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A green tea polyphenol, epigalocatechin-3-gallate, induces apoptosis of human hepatocellular carcinoma, possibly through inhibition of Bcl-2 family proteins

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***Background/Aims*: A major polyphenol of green tea, epigallocatechin-3-gallate (EGCG), has previously been shown to induce cell-cycle arrest and apoptosis in various cancers. However, little is known about its effects on hepatocellular carcinomas (HCCs).**

***Methods*: Four HCC cell lines, HLE, HepG2, HuH-7 and PLC/PRF/5, were treated with EGCG or vehicle. Cell viability was assessed by trypan blue staining and WST-8 assay. Cell-cycle, apoptosis and apoptosis-related proteins in HLE cells were evaluated by flow cytometry and Western blotting. The effect of EGCG was also studied in vivo using a xenograft model. The effect of co-treatment with EGCG and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was also assessed.**

***Results*: EGCG inhibited the growth of all HCC cell lines at concentrations of 50–100 μg/ml. In HLE cells, EGCG induced apoptosis but not cell-cycle arrest and appears to have down-regulated Bcl-2α and Bcl-xl by inactivation of NF-κB. Oral administration of EGCG showed similar effects in HLE xenograft tumors. Co-treatment with EGCG and TRAIL synergistically induced apoptosis in HLE cells.**

***Conclusions*: EGCG induced apoptosis in HLE cells, both in vitro and in vivo. Moreover, it enhanced TRAIL-induced apoptosis. Therefore, EGCG treatment may be useful for improving the prognosis of HCCs.**

***Keywords*: EGCG; Apoptosis; HCC; NF-κB; Bcl-2 family; TRAIL**

1. Introduction

Epidemiological studies have shown that green tea has anticarcinogenic and anticancer effects [1–5]. A major polyphenol of green tea, epigallocatechin-3-gallate (EGCG), is thought to be the main active ingredient. It is considered to suppress tumor growth indirectly by anti-angiogenic action and activation of immune function [6,7], and directly through cell-cycle arrest and induction of apoptosis mediated by various proteins including p53 and nuclear factor-kappa B (NF-κB) [8,9].

During the progression of hepatocellular carcinoma (HCC), cancer tissues gradually obtain various malignant features in a multistep fashion [10]. Most early-stage HCCs are small tumors with ill-defined boundaries, and consist of well-differentiated cancerous tissues. During the progression, the less differentiated lesions with more malignant properties occasionally arise and replace the preexisting parts. This process increases the malignant potential of a tumor. For this multistep dedifferentiation, HCCs in an advanced stage show poor prognosis, especially when tumors are too large to be treated by surgery or local interventions such as percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA).

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HCCs with poor prognosis are characterized by rapid cell proliferation and strong expression of antiapoptotic genes [11], which suggests that they are mainly due to incomplete cell-cycle arrest and apoptosis-resistance under conventional therapies. To overcome these problems a new or additional therapeutic strategy is needed. In addition, little is known about the anticancer effects and the basic mechanisms of EGCG against HCCs [12,13]. In the present study, we examined the effects of EGCG on growth of human HCC cell lines using an in vitro culture system and an in vivo animal xenograft model, and elucidated the mechanism of growth suppression by analysis of cell-cycle regulation and profiles of proapoptotic and antiapoptotic proteins.

2. Materials and methods

2.1. Cell lines and cell culture

Human HCC cell lines, HLE (an undifferentiated cell line) and HepG2, HuH-7 and PLC/PRF/5 (differentiated cell lines) were purchased from the Health Science Research Resources Bank, Osaka, Japan. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

2.2. Antibodies and inhibitors, ligand and EGCG

Antibodies for Bid, Bax, Bcl-2α, Bcl-xl, c-FLIP, AIF, and β-actin were purchased from Lab Vision Corporation (Fremont, CA). Antibodies for survivin, XIAP, cIAP-1/2 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were from R and D Systems Inc. (Minneapolis, MN). An antibody for RIP was from BioVision Research Products (Mountain View, CA). Antibodies for nuclear factor-kappa B (NF-κB) p65 and phospho-NF-κB p65 (Ser536) were from Cell Signaling Technology, Inc. (Beverly, MA). An NF-κB inhibitor, Bay 11-7085 was from Calbiochem (San Francisco, CA). A purified preparation of EGCG (>98% pure) was a kind gift from Dr Yukihiko Hara (Mitsui Norin Ltd, Shizuoka, Japan).

2.3. Cell growth assay

Cells were incubated in 6-well plates (1×10^5 cells/well) for 24 h, then treated with 50 μg/ml EGCG or vehicle. The time of EGCG addition was defined as 0 and cell numbers were counted by trypan blue staining every 24 h (24–96 h). Medium containing the appropriate concentration of EGCG or vehicle was changed every 2 days.

2.4. Cell cytotoxicity assay

Cells were incubated in 96-well plates (5×10^3 cells/well) for 24 h, then treated with EGCG (10, 25, 50 and 100 μg/ml), TRAIL (100 ng/ml) or vehicle. After 24 or 48 h of treatment, the number of viable cells was measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] assay (Nacalai Tesque, Kyoto, Japan). Ten microliters WST-8 solution was added into each well and the cells were incubated for another 1 h. The absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 600 nm using a microplate reader (Benchmark, Bio-Rad Laboratories, CA). Cell cytotoxicity was evaluated as the ratio of the absorbance of the sample to that of the control.

