Epigallocatechin gallate hinders human hepatoma and colon cancer sphere formation

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Abstract

Background and Aim: The long-term survival of patients with hepatocellular carcinoma remains unsatisfactory because of the presence of cancer stem cells (CSCs), which are responsible for tumor recurrence and chemoresistance after hepatectomy. Drugs that selectively target CSCs thus offer great promise for cancer treatment. Although the antitumor effects of epigallocatechin gallate (EGCG) have been reported in some cancer cells, its effects on CSCs remain poorly studied. In this study, we investigated the effects of EGCG on human hepatoma and colon CSCs.

Methods: HepG2 and HCT-116 cell lines were enriched by sphere formation, and their gene-expression profiles were analyzed by quantitative real-time polymerase chain reaction. EGCG-induced growth inhibition in the parental cells was determined by WST-8 assay, and protein expression was assessed by western blotting. Cell cycle profile and apoptosis analysis was performed using flow cytometer.

Results: Sphere-derived cells grown in serum-free, nonadherent cultures showed increased expression of stem cell markers, Nek2, and ATP-binding cassette transporter genes, compared with parental cells grown in conventional culture. EGCG induced growth inhibition in the parental cells in a dose-dependent manner. EGCG also inhibited self-renewal in hepatoma and colon CSCs, attenuated the expression of stem cell markers and ATP-binding cassette transporter genes, which are putative molecules associated with treatment resistance in CSCs, and decreased the transcription of Nek2 and p-Akt, resulting in the inhibition of Akt signaling. EGCG also altered cell cycle profile and apoptosis, which may in part play an important role in EGCG-induced cancer cell death.

Conclusions: Overall, these results suggest that EGCG could be a useful chemopreventive agent for targeting hepatocellular carcinoma and colon CSCs, in combination with standard chemotherapies.
Previous evidence demonstrated that many tumors are generated and maintained by cancer stem cells (CSCs) or tumor-initiating cells, which are capable of reinitiating tumors and generating a spectrum of differentiated cells through their ability to express anti-apoptotic and drug-resistant proteins. In addition, CSCs proliferate slowly and appear to be relatively drug resistant, thus surviving to encourage tumor recurrence after the completion of treatment. The existence of CSCs in HCC and colon cancer is likely to be one of the main reasons for the unsatisfactory effects of modern oncologic therapies. Eradication of CSCs is therefore essential for achieving stable, long-lasting remission and for potentially approaching a cure for HCC and colon cancers.

Epigallocatechin gallate (EGCG) is a major polyphenolic constituent of green tea, which has received considerable attention because of its many beneficial effects on human health and its ability to inhibit cancer growth via several mechanisms. Recent studies found that EGCG induced apoptosis in human head and neck squamous carcinoma and inhibited hepatocyte growth factor-induced tumor growth and invasion in oral and hypopharyngeal cancer. It was shown to cause cell cycle deregulation through nuclear factor-κB inhibition. EGCG is also an important preventive agent for reducing cancer incidence and has demonstrated synergistic effects on cancer cell responses to conventional chemotherapy.

Drugs that selectively target CSCs offer great promise for many cancer treatments; however, progress in the identification and development of such agents is lacking. There is therefore an urgent need to identify new treatment strategies for CSCs to allow the development of novel treatment strategies for HCC and colon cancer. The present study examined the in vitro effects of EGCG on human cancer cell sphere formation and investigated the possible underlying mechanisms.

Methods

Reagents and antibodies. Epigallocatechin gallate was purchased from Bio Verde (Kyoto, Japan). Mouse monoclonal anti-Nek2, mouse monoclonal anti-phospho-Akt, and horseradish peroxidase-linked anti-mouse IgG antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Abcam (ab9484; Cambridge, MA, USA).

