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ORIGINAL PAPER

Hepatocellular carcinoma patients with increased oxidative stress levels are prone to recurrence after curative treatment: a prospective case series study using the d-ROM test

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Abstract
Purpose Oxidative stress plays an important role in liver carcinogenesis. To determine the impact of oxidative stress on the recurrence of stage I/II hepatocellular carcinoma (HCC) after curative treatment, we conducted a prospective case series analysis.
Methods This study included 45 consecutive patients with stage I/II HCC, who underwent curative treatment by surgical resection or radiofrequency ablation at Gifu Municipal Hospital from 2006 to 2007. In these 45 cases, recurrence-free survival was estimated using the Kaplan–Meier method. The factors contributing to HCC recurrence, including the serum levels of derivatives of reactive oxygen metabolites (d-ROM) as an index of oxidative stress, were subjected to univariate and multivariate analyses using the Cox proportional hazards model.
Results The serum levels of d-ROM ($P = 0.0231$), α-fetoprotein (AFP, $P = 0.0274$), and fasting plasma glucose ($P = 0.0400$) were significantly associated with HCC recurrence in the univariate analysis. Multivariate analysis showed that the serum levels of d-ROM (hazard ratio [HR] 1.0038, 95 % confidence interval [CI] 1.0002–1.0071, $P = 0.0392$) and AFP (HR 1.0002, 95 % CI 1.0000–1.0003, $P = 0.0316$) were independent predictors of HCC recurrence. Kaplan–Meier analysis showed that recurrence-free survival was low in patients with high serum d-ROM (≥570 Carr U, $P = 0.0036$) and serum AFP (≥40 ng/dL, $P = 0.0185$) levels.
Conclusions The serum levels of d-ROM and AFP can be used for screening patients with a high risk for HCC recurrence. Patients who show increased levels of these factors require careful surveillance.

Keywords Hepatocellular carcinoma · Oxidative stress · d-ROM · Carcinogenesis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 750,000 annual cases; approximately the same number of people (700,000) die from this malignancy each year (Jemal et al. 2011). HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) (El-Serag 2002). Alcohol consumption, obesity, and related metabolic disorders such as diabetes mellitus are also involved in liver carcinogenesis (El-Serag 2002). The prognosis of patients with HCC is poor because the incidence of recurrence in patients with underlying cirrhosis is very high (Toyama et al. 2008). Therefore, careful surveillance of high-risk groups for HCC and early detection before progression to an advanced stage are important to improve the prognosis of this malignancy. It is therefore a task of pressing urgency to identify useful risk factors for HCC development or recurrence. Male gender, the presence of cirrhosis,

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high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells, and the presence of portal venous invasion of tumors are thought to increase the risk for HCC recurrence (Ikeda et al. 1993; Koike et al. 2000). The increased Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) value, which reflects insulin resistance, and high levels of serum leptin, one of the adipokines associated with obesity, are also independent risk factors for HCC recurrence (Imai et al. 2010; Watanabe et al. 2011).

Increased evidence indicates that continuous oxidative stress, which results from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms, plays a critical role in the development of various human malignancies, including HCC (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). As a major site of metabolism, the liver displays high levels of ROS resulting in increased oxidative stress. Oxidative stress is known to induce DNA damage, and accumulation of such genetic damage can eventually contribute to liver carcinogenesis (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). HCV infection is associated with elevated levels of ROS and decreased antioxidant levels in patients (Seronello et al. 2007). Oxidative stress has been associated with the development of steatosis and liver tumors in HCV core transgenic mice (Moriya et al. 2001). In addition, increased levels of ROS is also involved in migration, invasion, and metastasis of HCC cells (Hu et al. 2011; Chung et al. 2012). These findings suggest that oxidative stress biomarkers might potentially be useful for predicting the development and recurrence of HCC in patients with chronic liver disease. Clinical studies using liver specimens obtained by biopsy or surgery have shown the predictive power of oxidative stress biomarkers on HCC development (Chuma et al. 2008; Tanaka et al. 2011). However, serum oxidative stress biomarkers predictive for recurrence after curative treatment for HCC have not been investigated.

Quantification of derivatives of reactive oxygen metabolites (d-ROM) is a simple method for detecting hydroperoxide levels (Trotti et al. 2002), and clinical trials have shown that the d-ROM test is useful for evaluating oxidative stress (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011). In this study, we measured the serum d-ROM level in patients with HCC and designed a prospective case series analysis to examine the recurrence-free survival in consecutive patients with stage I/II HCC who received curative treatment by surgical resection or radiofrequency ablation (RFA), stratified according to the serum d-ROM level. Thus, the aim of the present study was to determine whether the d-ROM test is useful as a marker of oxidative stress for evaluating HCC recurrence risk in the clinical setting.

Patients and methods

Patients

We evaluated 45 consecutive primary HCC patients in Gifu Municipal Hospital from 2006 to 2007, all of whom met the following criteria: tumor stage classified as I or II and surgical resection or RFA as the initial treatment. Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (2010). HCC nodules were detected using imaging modalities including dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI), and abdominal arteriography. HCC was diagnosed from a typical hypervascular tumor stain on angiography and typical dynamic study findings of enhanced staining in the early phase and attenuation in the delayed phase.

Treatment, follow-up, and determination of recurrence

One patient was treated with surgical resection, 41 with RFA, and 3 with RFA after transarterial chemoembolization. The selection criteria for the initial treatments were determined according to the Clinical Practice Guidelines for HCC by the Japan Society of Hepatology (Clinical Practice Guidelines for Hepatocellular Carcinoma—The Japan Society of Hepatology 2009 update 2010). The response to treatment was defined as complete response when dynamic CT or MRI showed complete disappearance of the HCC imaging characteristics in all target lesions, according to the Response Evaluation Criteria in Cancer of the Liver (Kudo et al. 2010).

Patients were thereafter followed up on an outpatient basis by assessing the levels of serum tumor markers such as AFP and proteins induced by vitamin K absence or antagonist-II (PIVKA-II) every month and by using imaging modalities such as abdominal ultrasonography, dynamic CT scanning, or dynamic MRI every 3 months. Recurrent HCC was defined as the appearance of distant lesions to exclude local recurrence. Consequently, recurrent HCC was further classified into multicentric occurrence or intrahepatic metastasis by CT images according to the definition by the Liver Cancer Study Group of Japan (Liver Cancer Study Group of Japan 2010). The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence or until March 2012 if HCC did not recur.

Oxidative stress assay

Before curative treatment, oxidative stress was assessed by measuring the serum hydroperoxide concentration according to the d-ROM test (Diacron srl, Grosseto, Italy) by

Table 1 Baseline demographic and clinical characteristics

Variables	$n = 45$
Sex (male/female)	30/15
Age (years)	72 [50–82]
BMI (kg/m^2)	22.8 [15.6–33.5]
Etiology (B/C/B + C/other)	3/40/1/1
Follow-up period (days)	1,707 [305–2,231]
d-ROM (Carr U)	496 [295–869]
Child-Pugh classification (A/B/C)	33/12/0
ALB (g/dL)	3.5 [2.6–4.5]
ALT (IU/L)	51 [12–100]
T-Bil (mg/dL)	1.0 [0.5–3.7]
PLT ($\times 10^4$/μL)	9.8 [3.6–19.5]
PT (%)	71 [50–100]
FPG (mg/dL)	100 [41–224]
HbA_{1c} (%)[a]	5.7 [4.0–9.8]
AFP (ng/dL)	32.5 [1.7–16,931]
PIVKA-II (mAU/mL)	23.0 [5–1,860]
Stage (I/II)	21/24
Tumor size (cm)	1.7 [1.0–5.3]
Tumor number (1/2/3/4)	36/6/1/2
Portal vein invasion (yes/no)	0/45
Initial treatment for HCC (resection/RFA/TACE + RFA)	1/41/3

Values are presented as median [range]. *BMI* body mass index, *d-ROM* derivatives of reactive oxygen metabolites, *ALB* albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelet count, *PT* prothrombin time, *FPG* fasting plasma glucose, HbA_{1c} hemoglobin A1c, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *RFA* radiofrequency ablation, *TACE* transarterial chemoembolization

[a] HbA_{1c} is presented in National Glycohemoglobin Standardization Program units

using a free radical elective evaluator, FREE (Diacron srl), as described previously (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011).

