managing intermediate and advanced stage HCC cannot be easily drawn due to these disparities in clinical practices and guidelines among Asian countries. Thus, a new staging system suitable for Asian HCC patients and a corresponding optimal treatment algorithm should be further investigated using evidence-based data, which finally make way for an Asian consensus for the management of intermediate and advanced stage HCC.

Disclosure Statement

The authors declare that they have nothing to disclose.

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Correlation between Insulin Resistance and Outcome of Pegylated Interferon and Ribavirin Therapy, Hepatic Steatosis, Hepatic Fibrosis in Chronic Hepatitis C-1b and High Viral Load

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Key Words

Insulin resistance · Hepatic fibrosis · Hepatic steatosis · Genotype 1b · High viral load · Pegylated interferon · Ribavirin · Sustained virological response

Abstract

Background/Aims: Insulin resistance (IR) has been reported to be an independent predictor of treatment outcome in chronic hepatitis C patients. ***Methods:*** We analyzed the relationship between IR and the outcome of pegylated interferon and ribavirin (PEG-IFN/RBV) therapy, taking into account host factors of body mass index and histological index, such as rate of fatty change and fibrosis. Japanese patients (n = 30; 19 men and 11 women; median age 60.0 ± 8.7 years) with chronic hepatitis C-1b with a high viral load were treated with PEG-IFN-α2b/RBV for 48 weeks. ***Results:*** Sustained virological response (SVR) was seen in 60% (18/30) and non-SVR in 40% (12/30). HOMA-IR (homeostasis model of assessment-insulin resistance index) at the start and at 24 weeks of treatment showed no statistical difference between SVR and non-SVR. Correlation was observed between HOMA-IR and body mass index (r = 0.45, p = 0.013). Among 20 patients, steatosis and fibrosis were assessed by biopsy. Correlation was observed between HOMA-IR and steatosis (r = 0.57, p = 0.0093), whereas no correlation was observed between HOMA-IR and fibrosis. ***Conclusion:*** A larger prospective study is needed to clarify the role of IR in the outcome of PEG-IFN/RBV combination therapy and hepatic fibrosis in Japanese patients.

Introduction

Chronic hepatitis C genotype 1b and a high viral load are known to be highly refractory to interferon (IFN) therapy. In Japan, such patients account for approximate-

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Table 1. Patient baseline characteristics

Age, years	60.0 ± 8.7
Males/females	19/11
Body weight, kg	60.2 ± 10.9
BMI	22.9 ± 3.8
HCV-RNA, log IU/ml	4.5 ± 1.7
AST, IU/l	63.4 ± 44.8
ALT, IU/l	71.3 ± 63.3
FBS, mg/dl	92.8 ± 16.4
γ-GTP, IU/l	84.1 ± 99.1
T-Cho, mg/dl	170.0 ± 39.6
TG, mg/dl	85.7 ± 39.4
HOMA-IR	2.1 ± 1.1

Data are shown as mean ± SD.

ly 70% of chronic hepatitis C cases, and various strategies have been investigated to improve treatment outcome. Currently, the first choice for refractory patients is a 48-week combined administration of pegylated IFN and ribavirin (PEG-IFN/RBV). Nonetheless, sustained virological response (SVR) is achieved in at most 50% of such patients [1, 2].

Host factors including HLA class I [3] and II [4], ethnicity [5] and body mass index (BMI) [6] also influence SVR [7]. Indeed, overweight [6] patients have shown characteristic resistance to combination therapy, and increased insulin resistance (IR) has been identified as an independent variable associated with a poor response [8]. Experimental and clinical studies have shown the role of hepatitis C virus (HCV) infection in the development of IR [9]. Patients with mild chronic hepatitis demonstrate a higher homeostasis model of assessment-insulin resistance index (HOMA-IR) than do healthy controls matched for age and BMI [8]. IR has also been implicated in the progression of fibrosis [10] and the development of steatosis [11, 12]. The latter finding is, however, observed mainly in European and American, including Caucasian and African, patients. The aim of this study was to analyze the relationship between IR and the outcome of PEG-IFN/RBV therapy, taking into account host factors of BMI and histological index, such as rate of fatty change and fibrosis, in Japanese patients with chronic hepatitis C-1b and high viral loads.

Patients and Methods

Patients

A total of 30 patients (19 men, 11 women; age 60.0 ± 8.7 years) seen at Kobe Asahi Hospital and diagnosed with chronic HCV-1b infection on the basis of the presence of anti-HCV antibodies and HCV-RNA, were enrolled in the study. The patients were treated with PEG-IFN-α2b (1.5 μg per kilogram body weight, once a week subcutaneously) and RBV (600–1,000 mg daily, per os) for 48 weeks, according to the standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. The HCV genotype was determined according to the method of Okamoto et al. [13]. Informed consent in writing was obtained from each patient, and the study protocol conformed to the ethical guidelines approved by the Ethics Committee in Kobe Asahi Hospital. The baseline characteristics of 30 patients are listed in table 1.

Laboratory and Histological Tests

Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV-RNA based on the COBAS TaqMan HCV test (Roche Diagnostics Corp., Basel, Switzerland). Fasting glucose and insulin were obtained at the start of and at 24 weeks of the treatment with PEG-IFN/RBV; IR was assessed by HOMA: [fasting insulin [μU/ml] × (fasting glucose [mg/dl]/18)]/22.5 [14, 15]; BMI was calculated as weight divided by height (kg/m^2).

Twenty biopsies were assessed for staging fibrosis and grading steatosis: fibrosis was staged on a scale from 0 to 4 according to the new classification by Desmet et al. [16]: with 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; 3, severe fibrosis, and 4, cirrhosis. Steatosis was graded on a scale from 0 to 4 according to the percentage of cells with fat: with 0, <5%; 1, 5–32%; 2, 33–65%; 3, ≥66%.

Statistical Analysis

Statistical differences in treatment responses according to patient baseline parameters of age, sex, body weight, BMI, HCV-RNA load, aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting blood sugar (FBS), γ-glutamyl transpeptidase (γ-GTP), total cholesterol (T-Cho) and triglyceride (TG) were determined by Fisher's exact test or the Mann-Whitney U test. Differences between the start of and at 24 weeks of therapy were assessed by the Wilcoxon signed rank test. Correlation between IR and the staging of fibrosis, the grading of steatosis and BMI was assessed by single regression analysis. Variables with a p value of <0.05 were considered statistically significant.

Results

Among the 30 patients, SVR was seen in 60% (18/30) and non-SVR in 40% (12/30). The baseline characteristics and the clinical responses are shown in table 2. Sex, body weight, BMI, HCV-RNA load, AST, ALT, FBS, γ-GTP, T-Cho and TG showed no significant difference between SVR and non-SVR, but age did ($p = 0.03$).

HOMA-IR at the start and at 24 weeks of treatment was 2.2 ± 1.0 and 2.5 ± 3.9 in SVR, and 2.0 ± 1.2 and 1.4 ± 0.5 in non-SVR, respectively, with no statistical difference between the two groups. BMI was ≥25 in 20% (6/30) and <25 in 80% (24/30) of patients, and correlation

Table 2. Baseline characteristics and the clinical response in SVR and non-SVR

	SVR (n = 18)	Non-SVR (n = 12)	p value
Age, years	57.1 ± 8.8	64.3 ± 6.6	0.03
Males/females	12/6	7/5	0.80
Body weight, kg	62.3 ± 12.0	57.0 ± 8.4	0.21
BMI	23.8 ± 4.0	21.8 ± 3.2	0.28
HCV-RNA, log IU/ml	4.7 ± 1.9	4.2 ± 1.4	0.85
AST, IU/l	65.2 ± 51.3	60.0 ± 34.7	0.82
ALT, IU/l	82.3 ± 76.0	54.8 ± 33.7	0.46
FBS, mg/dl	95.4 ± 20.0	88.8 ± 8.1	0.63
γ-GTP, IU/l	108.1 ± 121.9	48.3 ± 24.4	0.66
T-Cho, mg/dl	176.6 ± 44.0	160.3 ± 31.2	0.23
TG, mg/dl	88.1 ± 47.8	82.3 ± 23.2	0.85
HOMA-IR	2.2 ± 1.0	2.0 ± 1.2	0.53

Data are shown as mean ± SD.