2.5. Cell cycle analysis

Cells were incubated for 24 h and then treated with EGCG (10, 25, 50 and 100 μg/ml) or vehicle. Cells (1×10^6) were fixed with 70% ethanol at −20 °C for 24 h, centrifuged, suspended in 1 ml PBS containing RNase A (10 μg/ml), incubated at 37 °C for 30 min, stained with propidium iodide (40 μg/ml), incubated on ice for 30 min and examined with a flow cytometer using Modfit LT software (Becton Dickinson, San Jose, CA).

2.6. Analysis of apoptosis

Cells (5×10^5) that had been treated for 24 h with EGCG (10, 25, 50 and 100 μg/ml), TRAIL (100 ng/ml) or vehicle were labeled with annexin V-FITC and PI using an apoptosis detection kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's protocol. The distribution of apoptotic cells was identified by a flow cytometer using CellQuest software (Becton-Dickinson, San Jose, CA). Cells that were annexin V(−) and PI(−) were considered viable cells. Cells that were annexin V(+) and PI(−) were considered early-stage apoptotic cells. Cells that were annexin V(+) and PI(+) were considered late-stage apoptotic cells or necrotic cells.

2.7. Caspase colorimetric assay

Cytoplasmic protein was extracted from 2×10^6 cells of each cell line that had been treated with EGCG (10, 25, 50 and 100 μg/ml) or vehicle for 24 h. Cytoplasmic protein (150 μg) and 5 μl fluorogenic substrate were added to 50 μl reaction buffer. The mixtures were placed in wells of a 96-well plate and assayed for caspase with a caspase-3, 8, 9 colorimetric assay kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's protocol.

2.8. Western blot

Cells that had been treated with EGCG (10, 25, 50 and 100 μg/ml), Bay 11-7085 (2.0 and 4.0 μg/ml), TRAIL (100 ng/ml) or vehicle for 24 h were lysed using standard procedures. The supernatant was used as cytoplasmic protein. Twenty to fifty micrograms cytoplasmic protein was electrophoresed in a 4–12% bis-tris gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk and incubated with primary antibody for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 h, antibody-stained bands were detected with SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL). The density of each band was measured using densitography software (ATTO, Tokyo).

2.9. Immunoprecipitation

Cells were treated with 100 μg/ml EGCG for 0, 3, 6, 12 and 24 h. Five hundred microgram of cytoplasmic protein, prepared as described above, was incubated with 60 μl Protein G Sepharose beads (Amersham Biosciences Corp, Piscataway, NJ) for 1 h and centrifuged. The supernatant was incubated with 2 μg *anti*-Bax antibody overnight and with 60 μl Protein G Sepharose beads for another 1 h. The beads were washed 4 times and boiled with 40 μl sample buffer. The immunoprecipitated Bcl-2α and Bcl-xl which bound to Bax were analyzed by Western blot analysis.

2.10. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells that had been treated with EGCG (10, 25, 50 and 100 μg/ml) or vehicle for 24 h and subjected to RT-PCR [14]. Transcripts of the gene for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as an internal control. The primer sequences and amplicon sizes were as follows: bcl-2α forward: 5′-TTGTGGCCTTCTTTGAGTTCG-3′, bcl-2α reverse: 5′-TAC TGCTTTAGTGAACCTTTT-3′ (332 bp product), bcl-xl forward: 5′-GGAGCTGGTGGTTGACTTTCT-3′, bcl-xl reverse: 5′-CCGGAA

