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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Cancer Prevention Research

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

Masahito Shimizu[1], Hiroyasu Sakai[1], Yohei Shirakami[1], Yoichi Yasuda[1], Masaya Kubota[1], Daishi Terakura[1], Atsushi Baba[1], Tomohiko Ohno[1], Yukihiko Hara[2], Takuji Tanaka[3], and Hisataka Moriwaki[1]

Abstract

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

Introduction

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

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

Authors' Affiliations: [1]Department of Medicine, Gifu University Graduate School of Medicine, Gifu; [2]Tea Solution, Hara Office Inc., Tokyo; and [3]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

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

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

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

Materials and Methods

Animals and chemicals

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

Experimental procedure

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

Histopathologic analysis

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

Protein extraction and Western blot analysis

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

RNA extraction and quantitative real-time reverse transcriptase PCR

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

Clinical chemistry

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

Hepatic lipid analysis

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

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

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

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

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

Statistical analysis

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

Results

General observations

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

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

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

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

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

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

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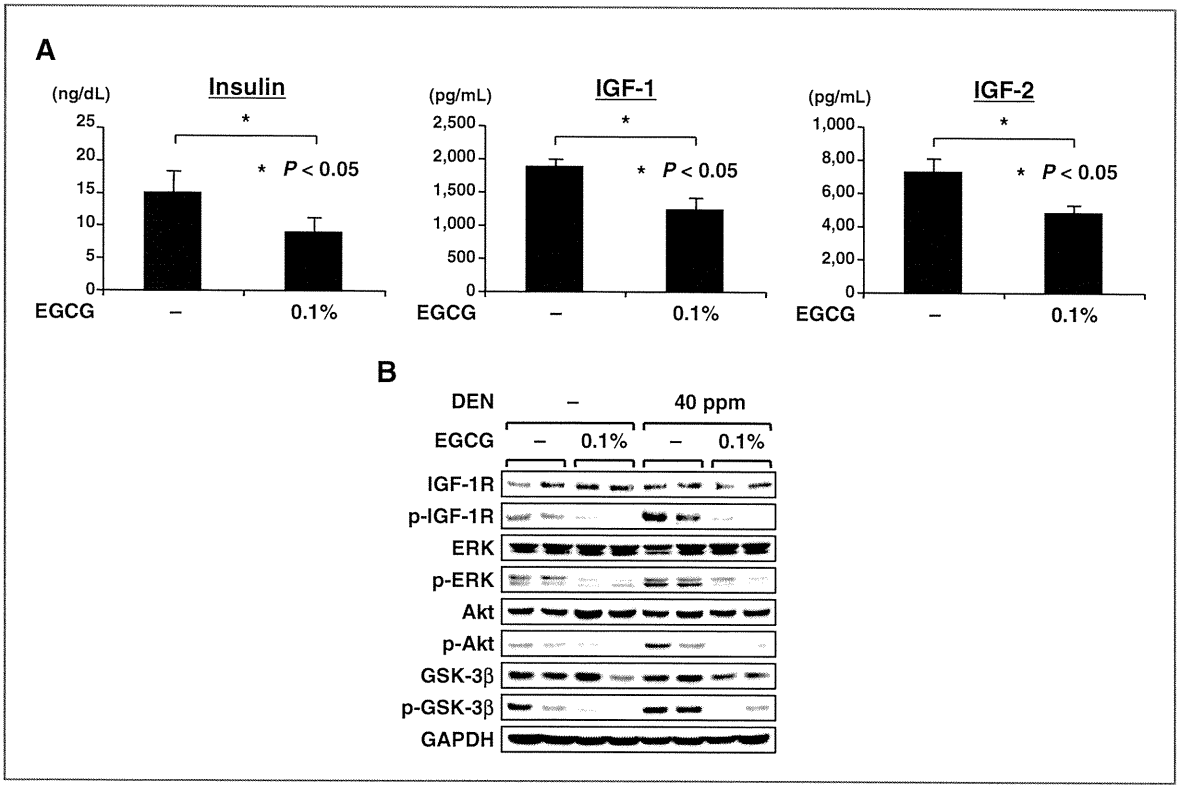

Figure 1. Effects of EGCG on the levels of serum insulin, IGF-1, and IGF-2 and on the activation of the IGF/IGF-1R axis in the liver of experimental mice. A, the serum concentrations of insulin, IGF-1, and IGF-2 in DEN-treated *db/db* mice were measured by an enzyme immunoassay. Values are the means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

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

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

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

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

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

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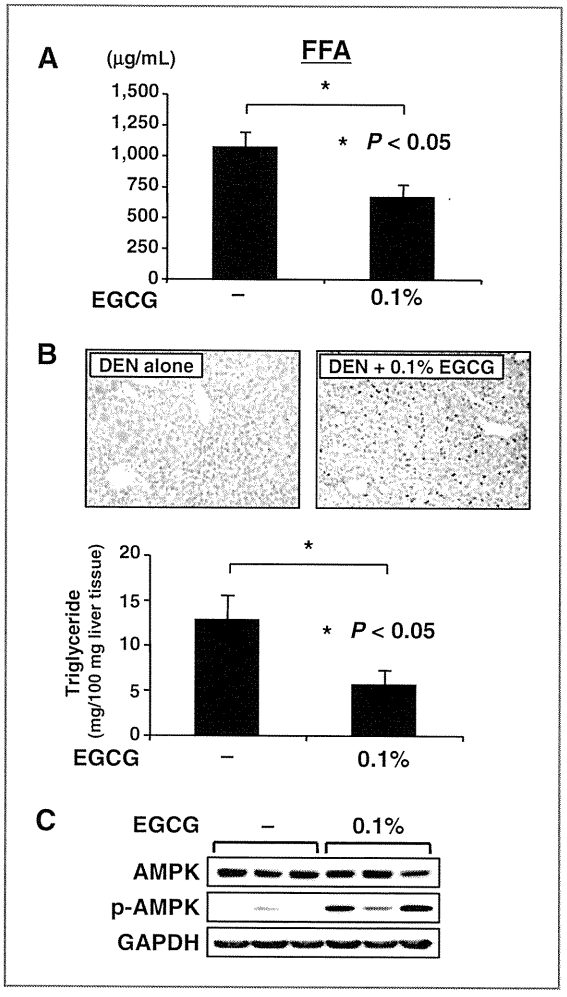

Figure 2. Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of the AMPK protein in the liver of DEN-treated *db/db* mice. A, the serum concentration of FFA was measured by an enzymatic method. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, frozen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

