Chemoprevention of spontaneous development of hepatocellular carcinomas in fatty liver Shionogi mice by a cyclooxygenase-2 inhibitor

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Cyclooxygenase 2 (COX-2) and retinoid X receptor α (RXRα) are suggested to have roles in carcinogenesis. COX-2 inhibitors have been reported to suppress growth of hepatocellular carcinoma (HCC) cell lines *in vitro*. However, little is known about the preventive effect of these drugs on spontaneous hepatocarcinogenesis *in vivo*. Etodolac exists in a racemic mixture containing S- and R-etodolac. S-etodolac is responsible for COX-2 inhibitory activity and R-etodolac is related to the downregulation of RXRα. Here, the effect of etodolac on spontaneous development of HCC in fatty liver Shionogi mice is evaluated. Etodolac was administered at a low (2 mg/kg) or high (10 mg/kg) dose three times a week for 16 months starting at the age of 3 months. The development of HCC was suppressed slightly in the high-dose group, and suppressed markedly in the low-dose group, although the development of fatty liver was not inhibited in either group. Plasma prostaglandin E_2 levels were also decreased significantly in the low-dose group, consistent with the suppression of HCC. The expression of RXRα and proliferating cell nuclear antigen in non-tumorous liver tissues was decreased significantly in both the low-dose and high-dose groups. These findings show that etodolac treatment at an optimum dose suppresses hepatocarcinogenesis *in vivo*, and may be useful for preventing the development of HCC in humans. (*Cancer Sci* 2006; 97: 768–773)

Hepatocellular carcinoma is a common malignancy worldwide, accounting for approximately 6% of all human cancers and up to 1 million deaths per year.[1,2] Epidemiological studies and clinical observations have indicated that some medicines, such as vitamin A, vitamin K2 and interferon-α, have chemopreventive effects for hepatocarcinogenesis.[3–5] Because these medicines are not enough to prevent hepatocarcinogenesis in humans, other efficient preventive tools are needed urgently.[6]

The use of COX-2 inhibitors is associated with a reduced development of certain types of tumors, such as colorectal cancer and prostate cancer.[7–9] COX-2 inhibitors suppress the growth of human HCC implants in nude mice and lung metastasis of HCC in F344 rats, and show preventive effects on chemically induced hepatocarcinogenesis in rats.[6,10–13] We reported previously that a specific COX-2 inhibitor, etodolac ([±]-1,8-diethyl-1,3,4,9,-tetrahydropyrano-[3,4-b] indole-1-acetic acid), decreases the levels of PGE_2 and inhibits the expression of PCNA in several HCC cell lines *in vitro*.[14] Etodolac exists in a racemic mixture containing S- and R-etodolac. S-etodolac has been shown to possess COX-2 inhibitory activity and R-etodolac was recently reported to bind RXRα and to inhibit the development of prostate cancer.[15–17] RXRα, which plays an important role in regulating cell proliferation and differentiation, is expressed abundantly in the liver and is involved in hepatic steatosis and hepatocarcinogenesis in HBV and HCV infection in humans.[18–20] However, little is known about the chemopreventive effect of COX-2 inhibitors on spontaneous hepatocarcinogenesis *in vivo*.

Fatty liver Shionogi mouse is an inbred strain that shows neither hyperphagia nor obesity but has an abnormal triglyceride accumulation in hepatocytes after birth.[21,22] Fifty percent of the mice show fatty liver grade I and II 9 weeks after birth, and all mice develop fatty liver grade III and IV after 15 weeks.[21] FLS mice develop severe fatty liver (hepatic steatosis) and chronic HCC under normal conditions, in which the incidence of HCC is reached to 52% at 16 months of age.[22] To explore the mechanism involved and to find a specific and effective medicine for the prevention of hepatocarcinogenesis, we studied the effect of a COX-2 inhibitor, etodolac, on spontaneous development of HCC in FLS mice.

Materials and Methods

Animals and experimental design

Thirty male FLS mice aged 2 months were obtained from Aburahi Laboratories, Shionogi Company (Shiga, Japan).

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Abbreviations: BW, bodyweight; COX-2, cyclooxygenase-2; E-HD, high-dose treatment group; E-LD, low-dose treatment group; FLS, fatty liver Shionogi; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PCNA, proliferating cell nuclear antigen; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription–polymerase chain reaction; RXRα, retinoid X receptor α.

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They were housed, one per cage, under specific pathogen-free conditions in a 12 : 12 h L : D cycle at 23 ± 1°C and 50 ± 10% humidity, and fed a standard CE-2 diet (CLEA Japan, Tokyo, Japan) and tap water *ad libitum*. The mice were divided randomly into three groups of 10 mice each. All animals received humane care and all experiments followed the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.[23] Two doses of etodolac (Nihon Shinyaku Company, Tokyo, Japan) were used: 2 mg/kg BW for the E-LD group and 10 mg/kg BW for the E-HD group. Etodolac was dissolved in 100% ethanol and diluted to suitable concentrations with a 5% aqueous solution of arabic gum. The solutions of etodolac were given to mice by oral gavage, three times per week (Monday, Wednesday and Friday) for 16 months from age 3–18 months. The control group was treated with the same amounts of 0.7% ethanol and 5% arabic gum. The mice were observed weekly for BW, skin damage and general condition. The animals that were still alive at 18 months were anesthetized with diethyl ether and blood was collected from the heart. The livers were immediately removed and weighed. Tumor nodules that had developed were measured for diameter and cut for formalin fixation and paraffin embedding or frozen storage.

Measurement of prostaglandin E_2

Prostaglandin E_2 levels in the plasma were assayed using the PGE_2 High Sensitivity Immunoassay Kit (R & D Minneapolis, MN, USA) as described previously.[14]

Histological examination

Tumor and non-tumorous liver tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into sections 5 μm thick, and stained with hematoxylin and eosin. The classification of liver histology was based on the criteria described by Frith and Ward.[24]

Immunohistochemical analysis

Paraffin sections from HCC and non-tumorous liver tissues were deparaffinized in xylene, rehydrated with graded concentrations of ethanol, and treated with antibodies against COX-2, RXRα and PCNA, as described previously.[25] All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at a dilution of 1/100. The RXRα-positive and PCNA-positive cells were counted microscopically in five high-power fields at magnitude × 400. The labeling index of RXRα and PCNA was expressed as the proportion of cells with positive RXRα and PCNA nuclear activity.

RNA extraction and reverse transcription–polymerase chain reaction

Total RNA was extracted from liver tissues using Isogen (Nippon Gene, Toyama, Japan), and mRNA was prepared using an Oligotex-dT30 mRNA Purification Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized using random 9-mers and an RNA PCR Kit (version 2.1; Takara). The primers for polymerase chain reaction were as follows: COX-2 forward, 5′-GGTCT GGTGC CTGGT CTGAT GATG-3′; COX-2 reverse, 5′-GTCCT TTCAA GGAGA ATGGT GC-3′;[9] RXRα forward, 5′-CTTTG ACAGG GTGCT AACAG AGC-3′; RXR α reverse, 5′-ACGCT TCTAG TGACG CATAC ACC-3′;[26] β-actin forward, ATGGT GGGAA TGGGT CAGAA GGAC-3′; and β-actin reverse, 5′-CTCTT TGATG TCACG CACGA TTTC-3′.[27] cDNA amplification was carried out under the conditions 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, using β-actin as an internal control. The products were analyzed on a 3% NuSieve 3 : 1 agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide and photographed under ultraviolet light.

Statistical analysis

Statistical analysis for the development of HCC was carried out using the Student's *t*-test or Fisher's exact test. Values are expressed as mean ± SE. $P < 0.05$ was considered statistically significant.

Results

Effects of etodolac on HCC development

Histological findings of fatty liver and HCC spontaneously developed in the liver of 18-month-old FLS mice are shown in Fig. 1A,B. The expression of COX-2 protein in the non-tumorous liver tissues was determined by immunohistological staining (Fig. 1C,D). The mRNA expression of COX-2 was confirmed by RT-PCR (Fig. 1E).

The incidence of HCC was evaluated after the administration of etodolac. The total numbers of HCC nodules were 11 in the control group (10 mice), 0 in the E-LD group (eight mice) and three in the E-HD group (nine mice). The numbers of mice that developed HCC were 5, 0 and 3 in the control, E-LD and E-HD groups, respectively. Development of HCC was suppressed completely by the administration of low-dose etodolac. The administration of high-dose etodolac also showed a suppressive effect, although it was not statistically significant (Table 1).

All of the 27 mice used in the present study developed fatty liver from grades II to IV at the end of experiments with no remarkable difference in the degree of fatty or inflammatory changes. A small number of mice in each group developed yellow nodules of 1–2 mm in diameter and reddish cysts, which were identified microscopically as fatty nodules and peliosis hepatis, respectively (data not shown). Liver cirrhosis was not observed in any of the mice. Liver weights also did not show significant differences among the control, E-LD and E-HD groups (data not shown). One mouse in the E-HD group died at the age of 17 months, and two mice in the E-LD group died at 13 and 14 months. No HCC was found in these mice and the cause of death was not clear.

Except for the livers, no abnormal findings were observed macroscopically in heart, lung, kidney, intestines and large vessels in any of the FLS mice. Five mice in the E-HD group had skin damage, including depilation and rash, and two among them had skin ulcers. The mean BW of mice at 18 months of age were 37.9 ± 1.11 g ($n = 10$), 36.88 ± 1.2 g ($n = 8$) and 35.56 ± 0.93 g ($n = 9$) in the control, E-LD and E-HD groups, respectively. No significant difference was found in BW among the control, E-LD and E-HD groups.

