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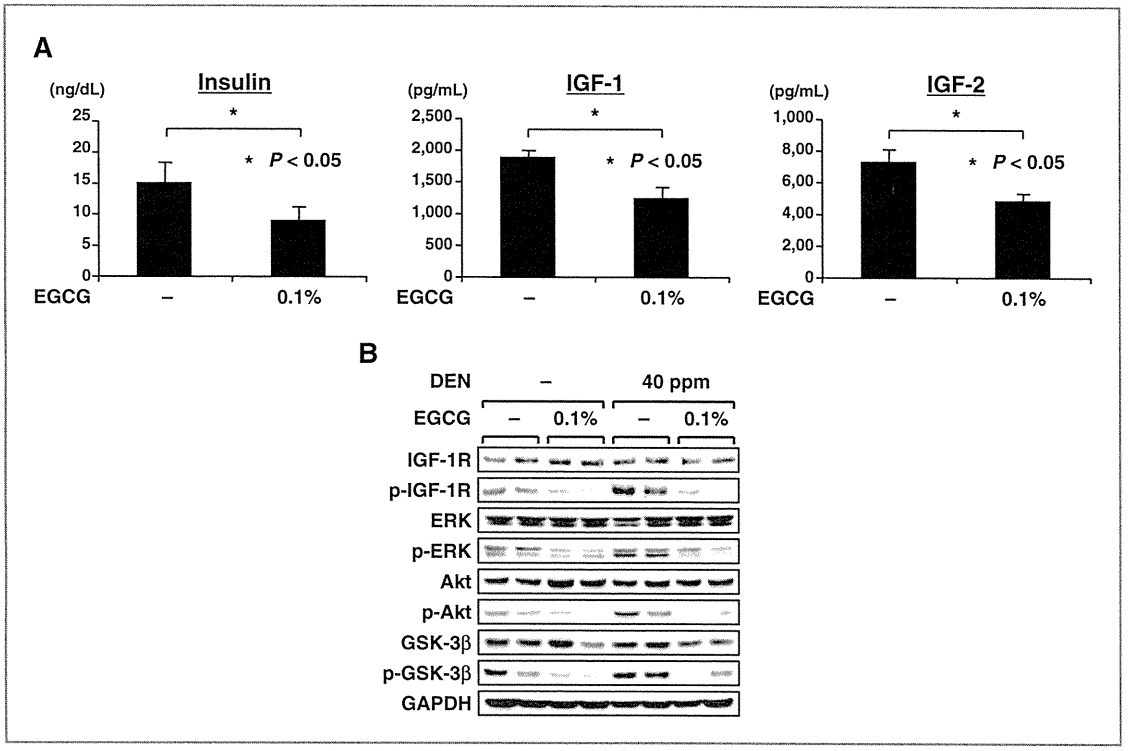

Figure 1. Effects of EGCG on the levels of serum insulin, IGF-1, and IGF-2 and on the activation of the IGF/IGF-1R axis in the liver of experimental mice. A, the serum concentrations of insulin, IGF-1, and IGF-2 in DEN-treated *db/db* mice were measured by an enzyme immunoassay. Values are the means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

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

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

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

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

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

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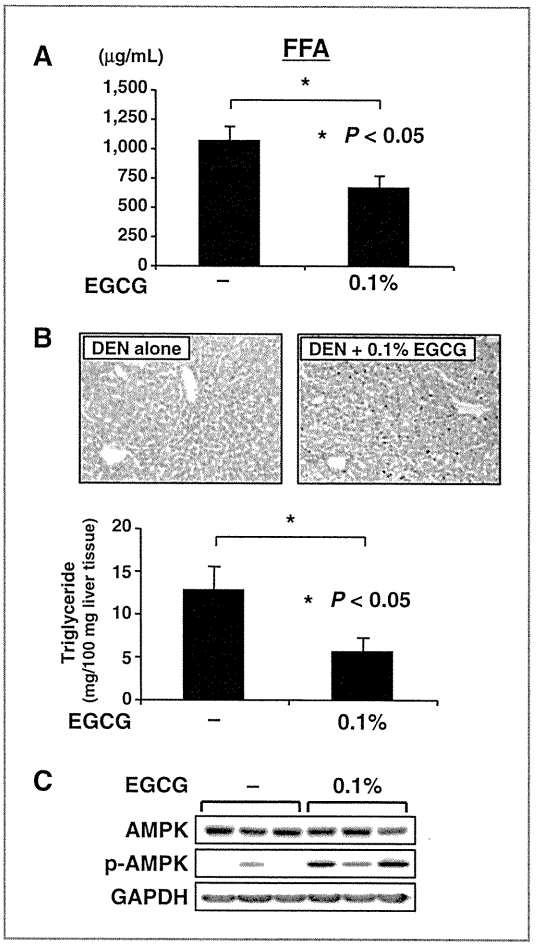

Figure 2. Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of the AMPK protein in the liver of DEN-treated *db/db* mice. A, the serum concentration of FFA was measured by an enzymatic method. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, frozen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

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

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

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

Discussion

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

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

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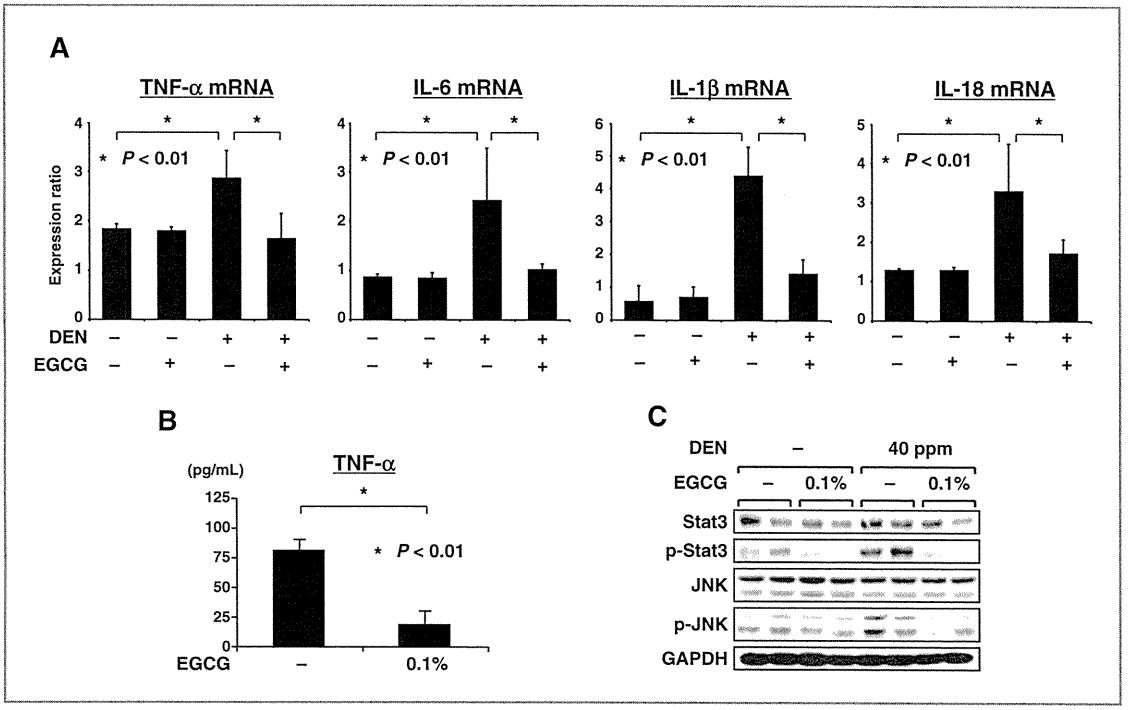

Figure 3. Effects of EGCG on the expression levels of TNF-α, IL-6, IL-18, and IL-1β mRNAs, the serum levels of TNF-α, and the activation of Stat3 and JNK proteins in the liver of experimental mice. A, the total RNAs were isolated from the livers of experimental mice, and the expression levels of TNF-α,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

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

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

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

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

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

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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RESEARCH ARTICLE Open Access

Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-*db/db* obese mice

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Abstract

Background: Obesity and related metabolic abnormalities, including inflammation and lipid accumulation in the liver, play a role in liver carcinogenesis. Adipocytokine imbalances, such as decreased serum adiponectin levels, are also involved in obesity-related liver tumorigenesis. In the present study, we examined the effects of pitavastatin - a drug used for the treatment of hyperlipidemia - on the development of diethylnitrosamine (DEN)-induced liver preneoplastic lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice.

Methods: Male *db/db* mice were administered tap water containing 40 ppm DEN for 2 weeks and were subsequently fed a diet containing 1 ppm or 10 ppm pitavastatin for 14 weeks.