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

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

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

Discussion

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

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

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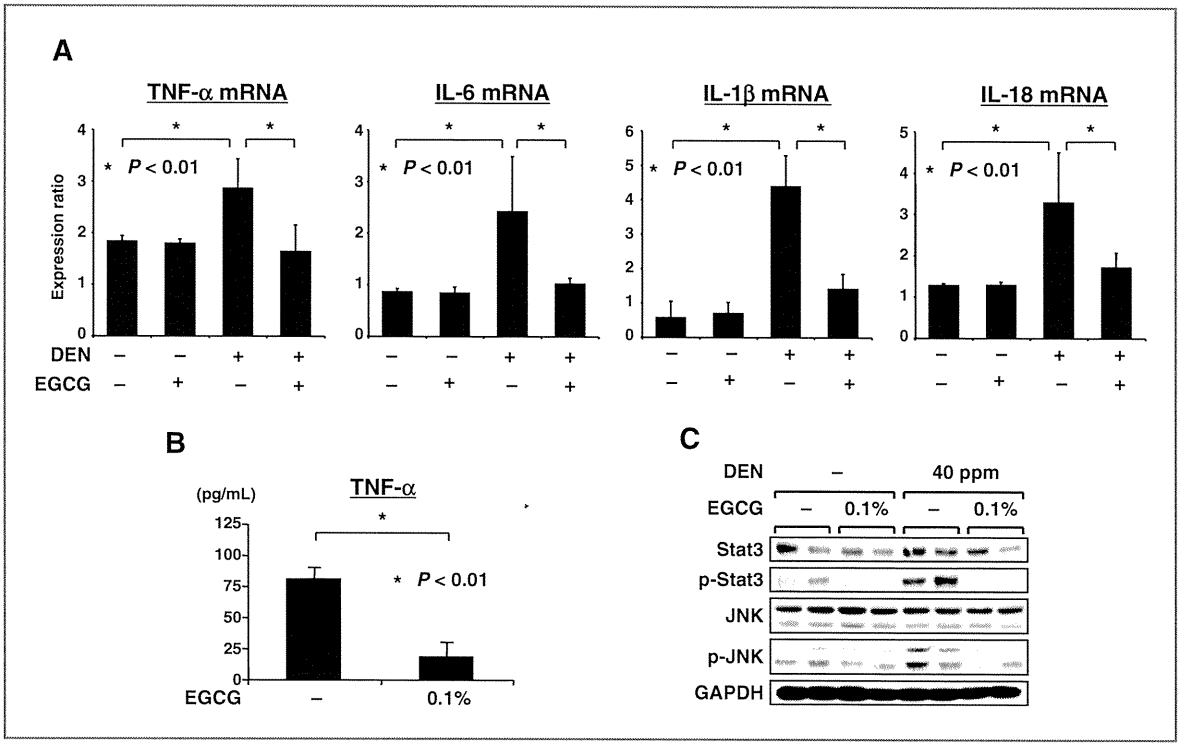

Figure 3. Effects of EGCG on the expression levels of TNF-α, IL-6, IL-18, and IL-1β mRNAs, the serum levels of TNF-α, and the activation of Stat3 and JNK proteins in the liver of experimental mice. A, the total RNAs were isolated from the livers of experimental mice, and the expression levels of TNF-α, 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.

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Ultraviolet Irradiation Can Induce Evasion of Colon Cancer Cells from Stimulation of Epidermal Growth Factor*

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Seiji Adachi[‡§1], **Ichiro Yasuda**[§], **Masanori Nakashima**[§], **Takahiro Yamauchi**[§], **Junji Kawaguchi**[§], **Masahito Shimizu**[§], **Masahiko Itani**[‡], **Momoko Nakamura**[‡], **Yumi Nishii**[‡], **Takashi Yoshioka**[¶], **Yoshinobu Hirose**[‖], **Yukio Okano**[¶], **Hisataka Moriwaki**[§], **and Osamu Kozawa**[‡]

From the Departments of [‡]*Pharmacology,* [§]*Gastroenterology, and* [¶]*Molecular Pathobiochemistry, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan and the* [‖]*Pathology Division, Gifu University Hospital, Gifu 501-1194, Japan*

Receptor down-regulation is the most prominent regulatory system of EGF receptor (EGFR) signal attenuation and a critical target for therapy against colon cancer, which is highly dependent on the function of the EGFR. In this study, we investigated the effect of ultraviolet-C (UV-C) on down-regulation of EGFR in human colon cancer cells (SW480, HT29, and DLD-1). UV-C caused inhibition of cell survival and proliferation, concurrently inducing the decrease in cell surface EGFR and subsequently its degradation. UV-C, as well as EGFR kinase inhibitors, decreased the expression level of cyclin D1 and the phosphorylated level of retinoblastoma, indicating that EGFR down-regulation is correlated to cell cycle arrest. Although UV-C caused a marked phosphorylation of EGFR at Ser-1046/1047, UV-C also induced activation of p38 MAPK, a stress-inducible kinase believed to negatively regulate tumorigenesis, and the inhibition of p38 MAPK canceled EGFR phosphorylation at Ser-1046/1047, as well as subsequent internalization and degradation, suggesting that p38 MAPK mediates EGFR down-regulation by UV-C. In addition, phosphorylation of p38 MAPK induced by UV-C was mediated through transforming growth factor-β-activated kinase-1. Moreover, pretreatment of the cells with UV-C suppressed EGF-induced phosphorylation of EGFR at tyrosine residues in addition to cell survival signal, Akt. Together, these results suggest that UV-C irradiation induces the removal of EGFRs from the cell surface that can protect colon cancer cells from oncogenic stimulation of EGF, resulting in cell cycle arrest. Hence, UV-C might be applied for clinical strategy against human colon cancers.

Members of the EGF receptor (EGFR)[2] family, which are frequently overexpressed in several types of human cancers, including cancers of the lung (1), head and neck (2), prostate (3), breast (4), pancreas (5), and colon (6), have been associated with abnormal growth of these tumors. It is well known that exposure of cells to EGF results in rapid autophosphorylation of EGFR molecules at the cell surface (7–10), which upon activation lead to cell proliferation, motility, and enhanced survival (11). There are several mechanisms by which EGFR becomes oncogenic including: 1) increased EGFR expression levels, 2) autocrine and/or paracrine growth factor loops, 3) heterodimerization with other EGFR family members and cross-talk with heterologous receptor systems, 4) defective receptor down-regulation, and 5) activating mutations (12). In clinical trials, increasing evidence shows the efficacy of EGFR-targeted agents, including monoclonal antibodies on the one hand and tyrosine kinase inhibitors on the other (13).

Following activation, the ligand-receptor complexes are internalized and then enter endosomes, where the receptors and their ligands are sorted to various intracellular destinations (14). Thus, some receptors can be recycled back to the cell surface via early endosomes, and others are targeted to late endosomes and lysosomes for proteolytic degradation. There is increasing evidence that not only does receptor internalization act to terminate signaling, but that internalized endosome-associated receptors are also able to stimulate specific signal transduction pathways (15–17). Some agents that induce ligand-independent internalization and degradation of EGFR, such as the 225 mouse antibody (18) and gemcitabine (5), could have promising potential for cancer therapies. By contrast, it has previously been reported that the other factors or agents, such as oxidative stress (19) and cisplatin (20), can induce internalization of the EGFR but not degradation. They differ in their effects on the fate of the receptors, downstream signaling, and cell proliferation.

Receptor down-regulation is the most prominent regulatory system of EGFR signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes. With the current knowledge of the mechanism underlying EGFR down-regulation, this molecular event involves several important phosphorylation sites in EGFR. One is the phosphorylation at Tyr-1045, which provides a docking site for the ubiquitin ligase c-Cbl resulting in ubiquitination of the EGFR (10). The others are the phosphorylation at serine or threonine residues that is thought to represent a mechanism for attenuation of the receptor tyrosine kinase activity (21–23). Among the major sites of serine and threonine phosphorylation of the EGFR, it has previously been shown that the Ser-1046/7 phosphorylation site is required for EGFR desensitization in EGF-treated cells (23). Moreover, mutant EGFR at Ser-1046/7

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[1] To whom correspondence should be addressed: 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: 81-58-230-6217; Fax: 81-58-230-6218; E-mail: seijiadachi0123@gmail.com.

[2] The abbreviations used are: EGFR, EGF receptor; UV-C, ultraviolet-C; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Rb, retinoblastoma protein; TAK-1, TGF-β-activated kinase-1; J, J/m^2; BrdU, bromodeoxyuridine; MKK, MAPK kinase; Ser-1046/7, Ser-1046/1047.

reportedly causes the inhibition of the EGF-induced endocytosis and down-regulation of cell surface receptors (22).

We have recently reported that phosphorylation of EGFR at serine 1046/7 via activation of p38 MAPK plays a pivotal role in down-regulation of EGFR induced by (−)-epigallocatechin gallate (24), anisomycin (25), and HSP90 inhibitor (26). Moreover, there is an evidence that cisplatin also induces EGFR internalization, which is mediated by p38 MAPK-dependent phosphorylation of the receptor at threonine 669 (20). Also, it has been shown that gemcitabine induces EGFR internalization and subsequent degradation, which may be a novel mechanism for gemcitabine-mediated cell death (5), whereas the activation of p38 MAPK is necessary for gemcitabine-induced cytotoxicity (27, 28). Taken together, serine phosphorylation of EGFR via p38 MAPK might be considered a new therapeutic target especially to counter cancer cells of the colon, lung, pancreas, and breast that highly express EGFR.