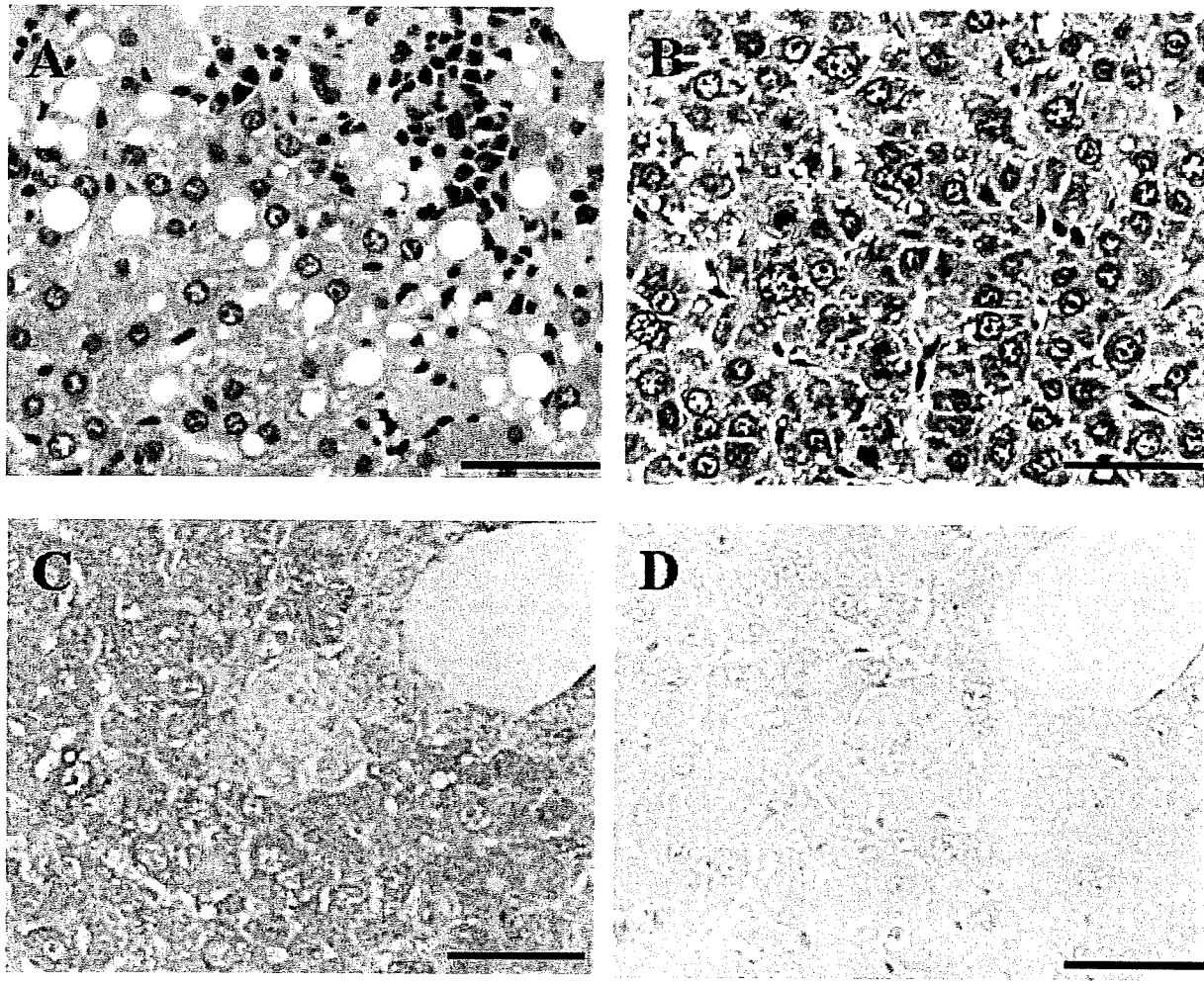

Fig. 1. Histopathological features of hepatocellular carcinoma (HCC) and expression of cyclooxygenase-2 (COX-2) in non-tumorous fatty liver of fatty liver Shionogi mice. (A) Non-tumorous liver tissue, (B) HCC, (C) expression of COX-2 protein in non-tumorous liver tissue by immunohistological staining, (D) negative control of (C). Scale bar = 50 μm. (E) Expression of COX-2 mRNA in a non-tumorous liver tissue by reverse transcription–polymerase chain reaction.

Table 1. Incidence of hepatocellular carcinoma (HCC) in fatty liver Shionogi mice

Group	No. mice	Etodolac (mg/kg)	Grade of steatosis			No. HCC nodules	No. mice that developed HCC
			II	III	IV		
Control	10	0	0	4	6	11	5 (50%)
E-LD	8	2	1	2	5	0*	0*
E-HD	9	10	0	3	6	3	3 (33%)

*$P < 0.05$ by Fisher's exact test. Grades of classification are according to the size and distribution pattern of the vesicles in the hematoxylin–eosin-stained sections.[24] E-HD, high dose of etodolac; E-LD, low dose of etodolac.

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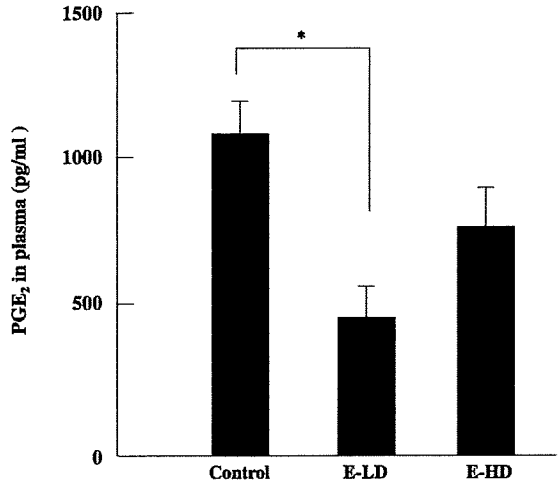

Fig. 2. Effect of etodolac on plasma prostaglandin E_2 (PGE_2) levels in fatty liver Shionogi mice. A marked decrease in plasma PGE_2 levels was observed after low-dose (2 mg/kg bodyweight) administration of etodolac (E-LD). E-HD, high-dose (10 mg/kg bodyweight) administration of etodolac. *$P < 0.05$. Bars indicate ±SE of mean.

Plasma PGE_2 levels after etodolac administration

The activity of etodolac can be estimated by analyzing the concentration of PGE_2 in the plasma. The concentrations of PGE_2 in the plasma were 1010.15 ± 120.22 pg/mL ($n = 10$), 443.33 ± 116.99 pg/mL ($n = 8$) and 773.8 ± 137.67 pg/mL ($n = 9$) in the control, E-LD and E-HD groups, respectively. We found that the plasma levels of PGE_2 of the E-LD group, but not the E-HD group, were significantly lower than in the control group ($P < 0.05$) (Fig. 2).

Effects of etodolac on RXRα expression

Hepatocarcinogenesis in FLS mice has been attributed to chronic inflammation in fatty liver. Therefore, we investigated the effects of etodolac administration on hepatocytes in non-tumurous fatty liver of FLS mice. In prostate cancer, R-etodolac has been shown to bind to RXRα, inducing its degradation via ubiqutin and the proteasome-dependent pathway.[17] Analysis by immunohistological staining showed that the expression of RXRα in hepatocytes in non-tumorous liver tissues was significantly lower in E-LD (10.98 ± 0.87%, $n = 6$) and E-HD (11.65 ± 1.72% $n = 4$) groups than in the control group (27.28 ± 2.91% $n = 5$) ($P < 0.01$; Fig. 3A–C). In contrast, semiquantitative RT-PCR analysis showed identical expression of RXRα mRNA among non-tumorous liver tissue of the control, E-LD and E-HD groups (Fig. 3D). These findings suggest that etodolac binds to RXRα and induces its degradation in the non-tumorous liver of FLS mice.

Effects of etodolac on PCNA expression

Proliferating cell nuclear antigen is expressed throughout the cell cycle, except during G_0 phase, and plays an important role in cell proliferation. The labeling index of PCNA in the non-tumorous liver tissues of the E-LD (2.15 ± 0.11%, $n = 8$) and E-HD groups (2.2 ± 0.27%, $n = 9$) was significantly lower than the control group (3.13 ± 0.26%, $n = 10$) ($P < 0.05$, Fig. 4). These findings show that the growth of hepatocytes in non-tumorous tissue was inhibited by etodolac administration.

Discussion

Fatty liver Shionogi mice develop serious fatty liver and HCC with age, providing a good animal model to study hepatocarcinogenesis from fatty liver *in vivo*.[21,22] Using FLS mice, we here examined the *in vivo* effects of a COX-2 inhibitor, etodolac, on spontaneous development of HCC.

Etodolac exists in a racemic mixture. S-etodolac possesses activity to inhibit COX-2, which catalyzes the conversion of arachidonic acid to PGE_2.[15,16] COX-2 and PGE_2 have been reported to be involved in carcinogenesis of the colon, prostate and liver.[7–9,28–30] Etodolac has been reported to reduce aberrant crypt foci in rat colon, and another selective COX-2 inhibitor, NS-398, has been reported to reduce rat colon carcinogenesis.[31–33] In the present study, we found that etodolac was effective in inhibiting PGE_2 synthesis and HCC development in FLS mice, particularly at a low concentration. A similar observation has been reported for aspirin, where a low dose has a better preventive effect than a high dose in human colorectal cancer.[7] On the other hand, NS-398 has been shown to inhibit aberrant crypt foci in F344 rats in a dose-dependent manner.[32]

In the present study, we observed that the plasma concentration of PGE_2 was higher in the E-HD group than in the E-LD group. The plasma concentration of PGE_2 was lower in the E-HD group than in the control group (not significantly). The plasma levels of etodolac in mice in the E-HD group were approximately five times higher than those in mice in the E-LD group (data not shown). Our previous study using HCC cell lines showed that PGE_2 generation by etodolac is not inhibited in a dose-dependent manner. Rather, PGE_2 levels in the culture medium were higher with the high-dose treatment than with the low-dose treatment.[14] The inhibition of PGE_2 generation by NS-398 was also dose-independent at doses higher than 100 mM in some HCC cell lines.[34] The dose-independency of plasma PGE_2 suppression by etodolac *in vivo* is compatible with these findings in the *in vitro* experiments. However, the precise mechanism of dose-independency has not yet been clarified. Another suggested explanation is that the higher levels of PGE_2 in the E-HD group may be attributable to adverse effects of a high dose of etodolac. Severe skin damage developed in mice in the E-HD group. This could be responsible for loss of the preventive effect of COX-2 inhibitor on HCC in the E-HD group. Furthermore, in the present study, the plasma PGE_2 levels were consistent with the HCC incidences. These data suggest that PGE_2 plays an important role in the development of HCC in FLS mice. The 2 mg/kg dose of etodolac three times a week used in this study is less than the usual dose in humans (200 mg orally twice a day), and no side effects were observed in this group. Thus, we consider that administration of a low dose of COX-2 inhibitor should be sufficient for liver cancer prevention in humans. Furthermore, it is necessary to evaluate the efficacy of etodolace doses lower than 2 mg/kg to elucidate the optimum dose for liver cancer prevention.