Statistical analysis

Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were evaluated using the logrank test. Baseline characteristics were compared using the Student's t test for continuous variables or the χ^2 test for categorical variables. Eleven possible predictors for HCC recurrence after initial curative treatment were selected as follows: sex, age, body mass index (BMI), Child-Pugh classification, serum albumin level, platelet count, fasting plasma glucose (FPG), serum AFP level, serum PIVKA-II level, tumor stage, and the serum d-ROM level. Parameters determined to be significant according to univariate analysis were then subjected to multivariate analysis using the Cox proportional hazards model. Receiver operating characteristic (ROC) analysis was used to identify the cut-off values for d-ROM and AFP that would best predict HCC recurrence. Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics and laboratory data of patients

The baseline characteristics and laboratory data of the 45 patients (30 men and 15 women, median age: 72 years) are shown in Table 1. The median follow-up period was 1,707 days (range 305–2,231 days). Thirty-three patients were classified as Child-Pugh class A, 12 patients as class B, and none as class C. The median d-ROM level of all the patients with HCC was 496 Carr U (range 295–869 Carr U).

Possible risk factors for HCC recurrence

In all 45 curative cases of stage I/II HCC, 41 patients experienced recurrence in the liver and 2 patients exhibited distant metastasis; 1 in the lung and the other in the bone. The 1-year, 3-year, and 5-year recurrence-free survival rates in the 45 patients were 60, 29, and 7 %, respectively (Fig. 1a). Among 41 cases that caused intrahepatic recurrence of HCC, 36 cases were diagnosed as multicentric occurrence and the others (5 cases) were as intrahepatic metastasis, respectively.

At first, we analyzed possible risk factors for total recurrence including both multicentric occurrence and intrahepatic metastasis by the Cox proportional hazards model using the 11 variables listed in Table 2. The serum d-ROM level (hazard ratio [HR] 1.0036, 95 % confidence interval [CI] 1.0005–1.0070, $P = 0.0231$), serum AFP level (HR 1.0001, 95 % CI 1.0000–1.0002, $P = 0.0274$), and FPG (HR 1.0008, 95 % CI 1.0004–1.0157, $P = 0.0400$) were significantly associated with HCC recurrence in univariate analysis. Among these variables, multivariate analysis indicated that serum levels of d-ROM (HR 1.0038, 95 % CI 1.0002–1.0071, $P = 0.0392$) and AFP (HR 1.0002, 95 % CI 1.0000–1.0003, $P = 0.0316$) were independent predictors of HCC recurrence (Table 3).

The cut-off values of d-ROM (570 Carr U) and AFP (40 ng/dL) for the prediction of HCC recurrence were determined by ROC analysis. Kaplan–Meier analysis showed that recurrence-free survival was lower in patients with high serum d-ROM levels (≥570 Carr U, $P = 0.0036$) (Fig. 1b) and in those with high serum AFP levels (≥40 ng/dL, $P = 0.0185$) (Fig. 1c). Table 4 shows the baseline characteristics and laboratory data of patients

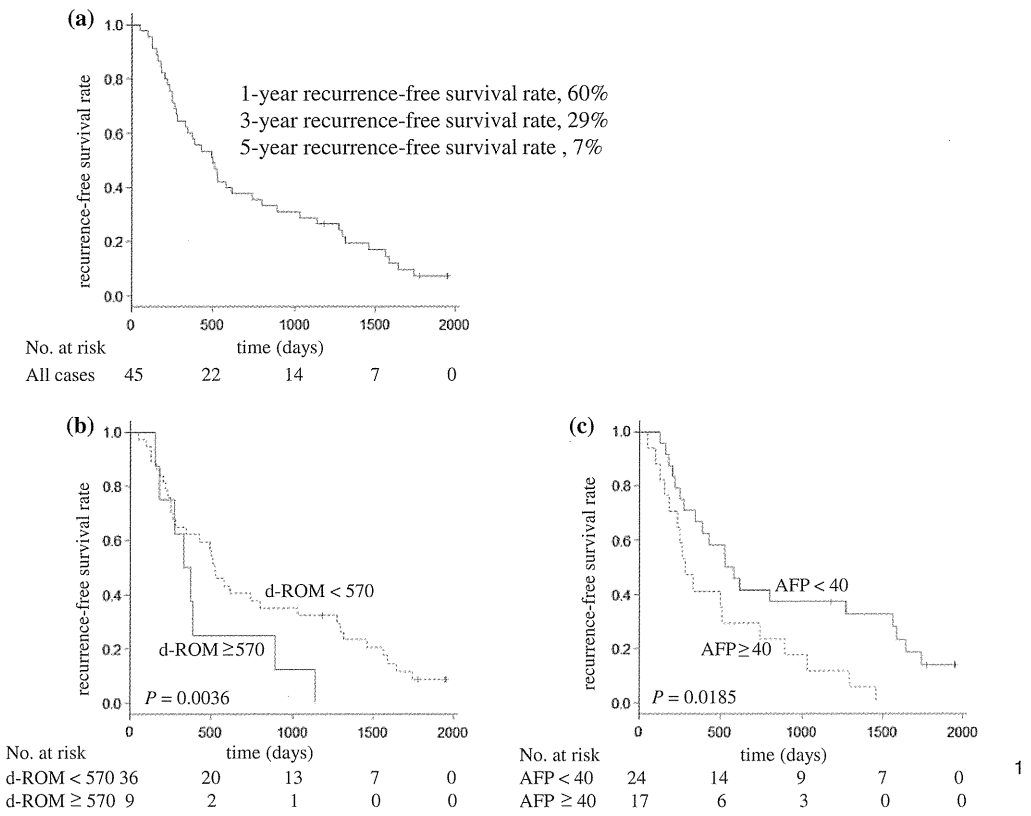

Fig. 1 Kaplan–Meier curves for recurrence-free survival in (**a**) all patients and in subgroups of patients, divided according to (**b**) d-ROM levels or (**c**) serum AFP levels

Table 2 Univariate analyses of possible risk factors for hepatocellular carcinoma recurrence according to a Cox proportional hazards model

Variable	HR	95 % CI		*P* value
		Lower limit	Upper limit	
Sex (male vs. female)	0.9142	0.4810	1.8307	0.7917
Age (years)	1.0086	0.9714	1.0473	0.6522
BMI (kg/m^2)	0.9878	0.9082	1.0696	0.7695
Child-Pugh classification (B vs. A)	0.9707	0.4643	1.8843	0.9329
ALB (g/dL)	0.9496	0.4600	1.9744	0.8893
PLT ($\times 10^4$/mL)	0.9817	0.9031	1.0613	0.6507
FPG (mg/dL)	1.0008	1.0004	1.0157	0.0400
AFP (ng/dL)	1.0001	1.0000	1.0002	0.0274
PIVKA-II (mAU/mL)	1.0005	0.9996	1.0012	0.2149
Stage (II vs. I)	1.1968	0.6468	2.2348	0.5664
d-ROM (Carr U)	1.0036	1.0005	1.0070	0.0231

CI confidence interval, *HR* hazard radio, *BMI* body mass index, *ALB* albumin, *PLT* platelets, *FPG* fasting plasma glucose, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *d-ROM* derivatives of reactive oxygen metabolites

divided on the basis of the serum d-ROM concentration (<570 Carr U and ≥ 570 Carr U). No significant differences were noted between the 2 subgroups.

The serum d-ROM levels were not significantly correlated with any clinical factors associated with hepatic functional reserve (serum total bilirubin, serum albumin,

Table 3 Multivariate analyses of possible risk factors for hepatocellular carcinoma recurrence according to a Cox proportional hazards model

Variable	HR	95 % CI		*P* value
		Lower limit	Upper limit	
FPG (mg/dL)	1.0004	0.9961	1.0126	0.2699
AFP (ng/dL)	1.0002	1.0000	1.0003	0.0316
d-ROM (Carr U)	1.0038	1.0002	1.0071	0.0392

CI confidence interval, *HR* hazard radio, *FPG* fasting plasma glucose, *AFP* α-fetoprotein, *d-ROM* derivatives of reactive oxygen metabolites

serum alanine aminotransferase, prothrombin time, and platelet count) and HCC (tumor size, AFP, and PIVKA-II) (Table 5). In addition, the d-ROM levels did not show significant relationships between the clinical factors for diabetes, including FPG, HbA_{1c}, fasting immunoreactive insulin, and HOMA-IR (Table 5), although several studies have reported that oxidative stress increases with the presence of diabetes and that the d-ROM level is correlated with diabetic factors (Vassalle et al. 2008; Hirose et al. 2009; Sugiura et al. 2011). Further, no significant differences in the median values of d-ROM were noted between (1) the Child-Pugh class A group (496 Carr U, range 295–869 Carr U) and the Child-Pugh class B group (500 Carr U, range 314–589 Carr U) and (2) the stage I group (516 Carr U, range 295–858 Carr U) and the stage II group (500 Carr U, range 314–869 Carr U), suggesting that d-ROM values were not associated with hepatic functional reserve, tumor factors, or the presence of diabetes in this study. These findings indicate that an increased d-ROM value was independently related to the recurrence of HCC.

