Table 1 Recommendations for nutritional management of liver cirrhosis: part 1

I. Assessment before nutrition and diet therapy
  (1) Evaluate clinical stage (compensated or decompensated liver cirrhosis) and the severity of liver damage (i.e. Child–Pugh classification) as well as presence of portal-systemic shunt.
  (2) Perform SGA† and anthropometry.‡
  (3) Evaluate impaired glucose tolerance, insulin resistance§ and postprandial hyperglycemia.
  (4) Evaluate oxidative stress conditions.¶
  (5) Examine dietary intake using a questionnaire.
  (6) Perform indirect calorimetry†† and trace element measurement.

†Subjective global assessment (SGA) is an effective method in the screening of malnourished patients. It examines age, sex, height, bodyweight, changes in bodyweight, changes in food intake, the presence of gastrointestinal symptoms, intensity of activities of daily living (ADL), the condition of loss of subcutaneous fat and muscles, the presence of edema/ascites, hair condition, among other factors.
‡In addition to height, bodyweight and body mass index (BMI: bodyweight [kg]/height [m]^2), arm circumference (AC) and triceps skinfold thickness (TSF) are measured using an insert tape and adipometer. Moreover, arm muscle circumference (AMC) is calculated by AC – 3.14 × TSF. Data are evaluated using standard values for the physical measurements of a Japanese individual (Japanese Anthropometric Reference Data: JARD 2001).[39] This allows the calculation of basal energy expenditure, resting energy expenditure and protein (amino acid) requirements according to age, sex difference and physical measurements. More detailed body composition analysis methods have recently become available, and these are based on bioelectrical impedance analysis.
§Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR = blood fasting insulin [μU/mL] × fasting blood glucose level [mg/dL] / 405) is used as an index for insulin resistance, with HOMA-IR ≥2.5 considered to indicate insulin resistance. However, this equation assumes that the fasting blood glucose levels are <140 mg/dL.
¶Although there are numerous biomarkers for evaluating oxidative stress, the measurement of serum ferritin levels should be used for the purpose of preventing hepatocellular carcinoma. In addition, the presence of anemia is examined using hemoglobin concentrations.
††Where indirect calorimeters are available, measurement of resting energy expenditure, non-protein respiratory quotient (npRQ) and oxidation rates for various nutrients (carbohydrate, fat, protein) after overnight fasting is useful in evaluating protein-energy malnutrition. Anthropometric values (%AC, %AMC) and the serum free fatty acid levels are useful indexes for npRQ during routine care; serum levels of tumor necrosis factor (TNF)-α and soluble TNF receptors and plasma ghrelin levels may also be used as references.

tolerance;[33–35] (ix) supplementation of BCAA granules and BCAA-enriched nutrients improve liver function and energy metabolism;[36,37] and (x) supplementation of BCAA granules inhibits carcinogenesis in a mouse model of NASH, possibly via improvement of insulin resistance.[38]

Based on these data and discussions among the members of JNUS, guidelines for nutritional management of Japanese LC patients were prepared and are shown in Tables 1 and 2. The guidelines consist of two parts. The first part (Table 1) describes essential nutritional assessments that should be performed before instituting nutritional and diet therapy. The second part (Table 2) describes the recommended dietary management for each nutrient, including energy, protein, fat, sodium chloride, iron and other nutrient requirements. Restriction of sodium chloride was decided based on the therapeutic guidelines for hypertension by the Japanese Society of Hypertension.[40] We also included supplemental descriptions in the tables in order to ensure that dietitians are able to perform nutritional assessment and therapy in accordance with these guidelines.

At this point, it is not clear whether supplementation with BCAA granules has any preventive effects on HCC recurrence after primary treatment for HCC, as the number of enrolled patients is small. A double-blind controlled study for zinc supplementation in LC patients with hyperammonemia is also still on-going. The final results of this study are expected to be available by the end of 2012.

DISCUSSION

IN ORDER TO establish new guidelines on nutritional management in LC patients, it is important to consider hepatocarcinogenesis. In this article, based on the results of JNUS projects between 2008 and 2010 and re-evaluation of previous publications concerning nutritional therapies in LC patients with or without HCC, we proposed new guidelines for nutritional management of Japanese LC patients, with the aim of preventing HCC.