Cell culture. Human hepatoma cell lines HLF, Huh7, and HepG2 and colon cancer cell lines HCT-116, HT-29, and HCT-115 were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells, except HCT-116, were maintained as a monolayer in RPMI (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of 5% CO₂ at 37°C. HCT-116 cells were maintained in McCoy’s 5A medium (Gibco, Life Technologies, Minatoku Shibaura 428, Tokyo, Japan). Human fibroblasts and hepatocytes were isolated from normal colon and hepatic tissues from patients undergoing colon and hepatic surgery, respectively, in our department, following standard protocols.

Cell proliferation assays. Cell proliferation was assessed by WST-8 assays using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Briefly, 5 × 10³ cells/well were seeded in 96-well plates and incubated for 24 h. EGCG dissolved in phosphate-buffered saline at various concentrations (0–75 μmol) was then added followed by incubation for up to 48 h. Each treatment was carried out in triplicate. After incubation, 10 μL of CCK-8 reagent was added to each well, and the plates were incubated at 37°C in an atmosphere of 5% CO₂ for 4 h. The optical density was then measured with a microplate reader (Multiskan JX; MTX Lab Systems, Vienna, VA, USA) at a wavelength of 450 nm.

Sphere formation. Cells were collected and washed to remove serum and then suspended in serum-free DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 100-μIU/mL penicillin, 100-μg/mL streptomycin, 20-ng/mL human recombinant epidermal growth factor, 10-ng/mL human recombinant basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA), 2% B27 supplement without vitamin A, and 1% N2 supplement (Invitrogen). The cells were subsequently cultured in six-well plates (Corning Inc., Corning, NY, USA) with or without 50-μmol EGCG for 1 week. After 1 week, the results were assessed by examination under an inverted microscope (×100) (Nikon TE2000-U, Japan).

Quantitative real-time polymerase chain reaction. Total RNA was extracted from parental cells and spheres after treatment with or without EGCG for 48 h using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2.5–μg total RNA by reverse transcription using a SuperScript RT kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed using the Applied Biosystems 7500 Real-Time PCR system, TaqMan Gene Expression Assays on demand, and TaqMan Universal Master Mix (gene-specific TaqMan probes with a StepOne Plus; Foster City, CA, USA). Human CD133 (Hs00195682_m1), NANOG (Hs02387400_g1), ABCG2 (Hs01053790_m1), ABCCI (Hs00219905_m1), and NEK2 (Hs00601227_mH) TaqMan primers were used. GAPDH was used as an internal control for normalization. Expression levels of all genes were calculated as the ratio to GAPDH. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1.

Western blot analysis. Cellular proteins were extracted according to standard protocols. Briefly, cells were lysed in ice-cold Radioimmunoprecipitation assay (RIPA) buffer (25-mM Tris–HCl pH 7.6, 150-mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (10 μL/mL), and protein concentrations were determined using the Bradford assay (Bio-Rad, Philadelphia, PA, USA). For immunoblotting, 30–50-μg protein was resolved over 7.5–12% polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking in 5% nonfat dry milk in Tris-buffered saline (TBS), the membranes were incubated with primary antibodies at 1:500 dilution in TBS overnight at 4°C, washed three times with
TBS Tween-20, and then incubated with HP-conjugated secondary antibodies at 1:2000 dilution in TBS for 2 h at room temperature. Membranes were washed again three times in TBS Tween-20 at room temperature. The samples were finally treated with enhanced chemiluminescence assay reagents (GE Healthcare, Buckinghamshire Hp7, 9NA, UK) and exposed to X-ray film to detect the protein bands.

**Cell cycle analysis.** HepG2 and HCT-116 cells were grown for 48 h, and different concentration of EGCG was added after changing the medium. Cells were incubated for 48 h before they were harvested. These cells were fixed gently with 80% ethanol in a freezer for 2 h and were then treated with 0.25% Triton X-100 for 5 min on ice. Cells were suspended in 150 μL of PBS containing 40-μg/mL propidium iodide (PI) and 0.1-mg/mL RNase. The cells were incubated in a dark room for 20 min at room temperature, and cell cycle analysis was performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

