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

		95% CI		
	OR	Lower	Upper	*P*-value
Men (vs women)	1.20	0.25	8.41	0.8242
Age (years)	1.06	0.93	1.23	0.3451
BMI	0.90	0.59	1.22	0.6036
Child B (vs A)	4.81	0.60	31.3	0.1253
ALB (g/dL)	0.08	0.01	0.45	0.0036
T-Bil (mg/dL)	2.75	0.27	19.7	0.3603
ALT (IU/L)	0.99	0.95	1.02	0.6923
PLT ($\times 10^4/\mu L$)	0.86	0.65	1.05	0.1770
PT (%)	0.95	0.87	1.01	0.1617
HOMA-IR	1.66	1.01	2.72	0.0420
HbA1c (%)	0.69	0.27	1.53	0.3850
AFP (ng/dL)	1.00	0.99	1.02	0.1242
PIVKA-II (mAU/mL)	1.00	0.96	1.03	0.6172

OR is shown with a unit increase in continuous variables.
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OR, odds ratio; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

95% CI = 0.0002–0.15, $P = 0.0001$) and HOMA-IR level (OR = 3.85, 95% CI = 1.57–14.2, $P = 0.0015$) as significant independent risk factors for recurrence.

Table 3 shows the patients' baseline characteristics and laboratory data divided according to HOMA-IR level (≤2.3 and >2.3). No significant differences were noted between the two subgroups except fasting plasma glucose and fasting immunoreactive insulin. Two patients in the HOMA-IR 2.3 or less subgroup took oral hypoglycemic drugs, sulfonylurea derivatives and voglibose. Three patients in the HOMA-IR more than 2.3 subgroup took oral hypoglycemic drugs; two took sulfonylurea derivatives and one took pioglitazone. No patient received insulin treatment.

We supplementally analyzed the data by excluding the patients under treatment with these oral hypoglycemics and also the patients with fasting plasma glucose above 140 mg/dL, in order to avoid possible unreliability in HOMA-IR evaluation. In nine patients, each remaining in HOMA-IR of 2.3 or less and HOMA-IR of more than 2.3, serum albumin (OR = 0.02, 95% CI = 0.0002–0.40, $P = 0.0060$) and HOMA-IR (OR = 3.49, 95% CI = 1.45–13.8, $P = 0.0033$) were still significant.

In a similar manner, evaluation of insulin sensitivity by QUICKI gave the results that lower QUICKI (≤0.33,

Table 3 Baseline demographic and clinical characteristics of patients classified according to HOMA-IR level

	HOMA-IR ≤2.3 ($n = 11$)	HOMA-IR >2.3 ($n = 13$)	*P*-value
Sex (male/female)	7/4	8/5	0.9157
Age (years)	70 (61–82)	74 (63–80)	0.1846
BMI	23.55 (19.5–33.5)	22.1 (19.5–25.1)	0.1219
Follow-up period (days)	393 (155–701)	337 (60–770)	0.2785
Child–Pugh classification (A/B)	10/1	10/3	0.3483
ALB (g/dL)	3.9 (2.4–4.4)	3.4 (2.7–4.4)	0.3304
ALT (IU/L)	40 (21–98)	53 (25–80)	0.6103
T-Bil (mg/dL)	0.8 (0.7–2.1)	1.0 (0.6–1.7)	0.6655
PLT ($\times 10^4/\mu L$)	8.5 (4.1–21)	8.9 (4.9–13.9)	0.5766
PT (%)	72 (56–95.5)	74 (58–118)	0.9953
FPG (mg/dL)	90 (75–119)	109 (86–155)	0.0151
FIRI (μg/dL)	7.98 (2.78–10.8)	14.1 (7.86–32.2)	0.0005
HOMA-IR	1.79 (0.76–2.27)	3.76 (2.91–7.39)	<0.0001
HbA1c (%)	5.05 (3.7–7.2)	5.3 (4.1–6.8)	0.6848
AFP (ng/dL)	23.2 (2.2–153.2)	28 (8–203)	0.7339
PIVKA-II (mAU/mL)	21 (9–127)	28 (9–67)	0.8071
Presence of oral hypoglycemic drugs (yes/no)	2/9	3/10	0.7678
Presence of insulin treatment (yes/no)	0/11	0/13	1.0000

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

i.e. impaired insulin sensitivity) was associated significantly with the increased risk of HCC recurrence (OR = 7.97, 95% CI = 1.32–152, $P = 0.0213$).

DISCUSSION

SEVERAL EPIDEMIOLOGICAL STUDIES have revealed the association of DM with cancer incidence and cancer mortality for various organs such as the liver, biliary tract, pancreas, endometrium, kidney, colon, bladder and breast.[7,18–21] The mechanism by which insulin acts as a carcinogenic factor is currently a focus of interests. First, insulin functions as a growth factor by phosphorylating insulin receptor substrate 1 and activating the downstream mitogen-activated protein kinase cascade, which affects cellular proliferation.[22,23] Second, hyperinsulinemia increases peripheral lipolysis and hepatic accumulation of free fatty acids, and the excess β-oxidation in mitochondria and microsome leads to the production of reactive oxygen species[24,25] that play a significant role in carcinogenesis.[26,27] Adipocyte-secreted cytokines (adipokines) such as tumor necrosis factor-α and interleukin-6 also play a significant role in both insulin residence and carcinogenesis.[28,29] Thus, these factors could cooperatively induce the insulin resistance and carcinogenesis.

We demonstrated in the present study that a higher HOMA-IR level increases the risk of early recurrence after initial curative RFA of stage I HCC in HCV-positive patients. This finding basically agrees with previous studies[7,8,18] that suggested an association between insulin resistance and carcinogenesis as described above, but HbA_{1c} level did not predict recurrence (Table 2). This might be explained by the clinical relevance that HbA_{1c} level in patients with liver cirrhosis is often underestimated because of anemia. Therefore, the results of the present study suggest that the role of hyperinsulinemia is more important than that of hyperglycemia as reflected by HbA_{1c} in the recurrence of HCC. We therefore should pay attention to levels not only of glucose and HbA_{1c} but also of insulin when we follow patients who are at risk for HCC.

Interventional modalities to improve insulin-resistance could be a key to prevent the primary or recurrent HCC in patients complicated with such metabolic disorders. For instance, metformin and thiazolidine derivatives could be potential candidates for this purpose.[30,31] Oral branched-chain amino acid (BCAA) granules might be a candidate for preventing HCC recurrence in DM cases because, in addition to improving hypoalbuminemia,[32,33] this agent improves insulin resistance without stimulating insulin secretion.[34] Improvements of insulin resistance and glucose tolerance by BCAA have been reported in clinical trials.[35,36] Furthermore, Muto *et al.*[37] described that oral supplementation with BCAA granules inhibited liver carcinogenesis in HCV-positive liver cirrhosis with DM and obesity. Such effect of BCAA is also supported in experimental models.[38,39] These reports,[32–39] together with our present findings (Table 2 and Fig. 1b), suggest that insulin resistance is a significant risk factor for early recurrence of HCC and thus might be a critical target to prevent the recurrence and development of second primary HCC.

A limitation of this study is that the therapeutic effect of the primary HCC was judged as curative by imaging diagnosis but not by surgical pathology. Although the recurrent HCC developed apart from the primary tumor, we could not totally differentiate the recurrent lesion and a second primary HCC. A higher recurrence rate in this study (Fig. 1a) might be explained by this fact. Advanced medical imagings such as positron emission tomography would help solving such limitations in future study of this kind. Furthermore, the basic question, if de novo, namely, the first or second primary liver carcinogenesis is regulated by insulin resistance, should be addressed by recruiting HCC-free cirrhotics. However, such study requires a larger sample size and a longer observation period. Instead, we focused on the recurrent HCC, including possible second primary tumors, which develop at a 2–3-fold higher incidence than the first primary one.