Recently, R-etodolac has been reported to bind specifically to RXRα and prevent prostate cancer.[17] In the present study,

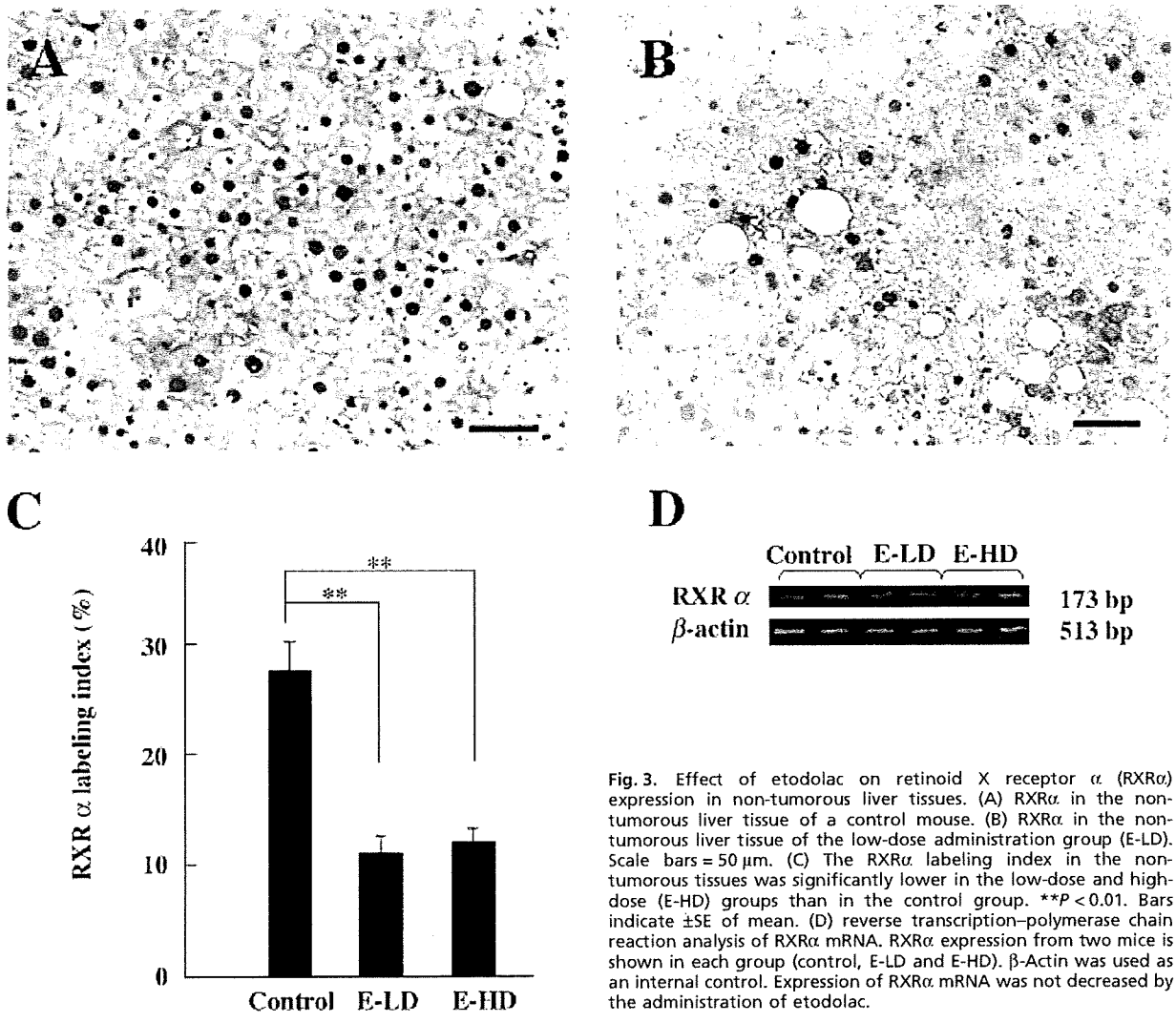

Fig. 3. Effect of etodolac on retinoid X receptor α (RXRα) expression in non-tumorous liver tissues. (A) RXRα in the non-tumorous liver tissue of a control mouse. (B) RXRα in the non-tumorous liver tissue of the low-dose administration group (E-LD). Scale bars = 50 μm. (C) The RXRα labeling index in the non-tumorous tissues was significantly lower in the low-dose and high-dose (E-HD) groups than in the control group. $^{**}P < 0.01$. Bars indicate ±SE of mean. (D) reverse transcription–polymerase chain reaction analysis of RXRα mRNA. RXRα expression from two mice is shown in each group (control, E-LD and E-HD). β-Actin was used as an internal control. Expression of RXRα mRNA was not decreased by the administration of etodolac.

we found significant decreases in RXRα protein expression in non-tumorous liver tissue in both the E-LD and E-HD groups, whereas RXRα mRNA expression was almost similar among the control, E-LD and E-HD groups. These results suggest that the degradation of RXRα induced by R-etodolac is also responsible for the preventive effect of hepatocarcinogenesis in FLS mice.

Hepatitis C virus stimulates the expression of COX-2 via oxidative stress.[35] HCV core protein induces fatty liver by binding to the DNA-binding domain of RXRα.[20] High levels of COX-2 and RXRα expression in hepatocytes may be involved in hepatocarcinogenesis following HBV and HCV infection.[18–20,36,37] Vitamin A has been reported to inhibit hepatocarcinogenesis by dephosphorylating RXRα.[38] Our results showed that both PGE_2 and RXRα levels were decreased by etodolac, indicated that etodolac may be useful for the prevention of HCC caused by HBV and HCV.

We have reported that COX-2 inhibitors (etodolac and NS-398) suppress PCNA expression and induce cell cycle arrest in HCC cell lines.[14,34] In the present study, we found that PCNA expression in non-tumorous fatty liver was significantly lower in both the E-LD and E-HD groups compared with the control group. The PCNA labeling index showed no difference between the E-LD and E-HD groups. Similar results have been reported with NS-398 in F344 rats.[32,33] These results suggest that low-dose administration of etodolac is sufficient to suppress cell cycle progression in FLS mice.

The present results suggest that low-dose administration of etodolac has a strong chemopreventive effect against hepatocarcinigenesis by inhibiting COX-2 activity, and RXRα and PCNA expression in mice. The prevention of hepatocarcinogenesis *in vivo* by COX-2 inhibitor may be caused by the primary suppression of malignant transformation from hepatocytes or inhibition of the growth of HCC cells in early stages, which have already developed in the liver but can not be detected as tumors. Etodolac may also prove to be of value in the prevention of HCC in humans.

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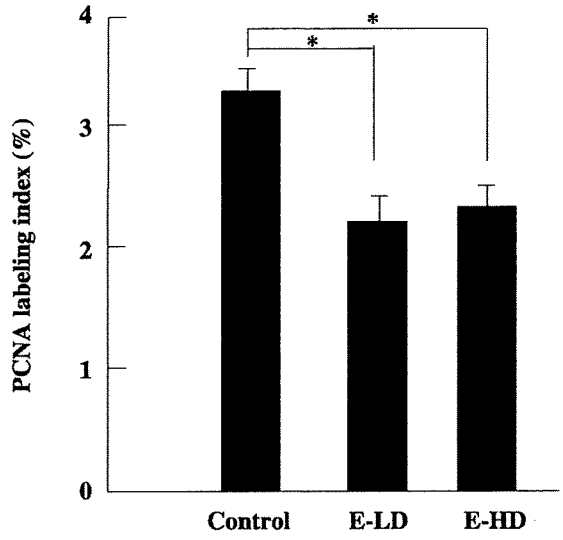

Fig. 4. Proliferating cell nuclear antigen (PCNA) labeling index in non-tumorous liver tissues. The PCNA labeling index was significantly suppressed by low-dose (E-LD) and high-dose (E-HD) administration of etodolac. *$P < 0.05$. Bars indicate ±SE of mean.

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Interferon-β plus ribavirin for patients with hepatitis C virus genotype 1: a randomised pilot trial

The rate of sustained eradication of hepatitis C virus (HCV) in response to a combination of interferon-α and ribavirin remains unsatisfactory in patients with genotype 1 infection.[1] No effective alternative treatment is currently available for non-responders. Interferon-β is also a type I interferon commonly used to treat chronic HCV infection in Japan. A previous study showed that a 24 week course of therapy with interferon-β plus ribavirin resulted in sustained loss of HCV in three of nine patients with chronic hepatitis C.[2] However, the efficacy and safety of interferon-β combined with ribavirin has yet to be fully evaluated.