**Apoptosis analysis.** HepG2 and HCT-116 cells were grown in a monolayer before treatment with the indicated concentrations of EGCG for 48 h. Cells were washed twice with cold PBS and then re-suspended in 1X Binding Buffer at a concentration of 1 × 10^7 cells/mL. Transfer of 100 μL of solution (1 × 10^7 cells) to a 5-mL culture tube was performed, and cells were stained with annexin V-FITC and PI according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA, USA). The cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Then, 400 μL of 1X Binding Buffer was added to each tube. Analysis was performed with a flow cytometer (Beckman Coulter, Brea CA, USA) to identify the subpopulations of the apoptotic cells within 1 h.

**Statistical analysis.** Data are expressed as the mean ± standard error of the mean from a minimum of three separate experiments. Differences between the means of two groups were analyzed by Student’s *t*-tests. One-way ANOVA with a Bonferroni multiple comparison post hoc test was used to compare two or more groups. Statistical analyses were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined at *P* < 0.05.

**Results**

**Epigallocatechin gallate decreased ABCC1 and ABCG2 transporter gene expression in cancer stem cells in vitro.** The chemoresistance of CSCs may be caused by the exclusion of exogenous and endogenous toxic materials by ATP-binding cassette (ABC) transporters. We therefore investigated the effects of EGCG on expression levels of multidrug resistance (*ABCC1*) and breast cancer resistance (*ABCG2*) genes in HepG2 and HCT-116 CSCs. *ABCC1* and *ABCG2* mRNA levels were increased in sphere-forming cells compared with the parental cells but were decreased by EGCG treatment (Fig. 3).

**Epigallocatechin gallate impaired NIMA-related kinase 2/Akt signaling of cancer stem cells.** Akt signaling is important for regulating the balance between cell proliferation, differentiation, and apoptosis. We therefore examined if the inhibitory effects of EGCG on hepatoma and colon cancer cells were mediated by the suppression of the Akt pathway. Gene and protein levels of Nek2 and protein levels of Akt were determined in cells treated with and without EGCG. EGCG successfully downregulated both gene and protein expression levels of Nek2 in CSCs (Fig. 4) and decreased Akt protein levels (Fig. 4c,d). Furthermore, inhibition of Akt by its inhibitor (Ly294002) affected the effect of EGCG on partial restoration of HepG2 and HCT-116 cell sphere formation; however, cell viability was not significantly affected (Fig. 5).

**Epigallocatechin gallate altered cell cycle profiles of HepG2 and HCT-116.** To evaluate the effect of EGCG on cell cycle, the cell cycle profile was assayed by flow cytometry. As shown in Figure 6, the cell cycle profile in HepG2 and HCT-116 control group was G1 54.5%, S 39.2%, and G2/M 6.4% and G1 64.6%, S 12.2%, and G2/M 23.3%, respectively. Treatment with 25 and 50 μmol of EGCG changed cell cycle profile. After treatment with 25 μmol of EGCG, the cell cycle profile was G1 44.4% and S 48.0% in HepG2 and G1 63.6% and S 11.6% in HCT-116 cells. When treatment concentration increased to 50 μmol, cell cycle profile was changed to G1 38.4% and S 69.6% in HepG2 and G1 49.8% and S 25.4% in HCT-116 cells. Thus, EGCG increased the number of cancer cells in the S phase, showing that EGCG alters the cell cycle profile.
Epigallocatechin gallate induced apoptosis in HepG2 and HCT-116 cells. To further explore the potential mechanisms by which EGCG-induced cell death, the apoptotic effects of EGCG were evaluated by flow cytometry after staining with annexin V and PI. Annexin V can be detected in both early and late stages of apoptosis; whereas PI stains cells only in late apoptosis or necrosis. Early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic or necrotic cells were stained for both annexin V and PI (upper right quadrant). The cytogram in Figure 7 shows that incubation with EGCG at 12.5 and 25 μmol for 48 h altered the number of apoptotic cells compared with the control group. With EGCG at 12.5 and 25 μmol, cells in early apoptosis were increased. Compared with control (14.47% and 2.87%) in HepG2 and HCT-116 cells, respectively, the percentage of apoptosis increased to 31.31% and 42.94% in HepG2 cells and 11.37% and 8.49% in HCT-116 cells after treatment with EGCG for 48 h at the concentration of 12.5 and 25 μmol, respectively. The results demonstrate that EGCG induces cell apoptosis.