Other study limitations are the short observation period, the small number of recruited patients and also the small number of detected events. Such a sample size essentially raises the possibility of β-error, including the absence of the statistical power of AFP and PIVKA-II for predicting the recurrence (Table 2). Previous reports agree that these tumor markers are risk factors of HCC recurrence,[2,6] although some criticisms remain.[4,5] In addition, we should state that the small number of events particularly restricts the reliability of multivariate analysis, while the calculation itself was possible in our study.

In conclusion, we presented for the first time that insulin resistance is significantly associated with the early recurrence of stage I HCC after curative RFA in HCV-positive patients. Increased HOMA-IR, which sensitively reflects insulin resistance, might be a useful biomarker for prediction of high-risk patients who cause early recurrence of HCC.

ACKNOWLEDGMENTS

THIS WORK WAS supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture (no. 17015016 to H. M.) and from the Ministry of Health, Labor and Welfare of Japan (to H. M.).

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Published OnlineFirst September 27, 2011; DOI: 10.1158/1940-6207.CAPR-11-0340

Research Article

Cancer Prevention Research

Possible Role of Visfatin in Hepatoma Progression and the Effects of Branched-Chain Amino Acids on Visfatin-Induced Proliferation in Human Hepatoma Cells

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Abstract

Obesity and related metabolic abnormalities, including adipocytokine dysbalance, are risk factors for hepatocellular carcinoma (HCC). Visfatin, an adipocytokine that is highly expressed in visceral fat, is suggested to play a role in the progression of human malignancies. Branched-chain amino acids (BCAA) reduce the incidence of HCC in obese patients with liver cirrhosis and prevent obesity-related liver carcinogenesis in mice. In this study, we investigated the possible role of visfatin on HCC progression and the effects of BCAA on visfatin-induced proliferation of HCC cells. In patients with HCCs, serum visfatin levels were significantly correlated with stage progression and tumor enlargement. Visfatin preferentially stimulated the proliferation of HepG2, Hep3B, and HuH7 human HCC cells compared with Hc normal hepatocytes. Visfatin phosphorylated extracellular signal–regulated kinase (ERK), Akt, and GSK-3β proteins in HepG2 cells. LY294002 [a phosphoinositide-3-kinase (PI3K) inhibitor], PD98059 [a MAP/ERK 1 kinase (MEK1) inhibitor], CHIR99021 (a GSK-3β inhibitor), and BCAA significantly inhibited visfatin-induced proliferation in HepG2 cells. BCAA also inhibited phosphorylation of GSK-3β, increased cellular levels of $p21^{CIP1}$, caused cell-cycle arrest in G_0/G_1 phase, and induced apoptosis in HCC cells in the presence of visfatin. These findings suggest that visfatin plays a critical role in the proliferation of HCC cells and may be associated with the progression of this malignancy. In addition, BCAA might inhibit obesity-related liver carcinogenesis by targeting and, possibly, by overcoming the stimulatory effects of visfatin. *Cancer Prev Res; 4(12); 2092–100. ©2011 AACR.*

Introduction

In addition to established risk factors such as hepatitis and alcohol consumption, obesity and its related metabolic abnormalities raise the risk of hepatocellular carcinoma (HCC; refs. 1–4). Several pathophysiologic mechanisms linking obesity and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade (5). In obese individuals, increased adipose tissue leads to the expression of a variety of adipocytokines. Recently, the role of obesity-associated dysfunctional adipose tissue and subsequent adipocytokine dysbalance in carcinogenesis has attracted attention (6). Clinical trials have shown that adipocytokine disorders, including increased levels of leptin and decreased levels of adiponectin in the serum, are implicated in hepatocarcinogenesis (7, 8). Leptin induces proliferation and inhibits apoptosis in human HCC cells (9). These findings suggest that adipocytokine dysbalance may play an important role in the development and progression of HCCs.

Visfatin/pre-B-cell–enhancing factor, which was originally isolated from peripheral lymphocytes, has been described as a secreted growth factor for early B-cell proliferation (10). More recently, visfatin has also been characterized as an adipocytokine that is highly expressed in the visceral fat of humans and rodents. Increased levels of visfatin, which are positively correlated with the size of visceral fat deposits, are observed in various clinical conditions such as obesity and diabetes mellitus (11, 12). Abnormalities in serum levels of visfatin have also been reported in nonalcoholic fatty liver disease, which is a hepatic manifestation of metabolic syndrome (13). These results are somewhat conflicting, however, as both increased and decreased serum levels of this adipocytokine have been found in patients with nonalcoholic fatty liver disease (14, 15).

Furthermore, previous studies have shown that visfatin may play a role in the development and progression of certain types of human malignancies (16). For instance,

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doi: 10.1158/1940-6207.CAPR-11-0340

Published OnlineFirst September 27, 2011; DOI: 10.1158/1940-6207.CAPR-11-0340

colorectal cancer, the development of which is associated with metabolic abnormalities (17), is accompanied by the overexpression of visfatin (18). Serum visfatin level is a good biomarker of colorectal malignant potential and stage progression (19). Visfatin stimulation increases cell proliferation in prostate and breast cancer cells (20, 21), whereas the use of visfatin inhibitor exerts an antitumor effect by inducing apoptosis (22). These findings suggest that visfatin is one of the key adipocytokines that links obesity and tumorigenesis and thus may be an effective target for the inhibition of obesity-related carcinogenesis. However, no detailed studies of the relationship between visfatin and HCCs have yet been conducted.

Branched-chain amino acids (BCAA; leucine, isoleucine, and valine) are used in patients with liver cirrhosis to improve protein malnutrition (23). Recent clinical trials have shown that oral supplementation with BCAA prevents progressive hepatic failure, improves event-free survival in patients with chronic liver diseases, and reduces the risk of HCCs in these patients who are obese (body mass index $\geq$ 25; refs. 4, 24). BCAA supplementation also prevents obesity-related carcinogenesis in both the liver and the colorectum of diabetic mice (25, 26). In the present study, we measured serum visfatin concentration in patients with HCCs and examined whether it was correlated with stage progression and tumor enlargement. We also examined in detail the effects of visfatin on the acceleration of HCC cell proliferation, focusing on the activation of signaling pathways, and investigated whether BCAA suppresses visfatin-induced growth of HCC cells.

Materials and Methods

Patients and measurement of serum visfatin concentration

Eighty-five primary HCC patients who underwent initial treatment at our hospital from January 2006 to December 2008 were enrolled in this study. Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (27). The greatest diameter of HCC was determined with dynamic computed tomography or magnetic resonance imaging. Fasting serum samples were collected at the time of diagnosis, and serum levels of visfatin were determined by ELISA (AdipoGen). The study protocol was approved by the Institutional Review Board for human research, and all patients gave written informed consents to enter the study.

Materials

Recombinant human visfatin was purchased from PeproTech Inc. BCAA (total amino acid content, 12.28 mmol/L), ΔBCAA (10.28 mmol/L), and neutral amino acid media (12.28 mmol/L) were obtained from Ajinomoto Pharmaceuticals Co. ΔBCAA serves as basal medium and contains 17 amino acids except BCAA. The concentrations of amino acids in the medium are as follows (in mmol/L): glycine, 0.40; alanine, 0.40; serine, 0.40; threonine, 0.80; cystine, 0.20; methionine, 0.20; glutamine, 4.00; asparagine, 0.40; glutamic acid, 0.40; aspartic acid, 0.40; phenylalanine, 0.40; tyrosine, 0.40; tryptophan, 0.08; lysine, 0.80; arginine, 0.40; histidine, 0.20; and proline, 0.40. BCAA medium was prepared by adding 2 mmol/L BCAAs (0.952 mmol/L leucine, 0.476 mmol/L isoleucine, and 0.572 mmol/L valine) to ΔBCAA medium. The composition of BCAA (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage used for the treatment of decompensated liver cirrhosis in Japan (4, 24). The neutral amino acid medium was prepared by adding 2 mmol/L neutral amino acids (0.667 mmol/L each of alanine, serine, and glycine) to the ΔBCAA medium and served as an amino acid content-matched control for BCAA medium. LY294002 was purchased from Cell Signaling Technology; PD98059, from Sigma; and CHIR99021, from Stemgent.