UV radiation from sunlight is sorted by wavelength regions: long wavelength UV-A (320–400 nm), medium wavelength UV-B (280–320 nm), and short wavelength UV-C (200–280 nm). In general, UV-A and UV-B are recognized the major carcinogenic components of sunlight (29). As for UV-C, it is used for studying DNA damage and cellular DNA repair process, although it does not actually exist in earth's surface because they are filtered out by the atmosphere. Although UV-C has been commonly applied for equipment such as water sterilization, recent studies show the possible application of UV-C against human cancer (30). However, its exact mechanism has not been fully clarified.

We have recently reported that the blockade of EGF stimulation significantly suppressed SW480 cell growth, suggesting that EGFR pathway plays an important role in proliferation of colon cancer cells (31). Therefore, we herein investigated the effect of UV-C on the down-regulation of EGFR in these cells and found that this induces the internalization and degradation of EGFR that indicates the removal of EGFR from cell membrane, and this action of UV-C can protect colon cancer cells from oncogenic stimulation such as EGF, resulting in cell cycle arrest.

EXPERIMENTAL PROCEDURES

Materials—SB203580, (5Z)-7-oxozeaenol, AG1478, and PD153035 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). BIRB0796 was obtained from Dr. Philip Cohen (University of Dundee, Dundee, UK). Antibodies against total EGFR and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-EGFR (Tyr-1045, Tyr-1068, and Ser-1046/7), phospho-p44/p42 MAPK, p44/p42 MAPK, phospho-p38 MAPK, p38 MAPK, phospho-SAPK/JNK, SAPK/JNK, phospho-TGF-β-activated kinase-1 (TAK-1), phospho-MKK3/6, cyclin D1, and phospho-retinoblastoma (Rb) were purchased from Cell Signaling (Beverly, MA). Antibodies against phospho-γH2A.X at Ser-139 (γH2AX) were purchased from Abcam (Cambridge, MA). An ECL Western blot detection system was purchased from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources.

Cell Culture—SW480 and HT29, human colon cancer cells were grown in DMEM (Invitrogen) containing 10% FCS with penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37 °C. DLD-1 cells, other human colon cancer cells, were grown in RPMI 1640 (Invitrogen). Two days after seeding, they were incubated in serum-free medium for 24 h as described previously (32).

Cell Proliferation Assay—The cells (3×10^3/well) were seeded onto 96-well plates, and 24 h later, the cells were exposed to the indicated doses (0–200 J/m^2 [J]) of UV-C after the aspiration of the growth medium. The cells were then incubated in DMEM with 1% FCS in a humidified 5% CO_2 incubator at 37 °C for 48 h. The remaining cells were finally counted by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit I (Roche Applied Science) in accordance with instructions of the manufacturer. BrdU incorporation was measured using cell Proliferation ELISA (Brd; Roche Applied Science). The cells (7×10^3/well) were seeded onto 96-well plates, and 48 h later, the cells were exposed to the indicated doses (0–50 J) of UV-C, just after the aspiration of the growth medium. The cells were then incubated in DMEM with 1% FCS for 24 h. They were then used for measurement according to the manufacturer's protocol. All of the assays were done in triplicate.

Clonogenic Survival Assay—The cells were grown in regular medium to 70% confluence and exposed to UV-C, AG1478, and PD153035 at the indicated doses. Twenty-four h after treatment, the cells were trypsinized and counted as usual. The cells (3×10^3) were reseeded into fresh tissue culture dishes and incubated for 7 days. Fresh media were added at day 4. At day 7, the media were removed, and the cells were fixed with 2 ml of clonogenic reagent (50% ethanol, 0.25% 1,9-dimethyl-methylene blue) for 45 min. They were then washed with PBS twice, and the blue colonies were counted.

UV-C Exposure—UV-C exposure of cells was performed in a UV-C 500 UV Crosslinker (GE Healthcare) by which we used 10–200 J/m^2 (J) of UV at 254 nm. After aspiration of the growth media, the cells were exposed to the indicated doses of UV-C in 20 s and then incubated for the indicated times.

Western Blot Analysis—The cells were lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 50 mM NaF, 50 mM HEPES, 1 mM Na_3VO_4, and 2 mM PMSF) and scraped from the Petri dishes. Protein extracts were examined by Western blot analysis as previously described (33). The protein was fractionated and transferred onto an Immune-Blot PVDF membrane (Bio-Rad). The membranes were blocked with 5% fat-free dry milk in PBS containing 0.1% Tween 20 for 30 min before incubation with the indicated primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as second antibodies. Peroxidase activity on the membrane was visualized on x-ray film by means of the ECL Western blot detection system.

Immunofluorescence Microscopy Studies—Immunofluorescence microscopy studies were performed as described previously (25). The cells grown on coverslip-bottomed dishes were first treated with either SB203580 or siRNA-p38 MAPK, followed by exposure to anti-EGFR antibodies for 15 min in RPMI

containing 1% BSA. They were then exposed to UV-C (30 J) and incubated in DMEM without FCS for additional 30 min. They were then fixed with 4% paraformaldehyde for 10 min on ice and then exposed to 0.1% Triton X-100 for 10 min to permeabilize the cell membrane. They were followed by exposure to Alexa Fluor 546®-conjugated donkey anti-mouse IgG antibodies (Invitrogen) and DAPI (Wako, Tokyo, Japan) for 1 h. The cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer's protocol.

siRNA Protocol—Predesigned siRNAs targeting p38 MAPK (On-TARGET plus Duplex J-003512–20, human MAPK14) was purchased from Thermo Fisher Scientific K.K. (Yokohama, Japan). Sequences are as follows: sense, GGAAUUCAAUGAUGUGUAUUU and antisense, AUACACAUCAUUGAAUUCCUU. Transfection was performed according to the manufacturer's protocol (Bio-Rad). In brief, 5 μl of siLentFect (Bio-Rad) and finally 100 nM of siRNA were diluted with Opti-MEM, preincubated at room temperature for 20 min, and then added to the culture medium containing 10% FCS. The cells were incubated at 37 °C for 48 h with siRNA-siLentFect complexes and subsequently harvested for preparation of Western blot analysis.

Quantification of Cell Surface EGFR by ELISA—Quantification of cell surface EGFR was performed as described previously (32). In brief, SW480 cells were pretreated with the indicated compounds and then exposed to the mouse anti-EGFR antibody (Santa Cruz Biotechnology) that recognizes the extracellular domain of the EGFR (1:50 dilution) in DMEM containing 1% BSA for 15 min at 37 °C. The cells were then incubated for the indicated times after exposure to UV-C (30 J) and then fixed with 4% paraformaldehyde for 10 min on ice. After blocking with 1% BSA in PBS for 1 h, the cells were exposed to an anti-mouse IgG, horseradish peroxidase-linked whole antibody (GE Healthcare) for 1 h at room temperature, followed by washing four times with PBS containing 1% BSA. Finally, the cells were exposed to 50 μl of 1-Step™ Ultra TMB-ELISA reagent (Pierce) for 5 min at room temperature. Fifty μl of 2 M sulfuric acid was then added to each well to stop the reaction. The absorbance of each sample at 450 nm was then measured.

Densitometric Analysis—The densitometric analysis was performed using scanner and image analysis software (Image J version 1.32). The background-subtracted signal intensity of each protein signal was normalized by the respective control signal. All of the data were obtained from at least three independent experiments.

