

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

栄養指導ツールの評価

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研究要旨：栄養治療をするうえで、身体計測は栄養障害のリスクを検索する指標として栄養評価の重要な評価基準のひとつとなる。肥満の診断基準においても体重中の体脂肪において皮下脂肪か、内臓脂肪かが重要な評価基準とされる。また体重が減量できれば、それだけで生活習慣病が改善することもある。最近肝臓病患者でも内臓脂肪型肥満の患者が増加し、特に食生活の改善は肥満の是正にむけ大きな鍵となる。今まで栄養教育により栄養状態が改善した患者の報告や肥満患者の体重評価の重要性を肝臓病学会地方会にて報告してきた。しかしながら肝臓病の患者は、糖尿病患者と違い栄養指導を受ける機会が少ないのが現状である。また患者自身に栄養指導を受けるという自覚も少ないうえに、栄養指導評価ツールも少ない。そこで誰でも簡便に理解でき、肝臓病の病態に応じた指導ツールを開発することにより、栄養療法の効果が高まることを検証する。

A. 研究目的

生活習慣を見直し、適正な食生活に導き肥満患者を減少させることは肝臓病予防に多い貢献できる。たとえば患者自身でも誰でも手軽に栄養診断・現状の栄養状態を評価でき、さらに患者が理解しやすい栄養指導評価ツールを作成する。

B. 研究方法

予備調査

現状の把握として2011年6月に肝友会の患者に協力していただき、アンケート調査を行った。

対象 肝友会に属する患者 20名
アンケート方式

1. メーカーの栄養指導用媒体や市販されている栄養指導用ツールを検証する。
2. 施設の管理栄養士にアンケート調査を行い、肝臓病患者用の栄養指導ツールを検証する。
3. 1と2を参考に栄養指導評価ツールを作成する。
4. 3を使い、実際の患者の効果を検証する。

（倫理面への配慮）

情報は個人が特定されないようにすべての個人にID番号をつける。

情報をコンピューターで処理する場へのセキュリティ管理についてはデータを保護

するため暗号化する。

C. 研究結果

予備調査より、栄養指導を受けていない、もしくはうけても1回のかたが大半を占めた。患者会の方々は栄養指導を受け、正しい知識を知り、食生活の改善をしたいと考えていたことがわかった。

1. 指導媒体

味の素ファルマ

○ 肝臓にやさしい食事と生活

慶應義塾大学医学部 石井裕正先生、
加藤眞三先生、武田純江、鈴木和子
(管理栄養士) 2010

合併症のない場合

30~35kcal/kg/day 1.2/kg/day

高アンモニア血症を伴う場合

30~35kcal/kg/day 0.8~1.0g/
kg/day

糖尿病を合併する場合

30kcal/kg/day 1.2g/kg/day

○ 肝硬変と食事のかかわり栄養療法編

山口大学医学部 坂井田功先生

リーバクト顆粒を服用される方へ

○ 肝硬変と上手につきあうために

慶応義塾大学日比先生 13-15ページ

MSD 株式会社 肝機能障害といわれ
たら食生活をみなおしましょう

監修ニューヨーク州立大学 桑山先生

聖マリアンナ 川島先生

等であった。

患者がダイエットするための具体的な指導媒体はないようである。

D. 考察

肝臓病患者は栄養指導を受けていないケースも多い。また古い時代の肝臓病食の内容や、自己流に市販の健康食品を利用するケースも多い。新しいガイドラインに沿い、適切に自分の身体評価をし、内臓脂肪減少にむけた食事療法ができるような媒体、栄養評価が自己で行えるような栄養指導評価ツールを作成する意義は大きいと考える。

E. 結論

多くの患者は正しい知識で食事療法への取り組みをしたいと考えている。

糖尿病指導媒体に比し、メーカーの栄養指導ツールは少なく、古いものも多いため現状にそぐわなものも少なくない。

F. 健康危険情報

特記事項なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的所有権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