We report the results of a randomised pilot trial comparing interferon-β plus ribavirin with interferon-α plus ribavirin in patients with HCV genotype 1 who poorly responded to interferon-α plus ribavirin. A total of 28 patients with HCV genotype 1 were given 6 MU of recombinant interferon-α2b (Schering-Plough, Kenilworth, New Jersey, USA) by intramuscular injection daily for four weeks. Twenty seven patients (16 men and 11 women; mean age 47 (±8) years) in whom HCV RNA was detected in serum on polymerase chain reaction at week 2 were included in this study and randomly assigned to receive one of two regimens from week 5. Fifteen patients continued to receive 6 MU interferon-α2b intramuscularly, given daily from week 5 to week 8, and three times weekly from week 9 to week 24 (interferon-α group). The other 12 patients were assigned to 6 MU natural interferon-β (Toray Industries Inc., Tokyo, Japan), given by intravenous injection daily from week 5 to week 8, and three times weekly from week 9 to week 24 (interferon-β group). Ribavirin (Schering-Plough) was concurrently administered at a daily dose of 600 mg to patients who weighed 60 kg or less and 800 mg to those who weighed more than 60 kg. At the time of this study, a 24 week course of interferon-α plus ribavirin was commonly used in Japan. The data were analysed according to intention to treat.

Baseline characteristics of the patients in the treatment groups were similar. At week 4 of therapy, when treatment was randomly assigned, the proportion of patients without detectable HCV RNA in serum did not differ between the interferon-α group and interferon-β groups (table 1). The proportion of patients without HCV RNA in serum was higher in the interferon-β group than in the interferon-α group at week 12, but did not differ between the groups at the end of treatment (week 24). However, 24 weeks later (week 48), the proportion of patients with a sustained virological response was significantly higher in the interferon-β group than in the interferon-α group. During treatment, neutralising antibodies to interferon were detected in two patients in the interferon-α group and in no patients in the interferon-β group. Leucocyte, neutrophil, and platelet counts and haemoglobin concentrations were similar in two groups. Therapy was discontinued because of serious adverse events (including depression) in three patients in the interferon-α group; all 12 patients in the interferon-β group completed 24 weeks of treatment. The dose of ribavirin was reduced because of anaemia in eight patients in the interferon-α group and in four in the interferon-β group.

We enrolled patients who did not have a favourable early response to treatment with interferon-α and ribavirin. Antibodies to interferon, which sometimes develop in

Table 1 Proportions of patients without detectable hepatitis C virus RNA in serum

	Interferon-α group (n = 15)	Interferon-β group (n = 12)	p Value (χ^2 test)
Week 4	4 (27%)	3 (25%)	0.92
Week 12	7 (47%)	10 (83%)	0.049
Week 24 (end of therapy)	10 (67%)	9 (75%)	0.64
Week 48	0 (0%)	3 (25%)	0.040

patients given recombinant interferon-α, can cause resistance to therapy. Both interferon-α and -β bind to a common type I interferon receptor but utilise different regions of the receptor subunits for specific signalling pathways,[3] potentially leading to distinct biological responses. An oligonucleotide array study has shown that some interferon stimulated genes are preferentially induced by interferon-β, but not by interferon-α.[4] We thus believe that interferon-β might be beneficial for some patients who are resistant to interferon-α. A large randomised trial of peginterferon-α plus ribavirin versus interferon-β plus ribavirin for 48 weeks is being conducted in patients with HCV genotype 1 who do not have a virological response[5] to 12 weeks of treatment with peginterferon-α and ribavirin.

In summary, a combination of interferon-β and ribavirin produced a significantly better sustained virological response than a combination of interferon-α and ribavirin in patients with HCV genotype 1 who were resistant to interferon-α plus ribavirin. Although the overall safety profiles of the two regimens were similar, the rates of treatment discontinuation and of reduction in the dose of ribavirin were lower in patients receiving interferon-β and ribavirin than in those receiving interferon-α and ribavirin.

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EDITOR'S QUIZ: GI SNAPSHOT

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Answer

From question on page 122

At explorative laparotomy, the pancreatic tumour involving the head and proximal body of the pancreas was judged to be resectable. Pylorus preserving proximal pancreaticoduodenectomy was performed. Histology of the tumour was consistent with a diagnosis of renal cell cancer (RCC) metastasis to the pancreas (fig 2). Metastases were not detected in peripancreatic lymph nodes. The patient did not receive any further adjuvant therapy and was discharged from hospital without any serious perioperative morbidity.

The vast majority of pancreatic carcinomas are primary, and among these, more than 90% are of ductal origin. Solitary pancreatic masses can be classified as secondary tumours to the pancreas in only 2% of all cases.[1] In the latter group, RCC seems to be the most common cancer. Within the last three years, 43 new cases of RCC metastases to the pancreas have been reported (Medline review). Median interval from nephrectomy to diagnosis of pancreatic metastases is 83 months, but time intervals as long as 10–20 years were also reported.[2] Complete resection of pancreatic metastases from RCC are associated with long term survival, particularly in cases of single tumours and/or a long disease free interval.[3]

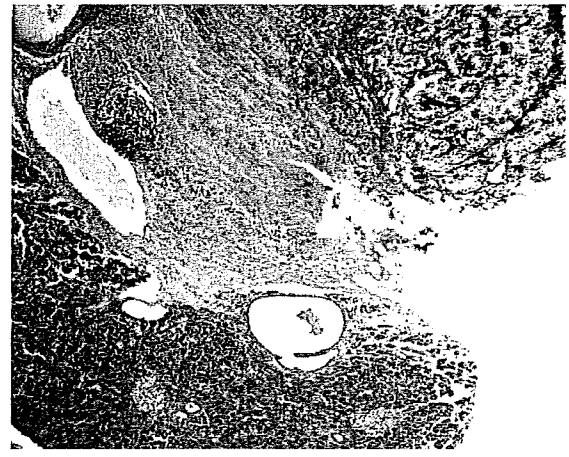

Figure 2 Histomorphological appearance of the pancreatic tumour (haematoxylin-eosin, ×40). From the lower left to the upper right corner, normal pancreatic glandular tissue, desmoplastic capsule, and clear cell carcinoma are visible.

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Does alcohol increase the risk of hepatocellular carcinoma among patients with hepatitis C virus infection?

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Abstract

We conducted a hospital-based case–control study to investigate the effects of alcohol drinking on hepatocellular carcinoma (HCC) among patients with hepatitis C virus (HCV) infection, with special reference to the disease course and changes in drinking habits. From among 1159 HCV-RNA positive patients under clinical follow-up at Osaka City University Hospital (OCUH), we identified 73 cases newly diagnosed with HCC during the past 3 years and selected 253 matched controls without HCC. The odds ratios were calculated for cumulative and average daily ethanol consumption, during three different periods (lifetime, before, and after the first identification of liver disease), using a logistic regression model. Among all subjects, there was a trend towards an inverse association between HCC and lifetime ethanol consumption ($P = 0.059$–0.066). The tendency was similar for ethanol consumption before the first identification of liver disease, while no associative trend was indicated after the first identification. Among those with minor changes on abdominal ultrasonography findings at the first OCUH visit, a positive association was suggested for ethanol intake after the first identification, although results were not statistically significant. In conclusion, our results did not demonstrate a strong positive association between alcohol drinking and HCV-related HCC in this population.

Keywords: Alcohol drinking; Hepatitis C; Hepatocellular carcinoma; Case–control study; Epidemiology; Disease course

1. Introduction

Chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) are well-studied causal factors for the development of hepatocellular carcinoma (HCC) [1]. It was recently estimated that 170 million people worldwide are infected with HCV [2]. Although this estimate is nearly half that for HBV infection [3], it is anticipated that HCV will play a major role in the future spread of HCC, because no effective vaccine is presently available. In France, it has been reported that the rise in mortality from liver cancer could be explained by HCV infection [4]. The report from the US has also shown that the HCV-related HCC has been significantly increased during recent years [5]. Similarly in Japan, where HCV infection is a major health problem [6], approximately 70% of patients with HCC were shown to be positive for anti-HCV antibody [7].

Alcohol drinking is also considered an independent risk factor for HCC [8]. However, the effect of alcohol intake on HCV-related HCC has not been sufficiently elucidated by analytical epidemiological studies. A few cohort studies confirmed a positive relation between the presence or absence

Abbreviations: CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OCUH, Osaka City University Hospital; OR, odds ratio

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of a previous heavy drinking habit and HCC [9–11]. Some case–control studies have examined the interaction between alcohol drinking and HCV [12–15] and the effect of alcohol among a subgroup with anti-HCV [14]. However, no case–control studies have been previously reported in which both cases and controls had HCV infection.

To further clarify the role of alcohol consumption on HCV-related HCC, we conducted a hospital-based case–control study in Osaka, Japan, where a high incidence of HCC has been reported [16]. Our study has two notable features: both the cases and controls were HCV-RNA positive, and changes in drinking habits during the disease course were assessed.

2. Materials and methods

2.1. Cases and controls

We reviewed the medical records of patients who visited the Department of Hepatology at Osaka City University Hospital (OCUH) for clinical follow-up from 1 November 2001 to 31 January 2002 (i.e., a 3-month recruitment period) to identify HCV-RNA positive patients. Patients of this department with HCV infection are requested to receive medical examinations at least every 3 months. Thus, we intended to list all these patients under the follow-up. Patients were excluded if they had additional types of liver diseases (e.g., HBs-Ag positive, autoimmune hepatitis, primary biliary cirrhosis, etc.) or were diagnosed with HCC at another hospital before referral for treatment or had a non-Japanese nationality. Consequently, we found a total of 1159 patients (male: 45%) aged 17–85 years (mean: 60 years) who were regarded as the study base for cases and controls. The conditions of the patients included healthy carrier, chronic hepatitis, cirrhosis of the liver, and HCC.