Detection of UV-C-caused DNA Damage—DNA damage caused by UV-C was examined by detection of phospho-H2A.X at Ser-139 (γH2AX). The cells were pretreated with or without 1 μM of BIRB0790 for 1 h and then exposed to UV-C (30 J), followed by incubation for the indicated periods. They were then lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, and complete protease inhibitor mixture tablets (Hoffmann-La Roche Inc. Nutley, NJ)). After sonication for 10 s, the protein extracts were quantified and examined by Western blot analysis. As for immunofluorescence study, the cells grown on coverslip-bottomed dishes were first treated with or without 1 μM of BIRB0790. They were then exposed to UV-C (30 J) and incubated for 3 h, followed by fixation, permeabilization, and exposure to anti-γH2AX for 1 h. They were then exposed to Alexa Fluor 546®-conjugated donkey anti-rabbit IgG antibodies and DAPI for 1 h. Finally, the cells were examined by fluorescence microscopy.

Statistical Analysis—The data were analyzed by analysis of variance followed by the Bonferroni method for multiple comparisons between the indicated pairs, and a $p < 0.05$ was considered significant.

RESULTS

UV-C and EGFR Kinase Inhibitors Inhibited Colon Cancer Cell Proliferation—We first investigated the effect of UV-C on the proliferation of SW480 cells using MTT. As shown in Fig. 1*A*, the IC_{50} value of UV-C in SW480 cells was ~80 J. Also, the Brd incorporation assay revealed that SW480 cell proliferation was significantly suppressed when the cells were treated with the increasing dose of UV-C (Fig. 1*B*, black bars). We also observed similar effects of UV-C in HT29 and DLD-1 cells, other colon cancer cells (Fig. 1*B*, *gray* and *white bars*). Therefore, it is likely that UV-C has suppressive effects on colon cancer cell proliferations. We have recently reported that anti-EGFR neutralizing antibodies significantly suppressed cell growth in SW480 colon cancer cells (31). In this study, we used two kinds of EGFR kinase inhibitor, AG1478 and PD153035, and examined the inhibitory effect of EGFR kinase activity on colon cancer cell proliferation. MTT assay shows that these inhibitors suppressed cell proliferation (Fig. 1*C*). In addition, Fig. 1*D* revealed the suppressive effects of UV-C as well as EGFR kinase inhibitors on colony formations, indicating the reduction of capability of SW480 cells to survive and replicate (34).

The ternary complex of cyclin D/cyclin-dependent kinase 4 and p27 Kip1 requires extracellular mitogenic stimuli for the release and degradation of p27 concomitant with a rise in cyclin D levels to affect progression through the restriction point and phospho-Rb-dependent entry into the S phase (35), indicating that increasing levels of cyclin D1 and phospho-Rb promote cell cycle, resulting in cell proliferation. Because EGFR kinase inhibitors also suppressed the phosphorylation of Rb as well as the protein level of cyclin D1 (Fig. 1*E*), it is likely that EGFR signaling plays a critical role in proliferation of colon cancer cells.

UV-C Induced Down-regulation of EGFR and Cell Cycle Arrest in Colon Cancer Cells—Overexpressed EGFR plays an oncogenic role in colon cancer cells, and its down-regulation is the most prominent regulatory system of EGFR signal attenuation (36). Whereas it has previously been reported that UV induces internalization and endosome arrest of EGFR in HeLa epidermal carcinoma cells (30, 37), we investigated the effect of UV-C on EGFR signaling pathway in SW480 cells. In Fig. 2*A*, UV-C caused a marked decrease in the amount of cell surface EGFRs within 10–30 min, whereas they were not affected in unstimulated SW480 cells. Moreover, UV-C markedly decreased the protein level of EGFR in SW480, HT29, and DLD-1 cells (Fig. 2*B*), thus suggesting that UV-C induces EGFR

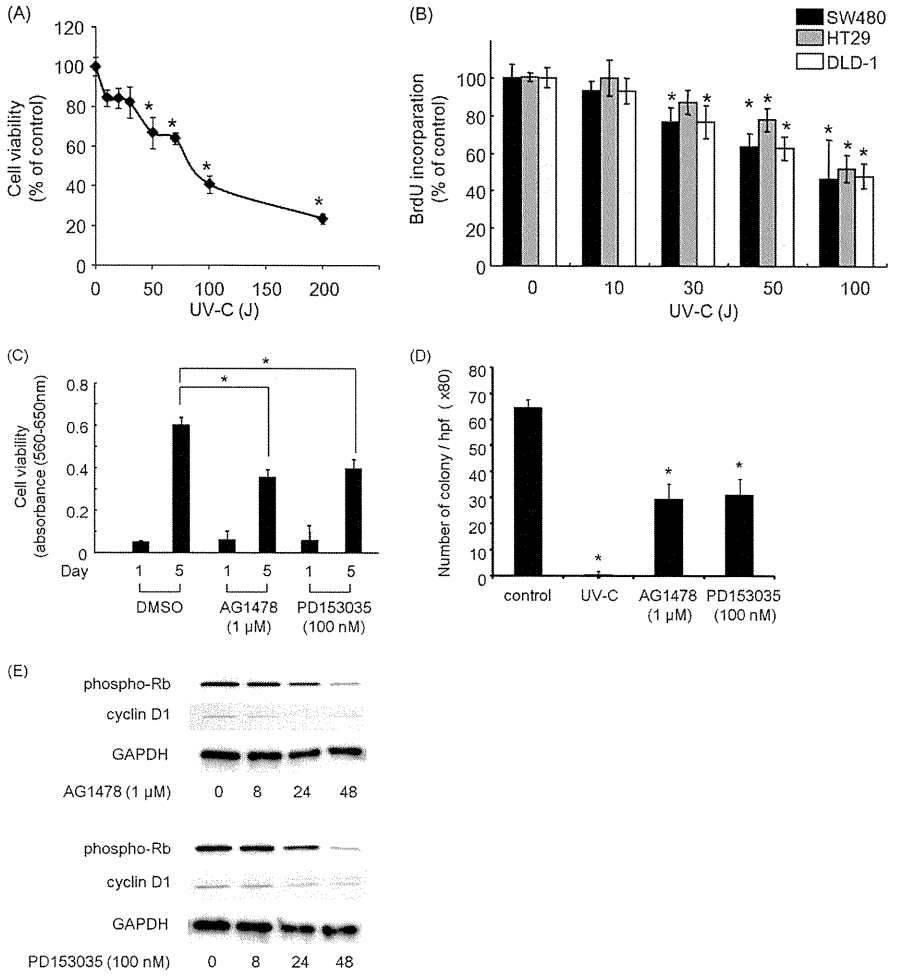

FIGURE 1. **UV-C and EGFR kinase inhibitors inhibited cell survival and proliferation in colon cancer cells.** *A*, SW480 cells were exposed to UV-C for 48 h under medium containing 3% FCS, and then the remaining cells were counted by MTT cell proliferation kit I. The results are expressed as percentages of growth, with 100% representing untreated control cells. *B*, the indicated cells (SW480, HT29, and DLD-1) were exposed to UV-C for 24 h under medium containing 3% FCS, and the measurement of BrdUrd incorporation during DNA synthesis was performed using cell proliferation ELISA (Brd). The results are expressed as percentage of incorporation, with 100% representing control cells. *C*, SW480 cells were treated with 1 μM of AG1478, 100 nM of PD153035, or vehicle for 4 days under medium containing 3% FCS, and then the remaining cells were counted by the MTT cell proliferation kit I. *D*, the attached SW480 cells were exposed to 30 J of UV-C, 1 μM of AG1478, or 100 nM of PD153035 and incubated for 24 h. After trypsinization, the counted cells (3×10^3) were reseeded into new culture dishes and incubated for 7 days. The cells were then fixed with clonogenic reagent (see "Experimental Procedures"), and the average numbers of colonies from five randomly chosen fields (×80) were counted, respectively. *E*, SW480 cells were treated with 1 μM of AG1478 or 100 nM of PD153035 for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-specific Rb, cyclin D1, and GAPDH. All of the assays were done in triplicate. The *error bars* designate S.D. of triplicate assays. The *asterisks* indicate a significant difference ($p < 0.05$), compared with the control.

down-regulation in colon cancer cells. Moreover, UV-C (30 J) time-dependently decreased the protein level of cyclin D1 in addition to phospho-Rb (Fig. 2*C*). Also, increasing dose of UV-C caused the decrease in both of cyclin D1 expression level and phospho-Rb, concurrently with the decrease in EGFR protein level (Fig. 2*D*). Therefore, it is probable that UV-C-induced EGFR down-regulation is correlated to cell cycle arrest in colon cancer cells.