Results: At sacrifice, feeding with 10 ppm pitavastatin significantly inhibited the development of hepatic premalignant lesions, foci of cellular alteration, as compared to that in the untreated group by inducing apoptosis, but inhibiting cell proliferation. Pitavastatin improved liver steatosis and activated the AMPK-α protein in the liver. It also decreased free fatty acid and aminotransferases levels, while increasing adiponectin levels in the serum. The serum levels of tumor necrosis factor (TNF)-α and the expression of *TNF-α* and *interleukin-6* mRNAs in the liver were decreased by pitavastatin treatment, suggesting attenuation of the chronic inflammation induced by excess fat deposition.

Conclusions: Pitavastatin is effective in inhibiting the early phase of obesity-related liver tumorigenesis and, therefore, may be useful in the chemoprevention of liver cancer in obese individuals.

Background

Hepatocellular carcinoma (HCC) is a serious healthcare problem worldwide because of its increasing morbidity and high mortality. Chronic inflammation of the liver and subsequent cirrhosis, which are highly correlated with hepatitis B and hepatitis C viruses infection and alcoholic liver disease, are the strongest risk factors for HCC development. Recent evidence also indicates that obesity and related metabolic abnormalities, especially diabetes mellitus and insulin resistance, raise the risk of HCC [1-4]. In obese individuals, high levels of free fatty acid (FFA) flux into the liver from excess adipose tissue. This in turn promotes hepatic steatosis and inflammation through the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and is closely associated with liver carcinogenesis [5-7]. Aberrant lipogenesis in the liver, which is closely linked to obesity and metabolic syndrome, is also a dominant event in liver carcinogenesis and human HCC progression [8]. Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the metabolic syndrome and a proportion of patients with this disease can progress to non-alcoholic steatohepatitis (NASH), which involves the risk of developing cirrhosis and HCC [9]. Therefore, in addition to lifestyle modification to reduce body weight, active pharmacotherapy is considered to be necessary for the management of NASH. For instance, metformin and thiazolidinediones,

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both of which increase insulin sensitivity, might be useful for the treatment of patients with NASH [10].

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used for the treatment of hyperlipidemia and have been shown to reduce the risk of cardiovascular disease [11]. Statins have recently also been suggested to be possible candidates for the management of NASH/NAFLD, which frequently coexist with hyperlipidemia and cardiovascular disease [12]. A pilot study revealed that treatment with atorvastatin decreases TNF-α serum levels and improves biochemical and histological features of disease activity in NASH patients with dyslipidemia [13]. The use of atorvastatin in hyperlipidemic patients complicated with NAFLD also improves serum transaminase levels and prevents hepatic fibrosis progression [14]. In a mice model, pitavastatin, a recently developed lipophilic statin, has been shown to ameliorate severe hepatic steatosis by enhancing hepatic free acid (FA) β-oxidation activity [15].

In addition to the lipid-lowering and anti-inflammatory effects, recent studies have revealed that statins appear to have anticancer and cancer chemopreventive properties [16,17]. A large cohort study showed that statin use is associated with a reduced risk of HCC in patients with diabetes [18]. Statins inhibit cell proliferation and induce apoptosis in human HCC-derived cells [19,20]. In addition, pitavastatin prevents obesity-related colorectal carcinogenesis by correcting adipocytokine imbalance and attenuating colonic inflammation in C57BL/KsJ-*db/db* (*db/db*) mice suffering from obesity and hyperlipidemia [21]. These findings suggest the possibility that long-term use of statins may also be effective for preventing the progression of obesity-related liver tumorigenesis. Our recent study showed that diethylnitrosamine (DEN)-induced liver tumorigenesis is significantly enhanced in *db/db* mice [22]. In the present study, we examined the effects of pitavastatin on the development of DEN-induced hepatic preneoplastic lesions, foci of cellular alteration (FCA), while focusing on the improvement of liver steatosis and inflammation using a *db/db* mice model.

Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and were humanely maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pitavastatin was obtained from Kowa Pharmaceutical Co. (Tokyo, Japan).

Experimental procedure

The animal experiment was approved by the Committee of Institutional Animal Experiments of Gifu University [22]. At 5 weeks of age, all 36 mice were administered tap water containing 40 ppm DEN for the first 2 weeks of the experiment. After DEN treatment, Groups 2 (n = 12) and 3 (n = 12) were given a basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) containing 1 and 10 ppm pitavastatin, respectively, until the end of the experiment. Group 1 (n = 12) acted as the control and was fed only a basal diet throughout the experiment. At 21 weeks of age (after 14 weeks of pitavastatin treatment), all the mice were sacrificed to analyze the development of FCA. Since neither C57B6 nor C57BL/KsJ-+/+ mice - the genetic controls for *db/db* mice - develop FCA and liver neoplasms by DEN administration during this period [22], control experimentation using these mice was not conducted in the present study.

Histopathology and immunohistochemical analysis for PCNA

Maximum sagittal sections of each lobe (6 sublobes) were used for histopathological examination. For all experimental groups, 4 μm-thick sections of formalin-fixed and paraffin-embedded livers were stained with hematoxylin & eosin (H&E) for histopathology. The presence of FCA, which are phenotypically altered hepatocytes showing swollen and basophilic cytoplasm and hyperchromatic nuclei, was judged according to the criteria described in a previous study [23]. The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a G_1-to-S phase marker, was performed to estimate the cell proliferative activity of FCA by using an anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark) [22]. On the PCNA-immunostained sections, the cells with intensively reacted nuclei were considered to be positive for PCNA, and the indices (%) were calculated in 20 FCA randomly selected from each group.

Protein extraction and western blot analysis

Equivalent amounts of extracted mice liver proteins (20 μg/lane) were examined by western blot analysis [22]. Previously described primary antibodies for AMP-activated kinase-α (AMPK-α), phosphorylated AMPK-α (p-AMPK-α), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used [21], with GAPDH serving as a loading control. The primary antibody for Bad was purchased from Cell Signaling Technology (Beverly, MA, USA). The intensities of the blots were quantified with NIH Image software version 1.62.

RNA extraction and quantitative real-time reverse transcription-PCR

Total RNA was isolated from the livers of experimental mice using the RNAqueous-4PCR kit (Ambion Applied

Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify *TNF-α*, *IL-6*, *Bcl-2*, *Bad*, and *GAPDH* genes, as described previously [21,24].

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 TNF-α (Shibayagi, Gunma, Japan), IL-6 (IBL, Gunma, Japan), adiponectin (Otsuka, Tokyo, Japan), and leptin (R&D Systems, Minneapolis, MN, USA) levels were determined by enzyme immunoassay according to the manufacturers' protocol. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), free fatty acid (FFA), total cholesterol, and triglyceride were measured with a standard clinical automatic analyzer (type 7180; Hitachi, Tokyo, Japan).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method [25]. The triglyceride levels in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co., Osaka, Japan) [22]. To visualize the intrahepatic lipids, Oil red O staining was utilized based on the standard procedure for frozen liver sections.

Statistical analysis

The results are presented as means ± SD, and were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software; San Diego, CA) for Macintosh. Differences among the groups were analyzed by either one-way ANOVA or, as required, by two-way ANOVA. When the ANOVA revealed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Bonferroni multiple comparisons test. The differences were considered significant when the two-sided P value was < 0.05.

Results

General observations

As presented in Table 1, administration of pitavastatin significantly ($P < 0.01$, Group 1 vs. Groups 2 and 3) and dose dependently ($P < 0.05$, Group 2 vs. Group 3) decreased the value of body mass index (BMI). The body weight and relative weights of liver and white adipose tissue (periorchis and retroperitoneum) of the mice that received 10 ppm pitavastatin were slightly lower than those of the untreated control mice, but the differences were not significant. During the experiment, pitavastatin administration did not cause any clinical symptoms for toxicity. Histopathological examination also revealed the absence of pitavastatin toxicity in the liver, kidney, and spleen (data not shown).

Effects of pitavastatin on DEN-induced liver preneoplastic lesions in db/db *mice*

Liver preneoplastic lesion FCA, which possesses basophilic cytoplasm and hyperchromatic nuclei (Figure 1A), was observed in the livers of mice from all groups at the termination of the experiment. Treatment with a high dose (10 ppm) of pitavastatin significantly inhibited the development of FCA in comparison to both the untreated control mice ($P < 0.001$) and low dose (1 ppm) of pitavastatin-treated mice ($P < 0.05$). Treatment with 1 ppm pitavastatin also demonstrated a tendency to suppress the development of FCA - the inhibition rate being 29% - in comparison to the untreated control mice, but the difference did not reach a statistical significance (Figure 1B).