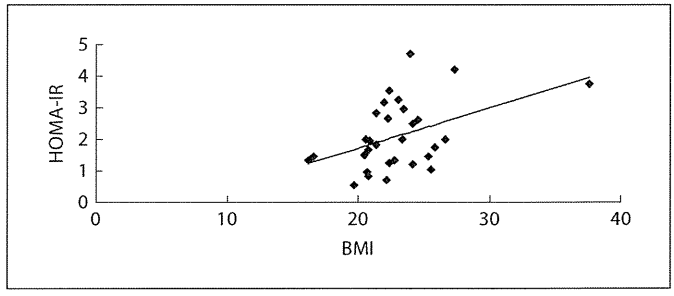

Fig. 1. Correlation observed between HOMA-IR and BMI (r = 0.45, p = 0.013).

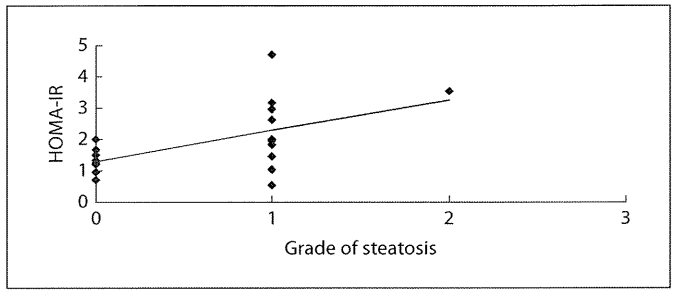

Fig. 2. Correlation observed between HOMA-IR and grade of steatosis (r = 0.57, p = 0.0093).

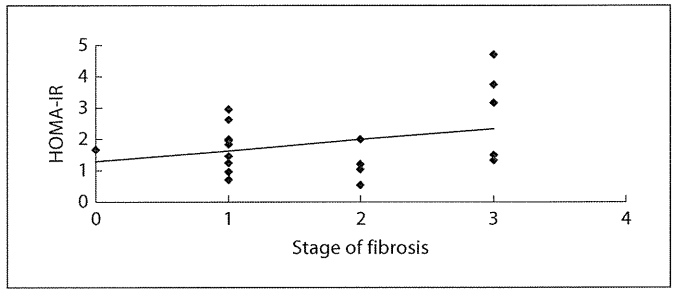

Fig. 3. No correlation was observed between HOMA-IR and stage of fibrosis (r = 0.32, p = 0.17).

was observed between HOMA-IR and BMI (r = 0.45, p = 0.013; fig. 1). Among 20 patients, steatosis assessed by histology revealed grade 0 in 45% (9/20), grade 1 in 50% (10/20), grade 2 in 5% (1/20), and grade 3 in 0% (0/20; fig. 2). Fibrosis was observed at stage F0 in 5% (1/20), F1 in 45% (9/20), F2 in 20% (4/20), F3 in 30% (6/20; fig. 3). Correlation was observed between HOMA-IR and steatosis (r = 0.57, p = 0.0093), whereas no correlation was observed between HOMA-IR and fibrosis (r = 0.32, p = 0.17).

Discussion

HCV genotype and HCV viral load remain the most important predictors of response to PEG-IFN/RBV combination therapy [17]. In contrast to HCV genotypes 2 and 3, which are significantly more susceptible to combination therapy with good outcome after standard or short-term treatment [18, 19], genotype 1 infection calls for developing more effective therapy and elucidating predictors of response conducive for optimizing individualized regimens. Therefore, the effect of host factors on the rate of SVR in anti-HCV therapy becomes a compelling concern for patients with unfavorable virological predictors.

IR, the stage of fibrosis and HCV genotype are independent predictors of response to anti-HCV therapy among Spanish patients [n = 159 (including genotype 1, n = 113); age, 41.7 years; BMI, 26.8]. In patients with genotype 1, a significantly lower SVR rate is observed in those with HOMA IR >2 than in those with HOMA-IR ≤2 (32.8%, 23/70 vs. 60.5%, 26/43, p = 0.007). The authors suggest that HOMA-IR might assist in further refining the prediction of antiviral response in genotype 1 patients [11]. In American, including African and Caucasian, patients with genotype 1 (n = 399; age, 47.7 years; BMI, 29.5), SVR rates of 49% for patients with HOMA-IR ≤2, and 36% for patients with HOMA-IR >2 have been

observed, the authors concluding that IR is independently associated with a low SVR rate [12]. HCV genotype 1b-infected Taiwanese patients (n = 150; age, 51.1 years; BMI, 23.5) with high IR demonstrated a lower SVR rate than those with low IR, suggesting the possible value of evaluating IR to predict response in HCV genotype 1b infection and a high pretreatment serum HCV-RNA level [20]. Noteworthy in that study is that the effect of HOMA-IR on response is observed in genotype 1b patients and particularly, for the first time, in those classified as 'difficult to treat' (genotype 1b infection and a high HCV-RNA level). Japanese patients with genotype 1b and a high viral load (n = 51; age, 57 years; BMI, 23.2) achieving SVR have lower HOMA-IR compared with non-SVR patients [21].

In the current study (BMI, 22.9), IR showed no difference between SVR and non-SVR patients, implying that the association of IR with response might be explained, in part, by ethnicity (Romero-Gomez, Spanish; Conjeevaram, American; the current study, Japanese), sample size (113, Spanish; 399, American; 150, Taiwanese, Dai's group; 51 Japanese, Mizuta, and 30 in the current study) and age (41.7, Spanish; 47.7, American; 51.1, Taiwanese, and 60.0 in the current study).

Since IR is a potentially modifiable factor, the response to the therapy might be improved by the modulation of insulin signaling and by improvements in IR and glucose control. The considerable potential for evaluating novel therapies and targets including insulin-sensitizing drugs for chronic hepatitis C patients deserves prospective investigation. Prospective studies for effective approaches resolving the IR issue before the initiation of combination therapy for chronic hepatitis C can significantly raise the SVR rate. HCV might induce IR irrespective of the severity of liver disease [8], and IR could be associated with severe hepatic fibrosis and might contribute to the progression of fibrosis in chronic HCV infection [8, 22, 23].

Around one to two thirds of liver biopsies from chronic hepatitis C patients show histological evidence of steatosis, which has been associated with being overweight, with hepatic fibrosis and TG levels [12, 24, 25]. Associations among IR, steatosis and liver fibrosis have been observed in chronic hepatitis C patients [25–29]. IR has been suggested as the cause, more than the consequence, of hepatic steatosis and fibrosis in patients with HCV, particularly in those with genotype 1 infection [30]. The mechanisms of the more obvious and crucial influence of IR, more than that of steatosis and fibrosis, need further study. In the current study, IR was found to be associated with BMI and steatosis, but not with hepatic fibrosis. The differences in the results regarding IR-associated hepatic fibrosis might also be explained by ethnic difference, sample size and age. Further large-scale study on Japanese patients is needed to clarify the role of IR in hepatic fibrosis.

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Disclosure Statement

None of the authors has any conflict of interest.

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Research Article

Cancer Prevention Research

Acyclic Retinoid Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} Mice

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Abstract

Obesity and the related metabolic abnormalities are associated with increased risk of hepatocellular carcinoma (HCC). Malfunctioning of retinoid X receptor (RXR) α due to phosphorylation by Ras/MAPK also plays a critical role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), which targets RXRα, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks, after which they were fed a diet containing 0.03% or 0.06% of ACR throughout the experiment. In mice treated with either dose of ACR for 34 weeks, the development of liver cell adenomas was significantly inhibited as compared with basal diet-fed mice. ACR markedly inhibited the activation of Ras and phosphorylation of the ERK (extracellular signal-regulated kinase) and RXRα proteins in the livers of experimental mice. It also increased the expression of *RARβ* and *p21^{CIP1}* mRNA while decreasing the expression of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA in the liver, thereby restoring RXRα function. Administration of ACR improved liver steatosis and activated the AMPK protein. The serum levels of insulin decreased by ACR treatment, whereas the quantitative insulin sensitivity check index (QUICKI) values increased, indicating improved insulin sensitivity. The serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were decreased by ACR treatment, suggesting attenuation of the chronic inflammation induced by excessive fatty deposits. ACR may be, therefore, useful in the chemoprevention of obesity-related HCC. *Cancer Prev Res; 4(1); 128–36. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is a serious health-care problem worldwide. The risk factors associated with the development of HCC include chronic hepatitis B and/or hepatitis C infection, particularly with subsequent cirrhosis. Recent evidence also indicates that obesity and the related metabolic abnormalities, especially diabetes mellitus, increase the risk of HCC (1–3). In a rodent model, the occurrence of diethylnitrosamine (DEN)-induced liver tumorigenesis was found to be significantly higher in obese and diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) mice than in genetic control mice (4). Diabetes mellitus has been shown to increase the risk of primary HCC in patients with viral hepatitis (5). Insulin resistance is also significantly associated with the recurrence of stage I HCC after curative treatment (6). Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the insulin resistance syndrome, and in a subset of NAFLD patients, the condition progresses to nonalcoholic steatohepatitis, which involves severe inflammation and therefore poses the threat of HCC (7, 8). Coexistent obesity or steatosis exacerbates liver injury and fibrosis and thus is involved in liver tumorigenesis (9). Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and their treatment must target the prevention of this malignancy.