UV-C Induced Phosphorylation of EGFR at Ser-1046/7, not Tyrosine Residues in Colon Cancer Cells—To elucidate how UV-C causes EGFR down-regulation in colon cancer cells, we examined the effects of UV-C on the phosphorylation of EGFR at several residues. Although UV-C had slight, but no appreciable effect on phosphorylation of EGFR at Tyr-1045 and Tyr-1068, both of which are known as autophosphorylation sites (8–10), UV-C markedly induced phosphorylation of EGFR at Ser-1046/7 in SW480 cells (Fig. 3). This phosphorylation at Ser-1046/7 appeared at 10 min after exposure to UV-C (30 J) and reached maximum at 60 min and decreased thereafter (Fig. 3*A*). In addition, this appeared when the cells were exposed to UV-C at a dose over 20 J (Fig. 3*B*).

p38 MAPK Was Involved in the Phosphorylation of EGFR at Ser-1046/7, and Subsequently Its Internalization Was Induced by UV-C in Colon Cancer Cells—To clarify how UV-C induces EGFR phosphorylation at serine residues, we next examined the effect of UV-C on the activations of MAPK cascades. UV-C induced phosphorylation of p44/p42 MAPK, SAPK/JNK, and p38 MAPK within 10–20 min in SW480 cells (Fig. 4*A*). These

results led us to further investigate which protein kinase plays a critical role in the phosphorylation of EGFR at serine residues induced by UV-C in colon cancer cells.

Although we showed that UV-C caused a rapid decrease in the amount of cell surface EGFR (Fig. 2*A*), we next examined whether UV-C also induces changes in the cellular localization of the EGFR in SW480 cells using fluorescence microscopy. As shown in Fig. 4*B*, EGFR was mainly localized on the cell surface (Fig. 4*B*, *panel 1*). As expected, UV-C caused the internalization of EGFR (Fig. 4*B*, *panel 2*), and moreover, when the cells were pretreated with SB203580, a p38 MAPK-selective inhibitor (38), the internalization of EGFR was virtually inhibited (Fig. 4*B*, *panel 4*). We also obtained similar results using siRNA-p38 MAPK (Fig. 4*C*). These results strongly suggest that activation of p38 MAPK truly mediates the UV-C effect on the cellular localization of EGFR in colon cancer cells.

As depicted in Fig. 4*D*, BIRB0790, another p38 MAPK-selective inhibitor (39) in addition to SB203580 significantly suppressed the phosphorylation of EGFR at Ser-1046/7 induced by UV-C (Fig. 4*D*), although that was not suppressed when the cells were pretreated with PD98059 (40) and SP600125 (41), which can inhibit the activation of MEK1/2 and SAPK/JNK, respectively (data not shown). To verify our results shown in Fig. 4*D*, we further performed siRNA experiment and observed that UV-C-caused phosphorylation of EGFR at Ser-1046/7 was clearly suppressed by siRNA-p38 MAPK, whereas negative control siRNA did not have any effects (Fig. 4*E*). We also verified that these findings were applicable in HT29 and DLD-1 cells (Fig. 4*F*). Together, we strongly suggest that phosphorylation of EGFR at Ser-1046/7 induced by UV-C was also mediated through the p38 MAPK pathway in colon cancer cells.

Phosphorylation of p38 MAPK Induced by UV-C Was Mediated through TAK-1 in Colon Cancer Cells—TAK-1 is activated by TGFβ as well as cytokines, including interleukin-1 (42, 43). TAK-1 is also known to be a kinase of MAPK kinase (MKK) 3/6 (44–46). Moreover, MKK3 can phosphorylate p38 MAPK (47). Therefore, we examined the effect of UV-C on TAK-1 to examine the upstream signaling for activation of p38 MAPK. Of interest, UV-C induced phosphorylation of TAK-1 as well as MKK3/6 (Fig. 5*A*). Moreover, the inhibition of TAK-1 using (5Z)-7-oxozeaenol restored UV-C-induced phosphorylation of EGFR (Ser-1046/7), MKK3/6, and p38 MAPK (Fig. 5*B*). Therefore, it is most likely that UV-C induces activation of p38 MAPK via TAK-1.

Degradation of EGFR and Cyclin D1 Induced by UV-C Was Blocked by the Inhibition of p38 MAPK in Colon Cancer Cells—We next investigated the involvement of p38 MAPK in UV-C-induced degradation of EGFR and cyclin D1. As depicted in Fig. 6*A*, the protein level of both EGFR and cyclin D1 were decreased when the cells were treated with UV-C (Fig. 6*A*, *lane 2* compared with *lane 1*), consistently with the above results shown in Fig. 2 (*B–D*). As expected, pretreatment of the cells with SB203580 significantly inhibited UV-C-induced degradation of cyclin D1 as well as EGFR (Fig. 6*A*, *lane 4* compared with *lane 3*). Similarly, the protein level of both EGFR and cyclin D1 were not decreased by UV-C in the cells that were transfected with p38 MAPK-siRNA (Fig. 6*B*, *lane 4* compared with *lane 3*), in comparison with the cells transfected with negative control (Fig. 6*B*, *lane 2* compared with *lane 1*). These results strongly suggest that activation of p38 MAPK plays a critical role in the down-regulation of EGFR induced by UV-C in colon cancer cells.

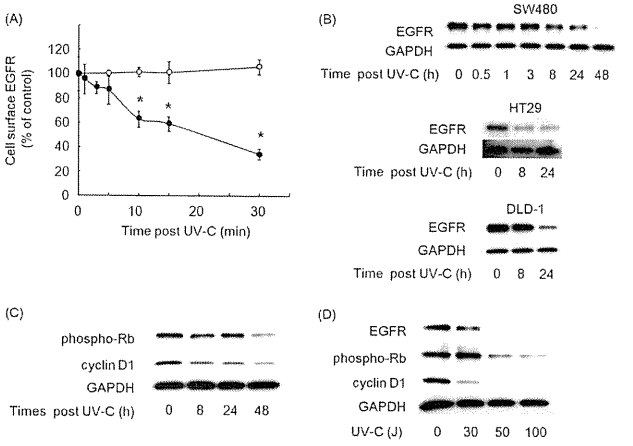

FIGURE 2. **UV-C caused down-regulation of the EGFR and cell cycle arrest in colon cancer cells.** *A*, UV-C induced the decrease in the cell surface amount of EGFR in SW480 cells. The *line graph* shows the quantification data for the cell surface amount of EGFR analyzed by ELISA (see under "Experimental Procedures"). ○, unstimulated SW480 cells; ●, SW480 cells exposed to 30 J of UV-C. *B*, the indicated cells (SW480, HT29, and DLD-1) were exposed to UV-C at a dose of 30 J and then incubated for the indicated periods. *C*, SW480 cells were exposed to 30 J of UV-C for the indicated periods. *D*, SW480 cells were exposed to UV-C at the indicated doses and then incubated for 24 h. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR, phospho-specific Rb, cyclin D1, and GAPDH. Representative results from triplicate independent experiments with similar results are shown.