Cell lines and cultures

HepG2, Hep3B, and HuH7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank and maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum. Hc human normal hepatocyte cell line was purchased from Cell Systems and maintained in a CS-S complete medium (Cell Systems). The cell lines have been characterized by each source, and any further authentication was not done in our laboratory. These cells were cultured in an incubator with humidified air with 5% CO_2 at 37°C.

Cell proliferation assay

Cell proliferation assays were conducted by a cell proliferation kit [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT); Roche)] according to the manufacturer's instructions. To examine the effects of visfatin on the proliferation of the HepG2, Hep3B, HuH7, and Hc cells, these cells were seeded on 96-well plates (1×10^4 cells per well). After 16 hours of serum starvation, the cells were treated with the indicated concentrations (0–400 ng/mL) of exogenous visfatin for 48 hours in the absence of serum. To investigate the effect of LY294002, PD98059, CHIR99021, and BCAA, HepG2 cells were treated with these agents in the absence and presence of visfatin (100 or 400 ng/mL) for 48 hours in serum-free medium. All assays were conducted in triplicate.

Protein extraction and Western blot analysis

Total cellular protein was extracted and equivalent amounts of protein were examined by Western blot analysis (28). The primary antibodies used to detect the respective protein bands have been described previously (28). An antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The intensities of the blots were quantified with NIH Image software, version 1.62.

Cell-cycle assays

Cell-cycle assays were conducted by a cell-cycle detection kit (Cayman) according to the manufacturer's instructions. HepG2 cells were treated with BCAA for 48 hours in the

Published OnlineFirst September 27, 2011; DOI: 10.1158/1940-6207.CAPR-11-0340

absence and presence of 100 ng/mL visfatin. After the harvested cells were fixed and stained, they were analyzed for DNA histograms and cell-cycle phase distribution with a FACScan flow cytometer (BD). The data were analyzed with the CellQuest computer program (BD) as described previously (28).

Apoptosis assays

The Annexin V-binding capacity of treated cells was examined with flow cytometry by the Annexin V-FITC Apoptosis Detection Kit I (BD) to evaluate the induction of apoptosis. HepG2 cells were treated with BCAA for 48 hours in the absence and presence of 100 ng/mL visfatin. After the cultured cells were washed with cold PBS, they were incubated in Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 minutes on ice. Stained cells were analyzed within 1 hour. Annexin V-FITC–positive and PI-negative cells were counted as apoptotic cells as described previously (29).

Statistical analysis

The data are expressed as mean ± SD. The statistical significance of the difference in mean values was assessed with one-way ANOVA, followed by the Scheffe *t* test. Values of $P < 0.05$ were considered significant.

Results

Association of serum visfatin concentration with HCC clinical stage and tumor size

We initially analyzed the possible association of serum visfatin concentration with the clinical stage and tumor size (greatest diameter) of HCCs in 85 patients (54 men and 31 women, median age 73 years). The median serum visfatin concentration was 5.8 ng/mL (range: 1.2–42.0). We found that the progression of clinical stage was correlated with serum visfatin concentration; the level of this adipocytokine was significantly increased in stage IV patients compared with levels in those with stage I and II disease ($P < 0.05$; Fig. 1A). In 85 patients, the mean Pearson product-moment correlation coefficient (r) and the P value (P) of tumor size with serum visfatin concentration were 0.315 and 0.003, respectively (Fig. 1B). Moreover, similar results ($r = 0.326$ and $P = 0.01$) were obtained when patients with diabetes mellitus (HbA1c ≥ 6%) and/or obesity were excluded ($n = 53$, Fig. 1C), indicating a positive correlation between HCC tumor size and serum visfatin levels regardless of complications with obesity and diabetes.

Effects of visfatin on cell proliferation and phosphorylation of extracellular signal–regulated kinase, Akt, and GSK-3β proteins in human HCC cells

We next examined whether visfatin stimulates the proliferation of HCC cells by XTT assay. When series of HCC cells (i.e., HepG2, Hep3B, and HuH7 cells) were treated with visfatin (25–400 ng/mL) for 48 hours, cell proliferation was significantly stimulated in a dose-dependent

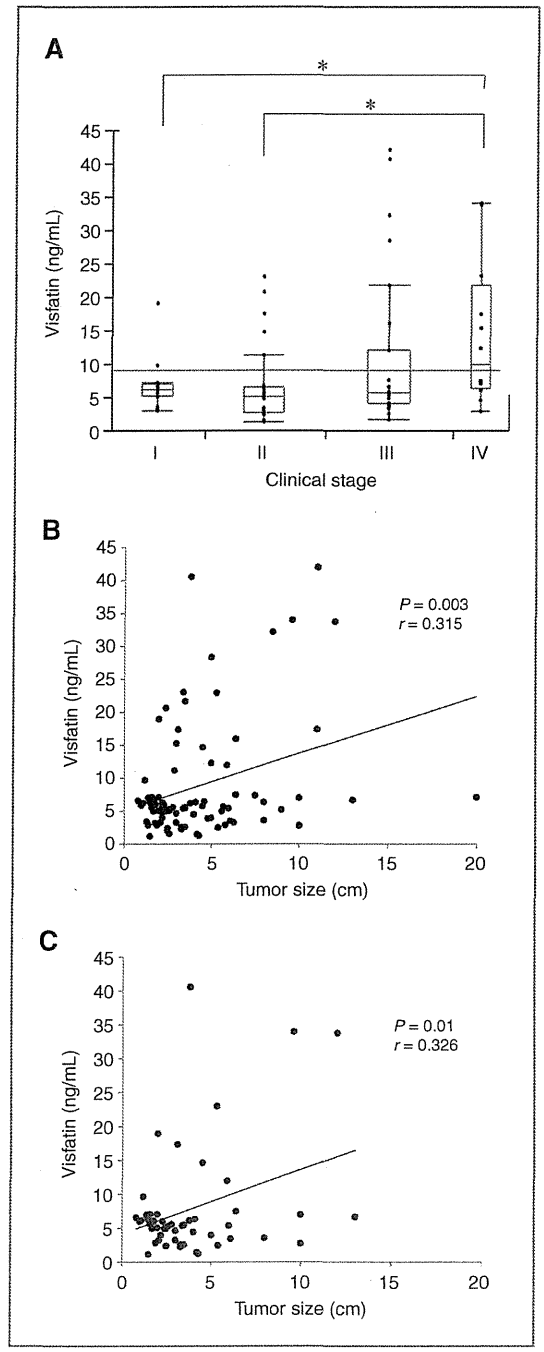

Figure 1. Correlation between serum visfatin concentrations and the clinical stage (A) and tumor size (B, C) of HCCs. A and B, the correlations were determined by analyzing 85 patients with primary HCCs. C, the correlation was determined by analyzing 53 HCC patients who are not obese and did not have diabetes mellitus. *, $P < 0.05$.