Acyclic retinoid (ACR, the same substance as NIK-333), a synthetic retinoid, apparently exerts chemopreventive effects on the development of HCC (10). It inhibits experimental liver carcinogenesis and suppresses the

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growth of HCC-derived cells by inducing apoptosis and causing cell-cycle arrest in G_0–G_1 (11–15). These effects of ACR are associated with its agonistic activity for distinct nuclear retinoid receptors—retinoid X receptors (RXR) and retinoic acid receptors (RAR), both of which have 3 subtypes (α, β, and γ; 16)—and subsequent expression of the ACR target genes *RAR*β and $p21^{CIP1}$ (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of postther-apeutic HCC recurrence and improved the survival rates of patients (17, 18). A phase II/III trial of ACR confirmed its effectiveness in preventing second primary HCC in hepatitis C virus–positive patients in a large-scale (n = 401) randomized, placebo-controlled trial; hazard ratio for recurrence-free survival with ACR 600 mg/d versus placebo was 0.27 (95% CI, 0.07–0.96) after 2 years randomization (19).

Among the retinoid receptors, RXRα is considered as one of the most important receptors with respect to the regulation of fundamental cell activities because it forms a heterodimer with other nuclear receptors and thereby acts as the master regulator of nuclear receptors (20). Recent studies indicate that phosphorylation of RXRα abolishes its ability to form a heterodimer with RARβ, and the accumulation of phosphorylated RXRα (p-RXRα, i.e., nonfunctional RXRα), which is caused by activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, plays a critical role in the development of HCC (10, 21, 22). On the other hand, the effects of ACR in suppressing growth and inducing apoptosis in HCC cells depend on the inactivation of Ras-ERK signaling system and subsequent RXRα dephosphorylation (15, 23, 24). In the present study, we examined the effects of ACR on obesity-related liver tumorigenesis by focusing on the inhibition of RXRα phosphorylation. We also examined whether ACR treatment improves the insulin resistance, liver steatosis, and inflammatory condition caused by obesity with DEN-treated *db/db* mice, a useful preclinical model, to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by chemopreventive drugs (4).

Materials and Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC, Inc. All mice received humane care and were housed at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. ACR was supplied by Kowa Pharmaceutical Co.

Experimental procedure

The experimental protocol, which was approved by the Institutional Committee of Animal Experiments of Gifu University, was as described previously (4). At 5 weeks of age, 40 *db/db* mice were randomly divided into 5 groups. All the mice in groups 1 (n = 10), 2 (n = 10), and 3 (n = 10) were given tap water containing 40 ppm of DEN for the first 2 weeks, which is sufficient to develop liver neoplasms in *db/db* mice (4). After DEN treatment, the mice in groups 2 and 3 were fed the basal diet CRF-1 (Oriental Yeast Co.) containing 0.03% ACR (group 2) or 0.06% ACR (group 3), respectively, with free access to the feed till the end of experiment. Group 4 (n = 5) was fed the CRF-1 diet containing 0.06% ACR. The mice in groups 1 and 5 (n = 5) were fed the CRF-1 diet throughout the experiment. The rationale for the doses (0.03% and 0.06%) selection of ACR was based on previous studies, in which similar doses of ACR inhibited experimental liver carcinogenesis induced by chemical agents (25, 26). At 41 weeks of age (after 34 weeks of ACR treatment), all the mice were sacrificed by CO_2 asphyxiation to check for the development of HCC, liver cell adenoma, and foci of cellular alteration (FCA).

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 lobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections of formalin-fixed, paraffin-embedded livers were stained routinely with hematoxylin and eosin (H&E) for histopathologic examination. The presence of HCC, liver cell adenoma, and FCA was judged according to previously described criteria (27). The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Ras activation assay

Ras activity was determined using a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Ras was precipitated in equivalent amounts of liver extract (50 μg) from DEN-treated mice (groups 1–3) by using Raf-1/Ras-binding domain-immobilized agarose, which was then subjected to Western blot analysis using anti-Ras antibody (24). The intensity of the blots was quantified using NIH imaging software Version 1.62.

Protein extraction and Western blot analysis

Total protein was extracted from the nontumor site of livers of DEN-treated mice, and equivalent amounts of proteins (30 μg per lane) were examined by Western blot analysis (4). Previously described primary antibodies for RXRα (ΔN-197 and D-20), ERK, phosphorylated ERK (p-ERK), Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, and GAPDH were used (15, 22, 28, 29). The ΔN-197 antibody is considered a specific antibody for the p-RXRα protein (22, 23). The GAPDH antibody served as a loading control.

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RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was isolated from the nontumor site livers of DEN-treated mice by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). cDNA was amplified from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative real-time reverse transcription PCR (RT-PCR) analysis was carried out as described previously (4). The specific primers used for amplification of the *TNF-α*, *IL-6*, *IL-1β*, and *β-actin* genes were as described previously (30). The primers for the amplification of *RARβ*, *$p21^{CIP1}$*, *cyclin D1*, *c-Jun*, and *c-Fos* genes are listed in Supplementary Table S1.

Clinical chemistry

Before sacrifice, the mice were fasted for 6 hours, and at sacrifice, blood samples were collected for assaying the serum concentrations of insulin, glucose, and TNF-α, which was as described previously (4, 29). The serum TNF-α (Shibayagi) levels were determined using an enzyme immunoassay according to the manufacturer's protocol. Insulin resistance was estimated by determining the quantitative insulin sensitivity check index (QUICKI) as follows: $\text{QUICKI} = 1/[\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin level and G_0 is the fasting glucose level, which correlates with the glucose clamp method (31).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (32). The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co.) according to the manufacturer's protocol. To visualize the intrahepatic lipids, Sudan III staining was conducted using the standard procedure with frozen sections.

Statistical analysis

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program Version 3.05 (GraphPad Software) for Macintosh. Differences among the groups were analyzed by either 1-way ANOVA or, as required, by 2-way ANOVA. When the ANOVA showed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Tukey–Kramer multiple comparisons test. The differences were considered significant when the 2-sided P value was less than 0.05.

Results

General observations

As shown in Table 1, no significant differences were observed in the body, kidney, and fat weights among the groups at the end of the study. A significant decrease in the liver weight was observed in the ACR-treated groups as compared with the basal diet-fed group ($P < 0.05$ or $P < 0.01$), irrespective of DEN treatment. Histopathologic examination showed the absence of ACR toxicity in the liver, kidney, and spleen (data not shown).

Effects of ACR on DEN-induced liver tumorigenesis in *db/db* mice

Table 2 summarizes the incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice from all groups. FCA developed in the livers of mice from all groups, irrespective of DEN treatment. On the other hand, liver cell adenomas developed only in the DEN-treated *db/db* mice. HCCs also developed in all DEN-treated groups; however, the incidence (10% in each group) wan not high. These findings might be associated with experimental protocol because the duration of the experiments (41 weeks) was sufficient to develop adenoma but not HCC. In mice treated with either dose (0.03% and 0.06%) of ACR, the incidence ($P < 0.01$ in each comparison) and multiplicity of adenoma ($P < 0.05$ or $P < 0.01$) were significantly inhibited compared to ACR-untreated mice. The number of FCA was also significantly decreased by ACR treatment, irrespective of DEN treatment ($P < 0.001$ or $P < 0.05$).

Effects of ACR on Ras activity and phosphorylation of RXRα, ERK, and Stat3 proteins in the livers of DEN-treated *db/db* mice

ACR prevents the growth of HCC cells by inactivating Ras-ERK and dephosphorylating RXRα, thereby restoring RXRα function (10, 15, 23, 24). Stat3 is also an ACR target for the inhibition of cancer cell growth (28). Therefore, the effects of ACR on the inhibition of Ras activity and phosphorylation of the RXRα, ERK, and Stat3 proteins were examined in this study by using an obesity-related liver tumorigenesis model. As shown in Figure 1A, the activity of Raf-1–bound Ras in the liver was significantly inhibited by treatment with either dose of ACR ($P < 0.01$). The expression levels of the p-ERK and p-RXRα proteins were also decreased by ACR treatment (Fig. 1B), indicating that ACR inhibits the development of obesity-related liver neoplasms, at least in part, by dephosphorylating RXRα and thereby restoring its function. At both doses, ACR also decreased the expression levels of the p-Stat3 protein in the livers of DEN-treated *db/db* mice (Fig. 1B).