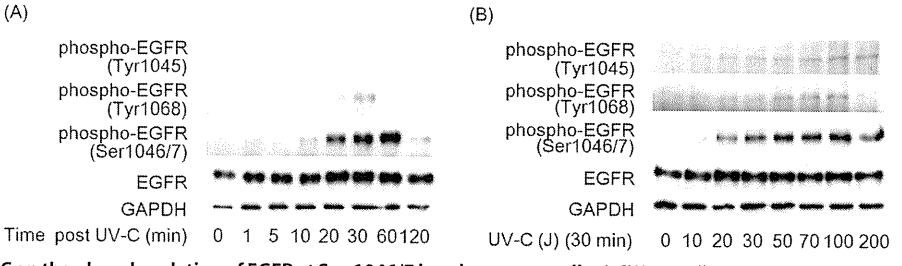

FIGURE 3. **Effect of UV-C on the phosphorylation of EGFR at Ser-1046/7 in colon cancer cells.** *A*, SW480 cells were exposed to UV-C at a dose of 30 J for the indicated periods. *B*, SW480 cells were exposed to UV-C at the indicated doses and then incubated for 1 h. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr-1045, Tyr-1068, and Ser-1046/7, anti-EGFR, and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

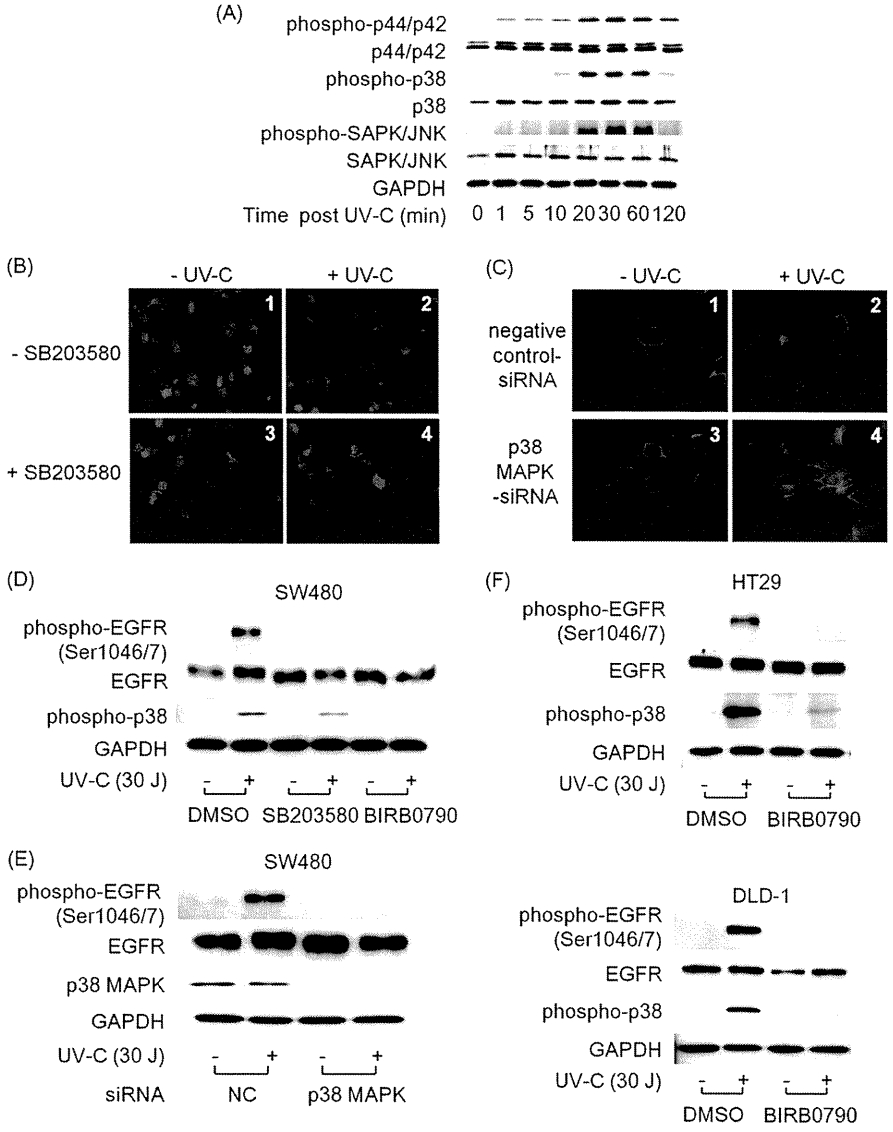

FIGURE 4. **Effects of p38 MAPK on the internalization and phosphorylation of EGFR at Ser-1046/7 in colon cancer cells.** *A*, SW480 cells were exposed to UV-C at 30 J and then incubated for the indicated periods. Protein extracts were then harvested and examined by Western blotting using anti-phospho-p44/p42 MAPK, anti-p44/p42 MAPK, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-SAPK/JNK, anti-SAPK/JNK, and anti-GAPDH antibodies. *B*, SW480 cells were pretreated with or without 10 μM of SB203580 for 1 h and then labeled for 15 min at 37 °C with anti-EGFR antibodies that recognize the extracellular domain of the EGFR. *C*, SW480 cells were incubated with 100 nM of p38 MAPK-siRNA or negative control siRNA at 37 °C for 48 h in FCS-free opti-MEM and then labeled for 15 min at 37 °C with anti-EGFR antibodies. They were then exposed to UV-C (30 J) and incubated for additional 1 h, followed by fixation with paraformaldehyde. After permeabilization of the cells with 0.1% Triton X-100, the cells were treated with Alexa 546-conjugated anti-mouse secondary antibody for EGFR (*red signal*) and DAPI (*blue signal*) for 1 h and then examined by fluorescence microscope. *D*, SW480 cells were pretreated with 10 μM of SB203580 or 1 μM of BIRB0790 for 1 h and then exposed to UV-C at 30 J and then incubated for additional 1 h. *E*, SW480 cells were incubated with 100 nM of p38 MAPK-siRNA or negative control siRNA at 37 °C for 48 h in FCS-free opti-MEM, followed by exposure to UV-C (30 J) for 1 h. *F*, HT29 and DLD-1 cells were pretreated with 1 μM of BIRB0790 for 1 h and then exposed to UV-C at 30 J and then incubated for additional 1 h. Protein extracts were then prepared and examined by Western blotting using antibodies against phospho-EGFR at Ser-1046/7, EGFR, phospho-p38 MAPK, and p38 MAPK, respectively. An antibody to GAPDH was used to control for protein loading. Representative results from triplicate independent experiments with similar results are shown.

Pretreatment with UV-C Can Protect Colon Cancer Cells from EGF Stimulation—It is well known that exposure of cells to EGF stimulation results in rapid autophosphorylation of EGFR molecules at the cell surface (7–10), which upon activation lead to cell proliferation, motility, and enhanced survival (11). Moreover, through EGF binding to cell surface EGFR, it activates an extensive network of signal transduction pathways including Akt pathways, which regulates multiple biological processes including survival, proliferation, and cell growth (48, 49). We next investigated the effect of pretreatment with UV-C on EGF-induced phosphorylation of EGFR and Akt in SW480 cells. When the cells were not exposed to UV-C or EGF, EGFR at any residue and Akt were not phosphorylated (Fig. 7*A*, *lane 1*). Indeed, UV-C caused phosphorylation of EGFR at Ser-1046/7 (Fig. 7*A*, *second panel*), consistently with the above results shown in Fig. 3. Interestingly, although stimulation with

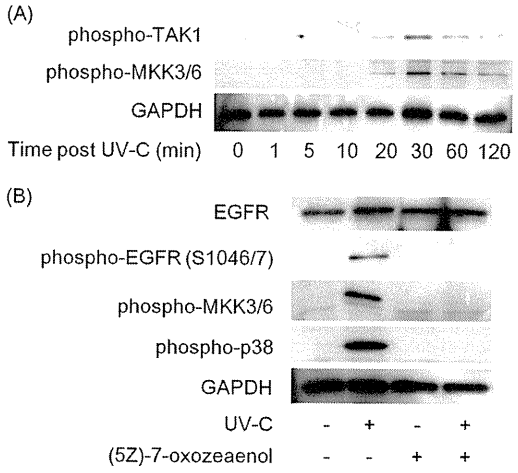

FIGURE 5. **The involvement of TAK-1 in UV-C-induced activation of p38 MAPK in colon cancer cells.** *A*, SW480 cells were exposed to UV-C at 30 J and then incubated for the indicated periods. *B*, SW480 cells were pretreated with or without the 300 nM of (5Z)-7-oxozeaenol, as a TAK-1 inhibitor, for 2 h and then exposed to UV-C (30 J) and incubated for additional 30 min. Protein extracts were then harvested and examined by Western blotting using anti-EGFR, phospho-EGFR (Ser-1046/7), phospho-TAK1, anti-MKK3/6, anti-p38 MAPK, and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