We hope these guidelines will form a basis for future discussions on nutritional management of LC by specialists such as hepatologists and dietitians.

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Table 2 Recommendations for the nutritional management of liver cirrhosis: part 2

II. Nutrition and diet therapy
- (1) Energy requirements[a]
  25–35 kcal/kg (ideal bodyweight) per day, based on *Standards for Dietary Intake* (2010 Edition, Recommended Dietary Allowance According to Intensity of Daily Activity).
  If any abnormalities are seen in glucose tolerance, intake should be 25 kcal/kg (ideal bodyweight) per day.
- (2) Required protein intake[b]
  If there is no protein intolerance: 1.0–1.5 g/kg/day (including oral BCAA granules).[c]
  If there is protein intolerance: 0.5–0.7 g/kg per day + BCAA-enriched enteral nutrient mixture.[d]
- (3) Required fat intake:[e] lipid energy ratio 20–25%.
- (4) Sodium chloride:[f] ≤6 g/day and <5 g/day if there are ascites and/or edema, respectively
- (5) Iron:[g] ≤7 mg/day if serum ferritin levels are above the upper limit of the reference interval.
- (6) Others: zinc supplementation,[h] adequate intake of vitamins and dietary fiber (e.g. vegetables, fruits).
- (7) LES as a divided meal (4 times/day) (amounts to 200 kcal).[i]

[a]Resting energy expenditure is often accelerated in liver cirrhosis patients and protein-energy malnutrition (PEM) is observed in approximately 80–90% of patients. However, approximately 30% of patients are obese, with a body mass index (BMI) of ≥25. Moreover, in cases of hepatitis C, there is a high frequency of insulin resistance exhibited. It is important to determine the required amount of energy by taking into account such nutritional conditions.
[b]Required protein intake includes the protein content of branched-chain amino acid (BCAA) formulation (BCAA granules or BCAA-enriched nutrient mixture for chronic liver failure). The majority of patients with decompensated liver cirrhosis (LC) often have protein intolerance, which is determined by referring to the blood ammonia levels.
[c]Patients in the decompensated state, including cases with hyperammonemia, are judged as having protein intolerance. The administration of BCAA granules (e.g. Livact Granules) is essential for the patient with serum albumin <3.5 g/dL, Fischer's ratio <1.8 and/or BTR < 3.5, and is usually administrated by dividing the dosage of 3 packs/day (12 g) into 3 administrations, but there is also a method whereby 2 packs are administrated (before sleep). Prevention of hepatocellular carcinoma (HCC) is expected in male hepatitis C patients with BMI >25 due to long-term administration of this formula. Improvement of the amino acid imbalance is also useful in recovering decreased dendritic cell functions.
[d]When administrating BCAA-enriched enteral mixtures (e.g. Aminoleban EN and Hepan ED), the amount of energy and protein present in this nutrient should be included in the total intake of energy and protein for the day. BCAA-enriched enteral mixtures should be the first choice in patients with PEM, regardless of the presence of protein intolerance.
[e]Ideal ratio of fatty acid composition for the inhibition of HCC has not been clarified, but a decline in n-6 and n-3 polyunsaturated fatty acids has been observed in patients with LC.
[f]Even patients who are not physically observed to have edema/ascites have a tendency for water retention, so fundamentally salt should be restricted.
[g]Excess deposition of iron in the liver causes oxidative stress and promotes hepatocarcinogenesis; thus, unless severe anemia is observed, an iron-restricted diet should be standard. Moreover, although the standard value of serum ferritin level differs with sex, phlebotomy in small amounts should be considered for patients with values ≥150 ng/mL.
[h]Zinc supplementation improves hyperammonemia and may suppress the occurrence of HCC in patients with LC over long-term administration.
[i]Lifestyle and eating habits of patients should examine. Late-evening snack (LES) is also useful for managing the blood glucose level in patients with impaired glucose tolerance, and combined use with α-glucosidase inhibitor enhances this effect. Usually, snacks such as rice balls (*onigiri*) are provided, but with the recommendation of using enteral nutrients, food products rich in BCAA are also used.
Fischer's ratio: BCAA/tyrosine + phenylalanine.
BTR: molar ratio of BCAA and tyrosine.