Effects of ACR on the expression levels of RARβ, $p21^{CIP1}$, cyclin D1, c-Fos, and c-Jun mRNA in the livers of DEN-treated *db/db* mice

ACR inhibits the growth of HCC cells by increasing the cellular levels of RARβ and $p21^{CIP1}$ but decreasing the levels of cyclin D1, and these effects might be associated with the restoration of RXRα function (12–15). It also suppresses the growth of cancer cells by inhibiting the activity of AP-1, which comprises the Jun and Fos oncoprotein families (28). Therefore, the effect of ACR on the mRNA levels of these molecules was examined next. As shown in Figure 1C, quantitative real-time RT-PCR analysis indicated that ACR treatment

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Table 1. Body, liver, kidney, and fat weights of the experimental mice

Group no.	Treatment	No. of mice	Weight, g			
			Body	Liver	Kidney	Fat[a]
1	DEN alone	10	71.2 ± 8.8[b]	4.5 ± 0.8	0.9 ± 1.0	7.5 ± 2.2
2	DEN + 0.03% ACR	10	65.7 ± 7.2	3.3 ± 1.1[c]	0.5 ± 0.1	6.0 ± 1.5
3	DEN + 0.06% ACR	10	66.0 ± 7.4	3.0 ± 0.7[d]	0.5 ± 0.1	5.7 ± 1.3
4	0.06% ACR alone	5	66.0 ± 7.4	3.0 ± 0.7[e]	0.5 ± 0.1	5.7 ± 1.3
5	Basal diet	5	67.9 ± 7.8	4.8 ± 1.0	0.6 ± 0.1	6.2 ± 1.4

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SD.
[c]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[e]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

significantly increased the expression levels of *RARβ* and $p21^{CIP1}$ mRNA, especially *RARβ* mRNA, in the livers of DEN-exposed *db/db* mice ($P < 0.01$). On the other hand, the expression levels of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were significantly decreased by ACR treatment ($P < 0.01$).

Effects of ACR on hepatic steatosis and the activation of AMPK in the livers of DEN-treated *db/db* mice

Hepatic steatosis is considered a promoter of the development of HCC (8, 9). Therefore, whether ACR treatment enhances the accumulation of lipids in the liver of experimental mice was examined. Examination of Sudan III–stained sections revealed that ACR treatment significantly improved macrovesicular steatosis in the livers of DEN-treated *db/db* mice (Fig. 2A, top panels). The triglyceride levels in the liver were also significantly decreased in mice treated with ACR at either dose ($P < 0.05$) in comparison with those fed the basal diet (Fig. 2A, bottom graph). Moreover, ACR markedly phosphorylated (activated) the AMPK protein, which is a critical serine/threonine kinase that monitors cellular energy status (33), in the livers of the experimental mice (Fig. 2B).

Effects of ACR on insulin resistance in DEN-treated *db/db* mice

Insulin resistance plays a critical role in the development of HCC (1–6). Therefore, the effects of ACR on the levels of serum insulin and QUICKI values, which indicate the degree of insulin sensitivity, were examined in DEN-treated *db/db* mice. As shown in Figure 2C, the serum insulin level was decreased ($P < 0.05$) whereas the QUICKI value was increased in mice treated with 0.06% ACR ($P < 0.05$)

Table 2. Incidence and multiplicity of hepatic neoplasms and FCA in the experimental mice

Group no.	Treatment	No. of mice	Incidence		Multiplicity[a]		FCA
			Adenoma	HCC	Adenoma	HCC	(No./cm^2)
1	DEN alone	10	7/10 (70%)	1/10 (10%)	1.3 ± 1.2[b]	0.1 ± 0.3	15.1 ± 3.5[c]
2	DEN + 0.03% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.2 ± 0.6[e]	0.1 ± 0.3	6.6 ± 2.5[f]
3	DEN + 0.06% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.1 ± 0.3[g]	0.1 ± 0.3	2.8 ± 1.8[f]
4	0.06% ACR alone	5	0/5 (0%)	0/5 (0%)	0	0	3.0 ± 2.8[h]
5	Basal diet	5	0/5 (0%)	0/5 (0%)	0	0	8.0 ± 1.2

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[d]Significantly different from group 1 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[g]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[h]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

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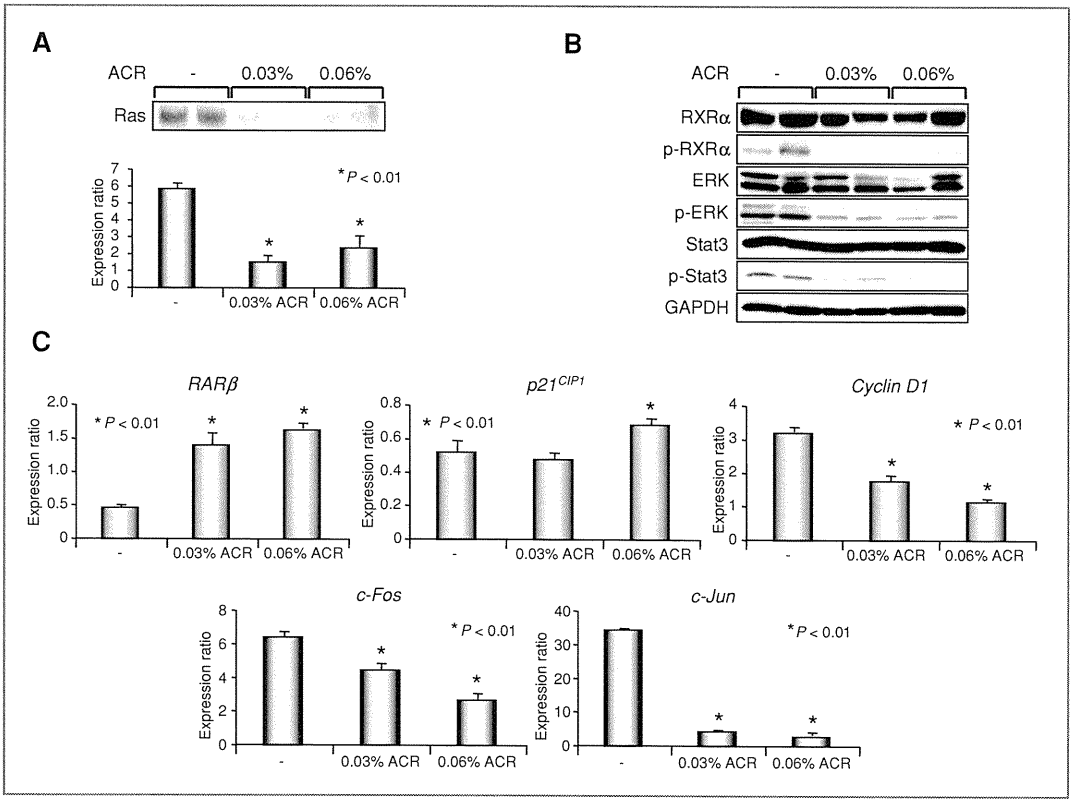

Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras activation assay kit (top). The relative intensity of the blots was quantified by densitometry and is displayed in the bottom graph. B, the expression levels of the RXRα, p-RXRα, ERK, p-ERK, Stat3, and p-Stat3 proteins were examined by Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from two different mice from each group. Repeat Western blots yielded similar results. C, the expression levels of *RARβ*, *p21^{CIP1}*, *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were examined by quantitative real-time RT-PCR using specific primers. β-Actin was used as a control. Each experiment was performed in triplicate, and the average value was calculated. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

compared with those in the basal diet–fed group. These findings suggest that ACR improves insulin resistance in obese and diabetic *db/db* mice.

Effects of ACR on the serum levels of TNF-α and hepatic expression of TNF-α, IL-6, and IL-1β mRNA in DEN-treated *db/db* mice

Because a state of chronic inflammation induced by excessive production of storage lipids and insulin resistance is associated with obesity-related liver carcinogenesis (34), the effects of ACR on the levels of the proinflammatory cytokines TNF-α, IL-6, and IL-1β in DEN-treated *db/db* mice were examined. As shown in Figure 3A, the serum levels of TNF-α were decreased after ACR treatment ($P < 0.01$). Furthermore, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were also significantly decreased by ACR treatment ($P < 0.01$). The decrease was most apparent in the levels of *IL-6* mRNA: the inhibition rates were about 85% at both doses of ACR (Fig. 3B).