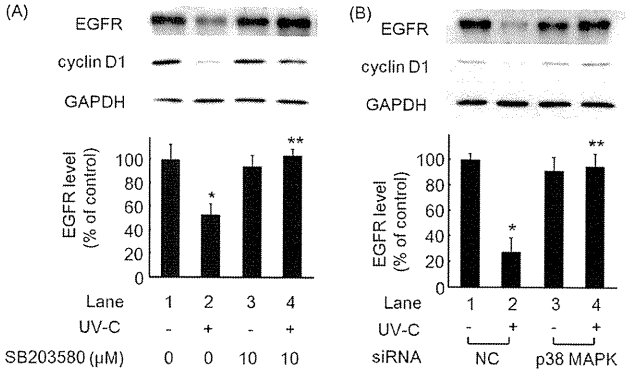

FIGURE 6. **The inhibition of p38 MAPK restored UV-C-induced degradation of the EGFR in colon cancer cells.** *A*, SW480 cells were pretreated with or without 10 μM of SB203580 for 1 h and then exposed to UV-C at 30 J, followed by incubation for 8 h. *B*, SW480 cells were incubated with 100 nM of p38 MAPK-siRNA or negative control (*NC*) siRNA at 37 °C for 48 h in FCS-free opti-MEM, followed by exposure to UV-C (30 J). Protein extracts were then prepared and examined by Western blotting using anti-EGFR and anti-cyclin D1 antibodies, respectively. An antibody to GAPDH was used to control for protein loading. The *bar graph* shows quantification data for the relative levels of EGFR, after normalization with respect to GAPDH, as determined by densitometry. The *asterisks* indicate significant increase (*, $p < 0.05$ compared with *lane 1*; **, $p < 0.05$ compared with *lane 2*). Representative results from triplicate independent experiments with similar results are shown.

EGF caused the phosphorylation of EGFR at tyrosine residues and Akt (Fig. 7*A*, *first* and *third panels*, *lane 2*), these phosphorylations were significantly suppressed when the cells were exposed to the increasing doses of UV-C before EGF stimulation (Fig. 7*A*, *first* and *third panels*). In cases of HT29 and DLD-1 cells, UV-C also had the suppressive effects on EGF-induced activation of EGFR (Fig. 7, *B* and *C*). These results strongly suggest that UV-C caused the evasion of colon cancer cells from EGF stimulation because UV-C can remove EGFR from cell membrane.

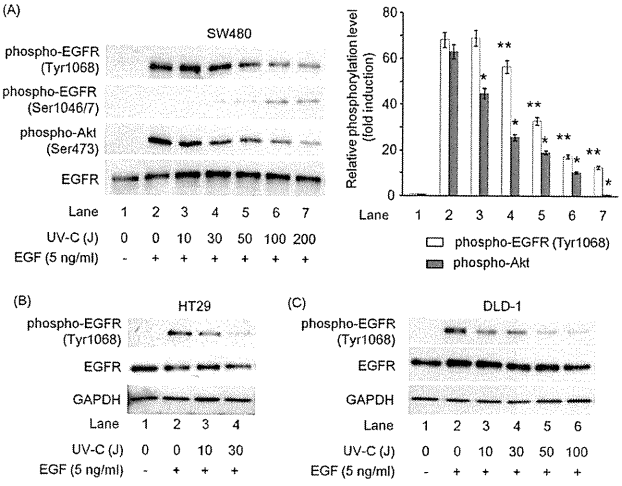

FIGURE 7. **Effects of exposure to UV-C before EGF stimulation on the phosphorylation of EGFR in colon cancer cells.** *A*, SW480 cells were pretreated with UV-C at the indicated doses of UV-C for 1 h and then exposed to 5 ng/ml of EGF for another 5 min. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr-1068 and Ser-1046/7, anti-phospho-Akt, and anti-EGFR antibodies. The *bar graph* shows quantification data for the relative phosphorylation levels of EGFR and Akt, after normalization with respect to EGFR, as determined by densitometry. *, $p < 0.05$ compared with the control (EGF-induced phosphorylation of EGFR at Tyr-1068 in *lane 2*); **, $p < 0.05$ compared with the control (EGF-induced phosphorylation of Akt in *lane 2*). *B* and *C*, HT29 (*B*) and DLD-1 (*C*) cells were pretreated with UV-C at the indicated doses of UV-C for 1 h and then exposed to 5 ng/ml of EGF for another 5 min. Protein extracts were then harvested and examined by Western blotting using anti-phospho-EGFR at Tyr-1068, anti-EGFR, and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

DNA Damage Is Not Involved in UV-C-induced EGFR Down-regulation in Colon Cancer Cells—DNA damage, provoked by UV radiation, evokes a cellular damage response composed of activation of stress signaling and DNA checkpoint functions (29). In addition, DNA double-strand breaks introduced into mammalian cells result in the specific phosphorylation of histone H2A.X at Ser-139, named γH2AX (50). We examined whether DNA damage correlates to UV-C-induced EGFR down-regulation via p38 MAPK. Although BIRB0790 clearly inhibited phosphorylation of p38 MAPK (Fig. 8*B*), immunofluorescence study and Western blotting showed that it had little effect on UV-C-induced γH2AX (Fig. 8, *A* and *B*). Therefore, it seems unlikely that DNA damage is involved in UV-C-induced EGFR down-regulation via p38 MAPK.

DISCUSSION

Whereas UV irradiation reportedly has many effects on skin, including inflammation, immunosuppression, and alterations in the extracellular matrix, in addition to accelerated skin aging (51), in the present study we demonstrated that UV-C has a potent anti-cancer effect by decreasing EGFR protein level in colon cancer cells. First, we showed that UV-C caused anti-survival and proliferative effects on SW480, HT29, and DLD-1 cells (Fig. 1). Because EGFR activation has been shown to be oncogenic (9), and we here showed that colon cancer cell proliferation depended on EGFR signaling (Fig. 1, *C–E*), we examined the effect of UV-C on the EGFR signaling and found that UV-C induced internalization and subsequent down-regula-

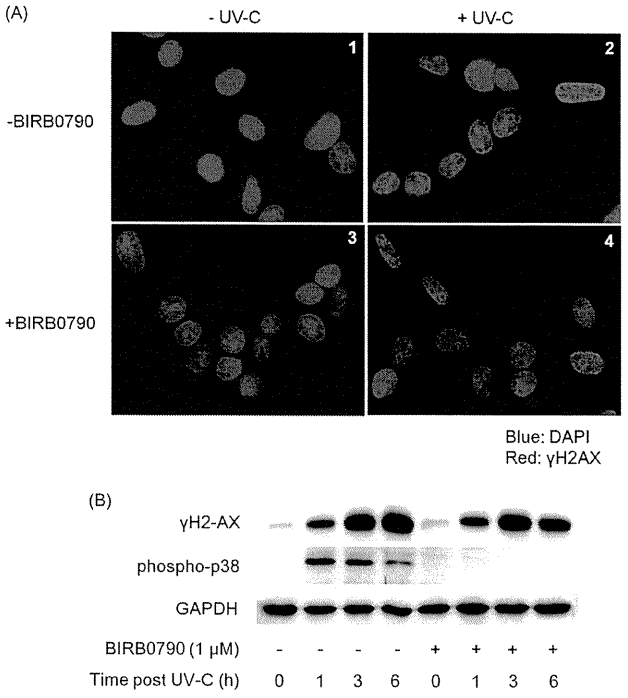

FIGURE 8. **The involvement of DNA damage in UV-C-induced activation of p38 MAPK in colon cancer cells.** *A*, SW480 cells grown on coverslip-bottomed dishes were pretreated with or without 1 μM of BIRB0790 and then exposed to UV-C (30 J) and incubated for another 3 h, and the cells were examined by fluorescence microscopy. *Red signals*, γH2AX; *blue signals*, DAPI. *B*, SW480 cells were pretreated with or without 1 μM of BIRB0790 for 1 h and then exposed to 30 J of UV-C, followed by incubation for another 3 h. Protein extracts were then harvested and examined by Western blotting using anti-γH2AX, anti-phospho-p38 MAPK, and anti-GAPDH antibodies. Representative results from triplicate independent experiments with similar results are shown.