Cases were defined as having HCC newly diagnosed at OCUH within 3 years before the recruitment period. The diagnosis of HCC was confirmed by either histology or radiological findings based on at least one imaging study (angiographic findings, computed tomography or magnetic resonance imaging). A total of 86 eligible cases were identified. Controls consisted of patients without HCC at recruitment and were individually matched with corresponding cases by sex, age (±2 years), and the date of first visit to OCUH (±2 years). The maximum case-to-control ratio was 1:5. A total of 333 eligible controls were identified.

The study protocol was approved by the ethics committee at the Osaka City University Graduate School of Medicine.

2.2. Information collection

Information was collected using three methods: a self-administered questionnaire for lifetime history, etc.; a personal interview for alcohol drinking history; and review of medical records for clinical data.

From 1 June, 2002 (i.e., the beginning of the study), eligible subjects who visited OCUH and consented verbally to participate in the present study were provided a self-administered, mail-back questionnaire. The questionnaire included the following questions: demographic and anthropometric characteristics; smoking habits; work experience and physical exercise; food intake frequency; past medical history (including blood transfusions or surgery); family history of liver diseases; reproductive history; oral contraceptive use. Subjects were also asked to be interviewed at the next visit concerning their drinking habits. A reminder letter was sent to the subject if the questionnaire was not returned within a month.

A well-trained interviewer who was blinded to case/control status conducted a personal interview regarding alcohol drinking. The interview was performed according to a structured questionnaire, which was modified version from a previously used one [17]. The questions included: (1) age when the subject first drank alcohol; (2) usual drinking frequency at that time; (3) usual daily drinking amount for each type of beverage (e.g., beer, Japanese *sake* or *shochu*, wine, and hard liquor, etc.) at that time; (4) age when the subject changed drinking habits thereafter; (5) return to the second question. Thus, we collected information on each participant's entire drinking history throughout his/her life. Subjects were also interviewed using a simple flushing questionnaire to determine aldehyde dehydrogenase-2 (ALDH 2) activity [18].

Laboratory data and findings of abdominal ultrasonography at or immediately after the first OCUH visit were collected from the medical records. At OCUH, abdominal ultrasonography findings had been conventionally scored by means of a semi-quantitative ultrasound scoring system [19], which has been shown to correlate highly with the degree of liver fibrosis as determined by the new European classification among patients with HCV infection [20]. The history of interferon treatment was also obtained.

2.3. Statistical analysis

We used three variables to characterize the disease time course: (1) the possible date of HCV exposure; (2) the date when liver disease was first identified; (3) the date of the first OCUH visit. For the date of HCV exposure, we used the date of transfusion or surgery. The midpoint between the first and the last transfusion or surgery was used if the subject underwent two or more procedures. The time when liver disease was first identified was defined as the age when liver dysfunction or HCV infection, not HCC, was first identified, whichever occurred first.

With respect to alcohol consumption, we defined drinkers as those who consumed alcoholic beverages once or more per week for at least 1 year throughout their lifetime. The drinking amount was converted to grams of ethanol according to the following formula: grams of ethanol = ingested volume × percentage of ethanol in the drink × specific

gravity/100. Two indices of ethanol consumption, cumulative and average daily amount, were estimated. First, we calculated cumulative ethanol consumption separately for three different periods: throughout the lifetime, before, and after the first identification of liver disease. Second, each amount was divided by each of those time intervals to estimate average daily ethanol consumption. For cases, the drinking amount until HCC diagnosis was calculated, while for controls, the drinking amount until the beginning of the study was used.

Significant differences between cases and controls were assessed by the Wilcoxon rank sum test or the Chi square test. The Mantel-extension method for the trend test was also used when appropriate. Changes in ethanol consumption were assessed by the Wilcoxon signed-rank test. For the analysis, ethanol consumption was categorized into three levels: non-drinker, and two consumption levels that divided the control group into two approximate sized groups.

For the analysis of all subjects, the adjusted odds ratios (ORs) for HCC and 95% confidence intervals (95% CIs) were estimated using the conditional logistic regression model. Subgroup analyses were also conducted to evaluate the effects of alcohol in the less-advanced or advanced stages of liver disease as determined by abdominal ultrasonography findings at the first OCUH visit (minor changes versus chronic hepatitis or liver cirrhosis). These analyses were performed using an unconditional logistic model, which included three additional matching variables. Variables with P values less than 0.1 in the univariate analysis were included in the multivariate model as potential confounders. When evaluating the effect of ethanol consumption before the first identification of liver disease, ORs were further adjusted for consumption after the first identification and vice versa. The test for trend was performed by including an exposure variable in the model that was assigned an ordinal number corresponding to the drinking level (i.e., a higher number indicated a higher drinking level). All reported P values were two-tailed. SAS Version 8.2 (SAS Institute Inc., Cary, NC) was used for all analyses.

3. Results

Among 419 eligible subjects (86 cases and 333 controls), 17 subjects (4 cases and 13 controls) were excluded because negative findings on HCV-RNA or positive findings on HBs-Ag became known. Sixty-four patients were lost after beginning of the study for the following reasons: 4 subjects (3 cases and 1 control) were deceased, 19 subjects (4 cases and 15 controls) were excluded because of physician or patient non-cooperation, and 41 subjects (2 cases and 39 controls) never re-visited OCUH after the beginning of the study. Thus, the remaining 338 subjects (73 cases and 265 controls) completed the questionnaire. Information collection rate among 402 subjects was 89% for cases and 83% for controls ($P = 0.17$). Twelve controls were further excluded because the corresponding cases were lost. Finally, 326 subjects (73 cases and 253 controls, 73 matched-sets) were included in the analysis.

Table 1
Demographic characteristics and disease chronology of cases and controls

Variables	Cases ($n = 73$)	Controls ($n = 253$)	P value[c]
Sex[a]: male (%)	34 (47)	131 (52)	0.433
Age: median (interquantile range)			
At viral exposure[b]	29 (17)	31 (16)	0.171
At first identification of liver disease	50 (20)	55 (14)	0.128
At first OCUH visit[a]	62 (11)	62 (9)	0.846
At beginning of the study[a]	69 (8)	69 (7)	0.364
At development of HCC	67 (9)	–	
Years: median (interquantile range)			
Viral exposure—first identification	21 (16)	20 (22)	0.971
Viral exposure—first OCUH visit	32 (13)	31 (16)	0.298
Viral exposure—beginning of the study	40 (13)	38 (16)	0.155
Viral exposure—development of HCC	38 (13)	–	
Years: median (interquantile range)			
First identification—first OCUH visit	8 (16)	5 (13)	0.093
First identification—beginning of the study	16 (14)	12 (12)	0.011
First identification—development of HCC	15 (14)	–	
Years: median (interquantile range)			
First OCUH visit—beginning of the study	8 (5)	7 (5)	0.328
First OCUH visit—development of HCC	5 (4)	–	

Abbreviations: OCUH, Osaka City University Hospital; HCC, hepatocellular carcinoma.

[a] Matching condition.

[b] Estimated by the date of transfusion or surgery before the first identification of liver disease.

[c] Wilcoxon rank sum test, except for sex with Chi square test.

Table 2
Selected characteristics of cases and controls

Variables	Cases *n* (%)	Controls *n* (%)	*P* value[c]
Relevant viral exposure[a]			
Transfusion	23 (50)	87 (52)	
Surgery	23 (50)	79 (48)	0.772
Drinking status			
Non-drinker	38 (52)	108 (43)	
Former drinker	19 (26)	58 (23)	
Current drinker	16 (22)	87 (34)	0.058
Flusher/non-flusher status (flusher)	41 (56)	143 (57)	0.990
Smoking status			
Non-smoker	36 (49)	125 (50)	
Former smoker	19 (26)	64 (25)	
Current smoker	18 (25)	64 (25)	0.960
Platelet counts ($<10 \times 10^4$ mm^{-3})	29 (40)	28 (11)	<0.0001
Aspartate aminotransferase (AST, ≧80 IU/l)	40 (55)	96 (38)	0.010
Alanine aminotransferase (ALT, ≧80 IU/l)	48 (66)	143 (57)	0.158
Gamma-glutamyltransferase (≧60 IU/l)	36 (49)	107 (42)	0.287
Albumin (<3.5 g/dl)	10 (14)	9 (4)	0.001
Fasting blood sugar (≧126 mg/dl)	13 (18)	18 (7)	0.004
Findings of abdominal ultrasonography[b]			
Minor changes	13 (18)	111 (44)	
Chronic hepatitis	33 (47)	114 (45)	
Liver cirrhosis	25 (35)	27 (11)	<0.0001
History of interferon therapy (yes)	18 (25)	91 (36)	0.071

[a] No information available for 27 cases and 87 controls.
[b] No information available for two cases and one control.
[c] Chi square test between two categories, and Mantel-extension method for the trend test among three categories.

3.1. Characteristics of study subjects

Table 1 shows the demographic characteristics and disease chronology of cases and controls. Age at the first OCUH visit and age at the beginning of the study were well-matched. The median duration from viral exposure to the first identification of liver disease and the first OCUH visit were about 20 and 30 years, respectively, for cases and controls. The time interval from first identification of liver disease and the first OCUH visit was longer for cases than controls ($P = 0.093$) and from the first identification and the beginning of the study ($P = 0.011$).

Table 2 shows selected characteristics of cases and controls. The first exposure to hepatitis C was estimated for approximately 60% of cases and controls. There were more drinkers among controls than cases ($P = 0.058$). The distribution of flushers and non-flushers or smokers and non-smokers was similar between the groups. Significantly more cases than controls had a lowered platelet counts ($P < 0.0001$) and albumin levels ($P = 0.001$), elevated fasting blood sugar levels ($P = 0.004$), and severe liver disease by abdominal ultrasonography findings ($P < 0.0001$). A higher proportion of cases also showed elevated aspartate aminotransferase (AST) levels ($P = 0.010$), but not alanine aminotransferase (ALT) levels and gamma-glutamyltransferase (GGT) levels ($P = 0.158$ and 0.287, respectively). Controls showed a tendency to receive interferon treatment more frequently than cases ($P = 0.071$).