Effects of pitavastatin on the cellular levels of Bad and Bcl-2 and the proliferation activity in FCA of DEN-treated db/db *mice*

We next examined the effects of pitavastatin on the induction of apoptosis in the liver and the inhibition of cell proliferation in FCA of DEN-treated *db/db* mice. Treatment with both low and high doses of pitavastatin increased the protein levels of Bad, a pro-apoptotic Bcl-2 family member, in the liver of experimental mice (Figure 2A, $P < 0.05$). The mRNA levels of this molecule

Table 1 Body, liver, kidney and white adipose tissue weights of the experimental mice

Group no.	Treatment	No. of mice	Body wt (g)	BMI[a]	Relative wt (g/100 g body wt) of:		
					Liver	Kidney	Fat[b]
1	DEN alone	12	63.1 ± 7.0[c]	7.2 ± 0.6	6.4 ± 1.5	0.9 ± 0.1	9.3 ± 1.0
2	DEN + 1 ppm Pitavastatin	12	59.7 ± 3.9	6.7 ± 0.4[d]	6.0 ± 0.8	0.9 ± 0.1	9.1 ± 0.8
3	DEN + 10 ppm Pitavastatin	12	55.2 ± 9.5	6.2 ± 0.6[d,e]	5.7 ± 1.2	1.0 ± 0.2	8.7 ± 1.0

[a]Body mass index.

[b]White adipose tissue of the periorchis and retroperitoneum.

[c]Mean ± SD.

[d]Significantly different from Group 1 ($P < 0.01$).

[e]Significantly different from Group 2 ($P < 0.05$).

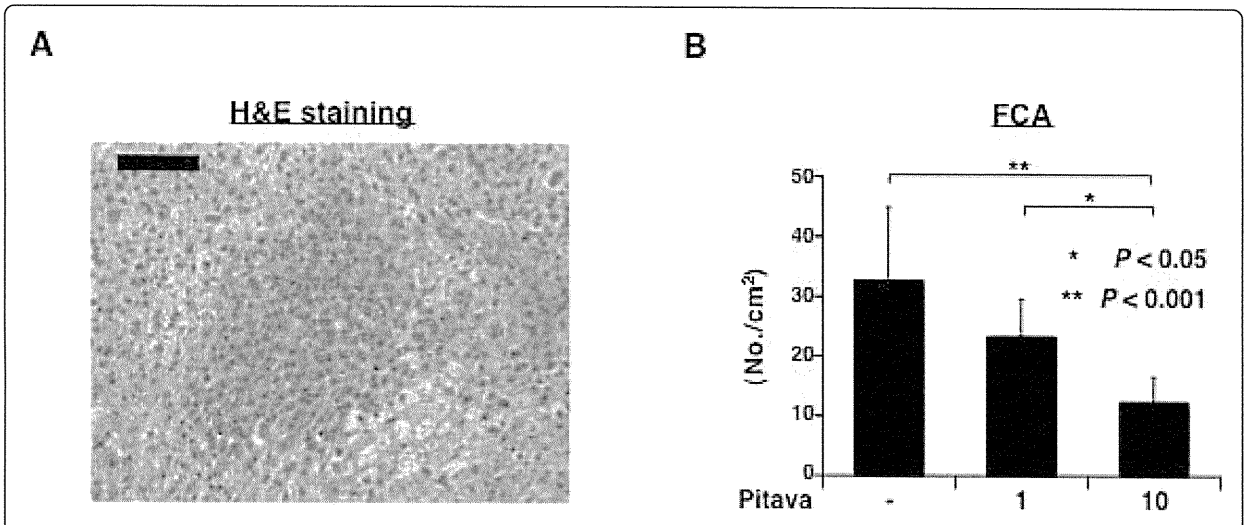

Figure 1 Effects of pitavastatin on DEN-induced FCA in *db/db* mice. (*A*) A representative photograph of FCA induced by DEN in *db/db* mice (H&E staining). Scale bar, 100 μm. (*B*) Average number of FCA in all groups (pitavastatin-untreated, 1 ppm pitavastatin-treated, and 10 ppm pitavastatin-treated groups). Each column represents the mean ± SD. * $P < 0.05$ vs. 1 ppm pitavastatin-treated group and ** $P < 0.001$ vs. the untreated group, respectively.

were also increased by 1 ppm pitavastatin administration (Figure 2B, $P < 0.05$). On the other hand, pitavastatin treatment induced a marked decrease in the levels of an anti-apoptotic molecule Bcl-2 mRNA (Figure 2B, $P < 0.05$). In addition, as shown in Figure 2C, the mean PCNA-labeling indices for FCA in mice treated with 1 ppm (23.9 ± 7.7%) and 10 ppm (16.6 ± 4.0%) pitavastatin were significantly lower than that in the mice which received only DEN (47.7 ± 11.0%; $P < 0.001$ for each comparison). These findings indicate that pitavastatin significantly suppresses FCA, at least in part, by inducing apoptosis and by reducing cell proliferation.

Effects of pitavastatin on hepatic steatosis, activation of AMPK-α protein in the liver, and serum levels of FFA, total cholesterol, and triglyceride in DEN-treated db/db *mice*

Accumulation of lipids in the liver, which is caused by dyslipidemia, is considered to play a role in liver tumorigenesis [5,6]. Therefore, we examined whether pitavastatin improved hepatic steatosis and hyperlipidemia in the experimental mice. Examination of Oil red O stained sections revealed severe hepatic steatosis in the DEN-treated *db/db* mice; however, the mice's conditions were markedly improved by pitavastatin administration (Figure 3A, upper panels). Similar to the histological findings, the levels of intrahepatic triglyceride were also significantly reduced by administration of pitavastatin (Figure 3A, lower panel, $P < 0.001$). Western blot analysis demonstrated that pitavastatin significantly phosphorylated (*i.e.*, activated) AMPK-α - a critical kinase that monitors cellular energy status [26] - in the livers of the experimental mice (Figure 3B, $P < 0.05$). In addition, treatment with both low ($P < 0.01$) and high ($P < 0.001$) doses of pitavastatin decreased the serum levels of FFA, while the levels of total cholesterol and triglyceride were not affected by administration of this agent (Figure 3C).

Effects of pitavastatin on serum levels of AST, ALT, adiponectin, and leptin in DEN-treated db/db *mice*

The serum levels of AST, ALT, adiponectin, and leptin in the experimental mice are listed in Table 2. The elevated serum AST and ALT levels, which might increase due to severe steatosis (Figure 3A), were significantly decreased by treatment with both low ($P < 0.001$) and high ($P < 0.05$) doses of pitavastatin. The serum leptin levels after pitavastatin administration demonstrated a downward trend, but the differences were not significant. However, treatment with this agent markedly increased the serum levels of adiponectin when compared to the control mice ($P < 0.05$).

Effects of pitavastatin on serum TNF-α levels and hepatic expression of TNF-α *and* IL-6 *mRNAs in DEN-treated* db/db *mice*

Chronic inflammation induced by excessive production of storage lipids is closely associated with obesity-related liver carcinogenesis [5-7]. Therefore, the effects of pitavastatin on the serum levels of TNF-α, a central mediator of chronic inflammatory disease, and on the expression of *TNF-α* and *IL-6* mRNAs in the liver of DEN-treated *db/db* mice were examined. Administration