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Cancer Prevention Research

Research Article

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

Soranobu Ninomiya[1], Masahito Shimizu[1], Kenji Imai[1], Koji Takai[1], Makoto Shiraki[1], Takeshi Hara[1], Hisashi Tsurumi[1], Sonoko Ishizaki[2], and Hisataka Moriwaki[1]

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,

Authors' Affiliations: [1]Department of Medicine, Gifu University Graduate School of Medicine, Gifu; and [2]Pharmacology, Exploratory & Applied Pharmaceutical Research Department, Pharmaceutical Research Center, Ajinomoto Pharmaceuticals Co., Ltd., Kawasaki, Japan

Corresponding Author: Masahito Shimizu, Department of Internal Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Phone: 81-58-230-6313; Fax: 81-58-230-6310; E-mail: shimim-gif@umin.ac.jp

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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

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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

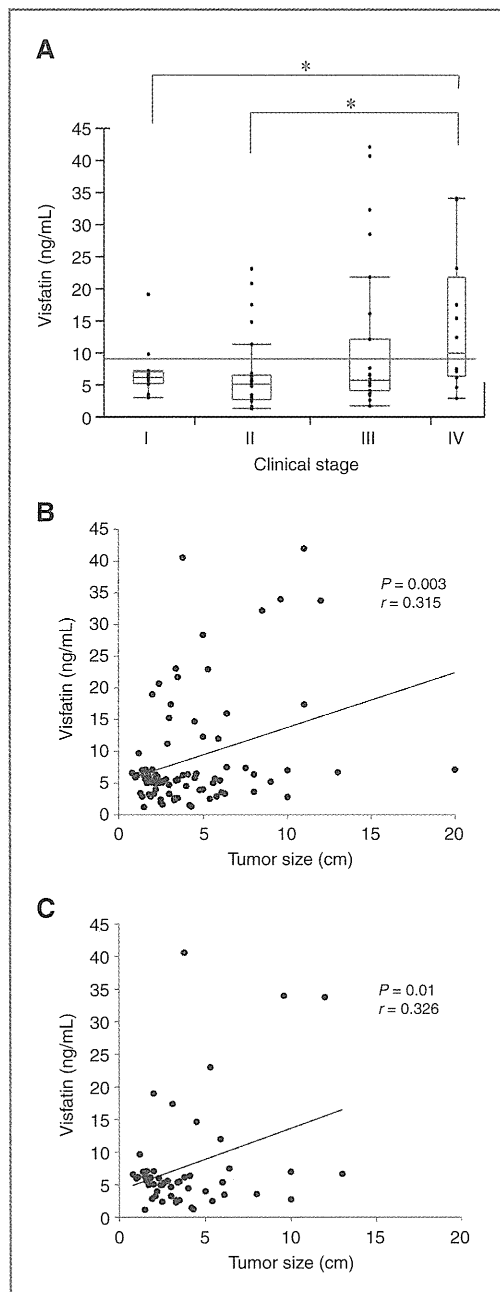

Figure 1. Correlation between serum visfatin concentrations and the clinical stage (A) and tumor size (B, C) of HCCs. A and B, the correlations were determined by analyzing 85 patients with primary HCCs. C, the correlation was determined by analyzing 53 HCC patients who are not obese and did not have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