3.2. Ethanol consumption during the disease course

Table 3 shows ethanol consumption during three different periods: lifetime, before, and after the first identification of liver disease. For all subjects, during each of the three periods, ethanol consumption was not significantly different between cases and controls. However, among both cases and controls, ethanol consumption significantly decreased after the first identification of liver disease ($P < 0.01$). We further stratified subjects based on ultrasonography findings at the first OCUH visit. In the subgroup that showed minor changes in abdominal ultrasonography findings, ethanol consumption was significantly decreased after the first identification among controls ($P < 0.01$) but not among cases. In the subgroup with chronic hepatitis or liver cirrhosis, reduced ethanol consumption was observed in both cases and controls ($P < 0.01$).

3.3. Effect of ethanol intake for HCC development

Table 4 shows the ORs for ethanol consumption among all subjects for the three time periods. In terms of lifetime consumption, increased ethanol intake was associated with a trend towards lower adjusted ORs in both cumulative and average daily ethanol consumption ($P = 0.066$–0.059). The tendency was similar for ethanol consumption before the first identification of liver disease. On the other hand, the ORs after the first identification showed no associative trend.

The results of subgroup analysis, stratified by ultrasonography findings at the first OCUH visit, are shown in Table 5. In the subgroup of cases and controls with chronic hepatitis or liver cirrhosis, the findings were similar to those of the analyses of all subjects: a negative association throughout the lifetime and before the first identification of liver disease, and no association after the first identification.

On the other hand, among cases and controls with minor changes, the adjusted ORs suggested a positive association throughout the lifetime and before the first identification, although no stepwise increase in ORs was shown. When we confined the analysis to ethanol consumption after the first identification of liver disease, a positive association became more plausible. The adjusted OR was 1.73 at <53 kg and 2.97 at ≧53 kg of cumulative ethanol consumption, and 1.14 at <13 g and 5.74 at ≧13 g of average daily ethanol consumption compared with non-drinkers, although the findings were not statistically significant.

Table 3
Ethanol consumption of cases and controls

Variables	All subjects ($n = 326$)		Findings of abdominal ultrasonoggraphy at the first OCUH visit[a]			
	Cases	Controls	Minor changes ($n = 124$)		Chronic hepatitis or liver cirrhosis ($n = 199$)	
			Cases	Controls	Cases	Controls
Cumulative ethanol consumption (kg): mean (S.D.)[b]						
Lifetime	226 (430)	333 (700)	220 (298)	238 (507)	235 (462)	411 (816)
Before the first identification of liver disease	160 (328)	233 (589)	160 (224)	136 (328)	166 (353)	311 (726)
After the first identification of liver disease	66 (173)[c]	100 (282)[c]	59 (141)	102 (315)[c]	70 (183)[c]	100 (255)[c]
Average daily ethanol consumption (g): mean (S.D.)[b]						
Lifetime	15 (28)	20 (39)	17 (24)	15 (31)	15 (29)	24 (45)
Before the first identification of liver disease	18 (33)	23 (45)	20 (25)	15 (31)	18 (35)	28 (52)
After the first identification of liver disease	10 (25)[c]	13 (31)[c]	13 (25)	12 (31)[c]	10 (25)[c]	14 (31)[c]

[a] Two cases and one control were not included because of missing value on findings of abdominal ultrasonography.
[b] The amount of ethanol consumption is expressed as mean and S.D. in spite of skewed distribution, because the median values were zero in nearly half of the cells due to a large number of non-drinker.
[c] Decreased in comparison to before the first identification of liver disease, by using Wilcoxon signed-rank test; $P < 0.01$.

Table 4
Odds ratios and their 95% confidence intervals for hepatocellular carcinoma among all subjects according to ethanol consumption

Variables	Cases n (%)	Controls n (%)	Crude OR (95% CI)	Adjusted[a] OR (95% CI)
Lifetime				
Cumulative ethanol consumption (kg)				
Non-drinker	38 (52)	108 (43)	1	1
<260	16 (22)	72 (28)	0.72 (0.35–1.49)	0.48 (0.18–1.31)
≧260	19 (26)	73 (29)	0.81 (0.37–1.80)	0.37 (0.13–1.07)
			(Trend: $P = 0.553$)	(Trend: $P = 0.066$)
Average daily ethanol consumption (g)				
Non-drinker	38 (52)	108 (43)	1	1
<20	17 (23)	73 (29)	0.75 (0.37–1.54)	0.49 (0.18–1.35)
≧20	18 (25)	72 (28)	0.77 (0.35–1.68)	0.36 (0.13–1.03)
			(Trend: $P = 0.478$)	(Trend: $P = 0.059$)
Before the first identification[b]				
Cumulative ethanol consumption (kg)				
Non-drinker	40 (55)	118 (47)	1	1
<200	14 (19)	67 (26)	0.66 (0.31–1.41)	0.48 (0.16–1.41)
≧200	19 (26)	68 (27)	0.99 (0.45–2.16)	0.55 (0.18–1.66)
			(Trend: $P = 0.887$)	(Trend: $P = 0.302$)
Average daily ethanol consumption (g)				
Non-drinker	40 (55)	118 (47)	1	1
<24	14 (19)	69 (27)	0.66 (0.31–1.41)	0.44 (0.15–1.26)
≧24	19 (26)	66 (26)	0.97 (0.45–2.10)	0.54 (0.17–1.68)
			(Trend: $P = 0.890$)	(Trend: $P = 0.290$)
After the first identification[b]				
Cumulative ethanol consumption (kg)				
Non-drinker	45 (62)	144 (57)	1	1
<53	13 (18)	54 (21)	0.90 (0.44–1.84)	1.22 (0.48–3.10)
≧53	15 (20)	55 (22)	0.99 (0.45–2.17)	1.09 (0.35–3.36)
			(Trend: $P = 0.928$)	(Trend: $P = 0.802$)
Average daily ethanol consumption (g)				
Non-drinker	45 (62)	144 (57)	1	1
<13	13 (18)	56 (22)	0.84 (0.41–1.72)	1.04 (0.40–2.67)
≧13	15 (20)	53 (21)	1.09 (0.50–2.35)	1.35 (0.47–3.87)
			(Trend: $P = 0.930$)	(Trend: $P = 0.611$)

[a] Adjusted for years since the first identification of liver disease, interferon treatment, abdomonal ultrasonography, platelet, AST, albumin, and fasting blood sugar, by using conditional logistic regression model.
[b] As for ethanol consumption before the first identification of liver disease, ORs were further adjusted for that after the first identification of liver disease, and vice versa.

Table 5
Adjusted odds ratios and their 95% confidence intervals for hepatocellular carcinoma according to ethanol consumption, stratified by findings of abdominal ultrasonography at first OCUH visit

Variables	Findings of abdonimal ultrasonography at the first OCUH visit[a]					
	Minor change (n = 124)			Chronic hepatitis or liver cirrhosis (n = 199)		
	Cases n (%)	Controls n (%)	OR[b] (95% CI)	Cases n (%)	Controls n (%)	OR[b] (95%CI)
Lifetime						
Cumulative ethanol consumption (kg)						
Non-drinker	7 (54)	50 (45)	1	29 (50)	57 (41)	1
<260	1 (8)	39 (35)	0.22 (0.01–4.78)	15 (26)	33 (23)	0.97 (0.38–2.49)
≧260	5 (38)	22 (20)	5.07 (0.40–64.3)	14 (24)	51 (36)	0.46 (0.16–1.30)
			(Trend: P = 0.174)			(Trend: P = 0.154)
Average daily ethanol consumption (g)						
Non-drinker	7 (54)	50 (45)	1	29 (50)	57 (40)	1
<20	1 (8)	37 (33)	0.16 (0.01–3.61)	16 (28)	36 (26)	0.92 (0.36–2.37)
≧20	5 (38)	24 (22)	2.71 (0.36–20.4)	13 (22)	48 (34)	0.48 (0.17–1.36)
			(Trend: P = 0.232)			(Trend: P = 0.177)
Before the first identification[c]						
Cumulative ethanol consumption (kg)						
Non-drinker	7 (54)	56 (51)	1	31 (53)	61 (43)	1
<200	2 (15)	36 (32)	0.42 (0.03–6.91)	12 (21)	31 (22)	0.86 (0.32–2.31)
≧200	4 (31)	19 (17)	10.6 (0.64–175)	15 (26)	49 (35)	0.62 (0.20–1.93)
			(Trend: P = 0.129)			(Trend: P = 0.420)
Average daily ethanol consumption (g)						
Non-drinker	7 (54)	56 (50)	1	31 (54)	61 (43)	1
<24	1 (8)	33 (30)	0.17 (0.01–4.18)	13 (22)	36 (26)	0.78 (0.30–2.05)
≧24	5 (38)	22 (20)	4.70 (0.55–40.0)	14 (24)	44 (31)	0.64 (0.20–2.06)
			(Trend: P = 0.194)			(Trend: P = 0.439)
After the first identification[c]						
Cumulative ethanol consumption (kg)						
Non-drinker	7 (54)	64 (58)	1	36 (62)	79 (56)	1
<53	3 (23)	28 (25)	1.73 (0.23–13.3)	10 (17)	26 (18)	1.38 (0.52–3.71)
≧53	3 (23)	19 (17)	2.97 (0.20–43.8)	12 (21)	36 (26)	1.15 (0.37–3.53)
			(Trend: P = 0.408)			(Trend: P = 0.700)
Average daily ethanol consumption (g)						
Non-drinker	7 (54)	64 (58)	1	36 (62)	79 (56)	1
<13	3 (23)	30 (27)	1.14 (0.13–10.0)	10 (17)	26 (18)	1.32 (0.50–3.49)
≧13	3 (23)	17 (15)	5.74 (0.36–90.9)	12 (21)	36 (26)	1.28 (0.40–4.11)
			(Trend: P = 0.282)			(Trend: P = 0.600)

[a] Three subjects were not included because of missing value on findings of abdominal ultrasonography.
[b] Adjusted for age, sex, years since the first OCUH visit, years since the first identification of liver disease, interferon treatment, platelet, AST, albumin, and fasting blood sugar, by using unconditional logistic regression model.
[c] As for ethanol consumption before the first identification of liver disease, ORs were further adjusted for that after the first identification of liver disease, and vice versa.