A separate analysis of 36 multicentric occurrence cases showed an inverse correlation between the serum d-ROM levels and the recurrence-free period, but the significance was marginal ($P = 0.0512$) due to the small number of patients (data not shown). In addition, intrahepatic metastasis cases ($N = 5$) showed a higher d-ROM levels (median 614 Carr U, range 496–869 Carr U) than non-recurrent cases ($N = 4$, median 474 Carr U, range: 461–509 Carr U) ($P = 0.112$).

Discussion

Increasing evidence suggests that oxidative stress plays a critical role in liver carcinogenesis (Sasaki 2006; Sakurai et al. 2008). Elevation of ROS can cause oxidative damage to important cellular macromolecules such as DNA, proteins, and lipids (Valko et al. 2007). Excessive ROS also disrupts the cell signaling pathways that are involved in cell growth and survival, leading further to the advanced stage of carcinogenesis, and cancer promotion and progression (Dreher and Junod 1996; Carmeliet 2000).

Table 4 Baseline demographic and clinical characteristics of patients classified according to the d-ROM level

	d-ROMs < 570 ($n = 36$)	d-ROMs ≥ 570 ($n = 9$)	*P* value
Sex (male/female)	24/12	6/3	1.0000
Age (years)	72 [50–82]	69 [58–76]	0.5325
BMI	22.5 [15.6–33.5]	23.0 [20.1–27.0]	0.7965
Etiology (B/C/B + C/other)	3/31/1/1	0/9/0/0	0.4968
Follow-up period (days)	1,712 [458–2,231]	1,643 [305–2,146]	0.2350
Child-Pugh classification (A/B)	26/10	7/2	0.7323
ALB (g/dL)	3.5 [2.6–4.5]	3.5 [3.0–4.4]	0.9312
ALT (IU/L)	48 [12–100]	53 [22–73]	0.5621
T-Bil (mg/dL)	1.0 [0.5–3.7]	1.0 [0.6–1.5]	0.8532
PLT ($\times 10^4/\mu L$)	10.4 [3.8–19.5]	6.6 [3.6–18.8]	0.0843
PT (%)	70 [50–100]	77 [59–90]	0.2293
FPG (mg/dL)	99 [41–224]	104 [83–140]	0.6272
HbA_{1c} (%)[a]	5.7 [4.0–9.8]	5.3 [4.8–7.0]	0.6447
AFP (ng/dL)	31 [1.7–16,931]	33 [16.7–210]	0.5130
PIVKA-II (mAU/mL)	19.5 [5–1,540]	57.0 [7–1,860]	0.2822
Stage (I/II)	17/19	4/5	0.8811
Initial treatment for HCC (resection/RFA/TACE + RFA)	1/32/3	0/9/0	0.3905

Values are presented as median [range]. *BMI* body mass index, *ALB* albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelets, *PT* prothrombin time, *FPG* fasting plasma glucose, *HbA_{1c}* hemoglobin A1c, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *HCC* hepatocellular carcinoma

[a] HbA_{1c} is presented in National Glycohemoglobin Standardization Program units

Table 5 Correlation between clinical factors and d-ROM using linear regression analysis

	Pearson's correlation coefficient	*P* value
ALB (g/dL)	−0.0073	0.9621
ALT (IU/L)	−0.1007	0.5153
T-Bil (mg/dL)	−0.0044	0.9771
PLT ($\times 10^4/\mu L$)	−0.2319	0.1254
PT (%)	0.2045	0.1777
FPG (mg/dL)	0.0013	0.9935
HbA_{1c} (%)	−0.1225	0.5043
FIRI (mg/dL)	−0.0740	0.6924
HOMA-IR	−0.1590	0.3271
AFP (ng/dL)	−0.1696	0.2892
PIVKA-II (mAU/mL)	−0.0263	0.8798
Tumor size (cm)	−0.1969	0.2074

ALB albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelets, *PT* prothrombin time, *FPG* fasting plasma glucose, *HbA_{1c}* hemoglobin A1c, *FIRI* fasting immunoreactive insulin, *HOMA-IR* Homeostatic Model Assessment of Insulin Resistance, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II

[a] HbA_{1c} is presented in National Glycohemoglobin Standardization Program units

In the present study, HCC recurrence was noted in patients with high serum d-ROM levels (≥570 Carr U, $P = 0.0036$, Fig. 1b) that reflect increased oxidative stress (Trotti et al. 2002). In particular, the 2-year recurrence rate was higher in patients with high serum d-ROM levels (Fig. 1b). We presume that this is primarily associated with the clinical characteristic mode of liver carcinogenesis, that is multicentric carcinogenesis, (occurrence) because when the whole liver was exposed to increased oxidative stress for a long duration, multiple malignant clones that can progress to HCC in the future may have been produced. In our multicentric occurrence cases ($N = 36$), an inverse correlation was actually found between d-ROM levels and recurrence-free period. In addition, intrahepatic metastasis cases showed higher d-ROM levels than non-recurrent patients. These results of the present study, together with recent reports showing the promoting effects of oxidative stress on migration, invasion, and metastasis of HCC cells (Hu et al. 2011; Chung et al. 2012), indicate that intrahepatic metastasis might also be involved, together with multicentric occurrence, in the increase in the 2-year recurrence rate. This finding suggests that increased oxidative stress is a risk factor for HCC development and that the d-ROM test could be a useful clinical diagnostic tool to predict the recurrence of HCC.

A recent clinical trial revealed that loss of the expression of CYP1A2, a major component of the hepatic cytochrome P450 oxidative system, in non-cancerous tissue is a predictive factor of recurrence after curative hepatectomy for early-stage HCC (Tanaka et al. 2011). High levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of DNA damage caused by ROS, in liver biopsy specimens is also a risk factor for HCC development in patients with chronic HCV infection (Chuma et al. 2008). These reports (Chuma et al. 2008; Tanaka et al. 2011), together with the results of the present study, strongly suggest that oxidative stress biomarkers are useful for evaluating patients at a high risk for HCC development. In particular, the results of this study are clinically relevant because the d-ROM test can be performed easily by using serum, whereas the methods used in previous studies involve the use of liver tissues obtained by invasive surgery or biopsy (Chuma et al. 2008; Tanaka et al. 2011).

Increased production of free radicals at the site of inflammation and subsequent oxidative DNA damage is a strong mechanistic link between chronic inflammation and carcinogenesis (Hussain et al. 2003). Oxidative stress is involved in chronic liver inflammation induced by viral hepatitis, alcoholic hepatitis, and non-alcoholic steatohepatitis (Day and James 1998; Loguercio and Federico 2003; Siegel and Zhu 2009). Oxidative DNA damage is enhanced in serum and liver specimens of patients with HCV infection (Sumida et al. 2000; Mahmood et al. 2004). A strong positive correlation between inflammation, intrahepatic oxidative stress, and oxidative DNA damage are also observed in the liver of HCV-associated HCC patients (Maki et al. 2007). In the present study, the median d-ROM level of all the patients with HCC was 496 Carr U (Table 1), and this value is much higher than that of healthy control individuals (250–300 Carr U) (Trotti et al. 2002). This finding may suggest that the systemic level of oxidative stress caused by liver inflammation is increased in HCC patients. The usefulness of the d-ROM test for evaluating the correlation between increased levels of systemic inflammation and oxidative stress has also been reported in previous clinical studies (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011).

The present study included 3 HCV-positive patients who received interferon therapy to eliminate the virus before HCC development. Two of these patients demonstrated a sustained virological response (SVR); however, all of them showed recurrence of HCC. Three additional HCV-positive patients also received interferon therapy to prevent HCC recurrence after the initial HCC development, but none of them showed a SVR, and 2 patients suffered a relapse. The serum d-ROM levels of the enrolled patients during the follow-up period, including after curative treatment as well as at the recurrence points, were not examined in the present study. However, these measurements seem to be significant and should be performed in future studies because the levels of d-ROM might be useful as a

biomarker for assessing the effectiveness of treatment for chronic liver diseases with interferon (Morisco et al. 2004). The d-ROM test was used in a clinical trial to evaluate oxidative status as a predictive factor of the therapeutic response of interferon and ribavirin treatment in patients with chronic hepatitis C (Morisco et al. 2004). In the study, the patients with a successive long-term response had lower d-ROM levels than non-responders (Morisco et al. 2004), suggesting that the serum levels of d-ROM might help to predict long-term response to interferon/ribavirin therapy in patients with chronic viral hepatitis. Moreover, this report also suggested that antiviral therapy could possibly attenuate oxidative stress because the mean d-ROM levels were significantly decreased during the treatment (Morisco et al. 2004). Iron depletion, which can decrease the production of ROS, improves the end-of-treatment biological and histological response to interferon therapy (Fontana et al. 2000). Iron reduction also decreases the levels of 8-OHdG and risk of HCC in HCV patients (Kato et al. 2001). Future studies to determine whether targeting oxidative stress is useful for the treatment of chronic liver disease, including the prevention of HCC, and whether the d-ROM test is applicable for evaluating oxidative stress should be conducted.