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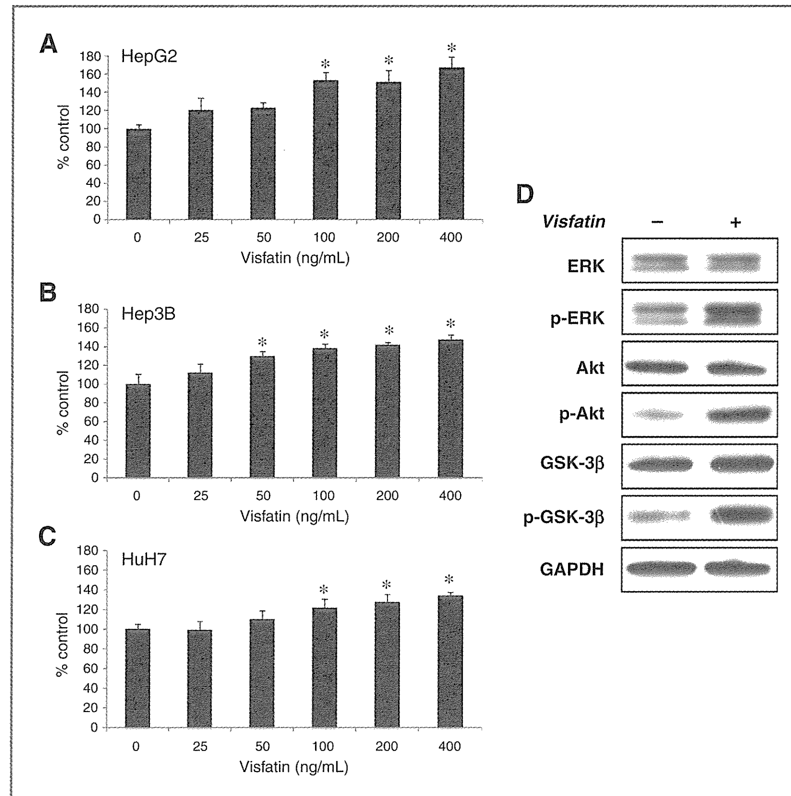

Figure 2. Effects of visfatin on the cell proliferation and phosphorylation of ERK, Akt, and GSK-3β proteins in HCC cells. HepG2 (A), Hep3B (B), and HuH7 (C) cells were treated with the indicated concentration of visfatin for 48 hours in serum-free medium. Cell proliferation was evaluated by an XTT assay. 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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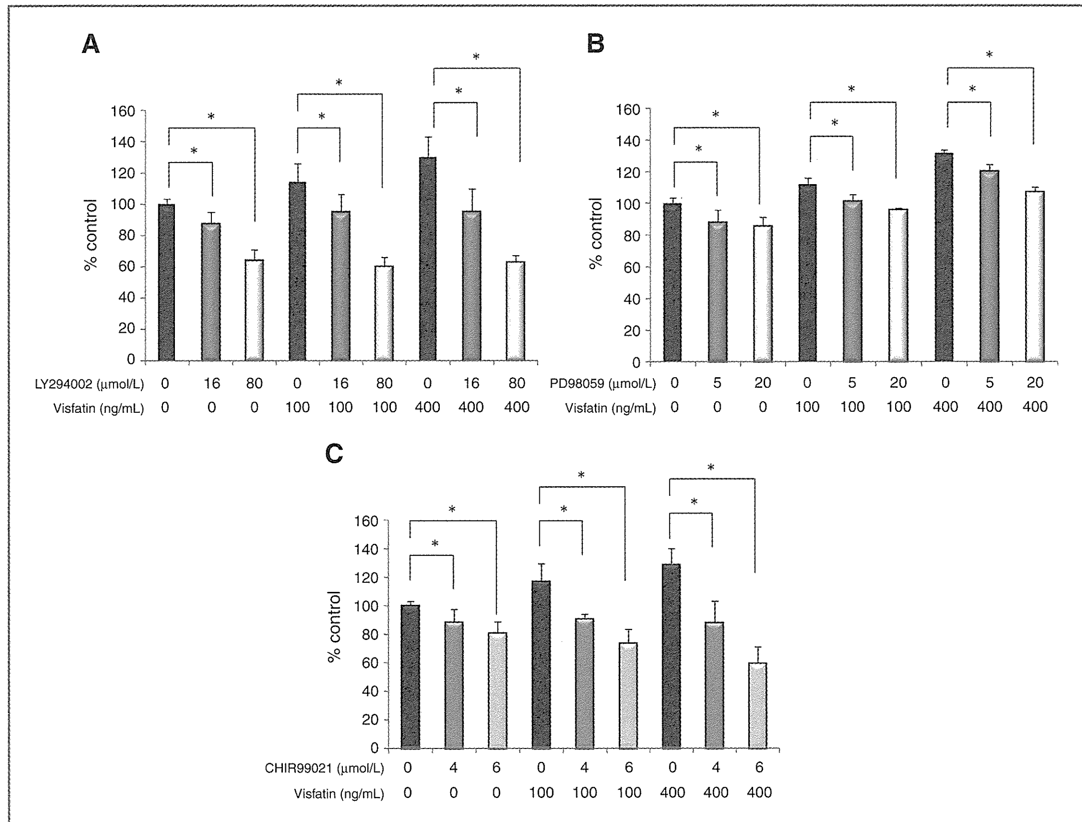