4. Discussion

This is the first case–control study that both cases and controls had HCV infection, which enabled us to make a straightforward interpretation regarding HCV-related HCC. It is important to explore risk factors for HCV-related HCC in the growing concern about HCV infection. We also aimed to minimize the potential effect of recall bias on drinking habits to some extent, since all subjects received similar recall stimuli. In addition, we could collect entire information on drinking history throughout lifetime.

To the date, many studies reported a positive association between alcohol and HCC ([12–14,21–27]). However, they were population-based cohort studies or case–control studies with controls those who were free from liver disease. Although some of these studies examined the synergism between alcohol drinking and HCV or the effect of alcohol among a subgroup with anti-HCV, their main objectives were to reveal independent effect of alcohol for HCC, not for HCV-related HCC.

In contrast, a few prospective cohort studies confined their participants to patients with HCV infection to clarify the risk factors for HCV-related HCC [9–11]. They mainly collected the information regarding a previous heavy drinking habit and reported a significant positive relation. It has also been suggested that heavy drinking enhances HCV-related

hepatocarcinogenesis [28,29]. A recent review has conclusively stated that alcohol use in chronic hepatitis C doubles the risk for HCC as compared with the risk in hepatitis C alone [8]. The present study, which has quite different design from previous ones, also aimed to confirm those findings with entire information on drinking history throughout lifetime. Unexpectedly, we did not find the similar results. Although it is difficult to clearly explain the lack of an association between HCC and alcohol, there are several ways to interpret our findings.

First, the impact of alcohol intake is truly trivial in this population. Similarly, some studies have suggested no association between alcohol drinking and HCC [30–34]. A few animal or clinical experiments also have provided biologically compatible findings [35–38].

Second, selection bias might have generated an inverse association. Studies among patients who are anti-HCV positive have reported that alcohol drinking significantly increases the risk of liver fibrosis progression [39–41] and that the cumulative survival rate was significantly lower among habitual drinkers than among non-habitual drinkers [42]. It is therefore likely that drinkers generally have a high risk for death from liver failure or severe complication of liver cirrhosis. Since our study base included patients who survived, alcohol consumption might have been underestimated. This underestimation seemed more likely to have occurred among cases than among controls due to the higher prevalence of liver cirrhosis at the first OCUH visit (Table 2). In fact, a previous study reported that heavy drinkers with liver cirrhosis do not usually survive long enough to develop HCC [43].

Third, it is also of concern whether subjects spontaneously reduced their alcohol intake as their general condition gradually worsened. Since this would have been more likely to occur among cases, a spurious protective effect of alcohol drinking might have been introduced. However, as indicated in Table 4, the inverse association was more marked for lifetime alcohol intake and intake before the first identification of liver disease, rather than after the first identification. Given that cases were more apt to change their drinking behavior than controls, the negative relationship should be more pronounced for alcohol intake after the first identification of liver disease.

To examine the preventive factor for HCC which might have masked the potential risk of drinking, we calculated crude ORs of each variable presented in Tables 1 and 2 among those with ≧260 kg of lifetime cumulative ethanol consumption. As a result, elevated platelet counts ($\geqq 10 \times 10^4$ mm^{-1}), decreased AST levels (<80 IU/l) and previous history of interferon treatment were shown to be preventive: crude OR (95% CI) were 0.17 (0.05–0.55), 0.32 (0.11–0.94), and 0.27 (0.07–1.00), respectively. All these variables were included as potential confounders in the multivariate model in Table 4. However, other unknown factors with stronger preventive effect for HCC might have offset the possible risk of heavy drinking. Since we failed to collect information concerning such factors, their effect could not be taken into consideration in the analysis. Thus, it is undeniable that no association between alcohol and HCC in the present study was spurious because of insufficient information collection.

Table 5 presents the results for the two subgroups. Among the group that showed minor changes in abdominal ultrasonography finidngs, the ORs suggested a positive association. On the other hand, among the subjects with chronic hepatitis or liver cirrhosis, the ORs were similar to those in the analysis of all subjects. It has been shown that the stage of fibrosis among patients with chronic HCV infection is a strong risk factor for HCC [44]: relative risk is 4.4 even at stage F2 (at least corresponding to chronic hepatitis on abdominal ultrasonography at OCUH [20]), 13.0 at stage F3 and 24.0 at stage F4. These risk estimates are greater than those for alcohol drinking which are generally reported to be as high as 4 in Asian populations [22–25]. Therefore, the effect of alcohol drinking among the subgroup with advanced liver disease seems to be masked by the overwhelming risk of fibrosis.

The finding in the subgroup comparison in Table 5 is a direct reflection of that in Table 3. In the strata of subjects with minor ultrasonography changes in Table 3, ethanol intake decreased after the first identification of liver disease among controls but not among cases. On the other hand, in the strata of subjects with chronic hepatitis or liver cirrhosis, both cases and controls reduced ethanol intake after the first identification. Thus, the associations with alcohol intake after the first identification were obscure.

We did not include the term for GGT levels or smoking amount in the multivariate model to calculate adjusted ORs. A positive association was significantly indicated between alcohol consumption and GGT levels or smoking amount, regardless of case/control status. Among cases, the median of GGT levels (IU/l) was 42.0 for non-drinkers, 53.5 for those with <260 kg and 100.0 for those with ≧260 kg of lifetime cumulative ethanol consumption ($P = 0.0005$). Among controls, the median was 36.5 for non-drinkers, 53.0 for those with <260 kg and 73.0 for those with ≧260 kg of lifetime cumulative ethanol consumption ($P < 0.0001$). A similar association was indicated for cumulative smoking amount (corresponding P value was <0.0001 and <0.0001, respectively). However, neither elevated GGT levels nor increased smoking amount was associated with HCC occurrence, regardless of drinking status. Adjusted ORs of those with ≧60 IU/l of GGT levels was 1.39 ($P = 0.660$) among non-drinkers and 0.73 ($P = 0.621$) among drinkers, compared as those with <60 IU/l. Similarly, adjusted ORs of those with <800 or ≧800 of cumulative smoking amount was 2.90 ($P = 0.272$) or 0.54 ($P = 0.681$) among non-drinkers and 0.45 ($P = 0.360$) or 0.49 ($P = 0.418$) among drinkers, compared as non-smokers. Thus, we did not consider both GGT levels and smoking as potential confounders in this study.

One major limitation of the study was the use of proxy variables concerning disease course: the time point of HCV exposure and the first identification of liver disease. However, it is not always possible to define the exact time point, such as

infection and subclinical or clinical onset, for any infectious disease. Moreover, HCV testing became widely available in the 1990s. Thus, it is not possible to estimate the time point of the disease course without using surrogate variables.

Another limitation is that prevalent HCC cases have been included, since the cases were those HCC patients who received the diagnosis within the last 3 years. In fact, the mean ethanol consumption in this study differed between those cases diagnosed within 1 year and those diagnosed earlier (e.g., average daily intake was 16.4 g versus 19.1 g or 8.1 g versus 11.2 g before and after the first identification liver disease, respectively). However, none of these differences were statistically significant. Thus, the influence of prevalent cases seems to be minimal.

Finally, information collection rate for the questionnaire was lower for controls than cases (83% versus 89%). In addition, 39 of eligible controls (12%) never re-visited OCUH after the beginning of the study. As shown in Table 2, controls had less sever liver disease than cases and might have been potentially easy to withdraw. It is therefore undeniable that our results were subjected to either direction of bias, depending upon the drinking habit among those who not participate. However, the extent of potential bias may not be serious enough to totally invalidate the present study, because the difference in information collection between cases and controls was not statistically significant ($P = 0.17$).

5. Conclusions

Our results did not demonstrate a strong positive effect of alcohol drinking on HCC among patients with HCV infection. However, this is not to say that those with HCV infection can drink without any restriction. Alcohol drinking should be still prohibited, because rather a positive association was suggested among the subgroup with minor changes in abdominal ultrasonography findings. In addition, the factor so called simply "alcohol" has many kinds of aspects, e.g., types of alcohol beverage, although we solely adopted cumulative and average daily ethanol consumption as the main exposure variables in the present study. Furthermore, there may be another threat that alcohol increases the risk of death from liver fibrosis progression. In other words, patients with HCV infection potentially have several kinds of critical outcomes affected by alcohol drinking. Further conclusive studies are needed to clarify the association between alcohol drinking and each of outcomes among those with HCV infection, desirably using a prospective cohort study design.