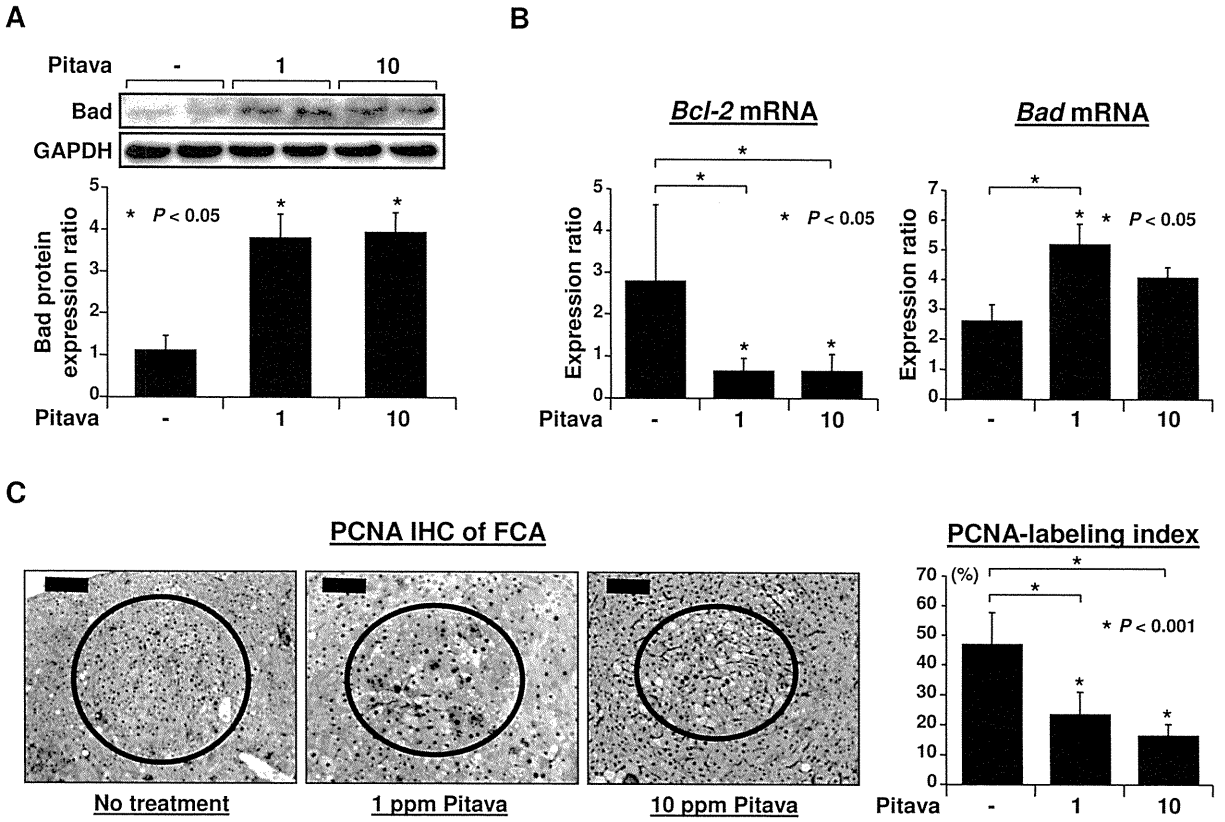

Figure 2 Effects of pitavastatin on the expression levels of Bad and Bcl-2 in the liver and cell proliferation in FCA induced by DEN in *db/db* mice. (*A*) The total proteins were extracted from the livers of experimental mice and the expression levels of the Bad protein were examined by western blot analysis (upper panel). The intensities of the blots were quantified by densitometry (lower panel). GAPDH antibody served as a loading control. Two lanes represent protein samples obtained from 2 different mice in each group. Repeat western blots yielded similar results. Values are the means ± SD. * $P < 0.05$ vs. the untreated group. (*B*) The total RNAs were isolated from the livers of experimental mice and the expression levels of *Bcl-2* and *Bad* mRNAs were examined by quantitative real-time RT-PCR using specific primers. The expression levels of each mRNA were normalized to the level of *GAPDH* mRNA. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group. (*C*) Representative pictures of the PCNA-immunohistochemical analysis of FCA (circled) developed in the livers of Groups 1, 2, and 3 (left panels). The PCNA-labeling indices of FCA developed in the livers of Groups 1 through 3 were determined by counting the PCNA-positive nuclei in FCA (right panel). Scale bars, 200 μm (no treatment) and 100 μm (1 and 10 ppm pitavastatin). * $P < 0.001$ vs. the untreated group.

of both doses of pitavastatin significantly decreased serum TNF-α levels (Figure 4A, $P < 0.05$). Further, quantitative real-time RT-PCR revealed that the expression levels of *TNF-α* and *IL-6* mRNAs in the livers of experimental mice were also significantly decreased after pitavastatin treatment (Figure 4B, $P < 0.05$, respectively), suggesting that pitavastatin attenuated liver inflammation in obese *db/db* mice.

Discussion and Conclusions

Statins lessen hyperlipidemia by competitively inhibiting HMG-CoA reductase, and thus, they are effective in preventing cardiovascular disease [11]. On the other hand, many studies have shown the anticancer and cancer chemopreventive effects of statins, such as the inhibition of cell proliferation, promotion of apoptosis, and inhibition of inflammation, angiogenesis, and metastasis [16,17,19,20]. The anticancer effects of statins also involve the inhibition of geranylgeranylation, primary of the Rho proteins [16,17]. These findings suggest the possibility of statins playing a role of cancer chemopreventive agents for certain malignancies.

The results of the present study clearly indicated that pitavastatin, which is widely used for the treatment of patients with hyperlipedemia, effectively prevents the development of DEN-induced liver preneoplastic lesions in obese *db/db* mice (Figure 1B). This is the first report that shows the preventive effect of statin analog on the development of obesity-related liver tumorigenesis. The unfavorable effects of obesity and related metabolic abnormalities are serious global healthcare problem. Among them, the promotion of HCC by obesity [1-4] is

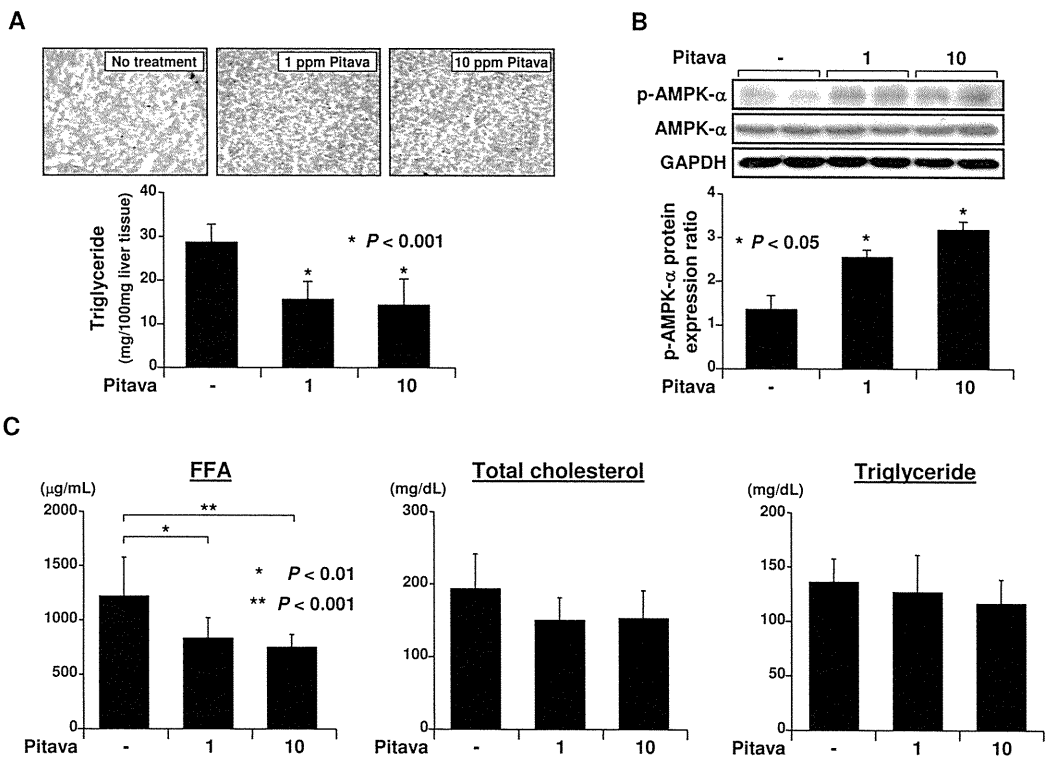

Figure 3 Effects of pitavastatin on hepatic steatosis, activation of the AMPK-α protein in the liver, and serum levels of FFA, total cholesterol, and triglyceride in DEN-treated *db/db* mice. (*A*) Frozen liver sections from experimental mice with or without pitavastatin treatment were stained with Oil red O to show steatosis (upper panels). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (lower panel). Values are the means ± SD. * $P < 0.001$ vs. the untreated group. (*B*) The total proteins were extracted from the livers of experimental mice and the expression levels of the AMPK-α and p-AMPK-α proteins were examined by western blot analysis (upper panel). The intensities of the blots were quantified by densitometry (lower panel). GAPDH antibody served as a loading control. Two lanes represent protein samples obtained from 2 different mice in each group. Repeat western blots yielded similar results. Values are the means ± SD. * $P < 0.05$ vs. the untreated group. (*C*) The serum concentrations of FFA, total cholesterol, and triglyceride in all groups. Values are the means ± SD. * $P < 0.01$ and ** $P < 0.001$ vs. the untreated group, respectively.

one of the critical issues that need to be addressed in the management of this malignancy. Therefore, our present finding seems to be clinically significant when considering the prevention of HCC in obese people, who are at an increased risk of developing HCC.