Finally, in addition to the production of ROS, alteration in the antioxidant activity is also implicated in imbalance of the normal redox state and subsequent liver carcinogenesis (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). Experimental studies have shown evidence that dietary antioxidants, for example, vitamin E, vitamin C, and selenium, play a possible role in the prevention of liver carcinogenesis (Glauert et al. 2010). Therefore, intervention trials to examine whether antioxidant supplementation decreases the serum d-ROM levels and, therefore, possibly inhibits the development of HCC should be conducted in the future. On the other hand, several clinical studies have shown that antioxidant activity is induced as an adaptive response to increased generation of ROS in patients with HCC (Clemente et al. 2007; Abel et al. 2009; Tsai et al. 2009). An increase in the activity of manganese superoxide dismutase, an antioxidant enzyme, occurs during the precancerous phase and serves as a potential biomarker for HCC (Clemente et al. 2007). Disruption of the redox balance, resulting in increased cellular antioxidant capacity, might also create an advantageous environment for the growth of HBV-associated HCC cells (Abel et al. 2009). These reports suggest that antioxidant activity could be a predictive factor for the development of HCC.

In conclusion, this is the first indication that stage I/II patients curatively treated using surgical resection or RFA who have increased serum d-ROM levels, which reflect increased oxidative stress, are liable to HCC recurrence. The d-ROM test can be used for screening patients at a high risk for HCC recurrence, and those who show increased d-ROM levels may require careful surveillance.

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Effects of Indoleamine 2,3-Dioxygenase Deficiency on High-Fat Diet-Induced Hepatic Inflammation

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Abstract

Hepatic immune regulation is associated with the progression from simple steatosis to non-alcoholic steatohepatitis, a severe condition of inflamed fatty liver. Indoleamine 2,3-dioxygenase (IDO), an intracellular enzyme that mediates the catabolism of L-tryptophan to L-kynurenine, plays an important role in hepatic immune regulation. In the present study, we examined the effects of IDO gene silencing on high-fat diet (HFD)-induced liver inflammation and fibrosis in mice. After being fed a HFD for 26 weeks, the IDO-knockout (KO) mice showed a marked infiltration of inflammatory cells, especially macrophages and T lymphocytes, in the liver. The expression levels of F4/80, IFNγ, IL-1β, and IL-6 mRNA in the liver and the expression levels of F4/80 and TNF-α mRNA in the white adipose tissue were significantly increased in IDO-KO mice, although hepatic steatosis, the accumulation of intrahepatic triglycerides, and the amount of oxidative stress were lower than those in IDO-wild-type mice. IDO-KO mice also developed marked pericellular fibrosis in the liver, accumulated hepatic hydroxyproline, and exhibited increased expression levels of hepatic TGF-β1 mRNA. These findings suggest that IDO-KO renders the mice more susceptible to HFD-induced hepatic inflammation and fibrosis. Therefore, IDO may have a protective effect against hepatic fibrosis, at least in this HFD-induced liver injury model.

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Introduction

Non-alcoholic fatty liver disease (NAFLD), which is strongly associated with obesity and metabolic syndrome, is one of the most common causes of chronic liver disease worldwide. NAFLD includes a spectrum of disturbances that encompasses various degrees of liver damage ranging from non-alcoholic steatohepatitis (NASH), a severe condition of inflamed fatty liver that can progress to hepatic fibrosis, cirrhosis, or even hepatocellular carcinoma. The critical features of NASH, in addition to simple steatosis, include forms of hepatocellular degeneration such as ballooning and Mallory hyaline degeneration, mixed inflammatory cell infiltration, and the development of fibrosis [1,2]. Obesity is associated with chronic low-grade systemic inflammation, which contributes to the progression from hepatic steatosis to NASH [3]. Among various immune cells, T lymphocytes play a critical role in the induction of inflammation both in the liver and in white adipose tissue (WAT) [4,5]. Macrophage infiltration into WAT is also an early contributing event in the development of systemic inflammation because it is accompanied by tumor necrosis factor (TNF)-α production, a central mediator of the inflammatory response [6]. These reports, therefore, indicate that chronic inflammation plays a key role in the pathogenesis of NASH [7].

Indoleamine 2,3-dioxygenase (IDO), an intracellular enzyme that degrades the essential amino acid L-tryptophan along the L-kynurenine pathway, is induced during inflammation by several immune factors, including interferon (IFN) γ [8]. IDO is considered to exert powerful immunomodulatory effects, including the promotion of immune tolerance, because L-kynurenine and some other metabolites derived from tryptophan by IDO can inhibit T cell activation and proliferation while increasing immunosuppressive regulatory T-cell (Tregs) activity [9–11]. The liver is a special lymphoid organ and is thus particularly susceptible to injury as a result of the immune response, which is primarily mediated by T lymphocytes [12].

IDO is activated in infectious, autoimmune, and malignant diseases that involve cellular immune activation in various organs, including the liver [13]. In fact, upregulation of the IDO expression in the liver and increased serum IDO activity have been found in chronic hepatitis C patients [14,15]. The IDO expression is also enhanced in the liver and adipose tissue in obese individuals [16].

Several rodent studies have revealed the role of IDO in liver injury. In hepatitis B virus (HBV) transgenic mice, the IDO expression in hepatocytes is enhanced in mice with liver injury caused by HBV-specific cytotoxic T lymphocytes [17]. Inhibition of IDO activity exacerbates liver injury in both α-galactosylceramide- and carbon tetrachloride (CCl_4)-induced acute hepatitis models and is associated with the induction of TNF-α [18,19]. These reports suggest that IDO plays a critical role in the regulation of liver inflammation and that targeting IDO activity might be an effective strategy for attenuating acute liver injury. However, the role of IDO in steatosis-induced liver injury has not yet been clarified. In the present study, we examined the effects of IDO on high-fat diet (HFD)-induced liver steatosis and subsequent hepatic inflammation and fibrosis using IDO-deficient mice.

Materials and Methods

2.1 Animals and experimental procedure

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Gifu University Life Science Research Center. The protocol was approved by the Committee on the Ethics of Animal Experiments of Gifu University (Permit Number: 24-65). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering. Five-week-old male IDO-wild-type (WT) mice and IDO-knockout (KO) mice with a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). HFD-60 (506.2 kcal/100 g) with 62.2% of the calories derived from fat was purchased from Oriental Yeast (Tokyo, Japan). The cholesterol content of the diet was 33.0 mg/100 g. After 1 week of acclimatization, 8 WT mice and 8 KO mice were given a pelleted HFD throughout the experiment (26 weeks) with free access to tap water and food. At the end of the experiment (32 weeks of age), all mice were sacrificed under sodium pentobarbital anesthesia and the livers were carefully removed.

2.2 Histopathological and immunohistochemical examinations

For all the experimental mice, 4 μm-thick sections of formalin-fixed and paraffin-embedded livers were stained with hematoxylin & eosin (H&E) for conventional histopathology or with Sirius Red stain to determine the presence of liver fibrosis. The histological features of the livers were evaluated using the NAFLD activity score (NAS) system [20]. The computer-assisted quantitative analyses of hepatic fibrosis development were carried out using the BZ-Analyzer-II software program (KEYENCE, Osaka, Japan) [21,22].

In order to evaluate the infiltration of inflammatory cells in the liver, immunohistochemical staining for Mac-1 (a macrophage marker), CD3 (a T-cell marker), and NIMP-R14 (a neutrophil marker) of paraffin-embedded sections was performed using the linked streptavidin-biotin method. Rat monoclonal anti-Mac-1 antibody (MAB1387Z, 1:50 dilution) was purchased from Chemicon Iuternational (Temecula, CA, USA). Rabbit polyclonal anti-CD3 (ab5690, 1:100 dilution) antibodies and rat monoclonal anti-neutrophil antibody (NIMP-R14, ab2557, 1:50 dilution) were obtained from Abcam (Cambridge, MA, USA). On the Mac-1-, CD3-, and NIMP-R14-immunostained sections, the inflammatory cells that intensively reacted to these antibodies were counted and the data are expressed as the percentage of total inflammatory cells in the liver. A positive cell index (%) was determined by counting at least 500 cells in a section from each mouse.