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Combined therapy of transcatheter hepatic arterial embolization with intratumoral dendritic cell infusion for hepatocellular carcinoma: clinical safety

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Summary

The curative treatments for hepatocellular carcinoma (HCC), including surgical resection and radiofrequency ablation (RFA), do not prevent tumour recurrence effectively. Dendritic cell (DC)-based immunotherapies are believed to contribute to the eradication of the residual and recurrent tumour cells. The current study was designed to assess the safety and bioactivity of DC infusion into tumour tissues following transcatheter hepatic arterial embolization (TAE) for patients with cirrhosis and HCC. Peripheral blood mononuclear cells (PBMCs) were differentiated into phenotypically confirmed DCs. Ten patients were administered autologous DCs through an arterial catheter during TAE treatment. Shortly thereafter, some HCC nodules were treated additionally to achieve the curative local therapeutic effects. There was no clinical or serological evidence of adverse events, including hepatic failure or autoimmune responses in any patients, in addition to those due to TAE. Following the infusion of ^{111}Indium-labelled DCs, DCs were detectable inside and around the HCC nodules for up to 17 days, and were associated with lymphocyte and monocyte infiltration. Interestingly, T lymphocyte responses were induced against peptides derived from the tumour antigens, Her-2/neu, MRP3, hTERT and AFP, 4 weeks after the infusion in some patients. The cumulative survival rates were not significantly changed by this strategy. These results demonstrate that transcatheter arterial DC infusion into tumour tissues following TAE treatment is feasible and safe for patients with cirrhosis and HCC. Furthermore, the antigen-non-specific, immature DC infusion may induce immune responses to unprimed tumour antigens, providing a plausible strategy to enhance tumour immunity.

Keywords: clinical safety, dendritic cells, hepatocellular carcinoma, immunotherapy, transcatheter hepatic arterial embolization

Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to either hepatitis C virus (HCV) or hepatitis B virus (HBV) infections [1–3]. The curative treatments for HCC, including surgical resection and percutaneous radiofrequency ablation (RFA), do not prevent tumour recurrence efficiently because active hepatitis and cirrhosis in the surrounding nontumour liver tissues exhibit high carcinogenetic potentials to develop *de novo* HCC [4–7]. In addition, their reduced hepatic reserve due to cirrhosis decreases the tolerance to these local treatments and reduces drug metabolism, including that of anti-cancer agents, and therefore limits their usefulness. Among many novel strategies targeting HCC recurrence, immune-based therapies are believed to enhance the sensitivity, specificity and self-regulation of the immune system to find and eradicate tumour cells wherever they reside [8].

Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [9–11]. During DC development, immature DCs exhibit the unique ability to take up and process antigens in the peripheral blood and tissues [12,13]. Subsequently, they migrate to draining lymph nodes, where they must mature to fully activated DCs to present the antigens to resting lymphocytes and elicit T cell responses [14–16]. During the maturation processes, they express high levels of cell-surface major

histocompatibility complex (MHC) antigen complexes and co-stimulatory molecules [17]. So far, most of the DC-based immunotherapies have been performed using intravenous (i.v.), intradermal (i.d.) and subcutaneous (s.c.) routes and lymph node injection following the predictive tumour antigen stimulation *ex vivo* [18,19]. Yet the clinical efficacy remained controversial because consistent tumour destruction or extended life span has not been observed in most treated cancer patients [20–22]. Accordingly, antigen stimulation may not be suitable for cancer treatments, or the proper tumour antigens may not be present to be taken up by DCs after the infusion.

In addition, DCs are reported to induce immune responses to target antigens by a cross-priming mechanism that is greatly enhanced when the target cells are apoptotic [23,24]. Apoptosis of tumour cells is induced by the standard treatments for HCC, i.e. transcatheter hepatic arterial embolization (TAE), percutaneous ethanol injection (PEI), RFA and intra-arterial chemotherapy [25,26]. Importantly, we have observed recently that immune responses specific for tumour antigens and peptides were enhanced during the course of the therapies, while anti-tumour responses were not enough to prevent HCC recurrence [27].

Based on these observations, we suggested a novel DC-based therapy in which immature DCs were injected through an arterial catheter into apoptotic tumour tissues following TAE. Immature DCs were delivered to HCC tumour tissues, by which we hypothesized that the physiological maturation steps including antigen ingestion, migration and presentation might proceed within the patient's body. In the current study, clinical safety was evaluated in patients with HCV-related cirrhosis complicated with HCC. The results suggest that immature DCs were infused successfully and safely to tumour tissues, and immune responses were induced to the tumour antigen peptides with human leucocyte antigen (HLA)-A24 binding motif, which are shared by most Asian individuals.

Patients and methods

Patients

Inclusion criteria were a radiological diagnosis of primary HCC by CT angiography, HCV-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent, the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/ml; platelet count $\geq$ 50 000/ml; creatinine < 1·5 mg/dl, and liver damage A or B [28].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

There were 10 patients enrolled in the study (one woman and nine men), with an age range of 45–79 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. Similarly, a group of 11 patients treated with TAE without DC administration was also enrolled in this study as a control. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors as reported previously [29,30]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2-ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in RPMI-1640 supplemented with 1% heat-inactivated autologous plasma, 100 U/ml penicillin G (GMP grade; Meiji, Tokyo, Japan), 100 μg/ml streptomycin sulphate (GMP grade; Meiji), 1000 U/ml recombinant human interleukin (IL)-4 (GMP grade; Cell Genix, Freiburg, Germany) and 100 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (GMP grade; Novartis, Basel, Switzerland) for 7 days. In the selected cases, the cells were pulsed with 10 μg/ml keyhole limpet haemocyanin (KLH) [depyrogenated, lipopolysaccharide (LPS) free; Calbiochem-Novabiochem Corp., San Diego, CA, USA] overnight 1 day before injection. On day 7, the cells were harvested for injection, 5×10^6 cells were reconstituted in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and $HLA\text{-}DR^+$.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies (MoAb) for 30 min on ice:

Table 1. Patient characteristcs and treatments.

Patient no.	Gender	Age (years)	HLA	TNM stages	No. of tumours	Largest tumour (mm)	Child-Pugh	KPS	Post-TAE Rx	Image complete Rx
1	M	45	A2 A11	IVB	Multiple	50	A	100	No	Complete
2	M	60	A26 A33	II	3	15	A	100	RFA	Incomplete
3	F	70	A3 A24	II	2	15	A	100	RFA	Complete
4	M	77	A2 A24	II	3	10	A	100	RFA	Complete
5	M	73	A24	III	Multiple	70	B	80	Chemo	Incomplete
6	M	62	A24 A26	III	Multiple	32	A	100	RFA	Complete
7	M	67	A11 A24	III	Multiple	35	A	100	Chemo	Incomplete
8	M	60	A2 A24	II	2	40	B	100	MCT	Complete
9	M	79	A2 A33	III	5	60	A	100	RFA	Complete
10	M	76	A11 A24	II	1	45	A	100	Ope	Complete
11	M	71	n.d.	II	1	35	B	100	No	Complete
12	M	68	A24	II	4	20	A	100	RFA	Complete
13	F	66	A2 A24	IVA	3	30	A	100	No	Complete
14	F	68	A11 A33	IVA	Multiple	50	B	100	Chemo	Incomplete
15	F	74	n.d.	II	2	20	B	100	RFA	Complete
16	F	67	A2 A24	III	3	25	A	100	RFA	Incomplete
17	F	70	A2 A11	I	1	20	B	100	No	Complete
18	M	59	n.d.	II	3	20	A	100	RFA	Complete
19	M	68	n.d.	III	Multiple	25	B	100	RFA	Complete
20	M	70	A2 A26	II	5	15	B	100	No	Complete
21	M	80	A2 A24	III	3	38	B	100	No	Complete

Chemo: chemotherapy; Child-Pugh: Child-Pugh classification; HCC: hepatocellular carcinoma; KPS: Karnovsky performance scores; n.d.: not determined; Ope: partial hepatectomy; RFS: percutaneous radiofrequency ablation; Rx: treatment; TAE: transcatheter arterial embolization; TNM: tumour-node metastasis.

anti-CD14-allophycocyanin (APC) (MÖP9), anti-HLA-DR-fluorescein isothiocyanate (FITC) (L243), anti-CD11c-APC (S-HCL-3), anti-CD123-phycoerythrin (PE) (9F5) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5·2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a FACSCalibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

^{111}Indium oxinate labeling and autoradiography

DCs were labelled with ^{111}Indium (In) oxinate at a specific activity of 32·5 μCi/10^6 cells according to the protocols supplied by the manufacturer (Nihon Medi-Physics, Hyogo, Japan). Scintigraphic images of the depot were acquired with a gamma camera 6, 24, 48 h and 7 days after injection. In one case, the treated HCC nodule was accidentally resected surgically 17 days after DC infusion. Autoradiography was conducted and analysed on a BAS 1000 image analyser (Fuji Photo Film, Tokyo, Japan).

Immunohistochemical analysis

The liver tissues were fixed in buffered zinc formalin (Anatech Ltd, Battle Creek, MI, USA), embedded in paraffin, sectioned (at 3 μm), and stained with haematoxylin and eosin. The paraffin sections were deparaffinized, treated in a pressure cooker for 1–40 min, and incubated with mouse anti-human CD1a (MTB1; 1 : 20 diluted), CD4 (1F6; 1 : 20), CD8 (1A5; 1 : 20), CD20 (7D1; 1 : 100), CD56 (CD564; 1 : 50), CD83 (1H4b; 1 : 20) (Novocastra, Newcastle, UK), CD14 (7; 1 : 20) or HLA-DR (LN3; 1 : 100) (LabVision, Fremont, CA, USA) antibody overnight at 4°C. The cells were then visualized using a Vectastain ABC Standard Kit (Vector Laboratories, Burlingame, CA, USA), and the tissue sections were counterstained with haematoxylin before mounting.

Interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay

The prevalence of activated, tumour antigen peptide-specific PBMCs was determined by IFN-γ ELISPOT analysis (Mabtech, Nacka, Sweden). Briefly, 96-well mixed cellulose ester membrane-backed plates (MAHA S4510; Millipore, Bedford, MA, USA) were coated with 100 μl of an anti-IFN-γ MoAb 1-D1K (15 μg/ml; Mabtech) overnight at 4°C. Peptides were added directly to the wells at a final concentration of 10 μg/ml. PBMCs were added to the wells at 3×10^5 cells/well. The plates were incubated at 37°C, 5% CO_2 overnight (14–16 h) and then processed as described [31,32]. ELISPOT assays were conducted in duplicate wells. IFN-γ producing cells were counted by direct visualization and are expressed