Discussion

In the present health care scenario, the effects of obesity, including the promotion of cancer, are critical issues that need to be resolved and HCC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities (1–3, 5, 6). A recent clinical trial revealed that supplementation of food with branched-chain amino acids (BCAA), which improves insulin resistance (35), reduced the risk of HCC in obese patients with chronic viral liver disease (3). BCAA supplementation also suppresses liver tumorigenesis in obese and diabetic *db/db* mice by improving insulin resistance and attenuating liver steatosis and fibrosis (4). The results of the present study clearly indicated that ACR also effectively

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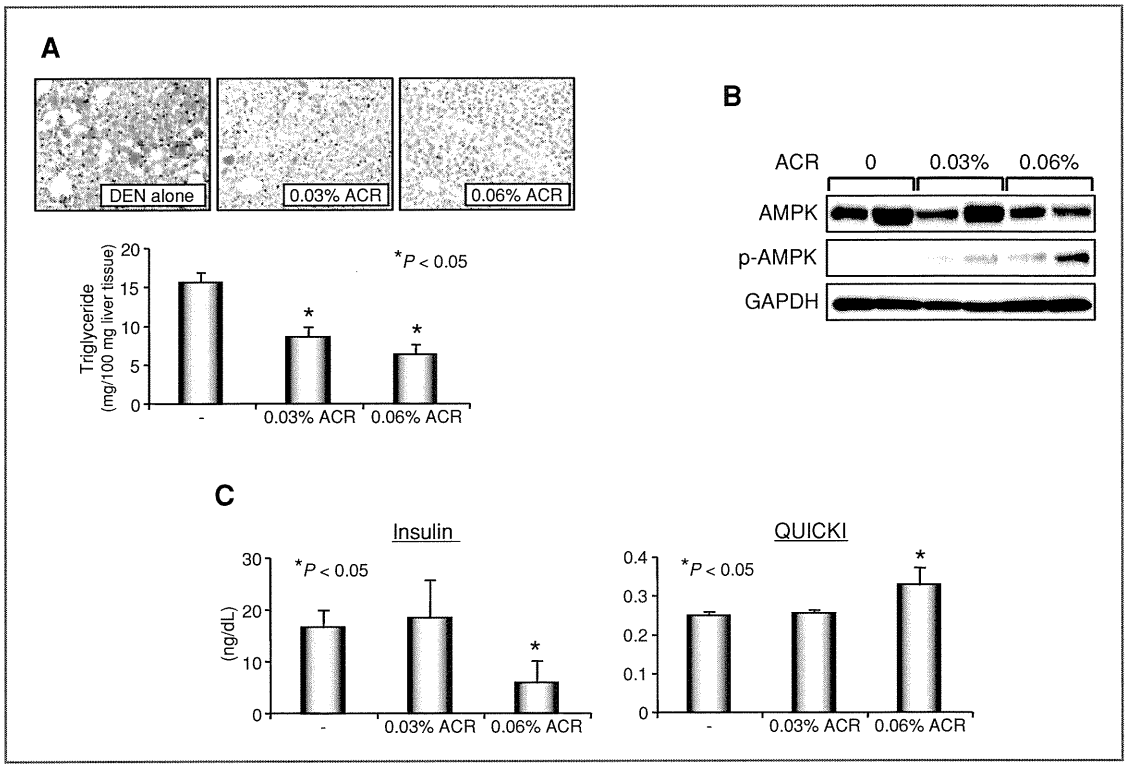

Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (top). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (bottom). B, the total proteins were extracted from the livers of DEN-treated mice, and the expression levels of the AMPK and p-AMPK proteins were examined by Western blot analysis, using the respective antibodies. A GAPDH antibody served as a loading control. C, the serum concentration of insulin was measured by enzyme immunoassay (left). The QUICKI value was calculated to evaluate insulin sensitivity (right). Values are the mean ± SD. *, $P < 0.05$ vs. ACR-untreated group.

prevents the development of obesity-related liver cell adenomas, and these effects are associated with improvement of hepatic steatosis and insulin resistance. Therefore, the findings of the present study, together with the results of previous studies using BCAA (3, 4), suggest that improvement of metabolic abnormalities by pharmaceutical or nutritional intervention might be an effective strategy for inhibiting obesity-related liver tumorigenesis.

Several biological effects of ACR are relevant to the prevention of obesity-related hepatotumorigenesis. First, it should be noted that ACR inhibits RXRα phosphorylation by suppressing the Ras/ERK signaling pathway in the livers of DEN-treated *db/db* mice. These findings are consistent with those of previous *in vitro* studies (15, 23, 24), but this is the first *in vivo* experiment, and the results seem to be significant because RXRα malfunction due to the phosphorylation by Ras-ERK plays a role in liver carcinogenesis and phosphorylated RXRα is therefore a critical target for HCC chemoprevention (10, 21). ACR suppresses the growth of HCC cells by inhibiting RXRα phosphorylation and restoring its original function as a master regulator of nuclear receptors (15, 22–24). Therefore, the expression levels of the *RARβ*, *$p21^{CIP1}$*, *cyclin D1*, *c-Fos*, and *c-Jun* genes, which are ACR targets (12–15, 28), were notably regulated by treatment with this agent. Among these molecules, RARβ seems to be the most important with respect to the induction of apoptosis (36). The upregulation of $p21^{CIP1}$, which negatively modulates cell-cycle progression, also activates the promoter region of the *RARβ* gene (37). Because RARβ can form a heterodimer with RXRα and thus synergistically inhibit the growth of HCC cells (14, 15), its induction might also have played a role in preventing the development of liver tumors in the present study. In addition, $p21^{CIP1}$ induction, which might be caused by activation of transforming growth factor (TGF)-β, also contributes to prevent the development of liver neoplasms because TGF-β induces senescence and inhibits growth in HCC cells by upregulating $p21^{CIP1}$ and ACR can activate latent TGF-β in liver stellate cells (38, 39).

Next, the effects of ACR in improving hepatic steatosis and insulin resistance, both of which accelerate HCC development (7–9), are discussed. These effects might also

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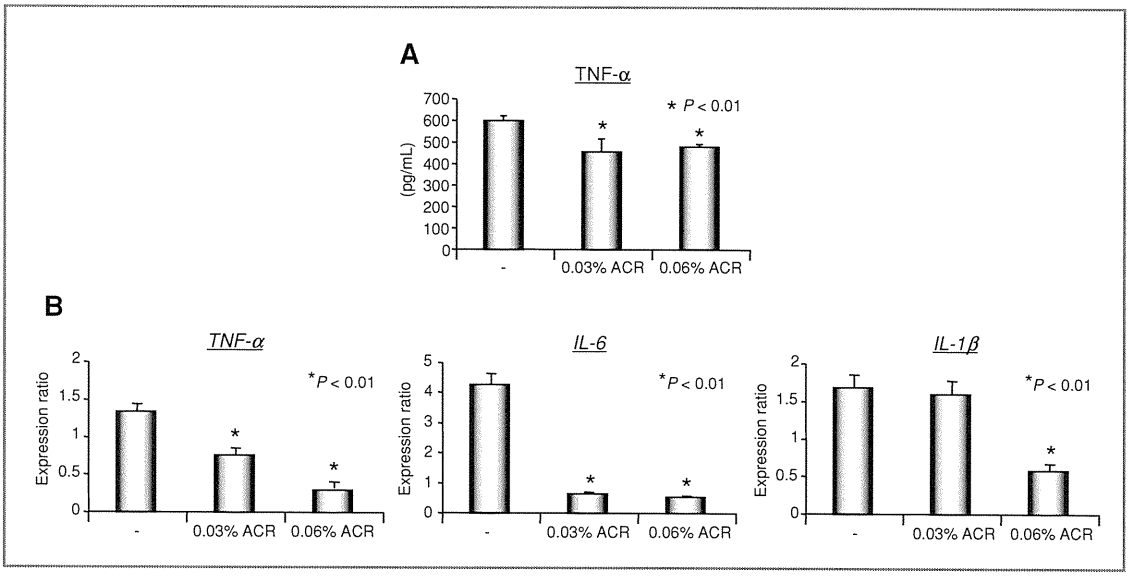

Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined by quantitative real-time RT-PCR using specific primers. The expression levels of these mRNAs were normalized to the level of the β-*actin* mRNA. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

be associated with RXRα dephosphorylation, as RXR can control insulin sensitization and lipid metabolism by forming a heterodimer with peroxisome proliferator-activated receptor (PPAR), an important molecule in the regulation of lipid homeostasis and energy metabolism (40). This speculation is interesting because the inhibition of RXRα phosphorylation and the activation of the RXR/PPAR heterodimer are also activities that cooperatively inhibit the growth of cancer cells (41). In addition, ACR might improve these metabolic abnormalities by activating AMPK, which increases glucose uptake and fatty acid oxidation but decreases fatty acid synthesis (33). This is another positive finding with regard to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a promising target for cancer chemoprevention (42, 43). For instance, a human study suggests that metformin, an AMPK activator used to treat type 2 diabetes mellitus, reduces the cancer risk in diabetic patients (44). Dietary energy restriction suppresses mammary tumorigenesis in rats by increasing the levels of activated AMPK (45). Pitavastatin, a lipophilic statin, was found to prevent obesity- and diabetes-related colon carcinogenesis in mice by activating AMPK in the colonic mucosa (29). These reports suggest the possibility that activation of AMPK by ACR aided in suppressing the development of obesity-related liver cells adenomas, as observed in the present study.