IV. 研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の 編集者名	書 籍 名	出版社名	出版地	出版年	ページ
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V. 研究成果の刊行物・別刷

Research Article

Acyclic Retinoid Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} MiceMasahito Shimizu¹, Hiroyasu Sakai¹, Yohei Shirakami¹, Junpei Iwasa¹, Yoichi Yasuda¹, Masaya Kubota¹, Koji Takai¹, Hisashi Tsurumi¹, Takuji Tanaka², and Hisataka Moriwaki¹**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 RXR α , on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks, after which they were fed a diet containing 0.03% or 0.06% of ACR throughout the experiment. In mice treated with either dose of ACR for 34 weeks, the development of liver cell adenomas was significantly inhibited as compared with basal diet-fed mice. ACR markedly inhibited the activation of Ras and phosphorylation of the ERK (extracellular signal-regulated kinase) and RXR α proteins in the livers of experimental mice. It also increased the expression of *RAR β* and *p21^{CIP1}* mRNA while decreasing the expression of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA in the liver, thereby restoring RXR α 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-1 β* mRNA in the livers of DEN-treated *db/db* mice were decreased by ACR treatment, suggesting attenuation of the chronic inflammation induced by excessive fatty deposits. ACR may be, therefore, useful in the chemoprevention of obesity-related HCC. *Cancer Prev Res*; 4(1); 128–36. ©2010 AACR.

Introduction

Hepatocellular carcinoma (HCC) is a serious health-care problem worldwide. The risk factors associated with the development of HCC include chronic hepatitis B and/or hepatitis C infection, particularly with subsequent cirrhosis. Recent evidence also indicates that obesity and the related metabolic abnormalities, especially diabetes mellitus, increase the risk of HCC (1–3). In a rodent model, the occurrence of diethylnitrosamine

(DEN)-induced liver tumorigenesis was found to be significantly higher in obese and diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) mice than in genetic control mice (4). Diabetes mellitus has been shown to increase the risk of primary HCC in patients with viral hepatitis (5). Insulin resistance is also significantly associated with the recurrence of stage I HCC after curative treatment (6). Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the insulin resistance syndrome, and in a subset of NAFLD patients, the condition progresses to nonalcoholic steatohepatitis, which involves severe inflammation and therefore poses the threat of HCC (7, 8). Coexistent obesity or steatosis exacerbates liver injury and fibrosis and thus is involved in liver tumorigenesis (9). Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and their treatment must target the prevention of this malignancy.

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

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

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growth of HCC-derived cells by inducing apoptosis and causing cell-cycle arrest in G₀-G₁ (11–15). These effects of ACR are associated with its agonistic activity for distinct nuclear retinoid receptors—retinoid X receptors (RXR) and retinoic acid receptors (RAR), both of which have 3 subtypes (α , β , and γ ; 16)—and subsequent expression of the ACR target genes *RAR β* and *p21^{CIP1}* (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of 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, RXR α 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 RXR α abolishes its ability to form a heterodimer with RAR β , and the accumulation of phosphorylated RXR α (p-RXR α , i.e., nonfunctional RXR α), 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 RXR α 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 RXR α 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 CO₂ 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- μ m 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 (cm²) 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 μ g) 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 μ g per lane) were examined by Western blot analysis (4). Previously described primary antibodies for RXR α (Δ N-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 Δ N-197 antibody is considered a specific antibody for the p-RXR α protein (22, 23). The GAPDH antibody served as a loading control.

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) was 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

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

^aWhite adipose tissue of the periorchis and retroperitoneum.

^bMean ± SD.

^cSignificantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).

^dSignificantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).

^eSignificantly 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 (No./cm ²)
			Adenoma	HCC	Adenoma	HCC	
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%) ^d	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%) ^d	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

^aNumber of neoplasms per mouse.

^bMean ± SD.

^cSignificantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.001$).

^dSignificantly different from group 1 by Fisher's exact probability test ($P < 0.01$).

^eSignificantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).

^fSignificantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.001$).

^gSignificantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).

^hSignificantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

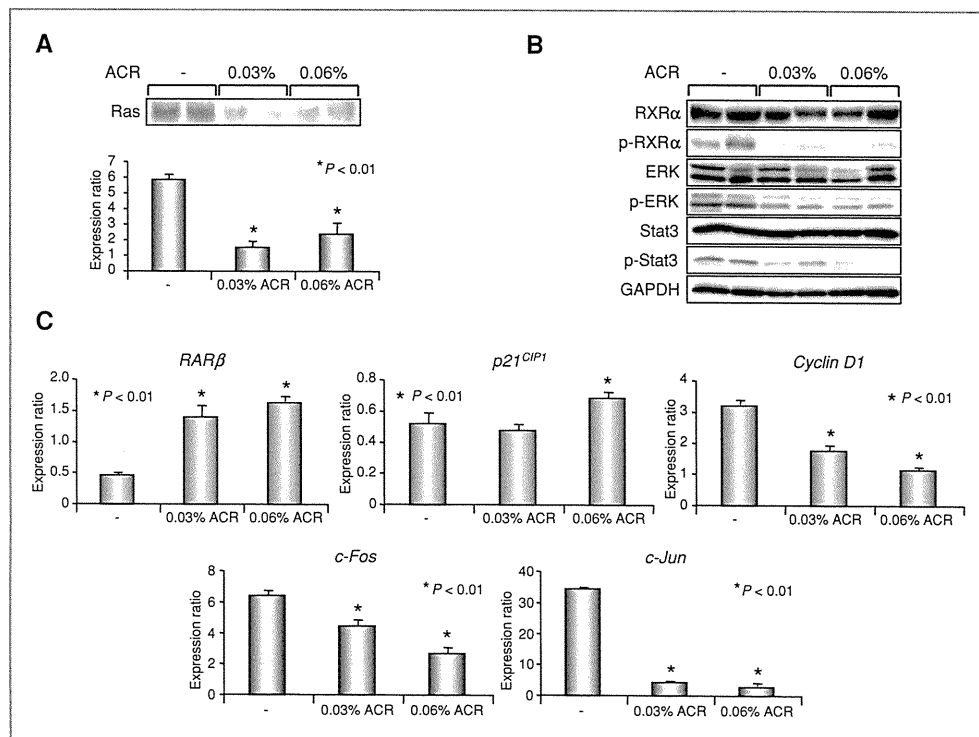


Figure 1. Effects of ACR on Ras activity; phosphorylation of RXR α , ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras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