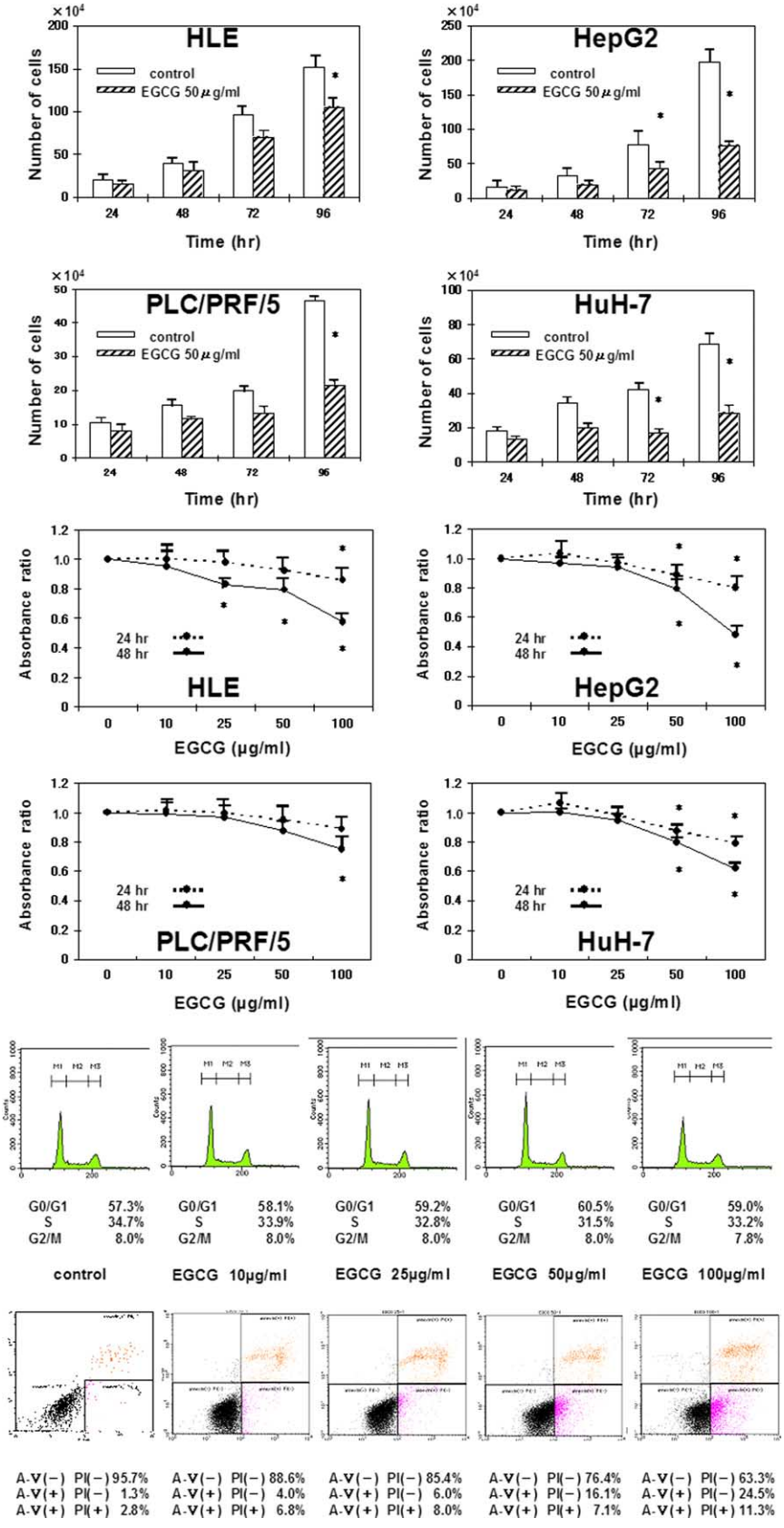

Fig. 1. EGCG inhibits growth of human hepatocellular carcinoma (HCC) cells in vitro and induces apoptosis. (a) Proliferation was assessed in human HCC cell lines, HLE, HepG2, PLC, and HuH-7. The cells were treated with 50 μg/ml EGCG or vehicle for 24, 48, 72 and 96 h, and counted by trypan blue staining. The data represent the mean and SD from five independent experiments. $^{*}P < 0.05$. (b) Cell cytotoxicity was assessed using the WST-8

GAGTTCATTCACTAC-3′ (379 bp product), G3PDH forward: 5′-GTCAACGGATTTGGTCGTATT-3′, G3PDH reverse: 5′-AGTCT TCTGGGTGGCAGTGAT- 3′ (540 bp product). The PCR protocol for bcl-2α or bcl-xl consisted of denaturation at 94 °C for 3 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, final extension at 72 °C for 5 min. The protocol for G3PDH was the same except for annealing at 54 °C for 45 s and amplification for 25 cycles. Amplification was exponential throughout all cycles. The PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide.

2.11. Growth inhibition of HCC by EGCG in a xenograft model

HLE cells (1×10^6) were injected into the dorsal subcutaneous tissue of twenty 8-week-old nude mice (male BALB/c Slc-nu/nu, Clea Japan, Inc., Tokyo, Japan) to form tumors. Tumor volumes were calculated daily with the formula $(4/3)\pi\{(\text{major axis}/2)\times(\text{minor axis}/2)\}^3$. When the tumor volumes reached 50–250 mm^3, the mice were randomly divided into 4 groups (groups A, B, C, D, $n=5$). The four groups were given sterile water containing 0, 0.8, 2.5 and 7.5 mg/ml EGCG ad libitum, respectively. Water consumption of each group was measured daily. The first day of EGCG water administration was defined as day 0. The mice were weighed every day until day 25 when they were sacrificed. None of the mice in any of the groups showed any obvious changes in body weight, in water consumption or in behavior throughout the experiment. Tumor tissues were collected and fixed in 4% buffered paraformaldehyde. Apoptotic cells were detected with a TUNEL assay and expressions of Bcl-2α and Bcl-xl in HLE cells were assessed with immunohistochemical staining [14]. Animals were treated humanely following the United Kingdom Coordinating Committee on Cancer Research Guidelines.

2.12. Statistical analysis

Differences between groups treated with each concentration of EGCG were assessed using Student's *t*-test. The synergistic effect of EGCG on TRAIL treatment was tested by ANOVA.

3. Results

3.1. EGCG inhibits growth of human HCC cell lines in vitro and induces apoptosis in HLE cells

EGCG treatment inhibited the growth of all of four HCC cell lines in a time-dependent manner (Fig. 1a). The cytotoxicity of EGCG was also observed in all of the cell lines in a dose- and time-dependent manner (Fig. 1b). We selected HLE cells for further investigations because undifferentiated HCC cells such as HLE cells generally show poor prognosis and require a new strategy in clinical practice [15]. EGCG treatment had no effect on cell-cycle progression but induced early-stage apoptosis in a dose-dependent manner (Fig. 1c). The number of cells that were either apoptotic in the delayed stage or necrotic also increased similarly by EGCG treatment. These results suggest that the anti-tumor effect of EGCG in HLE cells is mainly induced by apoptosis.