Insulin resistance and lipid accumulation in the liver produce inflammatory changes in the liver (7–9). ACR might decrease the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of experimental mice by improving hepatic steatosis and insulin resistance. These findings are significant because obesity-related HCC development clearly depends on enhanced production of TNF-α and IL-6, which cause hepatic inflammation and activate ERK and Stat3 (34). TNF-α, which lies at the core of the association between obesity and insulin resistance (46), contributes to obesity-induced IL-6 production and hepatocarcinogenesis (34). IL-6 is a major Stat3 activator in the liver, and the activation of the IL-6–Stat3 axis plays a critical role in HCC development (47, 48). In addition, uncontrolled activation of the Ras/ERK and Jak/Stat pathways is essential for HCC development (49). In the present study, ubiquitous activation of Ras-ERK signaling presumably caused accumulation of the p-RXRα protein in the liver of the obese mice. Our findings indicate that the effects of ACR in improving the inflammatory response and inhibiting Ras-ERK and Stat3 activation are crucial to prevent the development of obesity-related liver tumors.

Finally, it should be emphasized again that prevention of HCC by targeting hepatic steatosis, insulin resistance, and the state of chronic inflammation, which are caused by dysregulation of energy homeostasis, might be one of the promising strategies for the treatment of obese individuals who are at an increased risk of developing HCC (3, 4). ACR seems to be potentially effective and critical candidate for this purpose because it can improve hepatic steatosis and insulin resistance while also attenuating chronic inflammation. It inhibits RXRα phosphorylation induced by

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Ras-ERK activation, which might be associated with excess adipose tissue, and this effect is also important for preventing obesity-related liver tumorigenesis. The findings of the present study, together with the results of previous clinical trials indicating that ACR can significantly prevent the development of HCC in patients with viral cirrhosis without causing serious adverse effects (17–19), encourage the clinical usage of this agent for cirrhotic patients with obesity and diabetes. On the other hand, careful observation is required to apply a retinoid in clinical practice because of its potential toxicity. For instance, ACR may worsen hypertriglyceridemia in obese and diabetic subjects, which is a side effect observed in previous clinical trial (17), limiting the application of ACR to such subjects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Cancer Prevention Research

Preventive Effects of (−)-Epigallocatechin Gallate on Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BL/KsJ-*db/db* Mice

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Abstract

Obesity and related metabolic abnormalities, including insulin resistance and a state of chronic inflammation, increase the risk of hepatocellular carcinoma. Abnormal activation of the insulin-like growth factor (IGF)/ IGF-1 receptor (IGF-1R) axis is also involved in obesity-related liver tumorigenesis. In the present study, we examined the effects of (−)-epigallocatechin gallate (EGCG), a major biologically active component of green tea, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks and then they received drinking water containing 0.1% EGCG for 34 weeks. At sacrifice, drinking water with EGCG significantly inhibited the development of liver cell adenomas in comparison with the control EGCG-untreated group. EGCG inhibited the phosphorylation of the IGF-1R, ERK (extracellular signal-regulated kinase), Akt, GSK-3β (glycogen synthase kinase-3β), Stat3, and JNK (c-Jun NH_2-terminal kinase) proteins in the livers of experimental mice. The serum levels of insulin, IGF-1, IGF-2, free fatty acid, and TNF-α were all decreased by drinking EGCG, which also decreased the expression of TNF-α, interleukin (IL)-6, IL-1β, and IL-18 mRNAs in the livers. In addition, EGCG improved liver steatosis and activated the AMP-activated kinase protein in the liver. These findings suggest that EGCG prevents obesity-related liver tumorigenesis by inhibiting the IGF/IGF-1R axis, improving hyperinsulinemia, and attenuating chronic inflammation. EGCG, therefore, may be useful in the chemoprevention of liver tumorigenesis in obese individuals. *Cancer Prev Res; 4(3); 396–403. ©2011 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadly cancers worldwide. Chronic inflammation of the liver and subsequent cirrhosis, which are mainly induced by infection with hepatitis B and hepatitis C viruses, are risk factors for HCC development. Increasing evidence also indicates that obesity and related metabolic abnormalities, especially diabetes mellitus, raise the risk of HCC (1–3). Several pathophysiologic mechanisms linking obesity, steatosis, and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade. Insulin resistance leads to an increased expression of TNF-α, a central mediator of chronic inflammatory diseases, and its dysregulation is associated with the development of steatosis and inflammation within the liver (4, 5). Hyperinsulinemia also upregulates the levels of insulin-like growth factors (IGF) and abnormal activation of the IGF/IGF-1 receptor (IGF-1R) axis contributes to the development of various types of human malignancies, including HCC (6, 7). These findings suggest that targeting insulin resistance may be an effective strategy for preventing the development of obesity-related HCC. A recent animal experiment revealed that supplementation with branched chain amino acids, which is used to improve protein malnutrition in patients with liver cirrhosis, prevents obesity-related liver tumorigenesis by targeting insulin resistance and the IGF/IGF-1R axis (8).

Green tea, a beverage commonly consumed worldwide, possesses anticancer and cancer chemopreventive properties, and (−)-epigallocatechin gallate (EGCG) is the most potent of the green tea catechins (GTC) with respect to exerting these beneficial effects (9, 10). EGCG inhibits cell proliferation and induces apoptosis in cancer cells by inhibiting activation of some types of receptor tyrosine kinases (RTK) and related downstream signaling pathways (11, 12). Among such RTKs, the IGF-1R is one of the critical targets of EGCG with respect to its anticancer effects. In

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human HCC- and colon cancer–derived cells, EGCG suppresses cell growth by inhibiting the activation of the IGF/IGF-1R axis and its downstream ERK (extracellular signal-regulated kinase) and Akt proteins (13–15). EGCG also overcomes the activation of the IGF/IGF-1R axis and thereby inhibits the development of colonic premalignant lesions in an obesity-related colon carcinogenesis model (16).

In addition to anticancer and cancer chemopreventive effects, GTCs, especially EGCG, seem to have antiobesity and antidiabetic effects (17, 18). GTCs also possess anti-inflammatory properties because they inhibit the expression of proinflammatory cytokines TNF-α and interleukin (IL)-6, which are also associated with cancer prevention by GTCs (19–21). Supplementation with GTCs decreases plasma levels of insulin, TNF-α, and IL-6 in a high-fructose diet-induced rat insulin resistance model (22). These reports suggest the possibility that long-term treatment with GTCs may be effective for preventing the progression of obesity-related diseases, including the development of HCC. In the present study, we examined the effects of EGCG on obesity-related liver tumorigenesis in male C57BL/KsJ-*db/db* (*db/db*) mice initiated with diethylnitrosamine (DEN) by focusing on the inhibition of the activation of the IGF/IGF-1R axis. We also investigated whether EGCG treatment improves hyperinsulinemia, liver steatosis, and inflammatory condition in this preclinical mouse model that can be used to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by candidate chemopreventive agents (8).

Materials and Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC, Inc., and were humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. EGCG was obtained from Mitsui Norin Co. Ltd.