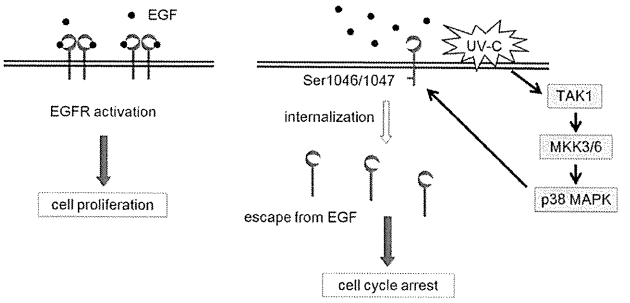

FIGURE 9. **Schematic representation of the effect of UV-C on EGF-induced proliferation of colon cancer cells.** After EGF binds to cell surface EGFR, it undergoes dimerization and autophosphorylation at tyrosine residues, and this triggers EGFR-related downstream signaling, leading to cell proliferation (7–10). By contrast, UV-C causes serial phosphorylation of TAK1, MKK3/6, and p38 MAPK and subsequent phosphorylation of EGFR at Ser-1046/7 but not tyrosine residues, and the serine-phosphorylated EGFR are internalized and eventually degraded. Therefore, UV-C can escape colon cancer cells from oncogenic stimulation of EGF, because EGF hardly binds to the internalized EGFR caused by UV-C.

tion of the EGFR and also decreased the protein level of cyclin D1 and phospho-Rb (Fig. 2), suggesting that the anti-cancer effect of UV-C could be due to cell cycle arrest in colon cancer cells.

In addition, we showed in Fig. 3 that UV-C caused a marked phosphorylation of EGFR at Ser-1046/7; however, UV-C failed to induce phosphorylation at Tyr-1045, the major c-Cbl binding site, as well as Tyr-1068, the Grb2 adaptor protein-binding site (8), in contrast to the prominent phosphorylation induced by EGF as we have previously shown (25). Moreover, although UV-C caused activation of p44/p42 MAPK, p38 MAPK, or SAPK/JNK (Fig. 4*A*), we observed that the inhibition of p38 MAPK suppressed EGFR internalization (Fig. 4, *B* and *C*). In addition, p38 MAPK was involved in phosphorylation at Ser-1046/7 (Fig. 4, *D–F*) and subsequent degradation (Fig. 6) of the EGFR induced by UV-C. Moreover, UV-C-induced activation of p38 MAPK was mediated through TAK-1 (Fig. 5). We also examined the effect of UV-C on apoptosis signal-regulating kinase 1, a MAPK kinase kinase, because apoptosis signal-regulating kinase 1 is activated in response to a variety of stress-related stimuli and activates MKK3, which in turn activate p38 MAPK (52). However, UV-C had no appreciable effect on phosphorylation of apoptosis signal-regulating kinase 1 at Ser-967 and Thr-845 (data not shown). Furthermore, we found in colon cancer cells that pretreatment with UV-C before EGF stimulation significantly suppressed the phosphorylation of EGFR at tyrosine residues and Akt (Fig. 7), indicating that UV-C can evade cells from oncogenic stimulation of EGF. In addition, as shown in Fig. 8, it seems unlikely that DNA damage is involved in UV-C-induced EGFR down-regulation via p38 MAPK. However, our present findings do not evaluate and cannot completely eliminate the possibility that DNA damage plays a role in UV-C-induced cell cycle arrest.

Whereas we have recently reported that the blockade of EGF stimulation significantly suppressed cell growth (31), we herein demonstrated that proliferation of colon cancer cells depended on the EGFR kinase activity, thus suggesting that the desensitization of EGFR signaling is a promising target against human colon cancer. In addition, an early work showed that exposure to UV light induced clustering and internalization of cell surface EGFR, and inhibition of clustering or receptor down-regulation attenuates UV responses (53), which is consistent with our present findings that internalization and subsequent degradation of EGFR induced by UV-C leads to cell cycle arrest of colon cancer cells by causing its phosphorylation at serine residues via p38 MAPK. Because it is generally understood that tyrosine phosphorylation results in cancer cell proliferation (9), these results also suggest the potential availability of UV-C for human colon cancer therapy because UV-C can cause EGFR down-regulation without oncogenic activation (Fig. 3). Moreover, Zwang and Yarden (30) previously reported that abrogating EGFR internalization reduces the efficacy of chemotherapy-induced cell death and EGFR internalization enhances the cytotoxic effect of cisplatin by preventing EGFR-mediated survival signaling, which may underlie interactions between chemotherapy and EGFR-targeting drugs. Therefore, our findings also provide the possibility of a new combination of conventional chemotherapy and UV-C for human colon cancer.

Our present study combined with previous findings is summarized in Fig. 9 as follows; when the cells are exposed to EGF stimulation, EGFR undergoes dimerization and tyrosine phosphorylation that directs the cells into cell proliferation (9). Subsequently, c-Cbl, a ubiquitin ligase, can bind to EGFR and cause ubiquitination and degradation of the EGFR (10, 54). By con-

trast, UV-C has little effect on EGFR phosphorylation at tyrosine residues, indicating that adequate dose of UV-C fails to exert the cell growth signals. However, UV-C induced serial phosphorylation of TAK1, MKK3/6, and p38 MAPK and subsequent phosphorylation of EGFR at Ser-1046/7. With time, EGFR molecules are internalized and eventually degraded. Therefore, pretreatment of the cells with UV-C can protect colon cancer cells from oncogenic stimulation such as EGF.

We have previously reported that (−)-epigallocatechin gallate, as well as HSP90 inhibitors, causes down-regulation of the EGFR via phosphorylation at Ser-1046/7 through p38 MAPK in human cancer cells (24, 26). Additionally, accumulating evidence shows that activation of p38 MAPK has an inhibitory effect on tumorigenesis (55, 56) and that a variety of agents, such as gemcitabine (5) and cisplatin (20), can also induce activation of p38 MAPK and internalization of EGFR into endosomal vesicles. Moreover, it has previously been reported that the Ser-1046/7 phosphorylation sites act to suppress the oncogenic signal transduction by the wild-type EGFR (21, 23). Hence, our present findings might provide a new therapeutic strategy for human colon cancer, although further investigations are necessary to elucidate the mechanism underlying EGFR down-regulation via its phosphorylation at serine residues.

Regarding our concern of how these findings would be translated into the clinic, the current limitation of the present study is the lack of practical tools to deliver UV-C irradiation onto the colon cancer tissue in the human body. Our hypothetical approaches would include a combination of extracorporeal generator and fiberscopic transmission of UV-C through colon endoscope or possible expansion of light emitting diode technique to exert shorter wavelength UV-C than with the currently available UV-A light emitting diode. However, substantial technical advances and some related time would be essentially required before practical application of UV-C for colon cancer management. Moreover, further studies are required to determine whether these effects of UV-C safely occur *in vivo*.

In summary, we found that UV-C induces EGFR down-regulation via p38 MAPK-mediated EGFR phosphorylation at Ser-1046/7. Moreover, our results strongly suggest that UV-C irradiation induces the removal of EGFR from cell surfaces that can evade colon cancer cells from oncogenic stimulation of EGF, resulting in cell cycle arrest in colon cancer cells.

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