Published OnlineFirst September 27, 2011; DOI: 10.1158/1940-6207.CAPR-11-0340

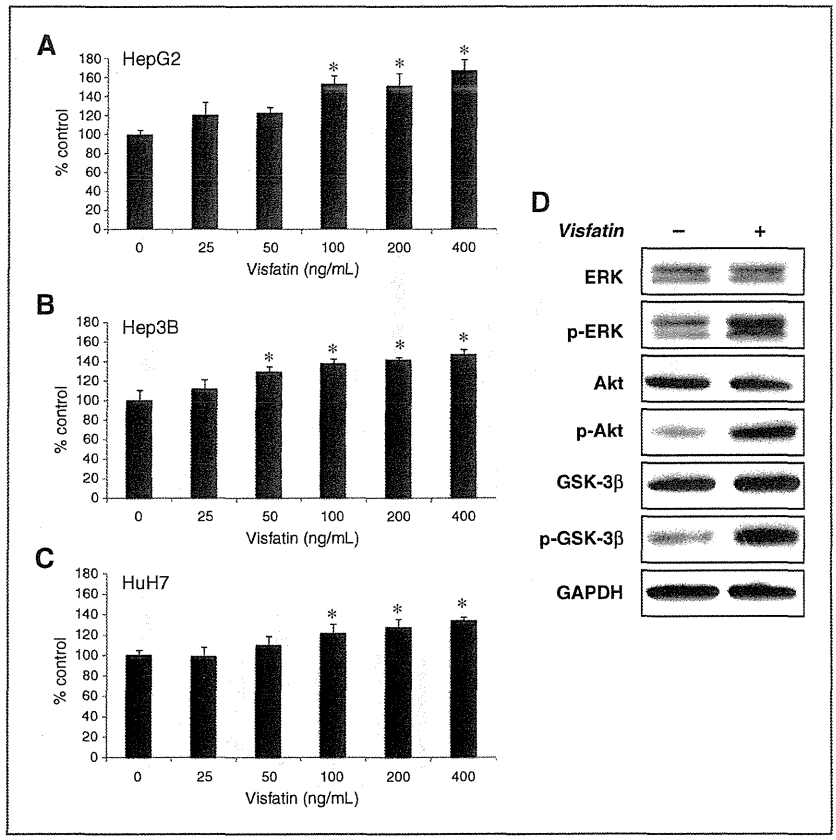

Figure 2. Effects of visfatin on the cell proliferation and phosphorylation of ERK, Akt, and GSK-3β proteins in HCC cells. HepG2 (A), Hep3B (B), and HuH7 (C) cells were treated with the indicated concentration of visfatin for 48 hours in serum-free medium. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *, $P < 0.05$. D, HepG2 cells were treated with and without 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed with a Western blot using respective antibodies. Equal protein loading was verified by the detection of GAPDH. Repeated Western blotting yielded similar results. p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; p-GSK-3β, phosphorylated GSK-3β.

manner ($P < 0.05$; Fig. 2A–C). In addition, treatment of HepG2 cells with 100 ng/mL of visfatin for 30 minutes caused a marked phosphorylation of extracellular signal–regulated kinase (ERK), Akt, and GSK-3β proteins (Fig. 2D), suggesting that visfatin might induce cell proliferation in HCC cells by activating PI3K/Akt and MAPK/ERK signaling pathways.

Effects of phosphoinositide-3-kinase, MAP/ERK 1 kinase, and GSK-3β inhibitors on visfatin-induced proliferation of HepG2 cells

We next examined whether pharmacologic inhibitors of phosphoinositide-3-kinase (PI3K; LY294002), MAP/ERK 1 kinase (MEK1; PD98059), and GSK-3β (CHIR99021) suppress visfatin-induced proliferation in HepG2 cells because the activation of PI3K/Akt and MAPK/ERK pathways might be involved in this proliferation (Fig. 2). As shown in Fig. 3, treatment with LY294002 (Fig. 3A), PD98059 (Fig. 3B), and CHIR99021 (Fig. 3C) significantly inhibited HepG2 cell proliferation both in the absence and presence of visfatin stimulation (100 and 400 ng/mL; $P < 0.05$). These findings suggest that PI3K and MAPK pathways could be effective targets for the inhibition of visfatin-induced proliferation in HepG2 cells.

Effects of BCAA on visfatin-induced proliferation of HepG2 cells

BCAA is reported to suppress obesity-related liver carcinogenesis (4, 25). Therefore, we next examined whether BCAA inhibits visfatin-stimulated proliferation of HepG2 cells because this adipocytokine, which is increased in obese individuals (11, 12), might play a role in the progression of HCCs (Fig. 1). As shown in Fig. 4A, the proliferation of HepG2 cells was significantly inhibited when the cells were treated in BCAA medium; meanwhile, this inhibition did not occur in neutral amino acid medium, which was served as an amino acid content-matched control for BCAA medium ($P < 0.05$). This finding possibly indicates that BCAA itself is specific in inhibiting the growth of HCC cells. In addition, a marked potentiation in the proliferative activity of HepG2 cells occurred after stimulation with 100 and 400 ng/mL visfatin, whereas BCAA treatment inhibited such proliferation in a dose-dependent manner regardless of visfatin stimulation ($P < 0.05$). The inhibition of proliferation with 2 mmol/L BCAA was greater (65% reduction) when the cells were cultured at higher concentration of visfatin (400 ng/mL) than that in the absence of the adipocytokine (41% reduction; Fig. 4B). In contrast, cell proliferation was not induced when Hc normal hepatocytes

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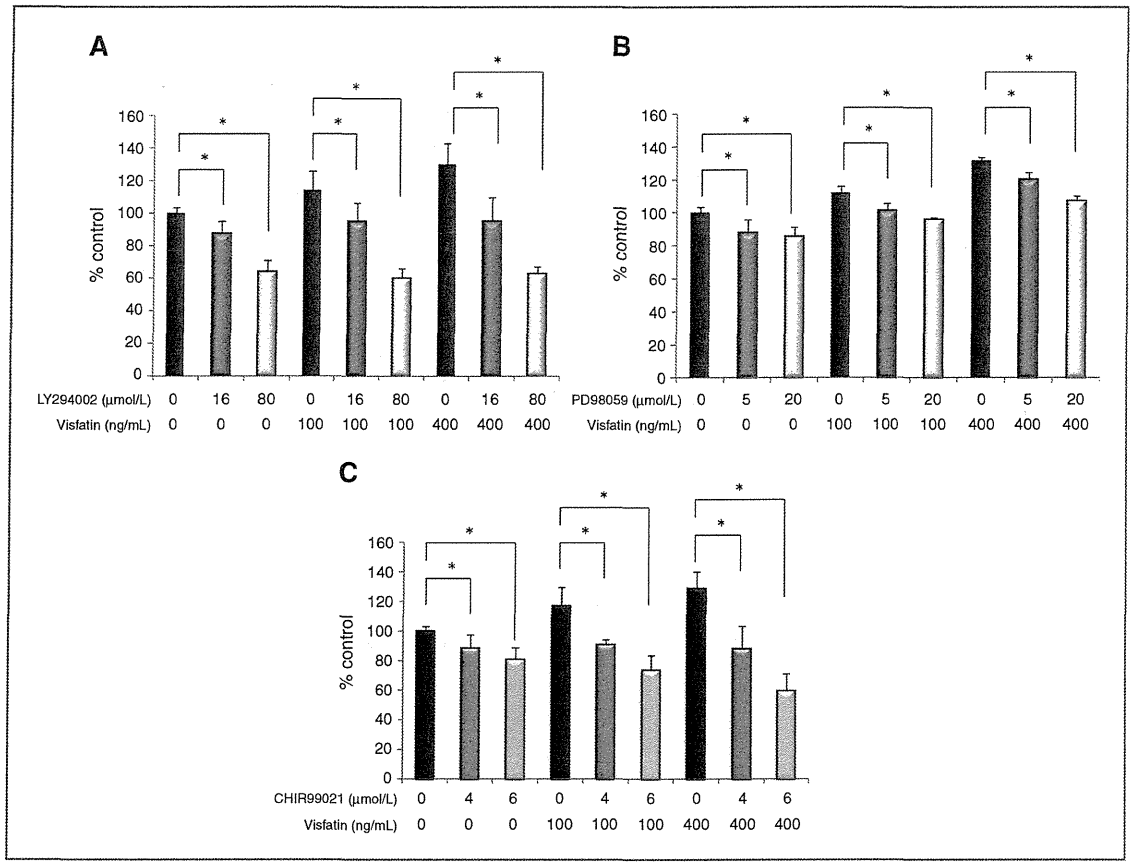

Figure 3. Effects of inhibitors of PI3K, MEK1, and GSK-3β on visfatin-induced proliferation in HepG2 cells. HepG2 cells were treated with LY294002, a PI3K inhibitor (A), PD98059, an MEK1 inhibitor (B), or CHIR99021, a GSK-3β inhibitor (C), in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *, $P < 0.05$.

were treated with similar concentrations of visfatin. BCAA also exerted no significant effect on the proliferation of Hc cells regardless of visfatin stimulation (Fig. 4C).

Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells

We next examined whether BCAA affected the phosphorylation of ERK, Akt, and GSK-3β proteins caused by visfatin in HepG2 cells. When the cells were stimulated by visfatin, the expression levels of phosphorylated (p)-GSK-3β protein were significantly decreased by BCAA treatment ($P < 0.05$; Fig. 5).

Effect of BCAA on cell-cycle progression, $p21^{CIP1}$ expression, and apoptosis induction in HepG2 cells in the presence and absence of visfatin

To determine whether the suppression of cell proliferation caused by BCAA (Fig. 4A and B) was associated with specific changes in cell-cycle distribution, we conducted cell-cycle analysis with DNA flow cytometry. When HepG2 cells were stimulated by visfatin for 48 hours, the percentage of cells in G_2/M phase (38%) was increased compared with that of cells not stimulated by visfatin (18%). Furthermore, regardless of visfatin stimulation, BCAA treatment increased the percentage of cells in G_0/G_1 phase; the percentage of cells in this phase was increased from 59% to 71% in the unstimulated cells and from 48% to 70% in the stimulated cells (Fig. 6A). Expression levels of $p21^{CIP1}$ protein, which suppresses tumors by promoting cell-cycle arrest (30), were also increased by BCAA treatment regardless of visfatin stimulation ($P < 0.05$; Fig. 6B). In addition, BCAA induced apoptosis in HepG2 cells because the percentage of Annexin V–positive cells was increased by the addition of BCAA in both the absence (2%–27%) and the presence (2%–10%) of visfatin stimulation (Fig. 6C).

Discussion

Obesity and related metabolic abnormalities are significant risk factors for the development of HCCs (1–5).

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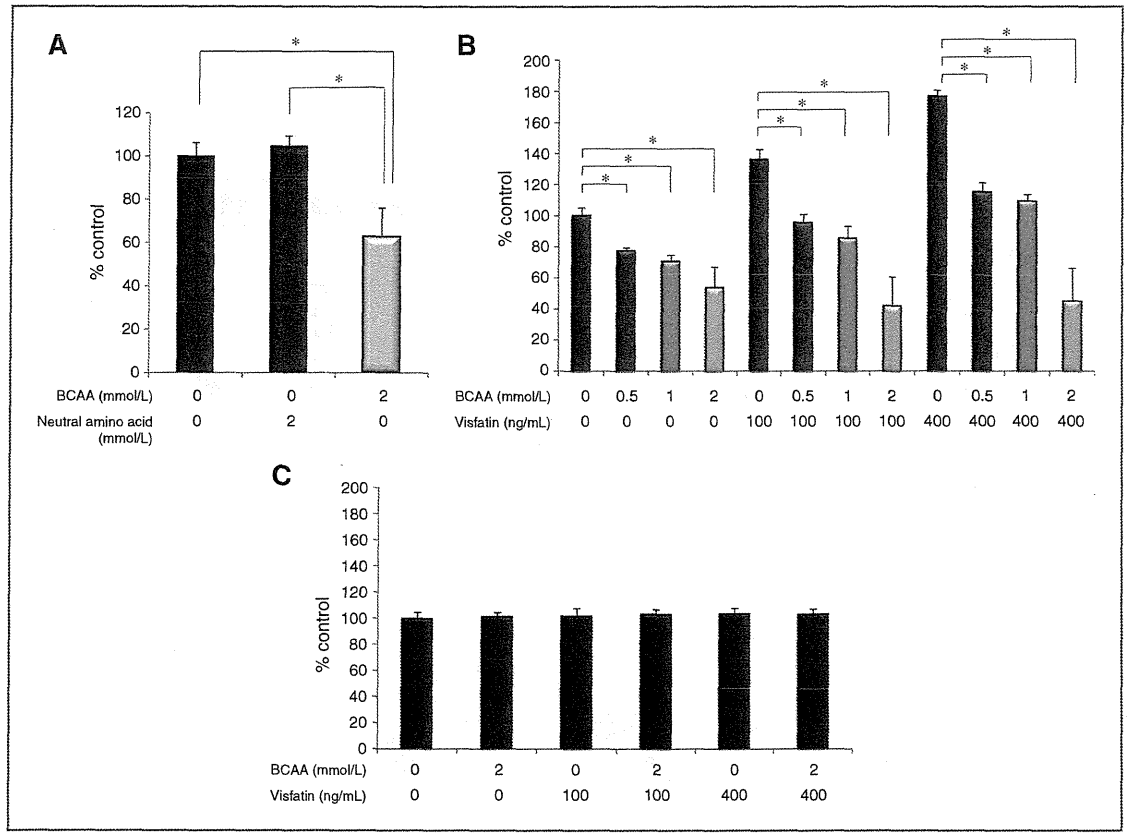

Figure 4. Effects of BCAA on visfatin-induced cell proliferation in HepG2 cells. A, HepG2 cells were treated in 2 mmol/L BCAA or 2 mmol/L neutral amino acid medium for 48 hours. Cell proliferation was evaluated by an XTT assay. HepG2 (B) and Hc (C) cells were treated with or without BCAA (0, 0.5, 1, and 2 mmol/L) in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars indicate SD values of triplicate assays. *, $P < 0.05$.

Among obesity-related metabolic disorders, adipocytokine dysbalance is considered to play a role in liver carcinogenesis (7–9); however, the detailed relationship remains unclear. The results of the present study provide the first evidence that higher levels of serum visfatin, which are frequently found in obese individuals (11, 12), are

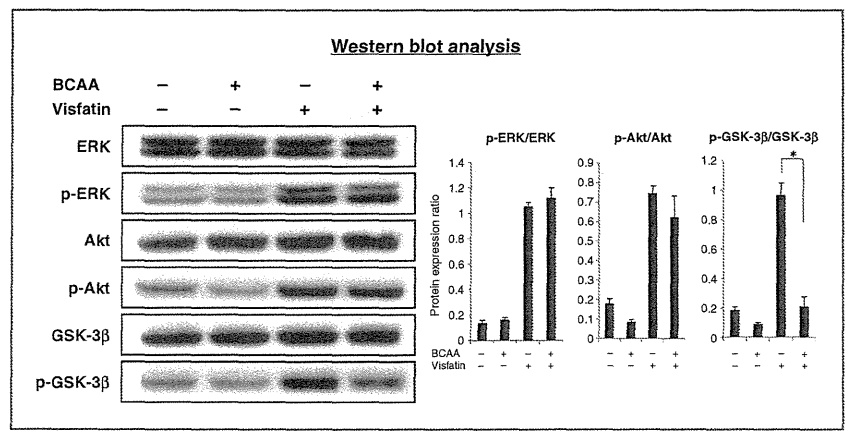

Figure 5. Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells. HepG2 cells were treated with or without BCAA in the absence or presence of 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed by Western blotting using corresponding antibodies (left). The intensities of the blots were quantified with densitometry. Columns and lines indicate mean ± SD (right). Repeated Western blotting produced similar results. *, $P < 0.05$. p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; p-GSK-3β, phosphorylated GSK-3β.