3.2. EGCG activates caspases and decreases expressions of Bcl-2α and Bcl-xl in HLE cells

EGCG treatment activated caspases-8, 9 and 3 in a dose-dependent manner (Fig. 2a). The caspase-9 pathway is mainly regulated by proteins of the bcl-2 family and inhibitor of apoptosis proteins (IAP) family. The expressions of Bcl-2α and Bcl-xl were gradually inhibited as the EGCG concentration increased. The expressions of Bid and c-IAP2 decreased at higher concentrations of EGCG (50, 100 μg/ml), the former probably due to the cleavage by activated caspase-8. EGCG had no influence on Bax, survivin, XIAP or c-IAP1 (Fig. 2b), c-FLIP and RIP (factors that control the activation of caspase-8), or Fas, the death receptor, or AIF, caspase-independent apoptosis-inducing factor (Fig. 2c). EGCG decreased the bindings of Bcl-2α and Bcl-xl to Bax, which may activate caspase-9 (Fig. 2d).

3.3. EGCG down-regulates the mRNAs of bcl-2α and bcl-xl by inhibition of NF-κB

The mRNA expressions of bcl-2α and bcl-xl decreased following EGCG treatment in a dose-dependent manner (Fig. 3a). Thus, EGCG decreases the expressions of Bcl-2α and Bcl-xl at the level of transcription. EGCG did not affect the protein level of the NF-κB p65 subunit in the cytoplasm but it decreased the active form, phospho-NF-κB p65 (Ser536), (Fig. 3b). When HLE cells were treated with an NF-κB inhibitor, Bay 11-7085 [16], the expressions of Bcl-2α and Bcl-xl decreased in a dose-dependent manner (Fig. 3c). These results appear to show that EGCG down-regulates both Bcl-2α and Bcl-xl expressions via NF-κB inhibition. To examine the time course of these effects, HLE cells were exposed to EGCG for up to 24 h. EGCG treatment inhibited the activation of NF-κB (P65) within 3 h and the effect continued for 24 h (Fig. 3d). The expressions of Bcl-2α and Bcl-xl decreased remarkably after 6 and 3 h of exposure, respectively and continued to decrease, while those of Bax and Fas showed no obvious changes.

3.4. EGCG inhibits growth of HLE in vivo, induces apoptosis and down-regulates Bcl-2α and Bcl-xl

The growth of HLE xenograft tumors in nude mice was suppressed by administration of EGCG compared with the control (Fig. 4a). The inhibitory effect was observed

assay. The cells were treated with EGCG at the indicated concentrations (0—100 μg/ml) and the absorbance ratio was measured in 24 and 48 h. The data represent the mean and SD from ten independent experiments. $^*P<0.05$. (c) Effects of EGCG on cell cycle progression and apoptosis in HLE cells were examined by a flow cytometer. The cells were treated as described under Section 2. The proportion of each phase of the cell cycle was analyzed using the Modfit LT software, and apoptosis was assessed using the CellQuest software. The data are from a representative experiment repeated three times with similar results. [This figure appears in colour on the web.]