2.3 Hepatic hydroxyproline analysis

The hepatic hydroxyproline content (μmol/g wet liver) was quantified colorimetrically in duplicate samples from approximately 200mg of the wet-weight liver tissues, as described previously [22].

2.4 RNA extraction and quantitative real-time RT-PCR analysis

Total RNA was isolated from the livers and adipose tissue of the mice using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), respectively [21]. cDNA was amplified from 0.5 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). A quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify F4/80, IFNγ, interleukin (IL)-1β, IL-6, TNF-α, superoxide dismutase (SOD)-1, SOD-2, glutathione peroxidase (GPx), transforming growth factor (TGF)-β1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the ribosomal protein large P0 (RPLP0) genes. The sequences of the primers for these genes, which were obtained from Universal ProbeLibrary Assay Design Center (Roche, Indianapolis, IN, USA), are shown in Table 1. The analysis to quantify the expression levels of tryptophan 2,3-dioxygenase (TDO) was performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and TOYOBO Real-time PCR Master Mix (TOYOBO, Osaka, Japan), as described previously [23]. Each sample was analyzed on a LightCycler Nano (Roche) with FastStart Essential DNA Green Master (Roche). The parallel amplification of GAPDH and RPLP0 was used as the internal control for liver and adipose tissue, respectively.

2.5 Clinical chemistry

The serum levels of alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer (type 7180; Hitachi, Tokyo, Japan).

Table 1. Primer sequences.

Gene	Primer sequence	
SOD1	F	5'- CAGGACCTCATTTTAATCCTCAC-3'
	R	5'- TGCCCAGGTCTCCAACAT-3'
SOD2	F	5'- TGCTCTAATCAGGACCCATTG-3'
	R	5'- GTAGTAAGCGTGCTCCCACAC-3'
GPx	F	5'- TTTCCCGTGCAATCAGTTC-3'
	R	5'- TCGGACGTACTTGAGGGAAT-3'
F4/80	F	5'- ACAAGACTGACAACCAGACGG-3'
	R	5'- TAGCATCCAGAAGAAGCAGGCGA-3'
IFNγ	F	5'- AGCAACAGCAAGGCGAAAAAG-3'
	R	5'- CGCTTCCTGAGGCTGGATTC-3'
IL-1β	F	5'- CAAGCAACGACAAAATACCTGTG-3'
	R	5'- AGACAAACCGTTTTTCCATCTTCT-3'
IL-6	F	5'- CCGGAGAGGAGACTTCACAGAG-3'
	R	5'- CTGCAAGTGCATCATCGTTGTT-3'
TNF-α	F	5'- TGGCCCAGACCCTCACACTCAG-3'
	R	5'- ACCCATCGGCTGGCACCACT-3'
TGF-β1	F	5'- ACCGGAGAGCCCTGGATACCA-3'
	R	5'- TATAGGGGCAGGGTCCCAGACA-3'
RPLP0	F	5'- ACTGGTCTAGGACCCGAGAAG-3'
	R	5'- CTCCCACCTTGTCTCCAGTC-3'
GAPDH	F	5'-GACATCAAGAAGGTGGTGAAGCAG-3'
	R	5'-ATACCAGGAAATGAGCTTGACAAA-3'

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2.6 Oxidative stress analysis

The serum hydroperoxide levels, one of the markers of oxidative stress, were determined using the derivatives of reactive oxygen metabolites (d-ROM) test (FREE Carpe Diem; Diacron s.r.l., Grosseto, Italy), according to the manufacturer's protocol.

2.7 Determination of the enzymatic activity of IDO

The IDO activity level in the serum was determined by calculating the ratio of the L-kynurenine/L-tryptophan concentrations [23]. Serum samples were deproteinized with 3% perchloric acid. Following centrifugation, aliquots of supernatant were collected to determine the concentrations of L-tryptophan and L-kynurenine using HPLC, as described previously [18].

2.8 Hepatic lipid analysis

After total lipids were extracted from the frozen livers (approximately 200 mg), the triglyceride levels were measured using the triglyceride E-test kit (Wako, Osaka, Japan) [21].

2.9 Statistical analysis

The data are expressed as the mean ± SD. Statistical significance of the difference between mean values was evaluated using the Mann-Whitney *U* test. Significance was defined as a *P* value less than 0.05.

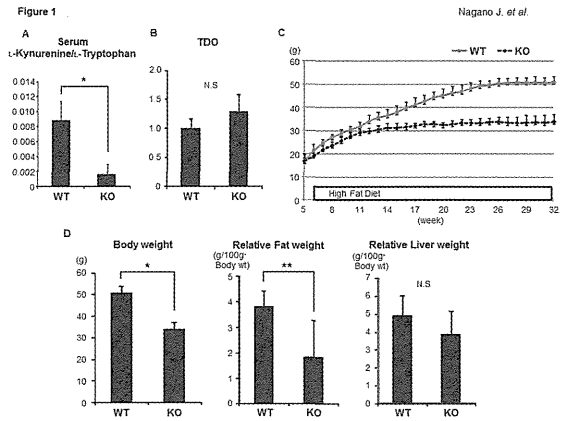

Figure 1. Effects of IDO deficiency on the serum L-kynurenine/L-tryptophan ratio, the expression levels of TDO in the liver, the growth curve, and the body, liver, and fat weights of the experimental mice. (A) The functional IDO activity level was determined by measuring the concentrations of L-kynurenine and L-tryptophan using HPLC. The L-kynurenine/L-tryptophan ratio indicates the IDO activity. (B) Total RNA was isolated from the livers of the experimental mice, and the expression levels of TDO mRNA were examined using quantitative real-time RT-PCR with specific primers. (C) The growth curve of the experimental mice. The body weights of all mice were measured once a week during the experiment. (D) The body weights and relative weights of the adipose tissues and livers of the experimental mice at the termination of the study. The values are expressed as the mean ± SD. * P <0.001, ** P <0.05.

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Results

3.1 General observations

We initially examined the enzymatic activity of IDO in the serum of the experimental mice by measuring the concentrations of L-kynurenine and L-tryptophan. The L-kynurenine/L-tryptophan ratios in serum of the IDO-KO mice were significantly lower than those in the serum of the IDO-WT mice (Figure 1A, $P < 0.001$), indicating that IDO activity was clearly inhibited in the IDO-KO mice. TDO, a hepatic enzyme that catalyses the first step of tryptophan degradation, was expressed in the liver in both the IDO-WT mice and the IDO-KO mice; however, IDO deficiency did not have a significant effect on the TDO mRNA expression (Figure 1B). Figure 1C shows the growth curves of the mice during this experiment. The body weight gain of the IDO-KO mice was smaller than that of the IDO-WT mice. At the end of the experiment, the body weights (Figure 1D, $P < 0.001$) and the relative weights of the adipose tissues of the IDO-KO mice (Figure 1D, $P < 0.05$) were also significantly lower than those of the IDO-WT mice.

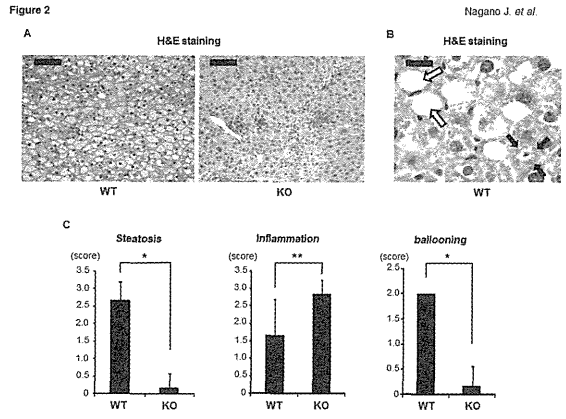

Figure 2. Effects of IDO deficiency on hepatic histopathology in the experimental mice. (A) and (B) H&E staining of liver sections from the experimental mice. (A) Representative photomicrographs of the liver sections of the IDO-WT mice and IDO-KO mice (low-power field). Black bar: 100 μm. (B) An enlarged photo (high-power field) of the liver sections from the IDO-WT mice. Ballooned hepatocytes (indicated by white arrows) and Mallory-Denk bodies (indicated by black arrows) were observed. Black bar: 20 μm. (C) The presence of NAS (steatosis, inflammation, and ballooning) was determined based on the histopathological analysis. The values are expressed as the mean ± SD. * P <0.001, ** P <0.05.