Experimental procedure

At 5 weeks of age, a total of 30 *db/db* mice were randomly divided into the following 4 experimental and control groups: DEN alone (group 1, $n = 10$); DEN plus 0.1% EGCG (group 2, $n = 10$); 0.1% EGCG alone (group 3, $n = 5$); and no treatment (group 4, $n = 5$). All of the mice in groups 1 and 2 were given tap water containing 40 ppm DEN for the first 2 weeks of the experiment, which is sufficient to develop hepatocellular neoplasms in *db/db* mice (8). After DEN treatment, the mice in group 2 were given free access to tap water containing 0.1% EGCG until the end of the experiment. The mice in group 3 were given 0.1% EGCG throughout the experiment. The concentration of EGCG (0.1%), which was established according to the findings of previous chemopreventive studies (16, 23), was within the physiologic range after daily intake of GTCs in human per unit body weight basis (24). The mice in groups 1 and 4 were given tap water without EGCG. At 41 weeks of age (after 34 weeks of EGCG treatment), all of the mice were sacrificed to analyze the development of liver neoplasms and preneoplastic lesions.

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 sublobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections, prepared from formalin-fixed and paraffin-embedded tissue blocks, were subjected to hematoxylin and eosin staining for histopathology. The presence of HCC, liver cell adenoma, and foci of cellular alterations (FCA) was judged according to previously described criteria (25). The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Protein extraction and Western blot analysis

Total protein was extracted from the nontumorous areas of livers and equivalent amounts of proteins (20 μg/lane) were examined by a Western blot analysis (8). Previously described primary antibodies for IGF-1R, phosphorylated IGF-1R (p-IGF-1R), ERK, p-ERK, Akt, p-Akt, Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, glycogen synthase kinase (GSK)-3β, p-GSK-3β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used (16, 26, 27). The primary antibody for c-Jun NH_2-terminal kinase (JNK) and p-JNK was obtained from Cell Signaling Technology. GAPDH served as a loading control.

RNA extraction and quantitative real-time reverse transcriptase PCR

Total RNA was isolated from the nontumorous areas of livers by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). The cDNA was amplified from 0.2 μg of total RNA, using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time reverse transcriptase PCR (RT-PCR) analysis was done using specific primers that amplify the *TNF-α*, *IL-6*, *IL-1β*, *IL-18*, and *β-actin* genes, as described previously (26, 28).

Clinical chemistry

The blood samples, which were collected at the time of sacrifice after 6 hours of fasting, were used for chemical analyses. The serum concentrations of insulin (Shibayagi), TNF-α, (Shibayagi), IGF-1 (R&D Systems), and IGF-2 (R&D Systems) were determined by an enzyme immunoassay according to the manufacturers' protocols. The serum levels of free fatty acid (FFA) were measured with a standard clinical automatic analyzer (type 7180; Hitachi).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (29). The triglyceride levels in the liver were measured using the triglyceride *E*-test kit (Wako Pure Chemical Co.) according to the manufacturers' protocol. To visualize the intrahepatic

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Table 1. Body, liver, and fat weights of the experimental mice

Group no.	Treatment	No. of mice	Body wt, g	Relative wt, g/100g body wt	
				Liver	Fat[a]
1	DEN alone	10	73.3 ± 8.8[b]	6.1 ± 1.6	10.6 ± 2.1
2	DEN + 0.1% EGCG	10	71.6 ± 8.1	6.1 ± 1.3	7.4 ± 1.5[c]
3	0.1% EGCG alone	5	61.1 ± 7.1	7.3 ± 1.5	9.3 ± 1.2
4	Tap water	5	67.9 ± 7.9	7.1 ± 1.5	9.0 ± 1.4

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SD.
[c]Significantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.01$).

lipids, Sudan III staining was carried out using the standard procedure with frozen liver sections.

Statistical analysis

The results are presented as the means ± SD and were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software) for Macintosh. Differences among the groups were analyzed by either 1-way ANOVA or, as required, by 2-way ANOVA. When the ANOVA showed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by the Tukey–Kramer multiple comparisons test. The differences were considered significant when the 2-sided P value was less than 0.05.

Results

General observations

During the experiment, EGCG treatment in drinking water did not cause any clinical symptoms for toxicity. No significant differences were observed in the body weights or relative weights of the livers among the 4 groups at the end of the study (Table 1). In the DEN-treated groups, drinking EGCG (group 2) significantly reduced the relative weights of white adipose tissue (periorchis and retroperitoneum) as compared with the untreated group (group 1, $P < 0.01$) at the termination of the experiment. Histopathologic examination revealed the absence of toxicity of EGCG in the liver, kidney, and spleen (data not shown).

Effects of EGCG on DEN-induced liver tumorigenesis in *db/db* mice

The incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice of all groups are summarized in Table 2. Irrespective of DEN treatment, FCA developed in the livers of mice from all groups. However, the number of this preneoplastic lesion was significantly increased by treatment with DEN ($P < 0.001$). In the DEN-treated mice, EGCG in drinking water significantly inhibited the development of FCA in comparison with the untreated control mice ($P < 0.001$). The incidence ($P < 0.01$) and multiplicity ($P < 0.01$) of adenoma, which developed only in the DEN-treated mice, were also significantly decreased by EGCG. HCC developed only in the DEN-treated groups, but the incidence (10% in each group) was not high. These findings might suggest that the duration of the experiments (41 weeks) was sufficient

Table 2. Incidence and multiplicity of hepatic neoplasms and FCA in the experimental mice

Group no.	Treatment	No. of mice	Incidence		Multiplicity[a]		FCA, no./cm^2
			Adenoma	HCC	Adenoma	HCC	
1	DEN alone	10	7/10 (70%)	1/10 (10%)	1.4 ± 1.2[b]	0.1 ± 0.3	14.9 ± 4.2[c]
2	DEN + 0.1% EGCG	10	1/10 (10%)[d]	1/10 (10%)	0.1 ± 0.3[e]	0.1 ± 0.3	7.7 ± 3.0[f]
3	0.1% EGCG alone	5	0/5 (0%)	0/5 (0%)	0	0	5.8 ± 1.3
4	Tap water	5	0/5 (0%)	0/5 (0%)	0	0	8.2 ± 1.1

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 4 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[d]Significantly different from group 1 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.01$).
[f]Significantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.001$).

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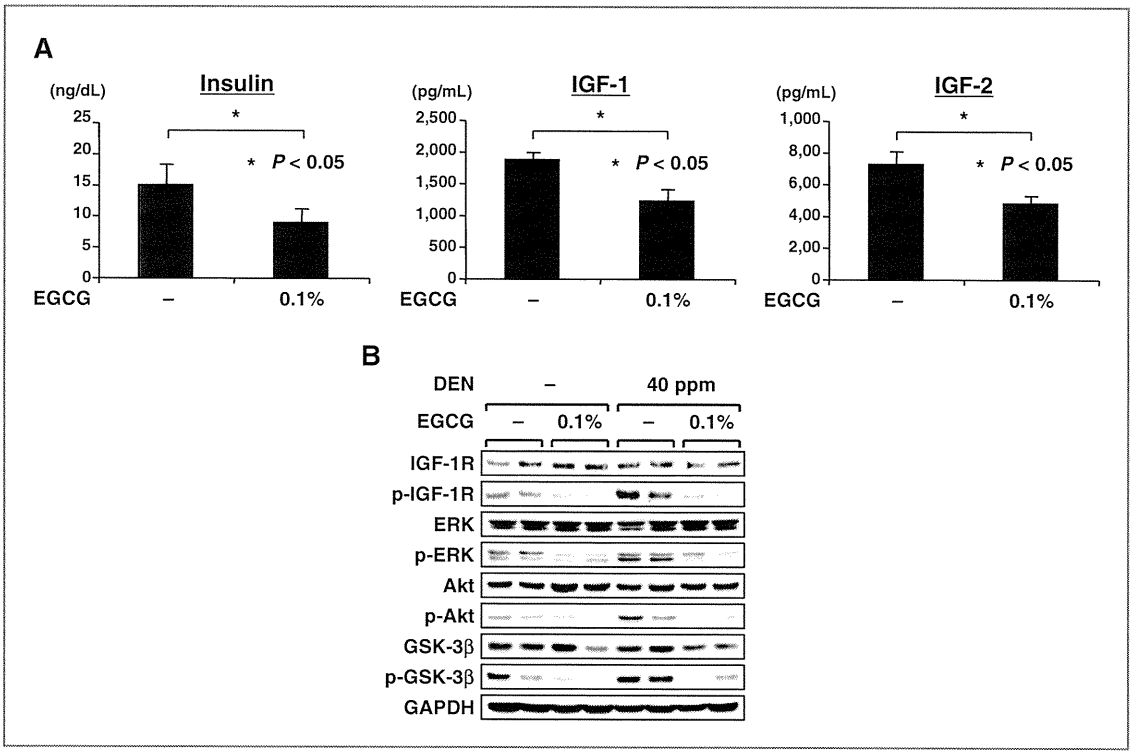

Figure 1. Effects of EGCG on the levels of serum insulin, IGF-1, and IGF-2 and on the activation of the IGF/IGF-1R axis in the liver of experimental mice. A, the serum concentrations of insulin, IGF-1, and IGF-2 in DEN-treated *db/db* mice were measured by an enzyme immunoassay. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, the total proteins were extracted from the livers of experimental mice and the expression levels of the IGF-1R, p-IGF-1R, ERK, p-ERK, Akt, p-Akt, GSK-3β, and p-GSK-3β proteins were examined by a Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from 2 different mice from each group. Repeat Western blots yielded similar results.

to develop adenoma but was relatively short to induce substantial number of HCC in the present study.