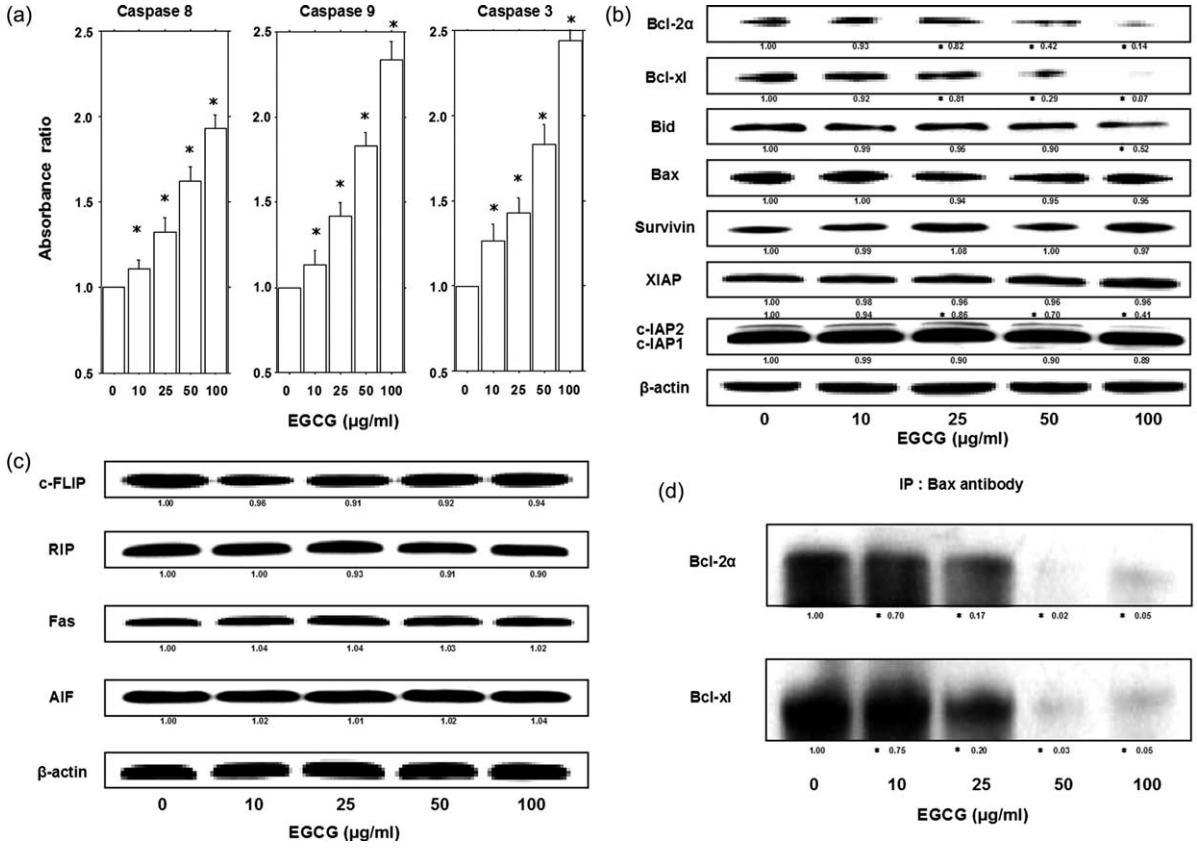

Fig. 2. EGCG activates caspases and decreases expressions of Bcl-2α and Bcl-xl in HLE cells. (a) Activation of caspase-8, -9 and -3 by EGCG in HLE cells. The cells were treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h and caspase activities in the cell lysates were measured with a colorimetric assay kit. The data represent the mean and SD from five independent experiments. $^{*}P<0.05$. (b, c) Western blots showing expressions of Bcl-2 family proteins and IAP family proteins (b) and c-FLIP, RIP, Fas and AIF (c) in cells treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h. (d) The quantities of Bcl-2α and Bcl-xl binding to Bax were analyzed by immunoprecipitation and Western blot assay. The data shown in each of (b,c) and (d) are representative of three experiments with similar results. Image intensities of a band for a protein p were defined as $(I_p^x/I_c^x)/(I_p^0/I_c^0)$ where I_p^x and I_c^x are the band intensities of the protein and the control (β-actin) at EGCG concentration x and I_p^0 and I_c^0 are the band intensities of the protein and control at EGCG concentration 0. $^{*}P<0.05$.

significantly from day 3 and increased as the concentration of EGCG increased. None of the mice showed any significant difference in body weight, water consumption, or behavior throughout the experiment. The tumor sections showed a large number of apoptotic cells and weak staining of Bcl-2α and Bcl-xl in EGCG-treated mice. In contrast, few apoptotic cells and strong staining of Bcl-2α and Bcl-xl were observed in the control mice (Fig. 4b). These results indicate that oral administration of EGCG induced apoptosis of tumor cells in vivo, probably by down-regulation of Bcl-2α and Bcl-xl.

3.5. EGCG sensitizes HLE cells to TRAIL-mediated apoptosis

HLE cells treated with TRAIL (100 ng/ml) for 24 h showed a weak cytotoxic effect compared with control. However, co-treatment with EGCG and TRAIL significantly enhanced the cytotoxicity in an EGCG dose-dependent manner (Fig. 5a). This effect was significantly different from that of treatment with EGCG alone, and was synergistic ($P<0.0001$ by ANOVA). An apoptosis assay demonstrated similar results (Fig. 5b). EGCG and TRAIL also had a synergistic effect on down-regulating the expressions of Bcl-2α and Bcl-xl in HLE cells (Fig. 5c).

4. Discussion

Advanced HCCs often show poor prognosis even following transcatheter arterial embolization (TAE) or surgical resection [17,18]. Our finding that EGCG inhibited the growth of not only well-differentiated cell lines but also an undifferentiated cell line, HLE (Fig 1a and b) suggests that EGCG has the potential to improve the prognosis of HCCs even in the advanced stage.

Although EGCG can induce apoptosis in various cancers, little is known about its effect on HCC. EGCG was shown to induce cell-cycle arrest and apoptosis through the activation of p53 and Fas-Fas ligand pathways in HepG2 cells [19]. In our experiments, EGCG failed to induce cell-cycle arrest