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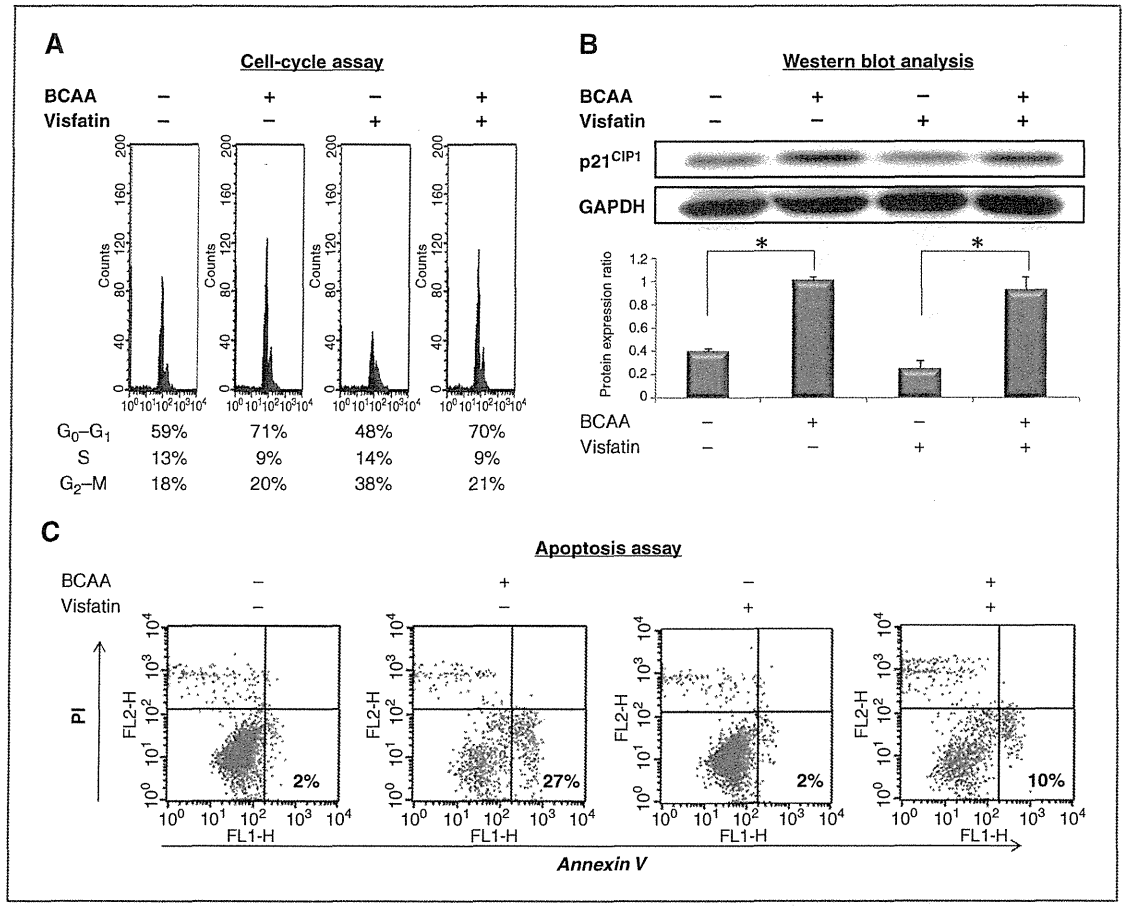

Figure 6. Effect of BCAA on the progression of cell cycle, expression of $p21^{CIP1}$, and induction of apoptosis in HepG2 cells in the presence and absence of visfatin. After treatment with and without BCAA in the presence and absence of 100 ng/mL visfatin for 48 hours, the cells were corrected and then used for cell-cycle assay (A), Western blot analysis (B), and apoptosis assay (C). A, the cells were stained with PI to analyze cell-cycle progression. B, total proteins were extracted from the cells, and the cell extracts were analyzed with a Western blot using anti-$p21^{CIP1}$ and GAPDH antibodies (top). The intensities of the blots were quantitated with densitometry. Columns and lines indicate mean and SD (bottom). *, $P < 0.05$. C, the cells were incubated with Annexin V–FITC to evaluate induction of apoptosis. Annexin V–FITC–positive and PI-negative cells were counted as apoptotic cells.

positively involved in stage progression and tumor enlargement in HCCs. On the other hand, the serum levels of other adipocytokines, including leptin, adiponectin, and resistin, are not associated with the stage progression of this malignancy (data not shown). Furthermore, visfatin stimulation strongly induced proliferation in a series of human HCC cells but not in Hc normal human hepatocytes. These findings suggest that visfatin, which might act as a growth factor in HCC cells, is one of the key adipocytokines that links obesity and the progression of HCCs. In addition, this study revealed that serum visfatin levels are significantly correlated with tumor enlargement of HCCs in patients who are not obese and do not have diabetes mellitus. A recent report has shown that visfatin is constitutively released from human HCC cells (31). This finding raises the possibility that visfatin is produced by HCC tissue itself, which might also explain the positive correlation between tumor size and serum visfatin levels observed in the present study. Therefore, our findings and the results of a previous report (31) together suggest that visfatin-dependent autocrine or paracrine loops contribute to abnormal proliferation in HCC cells.

The present study showed that visfatin induced cell proliferation in HepG2 cells by activating PI3K and MAPK signaling pathways because visfatin stimulation significantly increased phosphorylation of Akt, ERK, and GSK-3β proteins in these cells. These findings are consistent with previous reports that visfatin regulates a variety of signaling pathways, including PI3K/Akt, MAPK/ERK, and Stat3 (20, 32, 33). Visfatin stimulation also increases cell proliferation and ERK activity in prostate cancer cells (20). Moreover, recent experimental studies have shown that the activation of PI3K/Akt, MAPK/ERK, and Stat3 pathways is

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significantly associated with the development of liver tumors in obese mice, and that inhibiting the activation of these signaling pathways is critical to the prevention of obesity-related liver tumorigenesis (34, 35). These reports (34, 35), together with the present findings that specific inhibitors of PI3K, MEK1, and GSK-3β significantly suppress visfatin-induced proliferation in HCC cells, suggest that visfatin and its related signaling pathways might be effective targets for inhibiting obesity-related liver carcinogenesis.

BCAA, which was originally developed to improve protein malnutrition in patients with liver cirrhosis (23), produces improvements in metabolic abnormalities, especially insulin resistance and glucose tolerance (36, 37). BCAA supplementation also reduces the weights of white adipose tissue and improves liver steatosis in mice fed with a high-fat diet (38). In addition, long-term oral supplementation with BCAA is associated with a reduced frequency of HCCs in obese individuals (4). In rodent models, BCAA prevents obesity-related liver and colorectal carcinogenesis, and their beneficial effects are involved in the amelioration of insulin resistance and reduction of serum leptin levels (25, 26, 39). In the present study, BCAA significantly inhibited the proliferation of HCC cells stimulated by visfatin without affecting that of normal hepatocytes. This mechanism is a new one of BCAA that might explain the suppressive effects of this agent on obesity-related tumorigenesis. Therefore, the evidences in the present and previous studies (4, 25, 39) strongly support the active administration of BCAA as an HCC chemopreventive agent in patients with liver cirrhosis, especially obese patients who are at an increased risk for this malignancy. We are currently trying to gather evidence that BCAA prevents obesity-related liver carcinogenesis by targeting visfatin, in an ongoing animal study.

GSK-3β phosphorylation plays a critical role in cell survival, prevention of apoptosis, and progression of cell cycle in tumors (40). Therefore, the results of the present study suggest that BCAA might have inhibited visfatin-induced proliferation in HCC cells by, at least in part, inhibiting the phosphorylation of GSK-3β protein, which induces apoptosis and cell-cycle arrest in the G_0/G_1 phase in HepG2 cells. These findings are significant when considering the possibility of BCAA as a chemopreventive agent for HCCs because GSK-3β phosphorylation is closely associated with liver carcinogenesis (41). Phosphorylation of GSK-3β is also involved in the development of liver tumors in obese mice, and inhibition of this kinase effectively suppresses obesity-related liver tumorigenesis (35). Conversely, a recent study has shown that visfatin exerts antiapoptotic effects in HCC cells, and this might be associated with the enzymatic synthesis of NAD^+ (15). FK866, a visfatin inhibitor, effectively inhibited cell growth and induced apoptosis in human HCC cells by reducing cellular levels of NAD^+ (22). Further studies are required to clarify the effects of BCAA on the synthesis and regulation of NAD^+ and their relevance to the chemopreventive characteristics of this agent.

In summary, our data explained, for the first time, the molecular mechanisms responsible for HCC cell proliferation induced by visfatin, establishing a direct association between obesity and HCC progression. Because the evaluation of obesity-related metabolic disorders such as insulin resistance and hyperleptinemia are useful for predicting the risk of recurrence in HCCs (8, 42), we presume that, along with these metabolic abnormalities, measurement of serum visfatin levels might also have the potential to become a valuable biomarker for HCC development and progression. The results of the present study also indicate that targeting visfatin and related signaling pathways might be a promising strategy for the prevention or treatment of HCCs in obese patients with chronic liver disease. BCAA is potentially effective and critical candidate for this purpose because it can inhibit visfatin-mediated cell proliferation and activation of intracellular signaling pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Received July 5, 2011; revised September 8, 2011; accepted September 16, 2011; published OnlineFirst September 27, 2011.