The suppressive effect of pitavastatin on the development of obesity-related liver tumorigenesis was most likely associated with the induction of apoptosis in the liver (Figures. 2A and 2B) and the inhibition of proliferation in FCA (Figure 2C). This inhibition was also associated with the improvement of hepatic steatosis (Figure 3A) and the attenuation of inflammation (Figure 4) because excess accumulation of lipids in the liver accelerates hepatic tumorigenesis by inducing a chronic

Table 2 Serum levels of AST, ALT, adiponectin, and leptin in the experimental mice

Group no.	Treatment	No. of mice	AST[a] (IU/L)	ALT[b] (IU/L)	Adiponectin (μg/mL)	Kidney (ng/dL)
1	DEN alone	12	194 ± 47[c]	291 ± 112	15.5 ± 2.4	108.1 ± 33.4
2	DEN + 1 ppm Pitavastatin	12	111 ± 28[d]	180 ± 49[d]	19.2 ± 4.5[e]	104.3 ± 33.2
3	DEN + 10 ppm Pitavastatin	12	144 ± 28[e]	227 ± 96[e]	21.2 ± 7.4[e]	93.2 ± 31.2

[a]aspartate aminotransferase.
[b]alanine aminotransferase.
[c]Mean ± SD.
[d]Significantly different from Group 1 ($P < 0.001$).
[e]Significantly different from Group 1 ($P < 0.05$).

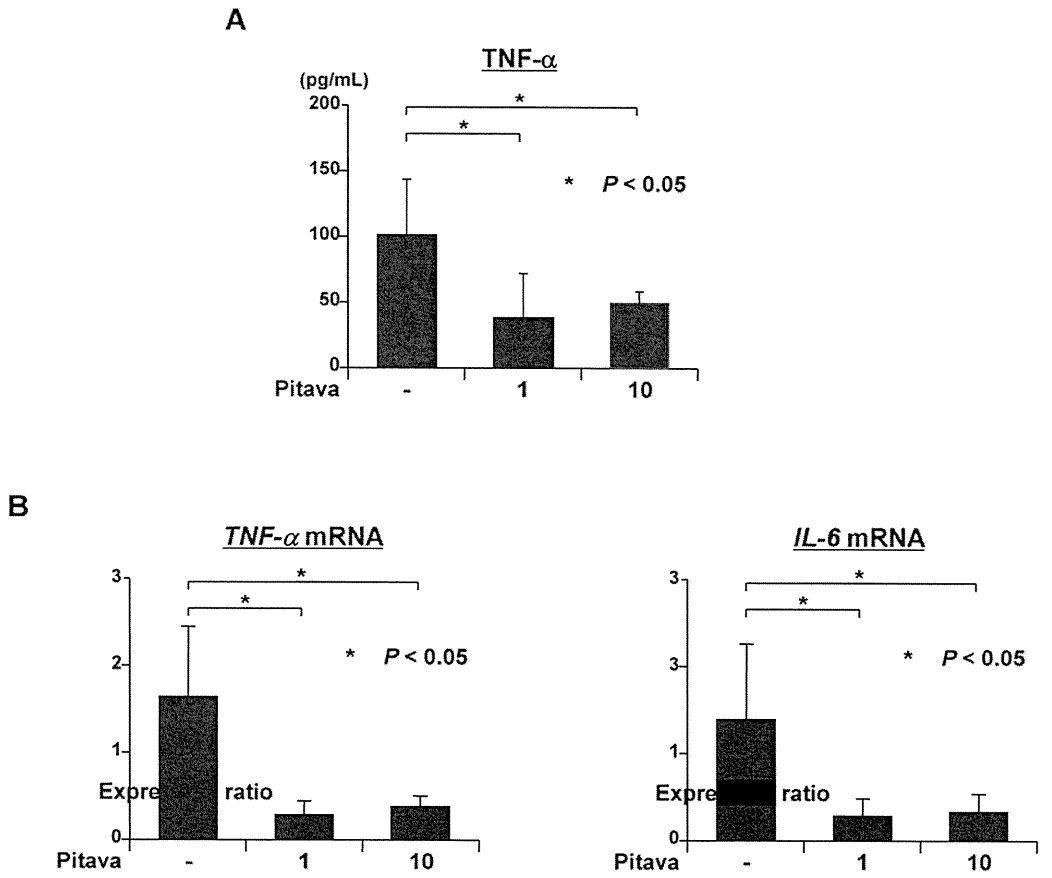

Figure 4 Effects of pitavastatin on the serum levels of TNF-α and the expression levels of *TNF-α* and *IL-6* mRNAs in the liver of DEN-treated *db/db* mice. (*A*) The serum concentration of TNF-α was measured by enzyme immunoassay. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group. (*B*) The total RNAs were isolated from the livers of experimental mice and the expression levels of *TNF-α* and *IL-6* mRNAs were examined by quantitative real-time RT-PCR using specific primers. The expression levels of each mRNA were normalized to the level of *GAPDH* mRNA. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group.

inflammatory reaction [5-7]. Pitavastatin mainly ameliorates hepatic steatosis by decreasing serum FFA levels (Figure 3C) since the high influx of FFA into the liver plays a major role in hepatic fat accumulation [5,6]. In addition, activation of AMPK-α by pitavastatin in the liver (Figure 3B), which increases FA oxidation, decreases FA synthesis, and improves hyperlipidemia [26], also contributes to the inhibition of lipid deposition in the liver. Further, these findings are significant when considering the prevention of obesity-related carcinogenesis because AMPK is regarded as a metabolic tumor suppressor and a promising target for cancer prevention and therapy [27]. AMPK activity is associated with the inhibition of lipogenesis, which has a pathogenic and prognostic significance for HCC [8], induction of apoptosis, and suppression of cell growth in human HCC-derived cells [28]. Pitavastatin has also been shown to inhibit obesity-related colorectal carcinogenesis through the activation of AMPK-α in the colonic mucosa [21].

In the present study, lipid-lowering effects of pitavastatin were positive on serum FFA but not significant on total cholesterol and triglyceride in DEN-treated *db/db* mice (Figure 3C). These findings are consistent with the results of a recent study indicating more high doses of pitavastatin (20 and 40 ppm) did not significantly decrease the serum levels of total cholesterol and triglyceride in Min mice, which show a hyperlipidemic state [29]. On the contrary, Egawa *et al.* [15] demonstrated that pitavastatin administration resulted in a significant reduction in the levels of plasma triglyceride and total cholesterol in aromatase-deficient mice. Treatment with both 1 and 10 ppm pitavastatin for 8 weeks also reduced serum levels of total cholesterol, but not triglyceride, in azoxymethane-treated *db/db* mice [21]. These reports [15,21,29], together with the results of the present study,

suggest that effects of pitavastatin on plasma lipids might depend on the animal strain and experimental procedure. In addition, it has been shown that pitavastatin potently inhibits *de novo* cholesterol synthesis without affecting serum lipid levels [30,31]. In rodents, cholesterol synthesis enzymes were remarkably induced by feedback regulation [32], suggesting that the effects of pitavastatin on reduction of plasma lipid and inhibition of HMG-CoA reductase activity might be masked by such feedback regulation.

Increases in TNF-α and IL-6 levels, which are accompanied by lipid accumulation in the liver, are involved in obesity-related liver carcinogenesis [5-7]. Therefore, reduction of serum TNF-α levels (Figure 4A) and inhibition of the expression of *TNF-α* and *IL-6* mRNAs in the liver (Figure 4B) by pitavastatin are important in preventing obesity-related liver tumorigenesis. These findings are consistent with previous reports that pitavastatin significantly suppresses inflammation- and obesity-related mouse colon carcinogenesis by attenuating chronic inflammation [21,33]. The effects of pitavastatin on decreasing the levels of TNF-α might be largely dependent on the reduction of BMI (Table 1) and serum FFA levels (Figure 3C). These phenomena may also be associated with the improvement of adipocytokine imbalance (Table 2) because TNF-α has been shown to decrease the levels of adiponectin, which is secreted by the adipose tissue, while increasing the levels of leptin in the adipocytes [34,35]. Moreover, up-regulation of serum adiponectin levels (Table 2) also plays a role in attenuating inflammation because this adipocytokine possesses the ability to down-regulate the production of TNF-α and IL-6 [36]. Adiponectin alleviates hepatic steatosis and ALT abnormalities in alcohol-induced fatty liver mice model and in *ob/ob* mice, a NAFLD mice model, by enhancing FA oxidation, while decreasing FA synthesis and TNF-α production in the liver [37]. Hypoadiponectinemia enhances the progression of steatosis and hepatic tumor formation in a mice model of NASH [38]. In addition, adiponectin inhibits cell proliferation and induces apoptosis in human HCC-derived cells by inducing AMPK activation [39]. Therefore, the elevation of adiponectin and activation of AMPK might be effective for the prevention of obesity-related tumorigenesis.

Hepatotoxicity is one of the critical concerns in treatment with statins. In the present study, however, pitavastatin did not cause significant toxicity in the liver as determined by histological examination. The serum aminotransferase (ALT and AST) levels were also decreased by treatment with this agent (Table 2). The safety of statins for patients with liver dysfunction has also been reported in several clinical trials [40]. In addition, patients with chronic liver disease, including NAFLD/NASH and HCV infection, may benefit from statins because cardiovascular risk is likely to be high in these diseases [12,41]. Therefore, statin use might be a promising therapy for NASH patients who have an increased risk of HCC [9], although periodic monitoring of serum aminotransferase levels should be conducted. The result of a recent epidemiological study revealing a significant relationship between the risk reduction of HCC and statin use among diabetic patients [18] may also encourage statin therapy for patients with chronic liver disease, especially NASH patients, who frequently have hyperlipidemia as well as insulin resistance.