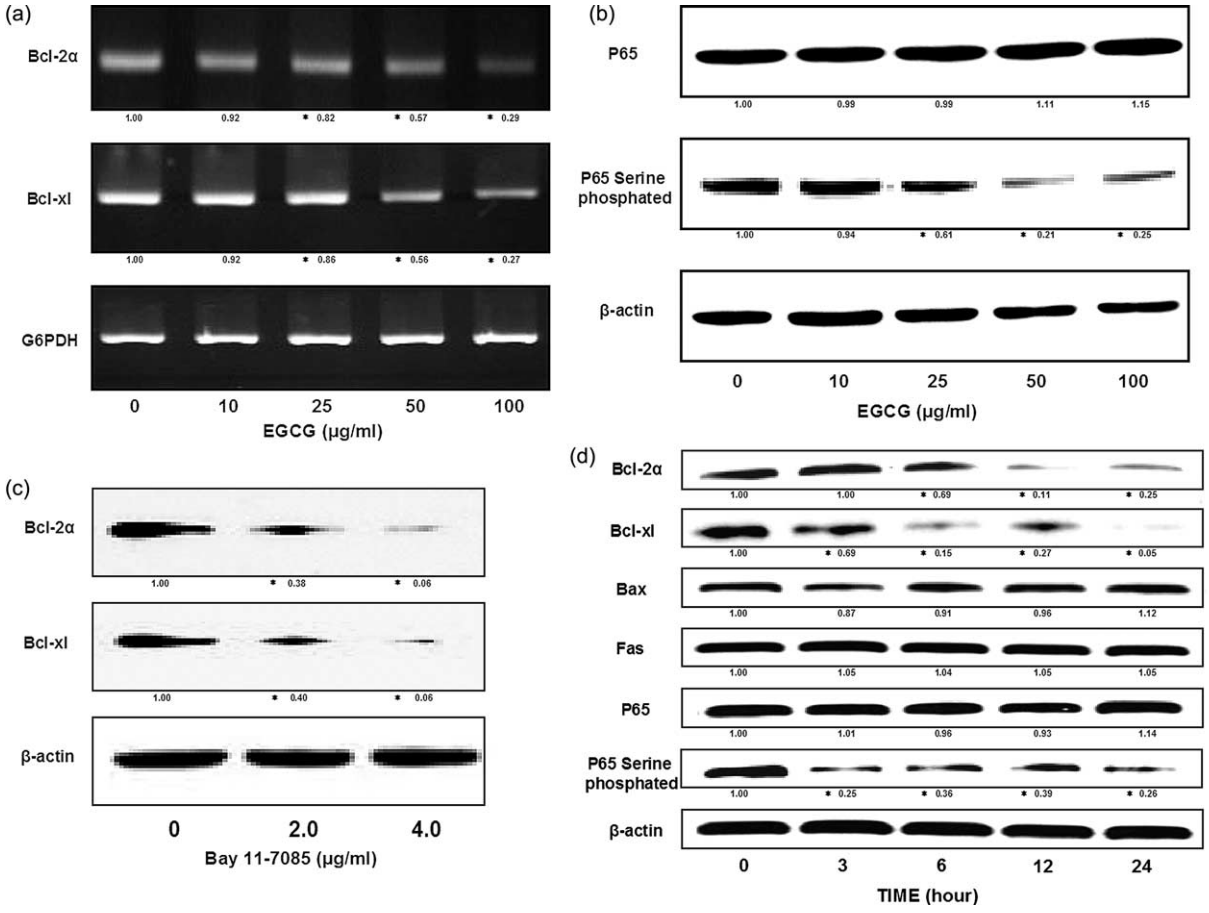

Fig. 3. EGCG inhibits mRNA transcriptions of Bcl-2α and Bcl-xl in HLE cells. (a) Both mRNA expressions of Bcl-2α and Bcl-xl in the cells treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h were analyzed by RT-PCR. (b) Activation of NF-κB in the cells was assessed by Western blot assay using antibodies for NF-κB p65 and phospho-NF-κB p65 (Ser536). The cells were treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h. (c) Expressions of Bcl-2α and Bcl-xl in the cells treated with the NF-κB inhibitor, Bay 11-7085 at the indicated concentrations (0–4.0 μg/ml) for 24 h. Those expressions were assessed by Western blot assay. (d) Western blot assay showing expressions of Bcl-2α, Bcl-xl, Bax, Fas, NF-κB p65 and phospho-NF-κB p65 (Ser536) in cells treated with 100 μg/ml EGCG for 0, 3, 6, 12 and 24 h. The data shown in each of (a)–(d) are representative of three experiments with similar results. See Fig. 2 for explanation of band intensities. $^{*}P < 0.05$.

but induced apoptosis in HLE cells (Fig. 1c). This is probably because p53 protein in HLE cells is dysfunctional due to a mutation of the p53 gene, whereas it is still functional in HepG2 cells. Additionally, EGCG treatment had no effect on expression of Fas in HLE cells (Figs. 2c and 3d). These results indicate that EGCG has more than one mechanism for inducing apoptosis in HCC. A Western blot assay of pro- and anti-apoptotic proteins showed that EGCG inhibits the expressions of Bcl-2α and Bcl-xl in HLE cells (Fig. 2b), which suggests that activation of caspase-9 by inhibition of Bcl-2α and Bcl-xl is a major mechanism for induction of apoptosis. The inhibition of c-IAP-2 also might contribute to activation of caspase-9. EGCG also activated caspase-8 in HLE cells (Fig. 2a) though it had no effect on the expressions of cFLIP, RIP and Fas (Fig. 2c). The overexpression of Bcl-2 in HCC cells was shown to reduce Fas-mediated apoptosis [20]. EGCG may activate caspase-8 through reactivation of the Fas-Fas ligand pathway by down-regulation of Bcl-2α expression. Another possibility is that EGCG directly activates caspase-8 through its binding to Fas [21].

NF-κB partially regulates the transcriptions of Bcl-2α and Bcl-xl [22]. In addition, our results suggest that EGCG down-regulates the expressions of Bcl-2α and Bcl-xl by inhibition of NF-κB (Fig. 3a–c). NF-κB is a transcriptional factor that is associated with various cellular processes, such as cytokine production, cellular adhesion, cell-cycle activation, apoptotic resistance, oncogenesis, and is activated in various solid tumors [23]. Thus, the inhibitory effect of EGCG on NF-κB may suppress the growth of various cancer cells in multifunctional ways. NF-κB activation is induced by reactive oxygen species [24] and inhibited by antioxidants [25]. Thus, the inhibitory effect of EGCG on NF-κB activation may be due to its strong ability to scavenge reactive oxygen species. Bcl-xl overexpression is frequently found in HCC tissues and is significantly correlated with a good prognosis [26,27]. On the other hand, Bcl-2α is not frequently expressed in HCCs [28], but TAE