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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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doi: 10.1158/1940-6207.CAPR-10-0331

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

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 ($P < 0.001$).

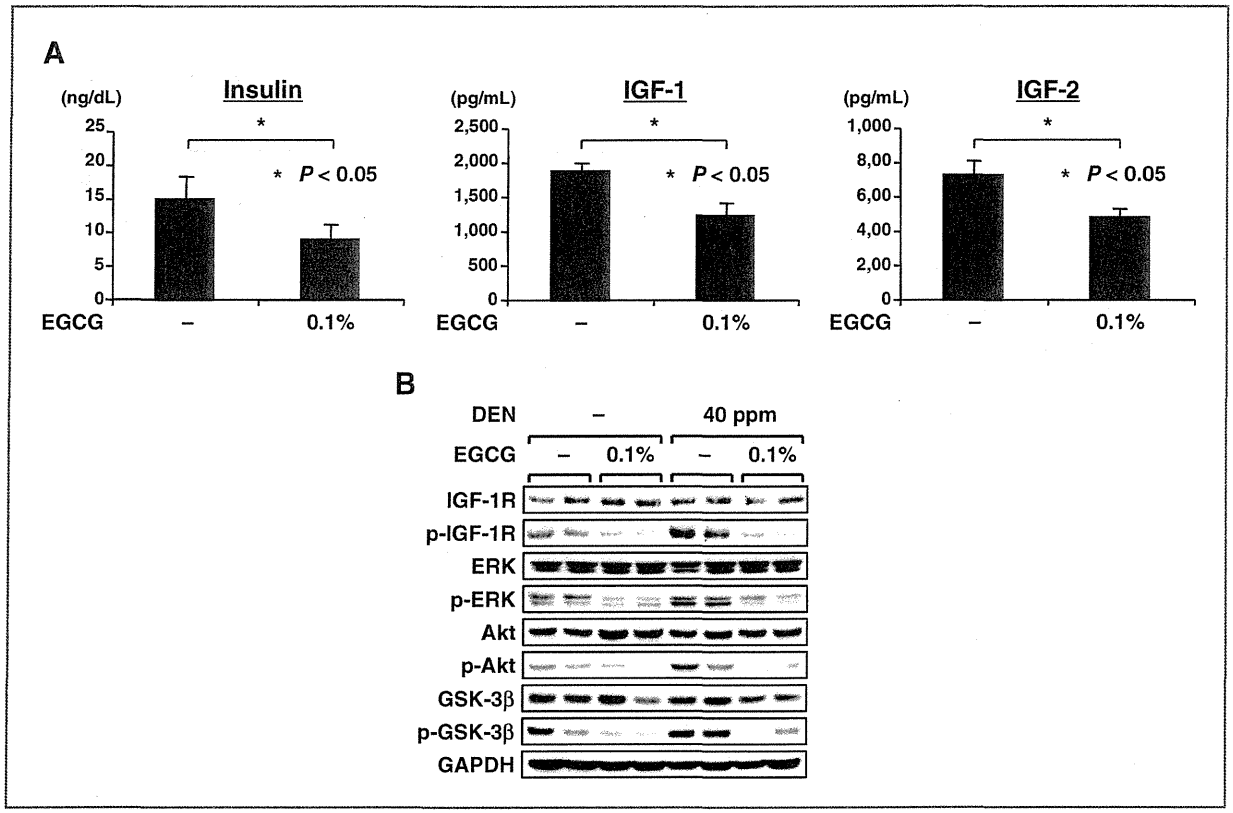

Figure 1. Effects of EGCG on the levels of serum insulin, IGF-1, and IGF-2 and on the activation of the IGF/IGF-1R axis in the liver of experimental mice. A, the serum concentrations of insulin, IGF-1, and IGF-2 in DEN-treated *db/db* mice were measured by an enzyme immunoassay. Values are the means ± 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

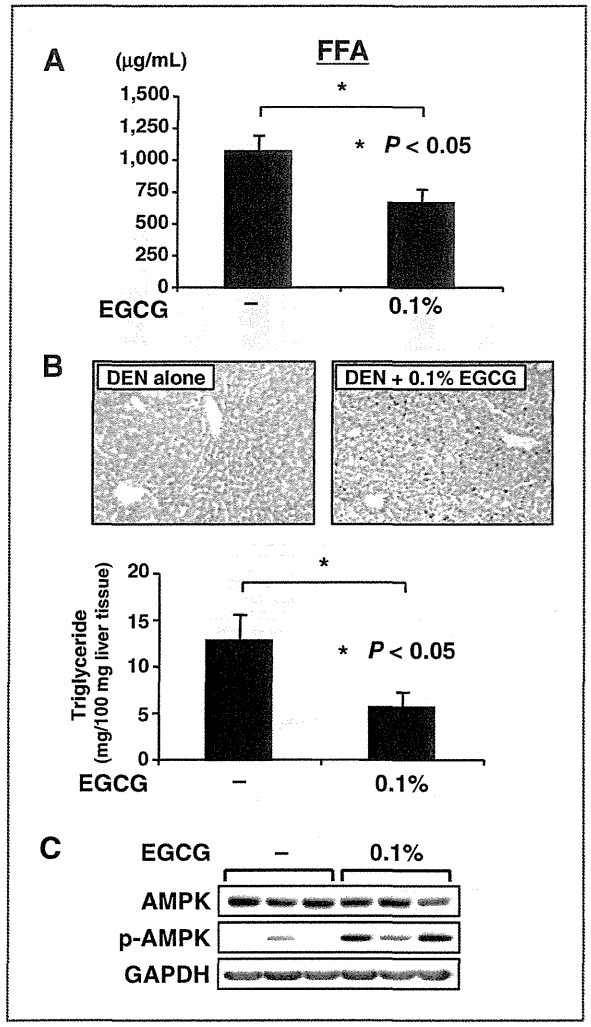

Figure 2. Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of the AMPK protein in the liver of DEN-treated *db/db* mice. A, the serum concentration of FFA was measured by an enzymatic method. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, frozen 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