Figure 3. Effects of inhibitors of PI3K, MEK1, and GSK-3β on visfatin-induced proliferation in HepG2 cells. HepG2 cells were treated with LY294002, a PI3K inhibitor (A), PD98059, an MEK1 inhibitor (B), or CHIR99021, a GSK-3β inhibitor (C), in the absence or presence of visfatin (100 or 400 ng/mL) for 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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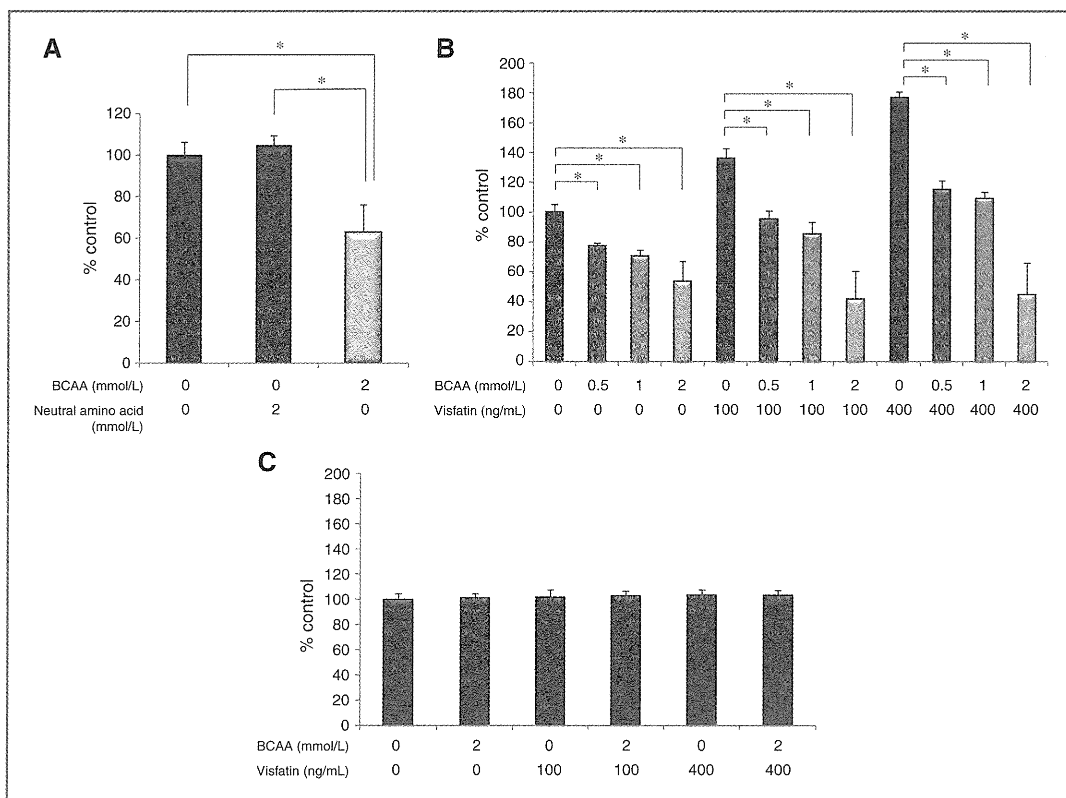

Figure 4. Effects of BCAA on visfatin-induced cell proliferation in HepG2 cells. A, HepG2 cells were treated in 2 mmol/L BCAA or 2 mmol/L neutral amino acid medium for 48 hours. Cell proliferation was evaluated by an XTT assay. HepG2 (B) and Hc (C) cells were treated with or without BCAA (0, 0.5, 1, 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