Finally, it should be noted that the results of recent studies indicating that supplementation with branched-chain amino acids and acyclic retinoid, both of which exert chemopreventive effects on the development of HCC in clinical trials [3,42], suppresses DEN-induced liver tumorigenesis in *db/db* mice by improving hepatic steatosis and attenuating chronic inflammation [22,43]. In summary, the results of the present study, together with those of the cited reports [22,43], suggest that the prevention of liver carcinogenesis by targeting hepatic steatosis, chronic inflammation, and adipocytokine imbalance, through either pharmaceutical or nutritional intervention, might be a promising strategy for obese individuals who are at an increased risk of developing HCC. Pitavastatin appears to be a potentially effective candidate for this purpose since it can improve liver steatosis and attenuate inflammation, at least in part, through the activation of AMPK-α and up-regulation of adiponectin.

List of abbreviations used

ALT: alanine aminotransferase; AMPK: AMP-activated kinase; ANOVA: analysis of variance; AST: aspartate aminotransferase; BMI: body mass index; DEN: diethylnitrosamine; FA: fatty acid; FCA: foci of cellular alteration; FFA: free fatty acid; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H&E: hematoxylin & eosin; HCC: hepatocellular carcinoma; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; IL: interleukin; PCNA: proliferating cell nuclear antigen; RT-PCR: reverse transcription-PCR; TNF-α: tumor necrosis factor-α.

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Authors' contributions

MS, YY, and TT conceived of the study, participated in its design, and drafted the manuscript. MS, YY, HS, MK, DT, AB, and TO performed *in vivo* experiment. TK performed statistical analysis. HT and HM helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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REVIEW

Cancer chemoprevention with green tea catechins by targeting receptor tyrosine kinases

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Recent studies indicate that receptor tyrosine kinases (RTKs), which play important roles in cell proliferation, are one of the possible targets of green tea catechins (GTCs) in cancer cell growth inhibition. (−)-Epigallocatechin-3-gallate (EGCG), the major catechin in green tea, inhibits cell proliferation and induces apoptosis in various types of cancer cells, including colorectal cancer and hepatocellular carcinoma cells, by blocking the activation of the epidermal growth factor receptor (EGFR) family of RTKs. EGCG inhibits the activation of insulin-like growth factor-1 receptor (IGF-1R) and VEGFR2, the other members of the RTK family, and this effect is also associated with the anticancer and chemopreventive properties of this agent. EGCG suppresses the activation of EGFR in part by altering membrane lipid organization and causing the subsequent inhibition of the dimerization and activation of this receptor. Preliminary trials have shown that GTCs successfully prevent the development and progression of precancerous lesions, such as colorectal adenomas, without causing severe adverse effects. The present report reviews evidence indicating that GTCs exert anticancer and chemopreventive effects by inhibiting the activation of specific RTKs, especially EGFR, IGF-1R, and VEGFR2, and concludes that targeting RTKs and their related signaling pathways by using tea catechins could be a promising strategy for the prevention of human cancers.

Keywords:
Colorectal cancer / EGCG / Hepatocellular carcinoma / Lipid rafts / Receptor tyrosine kinase

1 Introduction

Tea is one of the most popular beverages worldwide. In the recent years, tea polyphenols, which are known as catechins, have received great attention for their beneficial effects, in particular their involvement in cancer chemoprevention. Among tea catechins, green tea catechins (GTCs) are best known for their cancer-preventive properties. Rapidly increasing number of studies have reported that (−)-epigallocatechin-3-gallate (EGCG), the major biologically active component in green tea, is one of the most potent catechins capable of inhibiting cell proliferation and inducing apoptosis in cancer cells [1–6]. Recent studies have revealed that GTCs exert cancer chemopreventive and anticarcinogenic effects, at least in part, by modulating the activities of different receptor tyrosine kinases (RTKs) and their multiple downstream signaling pathways, including the Ras/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways [7, 8], which control the expression of the multiple target genes involved in cell proliferation and apoptosis [3–5].

The present report reviews the novel and updated mechanisms by which GTCs prevent carcinogenesis, with a special emphasis on colorectal cancer (CRC) and

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Abbreviations: AP-1, activator protein-1; **CRC,** colorectal cancer; **COX-2,** cyclooxygenase-2; **EGFR,** epidermal growth factor receptor; **EGCG,** (−)-epigallocatechin-3-gallate; **ERK,** extracellular signal-regulated kinase; **GTC,** green tea catechin; **GTE,** green tea extract; **HNSCC,** head and neck squamous cell carcinoma; **HCC,** hepatocellular carcinoma; **HGF,** hepatocyte growth factor; **IGF-1R,** insulin-like growth factor-1 receptor; **LR,** laminin receptor; **MEK,** mitogen-activated protein kinase; **NF-κB,** nuclear factor-κB; **PI3K,** phosphatidylinositol 3-kinase; **PDGF,** platelet-derived growth factor; **PGE_2,** prostaglandin E_2; **RTK,** receptor tyrosine kinase; **VEGF,** vascular endothelial growth factor; **VEGFR,** vascular endothelial growth factor receptor

hepatocellular carcinoma (HCC), and focusing on the effects of these agents on the activity of RTKs. Among the different types of RTKs, attention was particularly given to epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), and vascular endothelial growth factor receptor-2 (VEGFR2), because alterations in these RTKs and their ligands have been reported to be largely involved in the development of cancer [9–11]. In this review, the ability of GTCs, especially EGCG, to alter the activation status of these RTKs and their downstream signal transduction pathways is discussed. In addition, the potential for the clinical application of GTCs is also examined, particularly in reference to our recent pilot trial showing the preventive effect of GTCs on the recurrence of colorectal adenomas after polypectomy [12].

2 Green tea and cancer chemoprevention

Tea, especially green tea, produced from the leaves of the Camellia sinensis, is one of the most widely consumed beverages in the world. The health benefits of green tea are well documented, including its effect on cancer prevention. Green tea contains several polyphenolic compounds (catechins), including EGCG, (−)-epigallocatechin, epicatechin-3-gallate, and (−)-epicatechin. Among these GTCs, EGCG seems to be the most effective in the suppression of cell proliferation and induction of apoptosis in cancer cells [1–6]. Numerous animal experiments have shown the cancer chemopreventive effects of tea and its components [1, 2]. We recently reported that administration of EGCG through drinking water significantly suppresses chemically induced colonic and hepatic premalignant lesions in obese and diabetic mice [13, 14]. Treatment with EGCG and Polyphenon E, a standardized and well-characterized decaffeinated extract of green tea, significantly suppressed inflammation-related mouse colon carcinogenesis by attenuating the inflammatory reaction on the colonic mucosa [15]. EGCG consumption also significantly inhibited the growth of CRC and HCC xenografts in nude mice [16, 17].

Several properties of GTCs have been implicated in their anticancer and chemopreventive effects, such as their antioxidant [1], pro-oxidant [18], antimutagenic [19], antiinflammatory [15], and antiangiogenic effects [20]. EGCG also serves as a binding partner for many biomolecules, including the 67-kDa laminin receptor (LR) [21, 22] and the Bcl-2 proteins [23], which might be associated with inhibition of the activation of several types of intracellular signaling molecules and the induction of apoptosis [24, 25]. These biological effects exerted by GTCs may act cooperatively in preventing the development of human malignancies (for more details, see References [1, 2]).

Recently, we and other investigators revealed that targeting RTKs and their downstream signaling pathways might be one of the possible mechanisms mediating the effects of GTCs on the prevention of cancers [3–5]. Because abnormalities in the expression and/or function of cell surface RTKs and their specific signaling pathways are widely associated with the development of various types of human malignancies, targeting these aberrant molecules is an effective strategy for the prevention of certain types of cancers, including CRC and HCC. The following section will provide a detailed explanation of the relationship between abnormalities in specific RTKs and the development of CRC and HCC.

3 Abnormalities in RTKs and colorectal and liver cancers

All members of the RTK family show a similar structure consisting of an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain. The binding of specific ligands (growth factors and cytokines) to the extracellular domain of RTKs stimulates their intrinsic tyrosine kinase activity and triggers autophosphorylation of specific tyrosine residues, thereby resulting in the creation of docking sites for downstream targets. The major signaling pathways activated by RTKs are the Ras/ERK pathway and the PI3K/Akt pathway. In the Ras/ERK pathway, Ras activation by RTKs triggers its interaction with and activation of Raf-1 [7, 8]. The activation of cell surface RTKs and their downstream signalings play an important role in the control of many fundamental cellular processes in normal cells; however, tumor cells often show alterations in the activation of RTKs through several mechanisms, including mutations, overexpression, and the autocrine or paracrine production of their ligands [9–11].