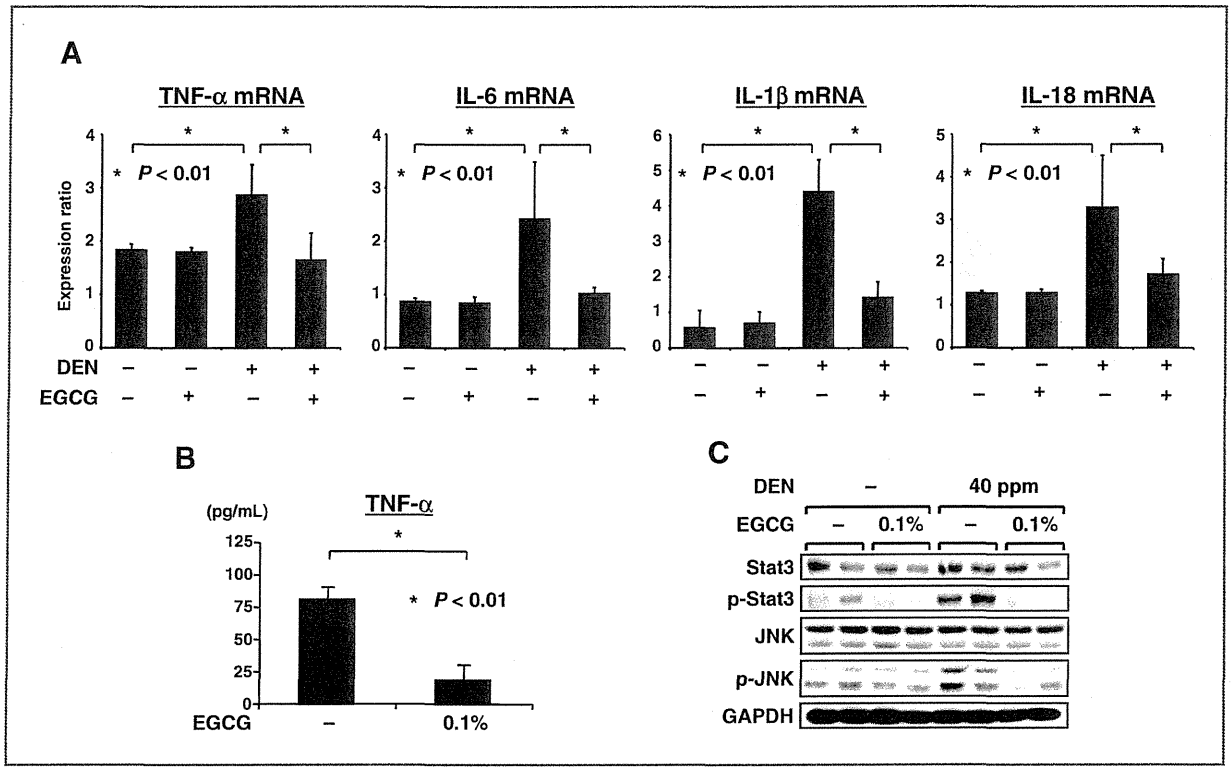

Figure 3. Effects of EGCG on the expression levels of TNF-α, IL-6, IL-18, and IL-1β mRNAs, the serum levels of TNF-α, and the activation of Stat3 and JNK proteins in the liver of experimental mice. A, the total RNAs were isolated from the livers of experimental mice, and the expression levels of TNF-α, IL-6, IL-1β, and IL-18 mRNAs 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 means ± SD. *, $P < 0.01$ versus the control groups. B, the serum concentration of TNF-α in DEN-treated *db/db* mice was measured by enzyme immunoassay. Values are the means ± SD. *, $P < 0.01$ versus the untreated group. C, the total proteins were extracted from the livers of experimental mice and the expression levels of the Stat3, p-Stat3, JNK, and p-JNK proteins were examined by a Western blot analysis. GAPDH antibody served as a loading control.

pathways, in the livers of experimental mice. These findings seem to be significant because the alteration of the IGF/IGF-1R axis, which is induced by insulin resistance, is involved in liver carcinogenesis and thus might play a critical role as a molecular target for HCC chemoprevention (6–8). In human HCC-derived cells, IGF-1 and IGF-2 activate IGF-1R, ERK, and Akt proteins and increase the expression of IGF-1 and IGF-2 mRNAs themselves but EGCG inhibits these sequences and thus suppresses growth and induces apoptosis in HCC cells (13). These findings, together with the results of the present study, suggest the possibility that EGCG overcomes the stimulatory effects of IGFs, disrupts the IGF/IGF-1R–related autocrine/paracrine loops, and thereby prevents the development of obesity-related liver tumorigenesis. In addition, the inhibition of GSK-3β phosphorylation by EGCG also plays a role in preventing the development of liver neoplasms because phosphorylation of this kinase, which is mediated by the IGF-1R/Akt axis, is closely associated with liver carcinogenesis (31).

Excess accumulation of lipids in the liver accelerates HCC development (4, 5). Therefore, the improvement of hepatic steatosis by EGCG is also significant when considering the inhibitory effects of this agent on obesity-related liver tumorigenesis. This effect of EGCG may be associated with reductions in white adipose tissue and serum FFA levels because host factors, particularly increased visceral fat and a high influx of FFA to the liver, lead to hepatic fat accumulation (4, 5). In addition, EGCG may also improve metabolic abnormalities by activating AMPK in the liver, which enhances insulin sensitivity and increases fatty acid oxidation but decreases fatty acid synthesis (32). This finding is consistent with recent studies showing that EGCG increases insulin sensitivity and fat oxidation and induces AMPK activity in the liver (36, 37). Furthermore, in addition to the improvement of metabolic disorders, activation of AMPK by EGCG also positively contributes to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a tumor suppressor and a promising target for cancer chemoprevention (38). In fact, EGCG has been shown to inhibit lipogenesis and cell-cycle progression through the activation of AMPK in human HCC-derived cells (39). The phosphorylation of LKB1, which is a tumor suppressor protein and a major AMPK kinase (38), is also increased by EGCG (37). Thus,

the antiobesity and cancer chemopreventive effects of EGCG might be mediated, at least in part, by the activation of AMPK.

Insulin resistance and lipid accumulation in the liver, which is mainly induced by the FFA flux, promotes liver inflammation through the production of proinflammatory cytokines such as TNF-α and IL-6, and this chronic inflammatory response is closely associated with activation of Stat3 and increased risk of HCC (4, 5, 33). Therefore, decreases in the expression of TNF-α, IL-6, IL-1β, and IL-18 mRNAs in the liver, reduced levels of serum TNF-α, and inhibited activation of Stat3 in the liver of *db/db* mice treated with EGCG are considered to be important in preventing obesity-related liver tumorigenesis. Among these targets, TNF-α, which links obesity with insulin resistance and contributes to obesity-induced IL-6 production (33, 34), has been shown to be a crucial target of EGCG that can inhibit cancer cell growth and prevent inflammation-related colorectal carcinogenesis (19–21). The inhibition of the activation of the IL-6/Stat3 axis by EGCG is also important because this axis plays a critical role in HCC development (40, 41). In addition, the effect of EGCG to inhibit JNK activation, which is caused by higher levels of TNF-α and FFA and is involved in obesity-mediated insulin resistance (42), also contributes to the prevention of obesity-related liver tumorigenesis by EGCG because JNK seems to be one of the most important kinases that is upregulated in HCC and could thus be a potential therapeutic target for this malignancy (43). Because JNK is located downstream of IGF-IR (30), the inhibition of the activation of the IGF/IGF-1R axis may also lead to the indirect inhibition of JNK.

One of the effective strategies for HCC chemoprevention is the deletion of latent malignant clones before they progress to detectable neoplasms, and improvement of whole liver condition might play a role in this prevention (44, 45). The liver accumulated with fat, which activates the IGF/IGF-1R axis and induces chronic inflammation, might be regarded as a hypercarcinogenic field (4, 5, 8, 33). Therefore, the findings that EGCG inhibits the activation of IGF-1R and related downstream signaling pathways and ameliorates inflammatory condition in nontumorous hepatic tissues seem to be significant when considering the practice of HCC chemoprevention. Presumably, EGCG reduces the number of FCA, at least in part, by improving the condition in the whole liver and thus preventing obesity-related field tumorigenesis of the liver in the present study.

The beneficial effects of GTCs have been reported in several clinical trials. For instance, supplementation with GTCs can significantly prevent the development of both colorectal adenomas and prostate cancers without causing adverse effects (46, 47). A double-blind, placebo-controlled pilot study showed that EGCG has the potential to increase fat oxidation in men (48), although more studies with a larger sample size are required to confirm this effect. The results of these trials may encourage the clinical usage of GTCs for obese patients to prevent pathogenesis of various chronic diseases that are caused by excessive body weights. In summary, the prevention of HCC by targeting the IGF/IGF-1R axis, hepatic steatosis, and chronic inflammation, which are caused by dysregulation of energy homeostasis, might represent a promising strategy for obese individuals who are at an increased risk of developing HCC (3, 8). GTCs, including EGCG, seem to be potentially effective and critical candidates for this purpose because, as shown in the results of the present study and those from previous reports, these agents can target metabolic abnormalities and may therefore restore metabolic homeostasis (16–22).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Mitsui Norin Co. Ltd. for providing EGCG. We also thank Ms. Yukari Nomura for her excellent technical assistance.

Grant Support

This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 22790638 to M. Shimizu and No. 21590838 to H. Moriwaki) and by grant-in-aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan.

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Received November 12, 2010; revised January 9, 2011; accepted January 20, 2011; published online March 3, 2011.

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