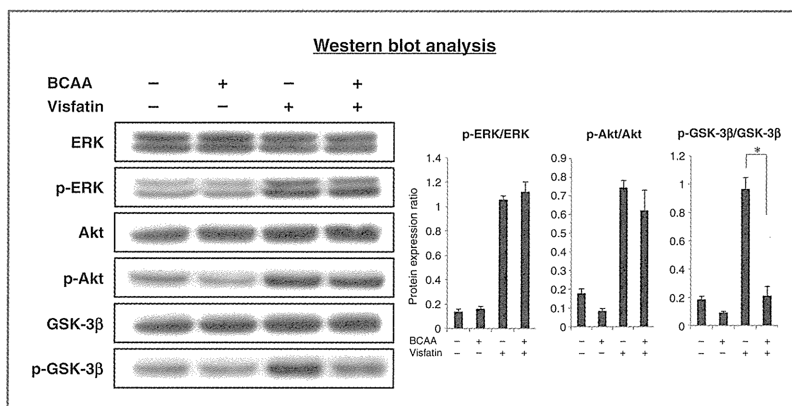

Figure 5. Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells. HepG2 cells were treated with or without BCAA in the absence or presence of 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed by Western 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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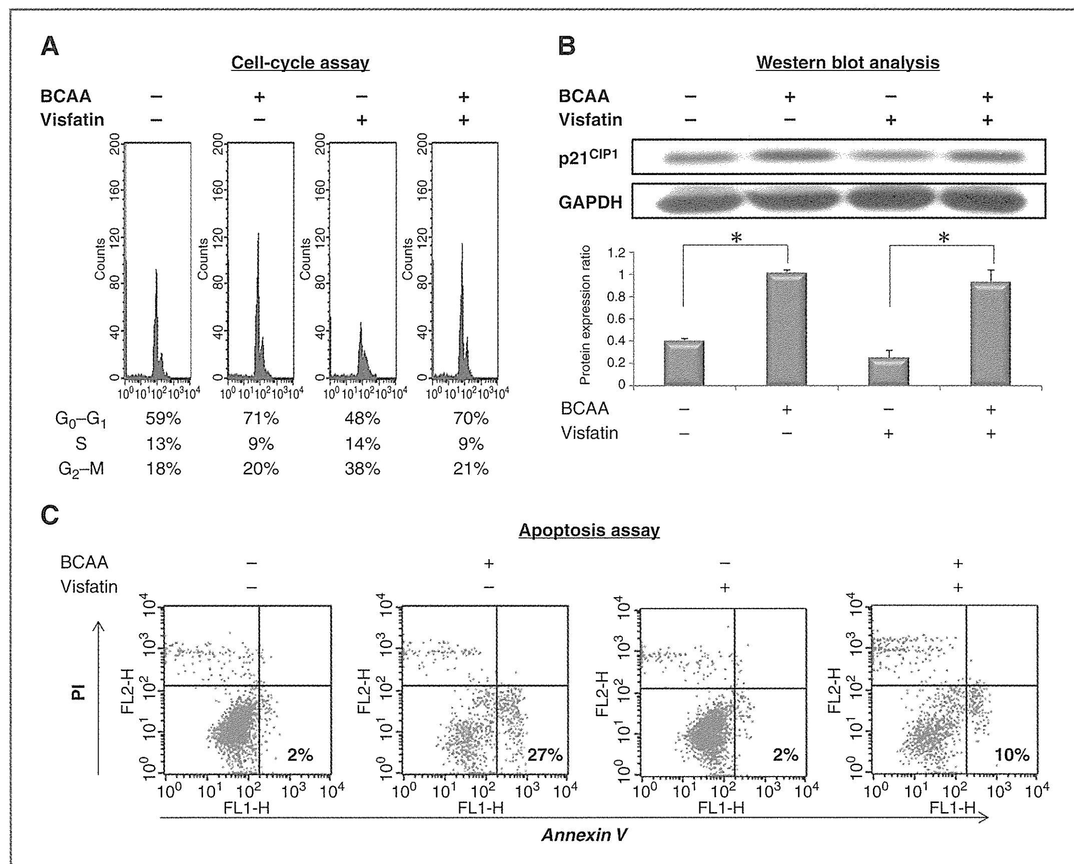