The EGFR family includes four members, namely EGFR (erbB1), HER2 (neu/erbB2), HER3 (neu/erbB3), and HER4 (neu/erbB4), which belong to subclass I of the RTK superfamily. IGF-1R and VEGFR2 belong to a separate family of RTKs. Although approximately 20 different RTK classes have been identified, abnormalities in certain RTKs, especially EGFR, IGF-1R, and VEGFR2, are largely associated with the acquisition of neoplastic properties in various organs, including the colorectum and liver [9–11]. Human CRC often displays an overexpression of EGFR, and the constitutive activation of this receptor and related downstream signaling pathways occurs in the early stages of human colorectal carcinogenesis [26–28]. EGFR is also overexpressed in HCC, and this phenomenon shows significant correlation with the proliferating activity, clinical stage, intrahepatic metastasis, and carcinoma differentiation [29]. Overexpression of IGF-1R is frequently observed in CRC when compared with its expression level in the normal colonic mucosa [30]. IGF-1R, which is expressed at low levels in normal hepatocytes, is also overexpressed in human HCC tissues, whereas the expression levels of IGFBP-3, a negative regulator of the IGF/IGF-1R axis, are

decreased in human HCC samples and this is associated with poor survival of HCC patients [31–33]. In addition, an overexpression and activation of the VEGF/VEGFR axis is observed in both human CRC and HCC, and this has also been shown to correlate with poor prognosis for these malignancies [34–37]. Therefore, targeting these RTKs and their downstream pathways may be a potentially effective strategy for the prevention and, in certain cases, treatment of some types of human malignancies, including CRC and HCC [9–11]. We will mainly discuss the effects of GTCs on EGFR, IGF-1R, and VEGFR in this review.

4 Effects of GTCs on the EGF receptor family of RTKs in CRC and HCC cells

Recent studies have revealed that several phytochemicals, including GTCs, exert antitumor activity by suppressing the activation of the EGF receptor family of RTKs and their downstream effectors in cancer cells [3–5]. Liang et al. [38] demonstrated that EGCG directly blocks epidermal growth factor (EGF) binding to the EGFR and thus inhibits the phosphorylation of this receptor and DNA synthesis in human A431 epidermoid carcinoma cells. We have extended this finding and reported that EGCG inhibits the activation of EGFR, HER2, and HER3, and their multiple downstream signaling pathways in human head and neck squamous cell carcinoma (HNSCC), breast cancer, and CRC cell lines [39–43]. EGCG and Polyphenon E preferentially inhibit the growth of CRC cells, which overexpress and activate EGFR and HER2, when compared with a normal human fetal colonic epithelial cell line. Treatment with these agents inhibits the activation of EGFR and HER2, the phosphorylation of Akt and ERK, and also the transcriptional activity of the activator protein-1 (AP-1), c-fos, nuclear factor-κB (NF-κB), and cyclin D1 promoters in the HT29 human CRC cell line [42]. In SW837 human CRC cells, EGCG also inhibits the activation of EGFR, HER2, and HER3, with the subsequent inhibition of the expression of cyclooxygenase-2 (COX-2) at the level of transcription, and it reduces the production of prostaglandin E_2 (PGE_2) by these cells [43]. These findings are of interest because both the EGF receptor family of RTKs and the COX-2/PGE_2 axis are critical targets for CRC chemoprevention and treatment [9, 44].

As described above, EGCG exerts its anticancer and chemopreventive effects in part through the inhibition of the EGFR family of RTKs. Therefore, there is growing interest in preventive and therapeutic strategies involving the combination of EGCG with other agents that inhibit EGFR activation because such a combination treatment targeting the same molecule might provide the potential for synergistic effects on growth inhibition in cancer cells [45]. Indeed, recent in vitro and in vivo studies with HNSCC cells revealed that the combination of EGCG and erlotinib, an EGFR-tyrosine kinase inhibitor, caused synergistic cell growth inhibition by inhibiting EGFR and Akt phosphorylation, inducing apoptosis, and suppressing the NF-κB signaling pathway [46, 47]. The combination of EGCG and erlotinib also resulted in a greater inhibition of both cell proliferation and growth rate of xenografts in non-small cell lung cancer cells than either agent alone [48]. These results suggest the possibility that a combined treatment with EGCG and EGFR-targeting agents provides a promising regimen for future chemoprevention and treatment of human malignancies, owing to the synergistic effects of these compounds.

5 Effects of GTCs on the IGF/IGF-1R axis in CRC and HCC cells

In addition to the EGFR family of RTKs, increasing evidence suggests that GTCs inhibit the tyrosine kinase activities of the other members of the RTK family, such as IGF-1R and VEGFR2. We recently reported that EGCG inhibits the activation of IGF-1R in HepG2 human HCC and SW837 CRC cells that display a constitutive activation of this receptor. In these studies, the inhibition of IGF-1R activation by EGCG was associated with a decrease in the expression levels of IGF-1/2, but an increase in the expression levels of IGFBP-3, which negatively controls the function of IGF-1/2 in these cancer cells [49, 50]. EGCG inhibits the expression of matrix metalloproteinases (MMPs)-7 and -9 in CRC cells and this may play a role in upregulating the expression of IGFBP-3 [49]. Because the IGF/IGF-1R axis, which forms autocrine and paracrine loops in cancer tissues, plays an important role in the development and growth of various types of cancer [10], disruption of these loops by GTCs might be an effective strategy for the prevention and treatment of certain cancers.

Similar effects of EGCG targeting the IGF/IGF-1R axis are also observed in in vivo studies. In an obesity-related colorectal carcinogenesis mice model, EGCG administration through drinking water effectively suppresses the development of premalignant CRC lesions by depressing the IGF/IGF-1R and COX-2/PGE_2 axes. In this study, EGCG caused the inhibition of the expression of COX-2 and the activation of IGF-1R on the colonic mucosa, and decreased the serum IGF-1 levels while increasing the serum IGFBP-3 levels in obese mice [13]. In accordance with this study, administration of EGCG through the drinking water also prevents obesity-related liver tumorigenesis in db/db mice by inhibiting IGF-1R, ERK, Akt, GSK-3β, Stat3, and JNK phosphorylation in the liver and decreasing the levels of insulin, IGF-1, and IGF-2 in the serum [14]. Other investigators have also demonstrated that the oral infusion of GTCs inhibits the development and progression of prostate cancer in mice by reducing the serum IGF-1 levels, inhibiting Akt and ERK activation, and increasing serum IGFBP-3 levels [51, 52]. Drinking EGCG also prevents carbon tetrachloride (CCl_4)-induced rat hepatic fibrosis by inhibiting IGF-1R expression [53]. This finding is significant when considering HCC

chemoprevention because inhibition of hepatic fibrosis, which is a precancerous condition to HCC, might be linked to the prevention of HCC development [54, 55].

6 Effects of GTCs on the VEGF/VEGFR axis in CRC and HCC cells

VEGF, which binds to and activates VEGFR, is a mitogen for endothelial cells that is often associated with pathological angiogenesis. Abnormal activation of the VEGF/VEGFR axis is therefore closely associated with tumor growth [11]. EGCG suppresses the growth of xenografts generated from the human HCC cell line Huh7 by decreasing serum VEGF levels and inhibiting the activation of VEGFR2, ERK, and Akt [16]. In CRC cell xenografts, the activation of VEGFR2, ERK, and Akt and the expression of VEGF are also inhibited by EGCG treatment and this might be associated with reduction of the expression of hypoxia-inducible factor (HIF)-1α, which strongly activates VEGF expression [17].

Several in vitro studies have also reported the inhibitory effects of GTCs on the VEGF/VEGFR axis. For instance, work from our group demonstrated that EGCG inhibits the production of VEGF in human HNSCC and breast cancer cells by blocking the activation of Stat3 and NF-κB [40]. EGCG also inhibits the phosphorylation of both VEGFR1 and VEGFR2 and induces apoptosis in chronic lymphocytic leukemia cells [56]. In addition, GTCs significantly inhibit HIF-1α protein accumulation and decrease VEGF expression in HepG2 cells by blocking both the PI3K/Akt and Ras/ERK signaling pathways [57]. EGCG inhibits ERK activation and suppresses the expression and promoter activity of VEGF in HT29 cells [58]. Similar to the findings showing the role of the IGF/IGF-1R axis in mediating the effect of GTCs, the above results suggest that the VEGF/VEGFR axis might also be a promising target of GTCs for the prevention and treatment of some types of human malignancies, including CRC and HCC.