Discussion

Cancer stem cells have recently attracted intense interest as key tumor-initiating cells that may also play an integral role in unlimited cancer growth, recurrence, and metastasis following chemotherapy. HCC and colon cancer development are attributed to the propagation of CSCs, which are capable of self-renewal, tumor initiation, and chemoresistance. However, the molecular events underlying the maintenance and expansion of CSCs require further investigation.
During cell division, Nek2 promotes centrosome splitting at the start of mitosis by phosphorylating multiple linker components and also regulates microtubule organization in the centrosome. High expression levels of Nek2 were shown to induce abnormal tumor proliferation and drug resistance in breast and ovarian cancers. Furthermore, upregulation of Nek2 was associated with tumor progression in a series of malignant tumors originating in different organs and tissues, including breast carcinoma and myeloma.

In accordance with the results of previous studies, we confirmed that Nek2 was highly expressed in HCC tumor tissues.
compared with adjacent nontumor tissues (data not shown). Furthermore, we demonstrated that Nek2 was overexpressed in cancer spheres compared with parental cells in vitro. These results thus confirm that Nek2 activation may contribute to tumorigenesis and progression in HCC and colon cancer. In addition, high expression levels of Nek2 in HCC and colon...
CSCs may represent a novel approach for developing drugs targeting CSCs. The tumorigenicity and invasive capacity of liver CSC were impaired by targeting the stem cell marker EpCAM, leading to the reduction of the CSC pool. In addition, inhibition of CD44 in HCC cells enhanced apoptosis and reduced tumorigenicity and invasiveness. CD133+ cells usually show upregulation of ABC transporters, leading to chemoresistance. The stemness gene NANOG has also been shown to regulate self-renewal and contribute to the expansion of CSCs.

In the present study, we focused on the surface marker CD133, which is highly expressed in liver metastasis and HCCs, pancreatic cancer, gallbladder carcinoma, lung adenocarcinomas, and colon and gastric cancers. CD133+ cells usually show upregulation of ABC transporters, leading to chemoresistance. The stemness gene NANOG has also been shown to regulate self-renewal and contribute to the expansion of CSCs.

The results of the present study showed that the expression levels of stemness markers (CD133 and NANOG) and ABC transporter genes (ABCC1 and ABCG2) were significantly increased in cancer spheres compared with parental cells, suggesting that these genes play regulatory roles in CSCs in HCC and colon cancer. In accordance with this, overexpression of Nek2 in cancer spheres
may contribute to the self-renewal, chemoresistance, and tumor-initiating capacities of HCC and colon cancer cells. This further suggests that the suppression of Nek2 could effectively inhibit self-renewal and chemoresistance in HCC and colon CSCs. Direct targeting of CSC-specific markers may also represent a promising therapeutic strategy for the eradication of CSCs. Tumor suppressor mechanisms that act as gatekeepers for cell growth, death, and lifespan or as caretakers of genome integrity are mainly involved at early stage in carcinogenesis by controlling mutation at the cell cycle process, suggesting that EGCG might affect the genome integrity to prevent the progression of pre-neoplastic formation to tumor.