Effects of EGCG on the serum levels of insulin, IGF-1, and IGF-2 and on the phosphorylation of IGF-1R, ERK, Akt, and GSK-3β proteins in the livers of experimental mice

Hyperinsulinemia and abnormal activation of the IGF/IGF-1R axis play a critical role in obesity-related liver carcinogenesis (6, 7). Therefore, the effects of EGCG on the serum levels of insulin, IGF-1, and IGF-2 and the activation of IGF-1R protein in the liver of experimental mice were examined. As shown in Figure 1A, the administration of EGCG in the drinking water significantly decreased the serum levels of insulin, IGF-1, and IGF-2 ($P < 0.05$, respectively) in DEN-treated mice. Western blot analysis revealed that IGF-1R protein was phosphorylated (i.e., activated) by the administration of DEN but EGCG drinking decreased the levels of p-IGF-1R protein in the livers of experimental mice irrespective of DEN treatment. The levels of the phospholylated forms of the ERK and Akt proteins, which are located downstream of IGF-1R (30), were also decreased by EGCG drinking. In addition, the phospholylation of GSK-3β, which is mediated by the IGF-1R/Akt signaling pathway (31), was significantly inhibited by EGCG drinking. DEN treatment increased the levels of p-ERK, p-Akt, and p-GSK-3β proteins, but the inhibitory effects of EGCG on the expression of these proteins were not affected by the administration of this carcinogen (Fig. 1B). These findings indicate that DEN enhances liver tumorigenesis in *db/db* mice, at least in part, by activating the IGF/IGF-1R axis and EGCG may inhibit the development of obesity-related liver neoplasms by targeting hyperinslinemia and the activation of the IGF/IGF-1R axis.

Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of AMPK protein in the livers of DEN-treated *db/db* mice

Hepatic steatosis, which is caused by hyperinsulinemia and an increased FFA concentration, is considered to be involved in liver tumorigenesis (4, 5). Therefore, the effects of EGCG on the serum levels of FFA and accumulation of lipids in the liver of DEN-treated *db/db* mice were examined. The levels of FFA in serum were significantly

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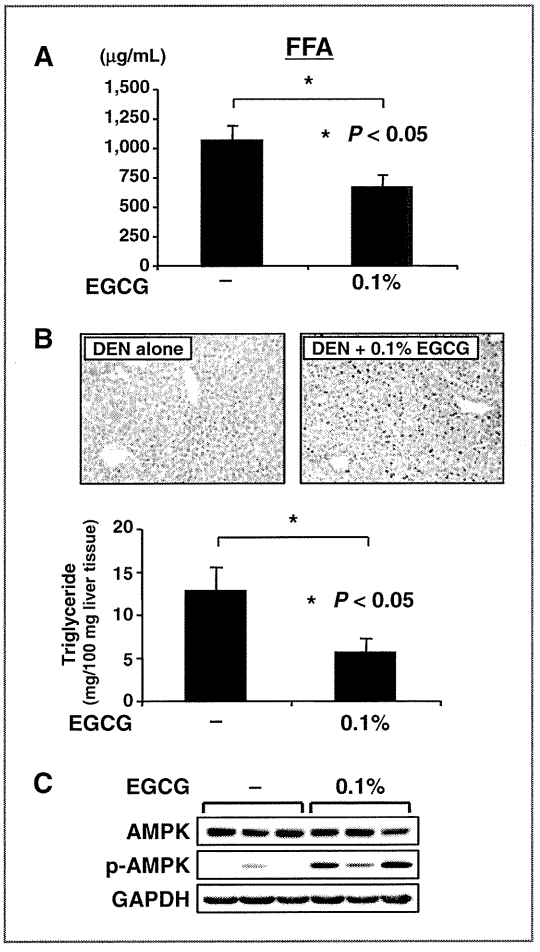

Figure 2. Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of the AMPK protein in the liver of DEN-treated *db/db* mice. A, the serum concentration of FFA was measured by an enzymatic method. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, frozen liver sections from DEN-exposed mice with or without EGCG treatment were stained with Sudan III to show steatosis (top). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (bottom). Values are the means ± SD. *, $P < 0.05$ versus the untreated group. C, the total proteins were extracted from the livers of DEN-treated mice, and the expression levels of the AMPK and p-AMPK proteins were examined by a Western blot analysis. GAPDH antibody served as a loading control. Three lanes represent protein samples from 3 different mice from the untreated and 0.1% EGCG-treated groups, respectively.

decreased by EGCG drinking (Fig. 2A, $P < 0.05$). The examination of Sudan III–stained sections showed that EGCG markedly improved the accumulation of lipids in the livers of DEN-treated mice (Fig. 2B, top panels). Similar to the histologic findings, the levels of triglyceride in the liver were significantly decreased by the administration of EGCG (Fig. 2B, bottom panel, $P < 0.05$). In addition, the expression levels of p-AMPK proteins were significantly increased by EGCG, thus indicating that the agent activated the AMPK protein, a central signaling system controlling the pathways of lipid metabolism (32), in the livers of the experimental mice (Fig. 2C).

Effects of EGCG on the hepatic expression of TNF-α, IL-6, IL-1β, and IL-18 mRNAs, serum levels of TNF-α, and the phosphorylation of Stat3 and JNK proteins in the livers of experimental mice

Obesity promotes liver tumorigenesis by inducing inflammation (33). Therefore, whether drinking EGCG altered the levels of the inflammatory mediators in the experimental mice was examined. As shown in Figure 3A, quantitative real-time RT-PCR revealed that the expression levels of TNF-α, IL-6, IL-1β, and IL-18 mRNAs in the livers, which were increased by DEN treatment ($P \leq 0.01$, respectively), were significantly decreased by EGCG ($P \leq 0.01$, respectively). The serum levels of TNF-α were also reduced after EGCG drinking in DEN-treated mice (Fig. 3B, $P < 0.01$). Furthermore, irrespective of DEN treatment, EGCG drinking decreased the expression levels of the p-Stat3 and p-JNK proteins, which play a role in obesity/TNF-α–mediated hepatic inflammation (34, 35) and are increased by DEN, in the livers of experimental mice (Fig. 3C). These findings suggest that EGCG improves hepatic steatosis and attenuates liver inflammation, which might be enhanced by DEN, in obese and diabetic *db/db* mice.

Discussion

Obesity and related metabolic abnormalities, particularly diabetes mellitus and insulin resistance, are significant risk factors for the development of HCC and therefore may be promising targets for the prevention of this malignancy in obese individuals (1–3, 8). The results of the present study clearly indicated that EGCG, which has been shown to improve dysregulation of energy homeostasis (17, 18), effectively prevents the development of liver tumorigenesis in obese and diabetic *db/db* mice by improving hyperinsulinemia and hepatic steatosis. A recent study showed that EGCG suppressed the development of colonic premalignant lesions induced by azoxymethane in *db/db* mice through improvement of hyperinsulinemia and inhibition of the IGF/IGF-1R axis on the colonic mucosa (16). These findings suggest that the improvement of metabolic abnormalities by either pharmaceutical or nutritional intervention may be an effective strategy to prevent certain types of obesity-related carcinogenesis and EGCG is a promising candidate for this purpose.

We showed that several biological activities of EGCG might contribute to the inhibition of obesity-related liver tumorigenesis in the present study. Among them, it should be emphasized first that EGCG decreases the serum levels of insulin, IGF-1, and IGF-2 while also inhibiting the activation of IGF-1R and related downstream signaling pathways, including the MAPK (mitogen-activated protein kinase)/ERK and PI3K (phosphatidylinositol 3-kinase)/Akt