7 Effects of GTCs on the hepatocyte growth factor (HGF)/c-Met and PDGF/PDGFR axes

In this review, we have mainly focused on a discussion of the inhibitory effects of GTCs on the activation of EGFR, IGF-1R, and VEGFR. However, it should be mentioned that GTCs also target other members of the RTK family, such as c-Met and platelet-derived growth factor receptor (PDGFR). c-Met is overexpressed in colon tumors and this is associated with poor prognosis [59, 60]. In human CRC cells, EGCG markedly suppressed the activation of c-Met in the presence of its ligand, HGF [61, 62]. In the liver of CCl_4-injected rats, EGCG significantly decreased the expression of PDGFR and thus attenuated hepatic fibrosis [53]. EGCG also inhibited PDGF-induced cell proliferation and reduced the autophosphorylation of the PDGFR by blocking the binding of PDGF to its receptor in human hepatic stellate cells; this might contribute to the prevention of liver fibrosis progression in patients with chronic liver diseases [63]. These reports suggest the possibility that GTCs can target certain types of RTKs in a variety of cell types; however, the precise mechanisms underlying the GTCs-mediated inhibition of RTKs activation in cancer cells remain to be elucidated.

8 Mechanisms mediating the inhibition of RTKs activation and intracellular signaling pathways by GTCs

One possible mechanism by which the inhibition of RTKs activation by GTCs could be explained is through the "sealing" and "trapping" effects of GTCs [64]. Namely, EGCG covers the cell surface and directly interrupts the binding of EGF to EGFR [38]. EGCG has also been shown to bind directly to EGF and VEGF, thus preventing these growth factors from interacting with their corresponding receptors and activating downstream signaling cascades [38, 65]. In addition, EGCG may also inhibit the activation of RTKs by affecting the expression levels of their ligands. The expression levels of the EGFR family ligands EGF and heregulin have been shown to be downregulated by EGCG treatment in CRC cells [17]. EGCG also decreases the levels of IGF-1, IGF-2, and VEGF, which might be associated with decreased ERK and Akt activities, in CRC and HCC cells [16, 17, 50]. These findings could partly explain the inhibitory effects of GTCs on the activation of RTKs in various types of cancer cells [16, 17, 38–43, 49, 50].

Several studies have also provided evidence that GTCs can directly target the kinase activity of RTKs and their intracellular signaling pathways and transcription factors. EGCG was shown to competitively bind to the ATP binding site of IGF-1R and block downstream signaling [66]. Sah et al. [67] demonstrated that EGCG directly inhibits ERK and Akt kinases in immortalized human cervical cells. In addition, EGCG was shown to play a role in the direct inhibition of the activation of ERK and mitogen-activated protein kinase kinase-1 (MEK1) and of the association with Raf-1 with MEK1, and in the inhibition of AP-1 activity in H-ras-transformed mouse epidermal cells [24, 25]. EGCG also exerted antiproliferative effects on H-ras-transformed rat intestinal epithelial cells [68]. These reports seem to be significant when considering the prevention of CRC by GTCs because Ras (KRAS) gene mutations occur frequently in this malignancy [69]. Moreover, administration of EGCG through the drinking water significantly decreased small intestinal tumor formation in $Apc^{Min/+}$ mice, a recognized mouse model for human intestinal cancer, by reducing the expression of the phosphorylated form of Akt and ERK proteins in small intestinal tumors [70]. Administration of EGCG through the drinking water also suppressed tumor

formation in $APC^{Min/+}$ mice by decreasing the levels of basic fibroblast growth factor in small intestinal tissue samples [71]. These reports are important because the mutation of the APC gene, a tumor suppressor gene, is critically implicated in human colorectal carcinogenesis [72]. Altogether, these results suggest that GTCs might exert antitumor and chemopreventive effects by binding, probably with relatively low affinity, to multiple cellular targets (Fig. 1). Moreover, these results also demonstrate the potential of GTCs as an effective chemopreventive agent against CRC in patients bearing APC and/or Ras gene mutations.

9 Lipid rafts: a promising target of EGCG

Evidence exists that several plasma membrane-associated RTKs, including EGFR, IGF-1R, and VEGFR2, are closely associated with the detergent-insoluble ordered membrane domains called "lipid rafts," which play a critical role as signal processing hubs. The localization of RTKs to lipid rafts appears to modulate both their ligand binding and tyrosine kinase activities [73–76]. Lipid organization is also considered to play a fundamental role in receptor internalization [77]. Recent studies show that lipid rafts provide a platform for a 67-kDa LR that binds EGCG, thus affecting the uptake of EGCG [21, 22, 78, 79]. The expression of the 67-kDa LR is found to be upregulated in various types of human cancers, including CRC [80], and to directly correlate with the malignant potential via activation of multiple signal transduction pathways such as MAPK [81, 82]. Therefore, EGCG may presumably mediate its cancer-preventive activity by targeting the 67-kDa LR [83].

In addition, on the basis of our recent series of studies [84–86], we presume that targeting lipid rafts is one of the most relevant mechanisms of EGCG in exerting its anticancer and chemopreventive properties (Fig. 2). EGFR activation was shown to only occur in the lipid raft fraction, whereas total cellular EGFR is present in the non-raft membrane fraction in HT29 cells. In these cells, EGCG

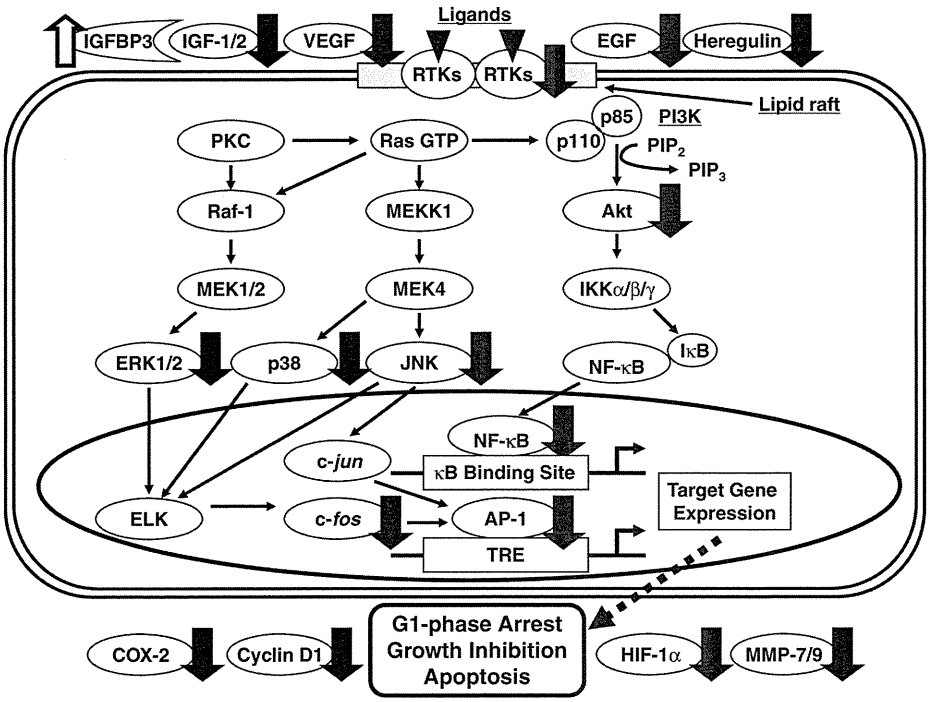

Figure 1. Effects of GTCs on RTKs and their intracellular signaling pathways. Activation of RTKs including EGFR, IGF-1R, and VEGFR2 by specific ligands leads to the induction of their intrinsic tyrosine kinase activities and autophosphorylation of tyrosine residues. The activated RTKs then create docking sites for downstream effector molecules such as Ras, Raf-1, and PI3K, which subsequently stimulate several intracellular processes. Activated Raf-1 stimulates MEK and its signaling cascade, resulting in the phosphorylation of the MAPK protein ERK. In its active state, MAPK activates a variety of transcription factors, including ELK and c-Jun, and subsequently promotes the expression of target genes by stimulating the transcriptional activity of AP-1, a dimeric complex that comprises members of the Jun and Fos families of transcription factors. The activation of PI3K triggers the synthesis of the lipid PIP_3, which activates the downstream pathways that involve Akt. The NF-κB family of transcription factors, which is important in cell survival, is one of the functional targets of Akt. EGCG inhibits the activation of certain RTKs, which takes place in lipid rafts (gray box), as well as the activation of the MAPK cascade, such as Ras/Raf/MEK/ERK/JNK pathways and PI3K pathways. Molecules that appear to be cellular targets for EGCG are indicated by a black arrow (downregulation) or by a white arrow (upregulation), respectively. These multiple effects of EGCG result in the induction of apoptosis and cell cycle arrest in the G_0–G_1 phase, thus inhibiting cell proliferation in cancer cells.