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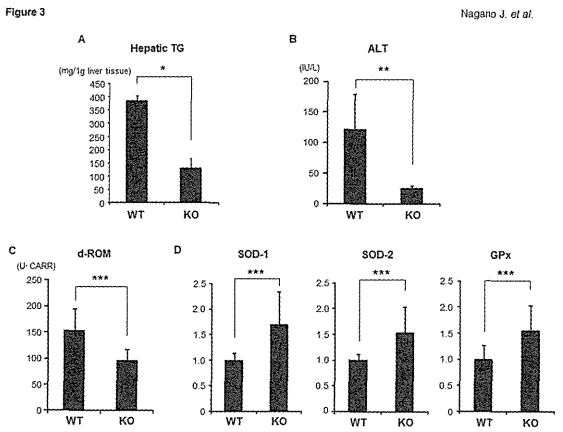

Figure 3. Effects of IDO deficiency on intrahepatic triglycerides, the serum ALT levels, and oxidative stress in the experimental mice. (A) Hepatic lipids were extracted from the frozen livers of the experimental mice, and the triglyceride levels were measured. (B) At sacrifice, blood samples were collected and the serum levels of ALT were assayed. (C) The hydroperoxide levels in the serum at the end of the experiment were determined using the d-ROM test. (D) Total RNA was isolated from the livers of the experimental mice, and the expression levels of SOD-1, SOD-2, and GPx mRNA were examined using quantitative real-time RT-PCR with specific primers. The values are expressed as the mean ± SD. * P <0.001, ** P <0.01, *** P <0.05.

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3.2 Effects of IDO deficiency on hepatic histopathology in the experimental mice

The H&E staining results of the livers of the IDO-KO mice and IDO-WT mice after 26 weeks of being fed the HFD are presented in Figure 2A and B. The infiltration of inflammatory cells was markedly increased in the livers of the IDO-KO mice, and the NAS inflammation scores were significantly higher than those in the IDO-WT mice (Figure 2C, $P < 0.05$). Interestingly, the hepatic steatosis and ballooning degeneration of hepatocytes were lower in the IDO-KO mice than in the IDO-WT mice at this experimental time point (Figure 2C, $P < 0.001$). In addition to the ballooned hepatocytes, Mallory-Denk bodies, which are a recognized feature of alcoholic hepatitis and NASH [24], were also observed in the liver of IDO-WT mice (Figure 2B).

3.3 Effects of IDO deficiency on the intrahepatic triglyceride levels, the serum ALT levels, and oxidative stress in the experimental mice

The histological findings were consistent with the measured intrahepatic triglyceride contents: the levels of triglycerides in the livers of the IDO-KO mice were significantly lower than those in the livers of the IDO-WT mice (Figure 3A, $P < 0.001$). The serum levels of ALT in the IDO-KO mice were also significantly decreased relative to those in the IDO-WT mice (Figure 3B, $P < 0.01$). In addition, the serum d-ROM levels, which reflect the serum hydroperoxide levels, were significantly lower in the IDO-KO mice than in the IDO-WT mice (Figure 3C, $P < 0.05$). Compared to the IDO-WT mice, there were also significant increases in the expression levels of SOD-1, SOD-2, and GPx mRNA, which encode antioxidant enzymes, in the livers of the IDO-KO mice (Figure 3D, $P < 0.05$). These findings indicate that hepatic triglyceride accumulation and oxidative stress are reduced, while antioxidant activity is increased, in mice lacking the IDO gene.

3.4 Effects of IDO deficiency on inflammation in the livers and WAT of the experimental mice

We next examined the expression levels of inflammatory mediators that are implicated in the progression of fatty liver to NASH [7] in the experimental mice. A quantitative real-time RT-PCR analysis revealed that the expression levels of F4/80, a marker of macrophages, were significantly increased in the livers of the IDO-KO mice in comparison to those observed in the livers of the IDO-WT mice (Figure 4A, $P < 0.01$). There were also significant increases in the expression levels of inflammatory mediators, including IFNγ, IL-1β, and IL-6 mRNA, in the livers of the IDO-KO mice compared to those observed in the livers of the IDO-WT mice (Figure 4A, $P < 0.05$). The expression levels of TNF-α mRNA were also higher in the livers of the IDO-KO mice than in the livers of the IDO-WT mice;

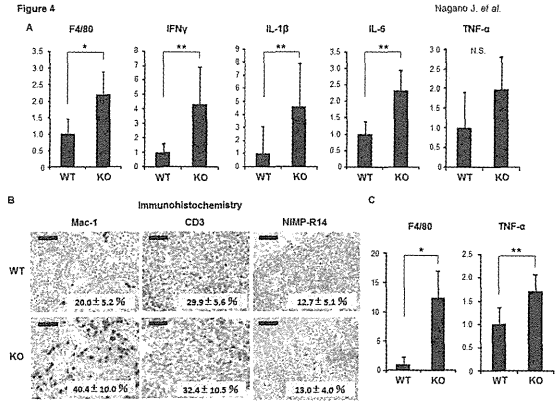

Figure 4. Effects of IDO deficiency on the inflammation in the liver and white adipose tissue of the experimental mice. (A) The expression levels of F4/80, IFNγ, IL-1β, IL-6, and TNF-α mRNA in the livers of the experimental mice. (B) The results of the immunohistochemical analyses of Mac-1, CD3, and NIMP-R14 in the livers of the experimental mice. A positive cell index (%) was shown in each photo. Black bar: 50 μm. (C) The expression levels of F4/80 and TNF-α mRNA in the WAT of the experimental mice. Total RNA was isolated from the livers (A) and WAT (C) of the experimental mice, and the expression levels of each mRNA were examined using quantitative real-time RT-PCR with specific primers. The expression levels of GAPDH mRNA and RPLP0 mRNA were used as internal controls for the liver and WAT, respectively. The values are expressed as the mean ± SD. * P <0.01, ** P <0.05.

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however, the difference was insignificant (Figure 4A). Furthermore, the immunohistochemical analyses demonstrated that the inflammatory cells that had infiltrated into the livers of the IDO-KO mice positively reacted with either the anti-Mac-1(40.4 ± 10.0%) or anti-CD3 (32.4 ± 10.5%) antibodies. On the other hand, the infiltration of neutrophils (13.0 ± 4.0%) was low compared to that of macrophages and T-cells. These findings suggest that macrophages and T lymphocytes were the predominantly increased cell populations in the livers of the IDO-KO mice. The infiltration of Mac-1 positive cells in the livers of IDO-KO mice (40.4 ± 10.0%) was high compared to that of IDO-WT mice (20.0 ± 5.2%) (Figure 4B, P < 0.05), and this is consistent with the results of RT-PCR analysis showing the increased levels of F4/80 mRNA in the livers of IDO-KO mice (Figure 4A).

Moreover, as shown in Figure 4C, the expression levels of F4/80 (P < 0.01) and TNF-α(P < 0.05) mRNA in WAT were both significantly increased in the IDO-KO mice compared to those observed in the IDO-WT mice, indicating that inflammation is augmented in WAT, in addition to the liver, in the IDO-KO mice [24].

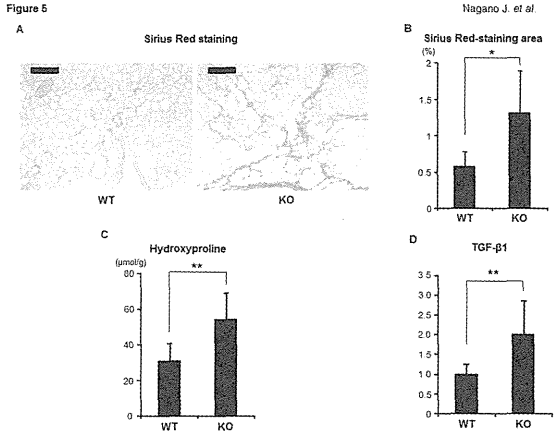

Figure 5. Effects of IDO deficiency on the hepatic fibrosis in the experimental mice. (A) Representative photomicrographs of liver sections stained with Sirius Red to show fibrosis. Black bar: 100 μm. (B) The Sirius Red-stained images of fibrosis were analyzed using a BZ-9000 fluorescence microscope, and the fibrotic area was measured using a BZ-Analyzer-II. (C) The hepatic hydroxyproline contents were quantified colorimetrically. (D) Total RNA was isolated from the livers of the experimental mice, and the expression levels of TGF-β1 mRNA were examined using quantitative real-time RT-PCR with specific primers. The values are expressed as the mean ± SD. * P <0.01, ** P <0.05.