The complex nature of human cancers seeks alternative management strategies to improve both the efficacy of current therapeutic agents and patient quality of life. The effectiveness of conventional therapies may be improved by combining them with drugs targeting CSCs. Green tea is a popular drink that is consumed by millions of people worldwide on a daily basis. In previous studies, EGCG demonstrated high cytotoxicity in cancer cells compared with normal cells and inhibited sphere formation by neuroblastoma cells.\textsuperscript{26} Takahashi \textit{et al.}\textsuperscript{27} reported that the inhibition of mechanical and biochemical Epithelial-mesenchymal transition (EMT) phenotypes with EGCG is a mechanism-based inhibition of cancer metastasis. In another pilot study in colorectal adenomas, Green tea extract (GTE) showed an effective chemoprevention of metachronous colorectal adenomas without serious adverse events occurred in the GTE group.\textsuperscript{28} However, studies on the regulatory effects of EGCG on CSCs are scarce, and to the best of our knowledge, there have been no reports on its effects on HCC and colon CSCs. In accordance with previous studies, the present results confirmed the cytotoxic effect of EGCG in cancer cells and its ability to attenuate hepatoma and colon cancer sphere formation by affecting overexpressed genes that are required for self-renewal.

In addition, ABC transporter family members, including \textit{ABCC1} and \textit{ABCG2}, were significantly upregulated in cancer spheres compared with parental cell lines and were consistently downregulated by EGCG. EGCG inhibited cancer sphere formation and regulated the chemoresistance of HCC and colon CSCs grown in serum free, nonadherent cultures.

Earlier studies showed that EGCG induced the expression of p21, p53, p16, and p27, associated with negative regulation of cell cycle progression.\textsuperscript{29,30} In the present study, EGCG caused cell cycle arrest in both HepG2 and HT-116 cells. The cells altered in G1/S phase were increased, indicating that cell cycle arrest induction may play an important role in EGCG-induced cancer cell death. In agreement with this, the cell cycle regulator Nek2 was significantly downregulated by EGCG in the current study, indicating that green tea may be an effective chemopreventive agent by acting on HCC and colon CSCs.

Inhibition of the key signaling pathways in CSCs represents a promising cancer-treatment strategy. In addition to Hedgehog\textsuperscript{31} and Notch\textsuperscript{32} signaling pathways, Akt signaling has also been implicated in stem cell self-renewal, cell-fate determination, and cell proliferation, differentiation, and apoptosis.\textsuperscript{33} The downstream target of Nek2 is protein phosphatase-1, which is a major phosphatase that directly dephosphorylates Akt at Thr450 to modulate its activation. Nek2 then binds directly to and phosphorylates protein phosphatase-1 on Thr320, resulting in the upregulation of phosphorylated Akt at Ser473.\textsuperscript{7} Downregulation of Akt signaling may thus provide a novel approach for targeting HCC and colon CSCs. The present study demonstrated that EGCG treatment led to the downregulation of Nek2 and hypophosphorylation of Akt, suggesting that EGCG can suppress the Akt signaling pathway in HCC and colon CSCs. To the best of our knowledge, this report provides the first evidence for EGCG suppression of the Akt pathway in HCC and colon CSCs. In addition, apoptosis is considered as an important pathway in the inhibition of tumor growth by many anticancer agents. We observed that EGCG induced cell apoptosis in HepG2 and HCT-116 cells, suggesting that the anti-proliferative effects of EGCG in human hepatoma and colorectal cancer cells could be also via apoptosis.

Lastly, our data demonstrated that addition of 40-μmol/L Akt inhibitor (Ly294002) showed partial restoration of HepG2 and HCT-116 cells cancer sphere formation in the presence of EGCG. In addition, in the presence of 30-μmol/L of Akt inhibitor (Ly294002), addition of EGCG did not affect cell viability significantly, showing Akt dependency effect of EGCG.

In summary, current treatment strategies for hepatoma and colon cancers are inefficient because of the chemoresistant nature of HCC and colon cancer cells. The present study elucidated the anticancer effects of EGCG, which was able to eliminate the stem cell characteristics of hepatoma and colon cancers and inhibit chemoresistance, partially by regulating the expression of ABC transporter-related genes. EGCG thus could have the potential to prevent the recurrence and metastasis of HCC and colon cancers, when combined with standard treatments. Further investigations of the effects of EGCG on HCC and colon CSCs could help in the development of novel drug combinations capable of eliminating HCC and colon CSCs.

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**References**

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