squares*) or pCMV (*closed diamonds*) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr-release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (*left*) or GKO mice (*right*) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$/group) and a representative picture of the liver in each group is shown at the bottom. *$p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFNγ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFNγ (data not shown).

IL-12-induced STAT4 signaling and IFNγ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFNγ in the presence or absence of anti-IFNγ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFNγ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFNγ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFNγ-dependent manner.

To examine STAT1 and STAT4 activation and IFNγ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells produced significant levels of IFNγ, but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFNγ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFNγ, which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

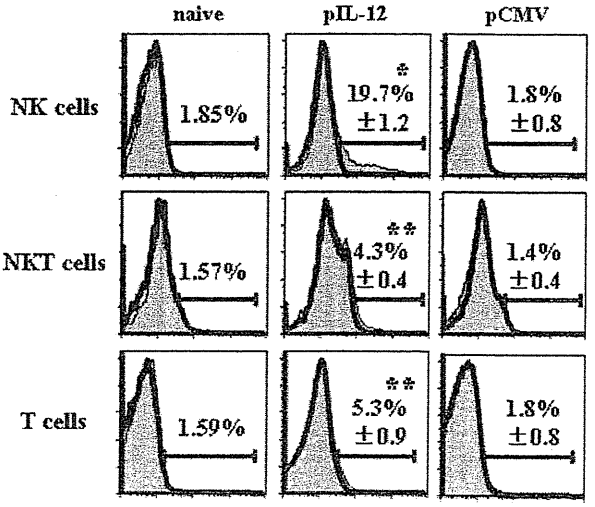

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations ($TCR\beta^-/DX5^+$ cells for NK cells, $TCR\beta^+/DX5^+$ cells for NKT cells and $TCR\beta^+/DX5^-$ cells for T cells). Isotype control stainings are shown by *open histograms*. Numbers in histograms represent averages ± SD of percentages of positive cells ($n = 3$ mice/group). $^*p < 0.0001$ vs. mock in NK populations. $^{**}p < 0.05$ vs. mock in each population

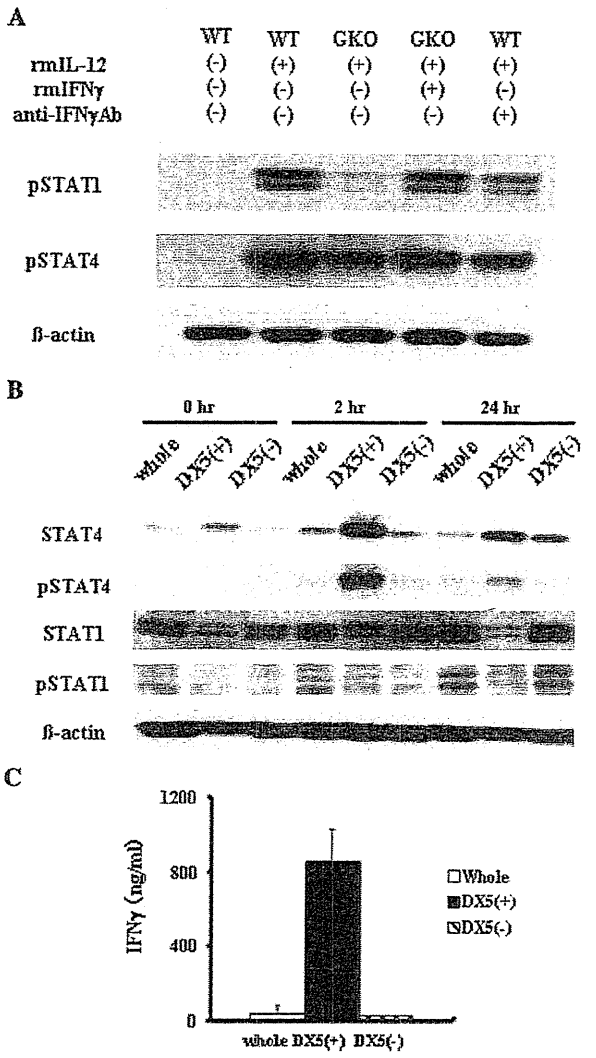

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFNγ (500 ng/mL) or anti- IFNγ antibody (20 μg/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β-actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into $DX5^+$ cells and $DX5^-$ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFNγ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFNγ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFNγ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFNγ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

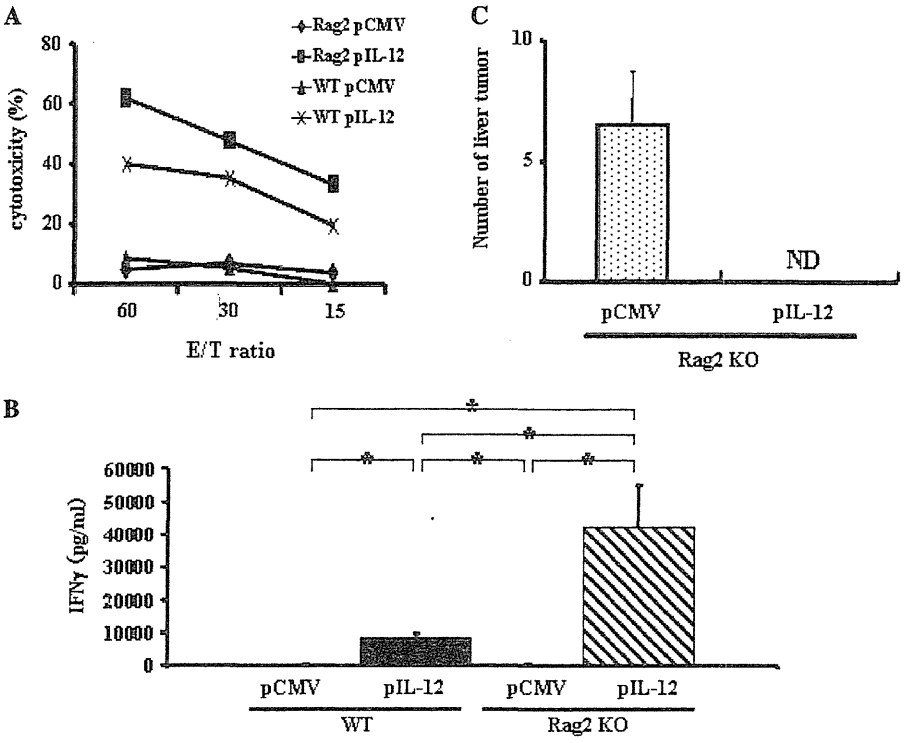

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ were determined by ELISA. Data are expressed as mean and SD ($n = 7$/group). $^*p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7$/group). *ND* not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFNγ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFNγ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFNγ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFNγ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12's anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,