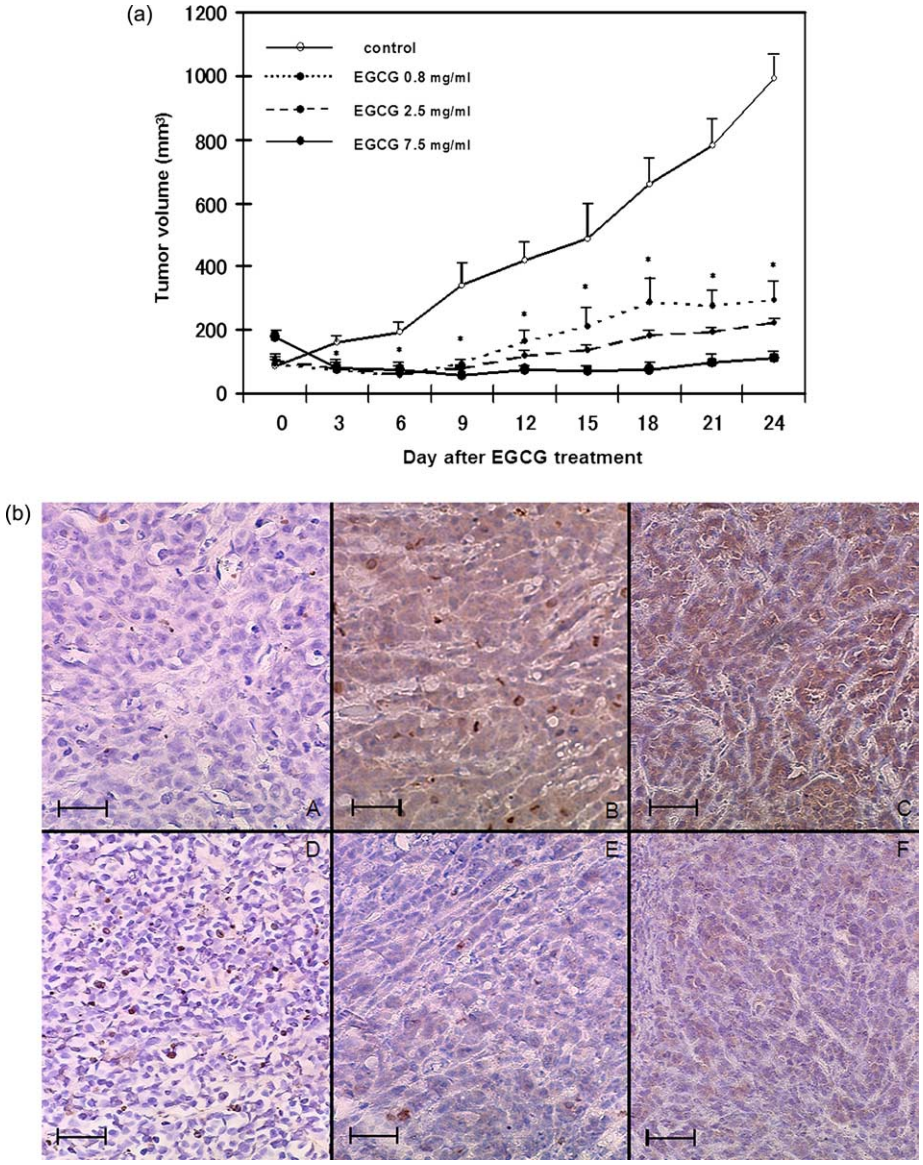

Fig. 4. (a) Growth curve of HLE xenografts in nude mice. Nude mice were injected with approximately 1×10^6 HLE cells into the dorsal subcutaneous tissue. When the tumor volumes reached 50–250 mm^3, the mice were divided into four groups and fed with sterile water or 0.8, 2.5, 7.5 mg/ml EGCG water respectively. The data represent the mean and SD from five nude mice. $^*P < 0.05$. (b) Apoptosis and expressions of Bcl-2α and Bcl-xl in HLE xenografts. Apoptosis was assessed by TUNEL assay (A, D) and expressions of Bcl-2α (B, E) and Bcl-xl (C, F) were assessed by immunohistochemistry. The upper row shows tumor sections in the control group and the lower row shows tumor sections in the group receiving 7.5 mg/ml EGCG. Each picture was taken at a magnification of 400×. Scale bars, 50 μm.

increases its expression in HCC tissues [29]. The induction of Bcl-2α by TAE may allow the cancer cells to escape from hypoxic injury. Furthermore, these antiapoptotic proteins are associated with resistance to chemotherapy [30] and to endogenous apoptotic signals such as TRAIL [31,32] both in vitro and in vivo. In our experiment, EGCG treatment down-regulated both Bcl-2α and Bcl-xl in HLE cells (Figs. 2b and 4b) both in vitro and in vivo and sensitized HLE cells to TRAIL-induced apoptosis in vitro (Fig. 5a and b). The sensitizing effect of EGCG appears to result from the down-regulation of Bcl-2α and Bcl-xl through inhibition of NF-κB (Fig. 5c).