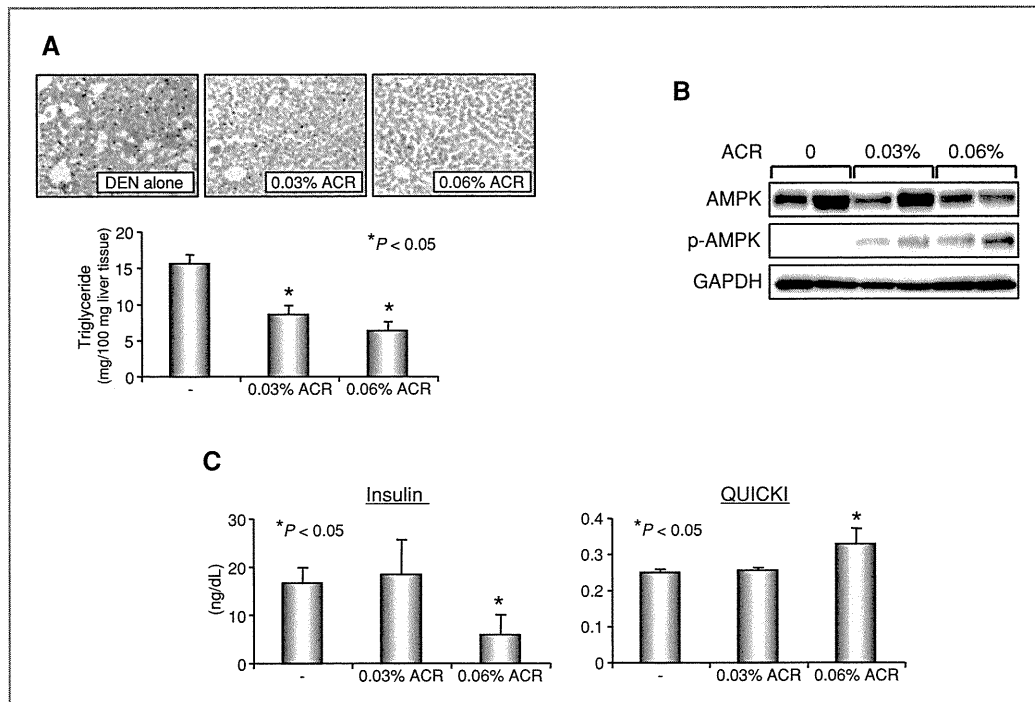


Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (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 \pm 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 RXR α 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 RXR α malfunction due to the phosphorylation by Ras-ERK plays a role in liver carcinogenesis and phosphorylated RXR α is therefore a critical target for HCC chemoprevention (10, 21). ACR suppresses the growth of HCC cells by inhibiting RXR α phosphorylation and restoring its original function as a master regulator

of nuclear receptors (15, 22–24). Therefore, the expression levels of the *RAR β* , *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, *RAR β* 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 *RAR β* gene (37). Because *RAR β* can form a heterodimer with RXR α 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

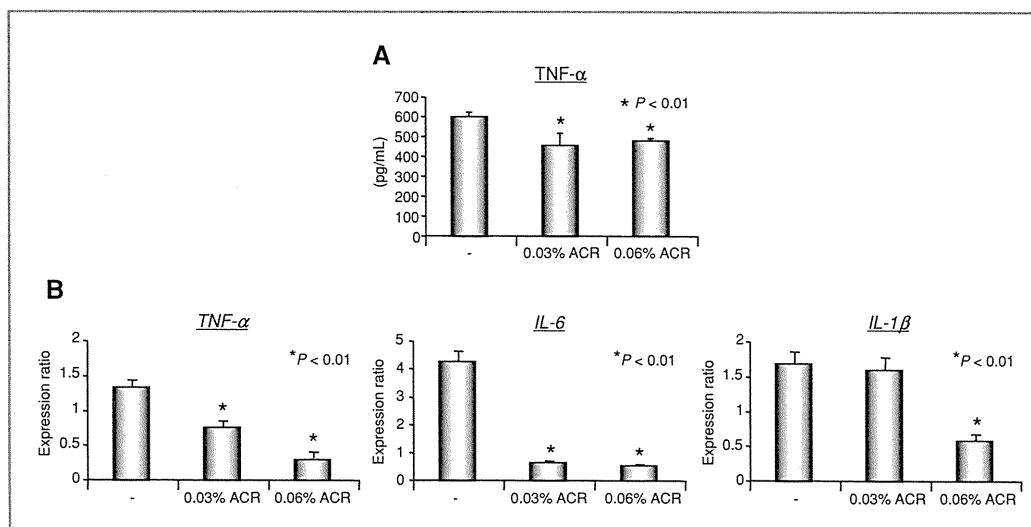


Figure 3. Effects of ACR on the serum levels of TNF- α and the expression levels of TNF- α , IL-6, and IL-1 β mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF- α was measured by enzyme immunoassay. B, the expression levels of TNF- α , IL-6, and IL-1 β mRNA were examined 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 \pm SD. *, $P < 0.01$ vs. ACR-untreated group.

be associated with RXR α 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 RXR α 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 expres-

sion levels of TNF- α , IL-6, and IL-1 β 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-RXR α 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 RXR α phosphorylation induced by