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3.5 Effects of IDO deficiency on hepatic fibrosis in the experimental mice

We next examined whether IDO deficiency has an effect on the development of steatosis-induced hepatic fibrosis. An examination of Sirius Red-stained sections indicated that, compared to the IDO-WT mice, the IDO-KO mice markedly developed pericellular fibrosis in the liver (Figure 5A and B, P < 0.01). Similar findings were observed in the measured hepatic hydroxyproline contents: the IDO-KO mice showed a significant increase in the amount of hydroxyproline observed in the liver (Figure 5C, P < 0.05). The expression levels of TGF-β1 mRNA, a central regulator of chronic liver disease contributing to fibrogenesis through inflammation [25], were also remarkably elevated in the livers of the IDO-KO mice compared to those observed in the livers of the IDO-WT mice (Figure 5D, P < 0.05). These findings may indicate that IDO-KO mice are susceptible to the development of steatosis-induced hepatic fibrosis.

Discussion

The results of the present study indicate that HFD-induced hepatic inflammation and fibrosis are significantly aggravated in IDO-KO mice, although the level of hepatic steatosis and amount of oxidative stress were lower compared to those in IDO-WT mice. Therefore, IDO deficiency is critically involved in

the acceleration of hepatic inflammation observed in the present study.

IDO is a rate-limiting enzyme that can degrade tryptophan via the kynurenine pathway. Because the IDO expression and its enzymatic activity, which are tightly controlled by several immune mediators such as IFNγ, play a key role in the suppression of the immune response [8–11], inhibiting the expression and activity of IDO might promote inflammatory signaling. Therefore, based on our present results, we consider that IDO-deficient mice are more susceptible to the induction of inflammation by HFD. Our results are consistent with those of recent reports showing that the inhibition of the enzymatic activity of IDO significantly exacerbated liver injury in α-galactosylceramide (α-GalCer)- and CCl_4-induced acute hepatitis animal models via the upregulation of IL-6 and TNF-α [18,19]. When the IDO-KO mice were treated with α-GalCer, the production of TNF-α from the infiltrating macrophages in the liver was significantly accelerated, and thus led to the development of severe hepatitis [18]. Therefore, in the present study, the increase in the number of hepatic macrophages might have been critically involved in the exacerbation of HFD-induced hepatic inflammation in the IDO-KO mice. These reports [18,19], together with the results of the present study, suggest that IDO may play a critical role in suppressing excess induction and progression of inflammation in the liver.

Innate immune cells, including Kupffer cells, natural killer T cells, and natural killer cells, play important roles in the excessive production of hepatic T helper 1 cytokines, which is associated with the development of steatohepatitis [4]. The regulation of the immune response by IDO is predominantly based on the ability of IDO to suppress the activation of lymphocytes [9–11]. An increased IDO activity inhibits proliferation and induces apoptosis in T cells and natural killer cells via tryptophan depletion and the production of toxic tryptophan metabolites [9]. In addition, recent studies have revealed that IDO inhibits T cell activation by driving the development of Tregs [10,11]. Tregs, which are actively engaged in the negative control of a variety of immune responses, are recognized as being one of the key players in hepatic immune regulation [26]. HFD-induced steatosis in mice is associated with the depletion of hepatic Tregs and leads to upregulation of the inflammatory pathway [27]. Therefore, an IDO deficiency may increase T cell activation, either directly or indirectly, by suppressing Tregs and thus contributed to a worsening of hepatic inflammation in the present study.

Obesity is associated with systemic low-grade inflammation and immune activation [5,6]. One clinical trial reported that activation of IDO is associated with reduced plasma tryptophan levels in obese patients [28]. IDO is also overexpressed in the liver and adipose tissue in obese subjects [16]. These reports indicate that the overexpression and activation of IDO are implicated in chronic immune activation in obese individuals. T cell infiltration into WAT and subsequent recruitment and activation of macrophages can induce TNF-α production, which is associated with the development of systemic inflammation [5,6]. The present study showed that the expression levels of F4/80 and TNF-α mRNA in WAT are elevated in IDO-KO mice compared to those observed in IDO-WT mice when the mice are fed an HFD, indicating that inflammation of WAT induced by HFD is worsened in IDO deficiency mice. Therefore, our findings suggest that IDO might have the ability to attenuate overactive immune responses caused by obesity in WAT in addition to the liver.

There are some possible limitations associated with the present study. For instance, a recent study demonstrated that neither the overexpression of IDO nor inhibition of its enzymatic activity affected the lipid accumulation in the liver, although the combination of L-tryptophan treatment and a high fat and high fructose diet exacerbated the hepatic steatosis [29]. Therefore, further experiments will be required to clarify the role of IDO and the L-kynurenine/L-tryptophan pathway in the development of hepatic steatosis. Furthermore, after 26 weeks of being fed the HFD, the IDO-KO mice showed lower steatosis and oxidative stress than the IDO-WT mice. The hepatocyte ballooning, which indicates hepatocyte injury, was also decreased in IDO-KO mice compared to IDO-WT mice. These findings seem paradoxical given the enhanced inflammation and fibrosis in IDO-KO mice in response to the HFD. A possible explanation might be that the liver inflammation proceeded earlier in IDO-KO mice, in a similar manner to NAFLD in the clinical setting, where many cases with NAFLD show the disappearance of steatosis during its natural history, while exhibiting severe fibrosis and cirrhosis in the late stages [30,31]. In order to verify this possibility, time course studies that evaluate the levels of hepatic injury, steatosis, and inflammation caused by HFD in the early phase should be conducted. In addition, a recent study revealed that hepatic fat deposits were broken down to provide energy for fibrogenesis in a CCl_4-treated mouse model [32]. Such a mechanism might have also been active in our HFD-fed IDO-KO mice, but again, further experiments will be required to confirm this hypothesis.

In conclusion, we herein demonstrated that IDO deficiency worsens hepatic and WAT inflammation in mice fed an HFD. Our findings suggest that regulation of the IDO-mediated immune response might be an interesting strategy for managing steatosis-related hepatic injury.

Author Contributions

Performed the experiments: JN YS TK NN HO. Analyzed the data: JN MS TT. Wrote the manuscript: JN MS TH HI TT HT KS MS HM.

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RESEARCH ARTICLE Open Access

Synergistic growth inhibition by acyclic retinoid and phosphatidylinositol 3-kinase inhibitor in human hepatoma cells

Atsushi Baba, Masahito Shimizu*, Tomohiko Ohno, Yohei Shirakami, Masaya Kubota, Takahiro Kochi, Daishi Terakura, Hisashi Tsurumi and Hisataka Moriwaki

Abstract

Background: A malfunction of RXRα due to phosphorylation is associated with liver carcinogenesis, and acyclic retinoid (ACR), which targets RXRα, can prevent the development of hepatocellular carcinoma (HCC). Activation of PI3K/Akt signaling plays a critical role in the proliferation and survival of HCC cells. The present study examined the possible combined effects of ACR and LY294002, a PI3K inhibitor, on the growth of human HCC cells.

Methods: This study examined the effects of the combination of ACR plus LY294002 on the growth of HLF human HCC cells.

Results: ACR and LY294002 preferentially inhibited the growth of HLF cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR and 5 μM LY294002, in which the concentrations used are less than the IC_{50} values of these agents, synergistically inhibited the growth of HLF, Hep3B, and Huh7 human HCC cells. These agents when administered in combination acted cooperatively to induce apoptosis in HLF cells. The phosphorylation of RXRα, Akt, and ERK proteins in HLF cells were markedly inhibited by treatment with ACR plus LY294002. Moreover, this combination also increased RXRE promoter activity and the cellular levels of RARβ and $p21^{CIP1}$, while decreasing the levels of cyclin D1.

Conclusion: ACR and LY294002 cooperatively increase the expression of RARβ, while inhibiting the phosphorylation of RXRα, and that these effects are associated with the induction of apoptosis and the inhibition of cell growth in human HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

Keywords: Acyclic retinoid, LY294002, Hepatocellular carcinoma, RXRα, Synergism

Background

Retinoids, vitamin A metabolites and analogs, are ligands of the nuclear receptor superfamily that exert fundamental effects on cellular activities, including growth, differentiation, and death (regulation of apoptosis). Retinoids exert their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are ligand-dependent transcription factors [1,2]. Among retinoid receptors, RXRs are regarded as master regulators of the nuclear receptor superfamily because they play an essential role in controlling normal cell proliferation and metabolism by acting as common heterodimerization partners for various types of nuclear receptors [1,2]. Therefore, altered expression and function of RXRs are strongly associated with the development of various disorders, including cancer, whereas targeting RXRs by retinoids might be an effective strategy for the prevention and treatment of human malignancies [3].

Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers worldwide. Recent studies have revealed that a malfunction of RXRα, one of the subtypes of RXR, due to aberrant phosphorylation by the Ras/mitogen-activated protein kinase (MAPK) signaling pathway is profoundly associated with liver carcinogenesis [4-9]. On the other hand, a prospective randomized study showed that administration of acyclic retinoid (ACR), a

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synthetic retinoid which targets RXRα, inhibited the development of a second primary HCC, and thus improved patient survival from this malignancy [10,11]. ACR inhibits the growth of HCC-derived cells via the induction of apoptosis by working as a ligand for retinoid receptors [12,13]. ACR also suppresses HCC cell growth and inhibits the development of liver tumors by inhibiting the activation and expression of several types of growth factors and their corresponding receptor tyrosine kinases (RTKs), which lead to the inhibition of the Ras/MAPK activation and RXRα phosphorylation [8,9,14-17]. These reports strongly suggest that ACR might be a promising agent for the prevention and treatment of HCC.

Phosphatidylinositol 3-kinase (PI3K) is activated by growth factor stimulation through RTKs and Ras activation, and plays a critical role in cell survival and proliferation in collaboration with its major downstream effector Akt, a serine-threonine kinase [18-20]. Increasing evidence has shown that aberrant activation of the PI3K/Akt pathway is implicated in the initiation and progression of several types of human malignancies, including HCC, indicating that targeting PI3K/Akt signaling might be an effective strategy for the treatment of cancers [18-22]. Several clinical trials have been conducted to investigate the safety and anti-cancer effects of therapeutic agents that inhibit the PI3K/Akt signaling cascade [18-20]. Combined treatment with a PI3K/Akt inhibitor and other agents, including MAPK inhibitors, might also be a promising regimen that exerts potent anti-cancer properties [23,24].

Combination therapy and prevention using ACR as a key drug is promising for HCC treatment because ACR can act synergistically with other agents in suppressing growth and inducing apoptosis in human HCC-derived cells [17,25-30]. The aim of the present study is to investigate whether the combination of ACR plus LY294002, a PI3K inhibitor, exerts synergistic growth inhibitory effects on human HCC cells, and to examine possible mechanisms for such synergy, predominantly focusing on the inhibitory effects on RXRα phosphorylation by a combination of these agents.

Methods

Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical (Tokyo, Japan). LY294002 was purchased from Wako (Osaka, Japan). Another PI3K inhibitor NVP-BKM120 (BKM120) was from Selleck Chemicals (Houston, TX, USA).

Cell lines and cell culture conditions

HLF, Huh7, Hep3B, and HepG2 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin. The Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in CS-S complete medium (Cell Systems). These cells were cultured in an incubator with humidified air containing 5% CO_2 at 37°C.

Cell proliferation assays

Three thousand HCC (HLF, Huh7, Hep3B, and HepG2) or Hc cells were seeded on 96-well plates in serum-free medium. Twenty-four hours later, the cells were treated with the indicated concentrations of ACR or LY294002 for 48 hours in DMEM supplemented with 1% FCS. Cell proliferation assays were performed using a MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The combination index (CI)-isobologram was used to determine whether the combined effects of ACR plus LY294002 were synergistic [25,27,30,31]. HLF cells were also treated with a combination of the indicated concentrations of ACR and BKM120 for 48 hours to examine whether this combination synergistically inhibited the growth of these cells.

Apoptosis assays

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 activity assays were conducted to evaluate apoptosis. For the TUNEL assay, HLF cells (1×10^6), which were treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 48 hours, were stained with TUNEL methods using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) [25]. The caspase-3 activity assay was performed using HLF cells that were treated with the same concentrations of the test drugs for 72 hours. The cell lysates were prepared and the caspase-3 activity assay was performed using an Apoalert Caspase Fluorescent Assay Kit (Clontech Laboratories, Mountain View, CA, USA) [30].

Protein extraction and western blot analysis

Protein extracts were prepared from HLF cells treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 12 hours because this treatment time was appropriate for evaluating the expression levels of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated Akt (p-Akt), and phosphorylated RXRα (p-RXRα) proteins [25,29,30]. Equivalent amounts of extracted protein were examined by western blot analysis using specific antibodies [25]. The anti-RXRα and anti-RARβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for ERK, p-ERK, Akt, p-Akt, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Beverly, MA, USA). The antibody for p-RXRα was kindly provided by Drs. S. Kojima

and H. Tatsukawa (RIKEN Advanced Science Institute, Saitama, Japan).

RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from HLF cells using an RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III Synthesis system (Invitrogen, Carlsbad, CA, USA) [32]. Quantitative real-time reverse transcription PCR (RT-PCR) analysis was performed using specific primers that amplify the RARβ, $p21^{CIP1}$, cyclin D1, and β-actin genes. The specific primer sets used have been described elsewhere [25,30].

RXRE reporter assays

HLF cells were transfected with RXR-response element (RXRE) reporter plasmids (100 ng/well in 96-well dish), which were kindly provided by the late Dr. K. Umesono (Kyoto University, Kyoto, Japan), along with p*RL*-CMV (*Renilla* luciferase, 10 ng/well in 96-well dish; Promega) as an internal standard to normalize transfection efficiency. Transfections were carried out using Lipofectamine LTX Reagent (Invitrogen). After exposure of cells to the transfection mixture for 24 hours, the cells were treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 24 hours. The cell lysates were then prepared, and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega) [25].

Statistical analysis

The data are expressed in terms of means ± SD. The statistical significance of the differences in the mean values was assessed using one-way ANOVA, followed by Tukey-Kramer multiple comparison tests. Values of <0.05 were considered significant.

Results

ACR and LY294002 cause preferential inhibition of growth in HLF human HCC cells in comparison with Hc normal hepatocytes

In the initial study, the growth inhibitory effect of ACR and LY294002 on HLF human HCC cells and on Hc hepatocytes was examined. ACR (Figure 1A) and LY294002 (Figure 1B) inhibited the growth of HLF cells with IC_{50} values of approximately 6.8 μM and 15 μM, respectively. On the other hand, Hc cells were resistant to these agents because the IC_{50} values of ACR and LY294002 for the growth inhibition of Hc cells were each greater than 50 μM (Figure 1). These results suggest that ACR and LY294002 preferentially inhibit the growth of HCC cells compared with that of normal hepatocytes.

ACR along with LY294002 causes synergistic inhibition of growth in HCC cells

Next, the effects of the combined treatment of ACR plus LY294002 on the growth of HCC-derived cells and Hc hepatocytes were examined. When HLF human HCC cells were treated with a range of concentrations of these agents, the CI indices for less than 1 μM ACR (0.5 or 1 μM) plus less than 10 μM LY294002 (5 or 10 μM) were 1+ (slight synergism), 2+ (moderate synergism), or 3+ (synergism). In particular, the combination of as little as 1 μM ACR (approx. IC_{15} value) and 5 μM LY294002 (approx. IC_{25} value) exerted synergistic growth inhibition because the CI-isobologram analysis yielded a CI index of 0.54 (3+), which indicates synergism [25,27,30,31], with this combination (Figure 2A,B, and Table 1). In other HCC cell lines, including Huh7, Hep3B, and HepG2 cell lines, similar findings were also obtained using Huh7 and Hep3B cells; the combination of 1 μM ACR plus 5 μM LY294002 significantly suppressed the growth of these cells (Figure 2C). In contrast, the growth of Hc normal hepatocytes was not affected by the combination of these agents; even a

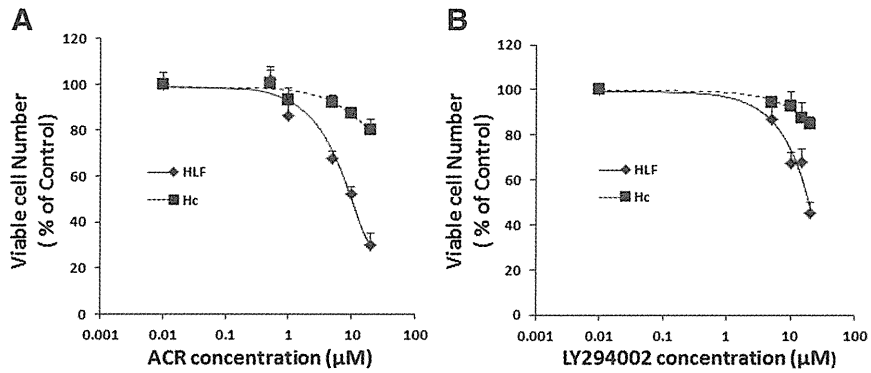

Figure 1 Inhibition of cell growth by ACR and LY294002 in HLF human HCC cells and Hc normal hepatocytes. HLF and Hc cells were treated with the indicated concentrations of ACR **(A)** or LY294002 **(B)** for 48 hours. Cell viability was determined by the MTS assay and expressed as a percentage of the control value. Error bars present the SD of triplicate assays.