Our findings that EGCG suppresses the growth of HLE cells (Fig. 4a) and down-regulates the expressions of Bcl-2α and Bcl-xl in vivo (Fig. 4b) raise the possibility that oral administration of EGCG can help overcome the chemotherapy resistance of HCCs. However, the concentrations of EGCG that were needed to significantly down-regulate *anti*-apoptotic proteins and induce apoptosis in vitro (50–100 μg/ml) were much higher than the concentration that is physiologically achieved by ordinary green tea consumption. Consumption of six or seven cups of green tea per day (corresponding to a dose of ~30 mg EGCG/kg per day) will result in a plasma EGCG concentration of approximately 1 μg/ml [33]. However,

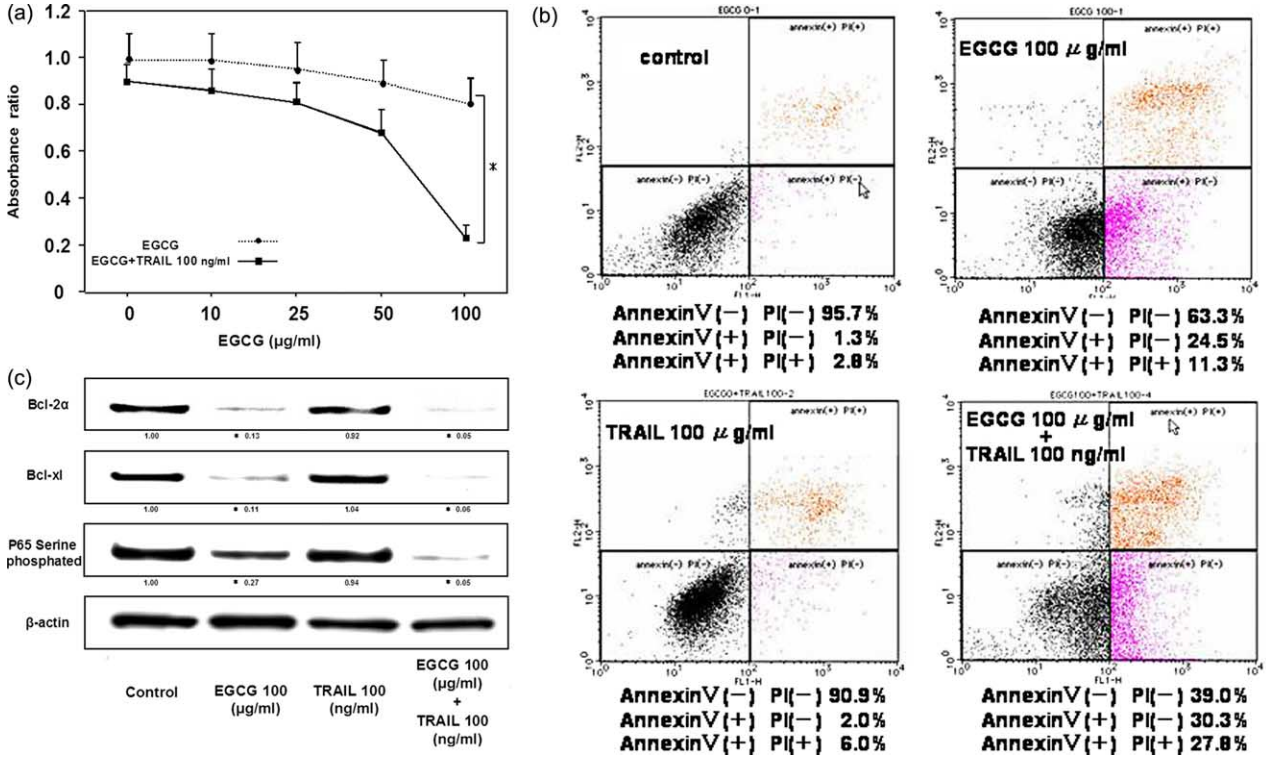

Fig. 5. EGCG sensitizes HLE cells to TRAIL-mediated apoptosis. (a) Cell cytotoxicity was assessed using the WST-8 assay in the cells treated with vehicle or 100 μg/ml EGCG or 100 ng/ml TRAIL or combinations of these agents for 24 h. The combination treatment significantly enhanced the cell cytotoxicity compared with EGCG treatment alone. The data represent the mean and SD from five independent experiments. $^{*}P < 0.0001$ by ANOVA. (b) Apoptosis in the cells treated as described above was examined by flow cytometry. The experiment was repeated three times with similar results. (c) Western blot assay showing expressions of Bcl-2α, Bcl-xl and phospho-NF-κB p65 (Ser536) in cells treated with vehicle or 100 μg/ml EGCG or 100 ng/ml TRAIL or combinations of these agents for 24 h. The data shown in (c) is representative of three experiments with similar results. See Fig. 2 for explanation of band intensities. $^{*}P < 0.05$. [This figure appears in colour on the web.]

higher plasma EGCG concentrations can be achieved by taking EGCG supplements [34]. In our study, EGCG had no obvious adverse effects on the growth or behavior of mice, even at a dose of 1125 mg/kg per day. EGCG also has an antiangiogenic effect and activates an immune response against tumors, which may increase its antitumor effect in vivo.

In conclusion, our results show that EGCG treatment inhibited the growth of HLE cells in vitro and in vivo. The inhibition was caused by the induction of apoptosis as a result of the activations of caspase-8, -9 and -3. These caspases appear to be activated by the down-regulation of Bcl-2α and Bcl-xl through inhibition of NF-κB. Our results indicate that EGCG treatment may be useful in clinical cases to improve the prognosis of advanced HCCs by direct induction of apoptosis and by enhancing the effects of other factors such as TRAIL.

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