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

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Preventive Effects of (–)-Epigallocatechin Gallate on Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BL/KsJ-*db/db* MiceMasahito Shimizu¹, Hiroyasu Sakai¹, Yohei Shirakami¹, Yoichi Yasuda¹, Masaya Kubota¹, Daishi Terakura¹, Atsushi Baba¹, Tomohiko Ohno¹, Yukihiko Hara², Takuji Tanaka³, and Hisataka Moriwaki¹

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-3 β (glycogen synthase kinase-3 β), Stat3, and JNK (c-Jun NH₂-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-1 β , and IL-18 mRNAs in the livers. In addition, EGCG improved liver steatosis and activated the AMP-activated kinase protein in the liver. These findings suggest that EGCG prevents obesity-related liver tumorigenesis by inhibiting the IGF/IGF-1R axis, improving hyperinsulinemia, and attenuating chronic inflammation. EGCG, therefore, may be useful in the chemoprevention of liver tumorigenesis in obese individuals. *Cancer Prev Res*; 4(3); 396–403. ©2011 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadly cancers worldwide. Chronic inflammation of the liver and subsequent cirrhosis, which are mainly induced by infection with hepatitis B and hepatitis C viruses, are risk factors for HCC development. Increasing evidence also indicates that obesity and related metabolic abnormalities, especially diabetes mellitus, raise the risk of HCC (1–3). Several pathophysiologic mechanisms linking obesity, steatosis, and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade. Insulin resistance leads to an increased expression of TNF- α , a central

mediator of chronic inflammatory diseases, and its dysregulation is associated with the development of steatosis and inflammation within the liver (4, 5). Hyperinsulinemia also upregulates the levels of insulin-like growth factors (IGF) and abnormal activation of the IGF/IGF-1 receptor (IGF-1R) axis contributes to the development of various types of human malignancies, including HCC (6, 7). These findings suggest that targeting insulin resistance may be an effective strategy for preventing the development of obesity-related HCC. A recent animal experiment revealed that supplementation with branched chain amino acids, which is used to improve protein malnutrition in patients with liver cirrhosis, prevents obesity-related liver tumorigenesis by targeting insulin resistance and the IGF/IGF-1R axis (8).

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

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

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

Materials and Methods

Animals and chemicals

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

Experimental procedure

At 5 weeks of age, a total of 30 *db/db* mice were randomly divided into the following 4 experimental and control groups: DEN alone (group 1, $n = 10$); DEN plus 0.1% EGCG (group 2, $n = 10$); 0.1% EGCG alone (group 3, $n = 5$); and no treatment (group 4, $n = 5$). All of the mice in groups 1 and 2 were given tap water containing 40 ppm DEN for the first 2 weeks of the experiment, which is sufficient to develop hepatocellular neoplasms in *db/db* mice (8). After DEN treatment, the mice in group 2 were given free access to tap water containing 0.1% EGCG until the end of the experiment. The mice in group 3 were given 0.1% EGCG throughout the experiment. The concentration of EGCG (0.1%), which was established according to the findings of previous chemopreventive studies (16, 23), was within the physiologic range after daily intake of GTCs in human per unit body weight basis (24). The mice in groups

1 and 4 were given tap water without EGCG. At 41 weeks of age (after 34 weeks of EGCG treatment), all of the mice were sacrificed to analyze the development of liver neoplasms and preneoplastic lesions.

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 sublobes) were used for histopathologic examination. For all experimental groups, 4- μ m 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 μ g/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)-3 β , p-GSK-3 β , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used (16, 26, 27). The primary antibody for c-Jun NH₂-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 RNeasy RNeasy-4PCR kit (Ambion Applied Biosystems). The cDNA was amplified from 0.2 μ g 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-1 β* , *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

^aWhite adipose tissue of the periorchis and retroperitoneum.^bMean ± SD.^cSignificantly 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

^aNumber of neoplasms per mouse.^bMean ± SD.^cSignificantly different from group 4 by Tukey–Kramer multiple comparison test ($P < 0.001$).^dSignificantly different from group 1 by Fisher's exact probability test ($P < 0.01$).^eSignificantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.01$).^fSignificantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.001$).