Figure 6. Effect of BCAA on the progression of cell cycle, expression of $p21^{CIP1}$, and induction of apoptosis in HepG2 cells in the presence and absence of visfatin. After treatment with and without BCAA in the presence and absence of 100 ng/mL visfatin for 48 hours, the cells were corrected and 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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Cancer Prevention Research

Research Article

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

Masahito Shimizu[1], Hiroyasu Sakai[1], Yohei Shirakami[1], Junpei Iwasa[1], Yoichi Yasuda[1], Masaya Kubota[1], Koji Takai[1], Hisashi Tsurumi[1], Takuji Tanaka[2], and Hisataka Moriwaki[1]

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- $+\mathrm{Lepr}^{db}/+\mathrm{Lepr}^{db}$ (*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- $+\mathrm{Lepr}^{db}/+\mathrm{Lepr}^{db}$ (*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

Authors' Affiliations: [1]Department of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan, and [2]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Corresponding Author: Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Phone: 81-(58)-230-6313; Fax: 81-(58)-230-6310; E-mail: shimim-gif@umin.ac.jp

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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 *RARβ* and $p21^{CIP1}$ (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of posttherapeutic 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: 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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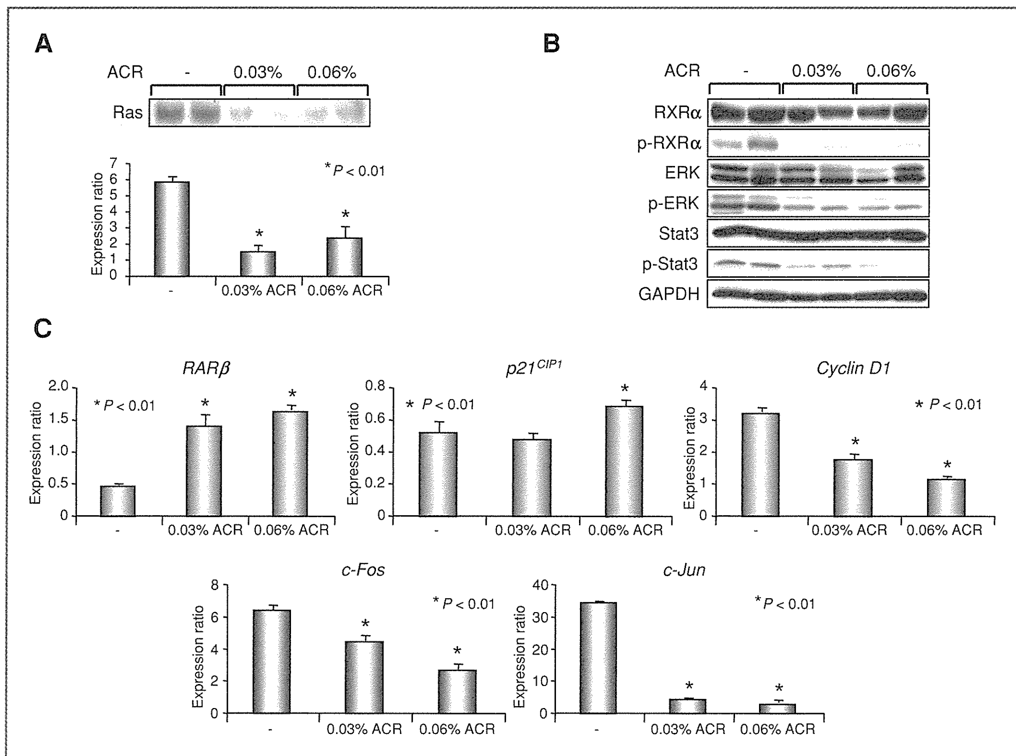

Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras 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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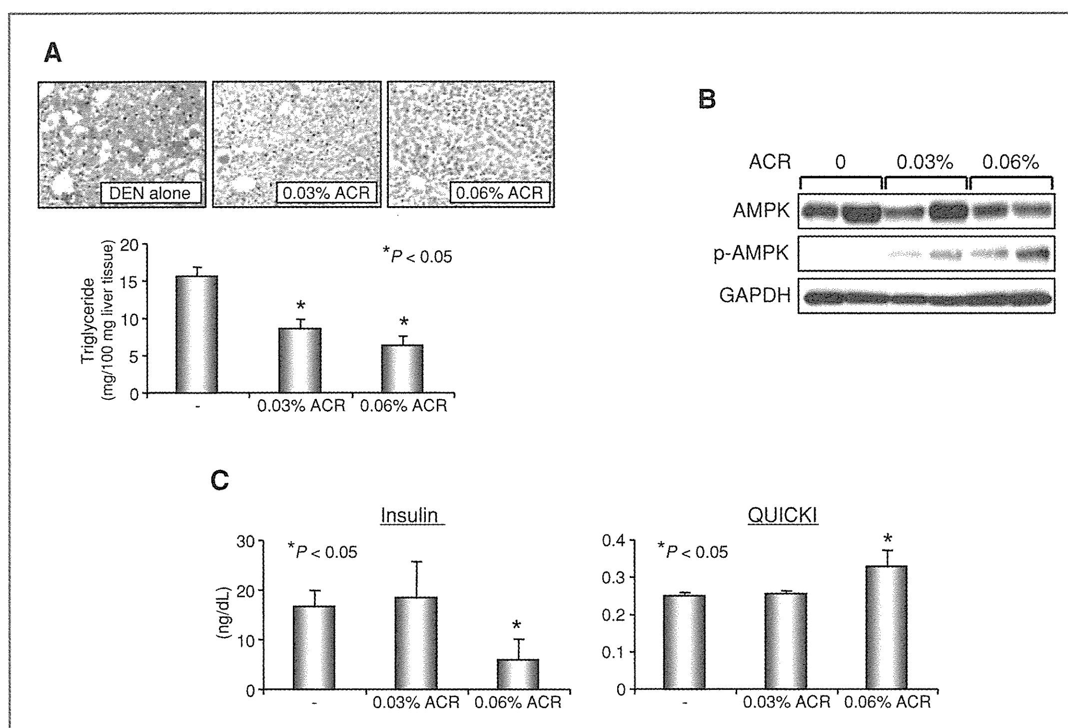

Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (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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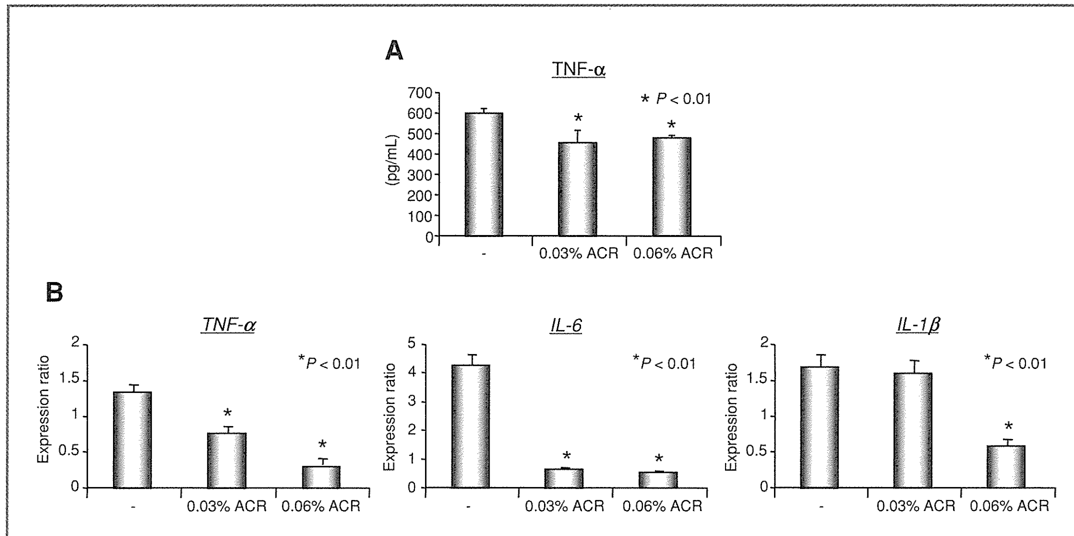

Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined 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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