Comparative Examination of Anti-proliferative Activities of (−)-Epigallocatechin Gallate and (−)-Epigallocatechin against HCT116 Colorectal Carcinoma Cells

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We compared anti-proliferative activities of (−)-epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) against HCT116 colorectal carcinoma cells. These catechins inhibited cell growth to nearly the same extent at low cell confluency in plates. However, their inhibitory effect grew weaker as cell confluence increased, and this tendency was more conspicuous for EGC than for EGCG. Both EGCG and EGC activated the phosphorylation of the major MAPKs, ERK, JNK, and p38, in the HCT116 cells as in many other established human cancer cells though to different extents. Cell cycle analyses, DNA fragmentation assays, and TUNEL assays as well as Western blot assays suggested that these catechins inhibited cell growth through mitogen-activated protein kinase (MAPK)-mediated apoptosis rather than cell cycle regulation.

Key words HCT116 colorectal carcinoma cell; (−)-epigallocatechin gallate; (−)-epigallocatechin; confluency; apoptosis; mitogen-activated protein kinase

The green tea catechins have diverse beneficial effects on human health. For example, (−)-epigallocatechin gallate (EGCG) exhibits inhibitory activities against carcinogenesis and established cancer cells.1–4) After the ingestion of green tea, substantial amounts of catechins are present in the gastrointestinal tract.5–8) Furthermore, in plasma, EGCG mostly exists in the free form, whereas other components such as (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) are present in conjugated forms such as glucuronides. Although the levels of EGC and EC in green tea are about 50% and 10% of the level of EGCG, the area under the blood concentration–time curve (AUC) of total EGC and EC were approximately two-fold higher and equivalent to the AUC of total EGCG, respectively, after ingestion of a water extract of green tea.9) In addition, a recent study led to results suggesting that some glucuronides of EGCG and EGC retain radical quenching and other biological activities of the aglycone.10,11) It is therefore probable that not only EGCG but also other green tea catechins play an important role in the chemoprevention of colon cancer. However, few investigations have so far been reported on the feasibility of using catechins other than EGCG for cancer chemoprevention.

In the present study, we have examined the correlation between the anti-proliferative activity of EGCG and EGC against HCT116 colorectal carcinoma cells and the confluent status of the cells in plates, as well as the molecular mechanisms underlying this activity. It was found that EGCG and EGC had equivalent levels of anti-proliferative activity at low cell confluence. Furthermore, the major mitogen-activated protein kinase (MAPK)s, ERK, JNK, and p38, were activated by EGCG and EGC in the cells though to different extents and the cell growth inhibition by these catechins was caused by MAPK-mediated apoptosis rather than cell cycle regulation.

MATERIALS AND METHODS

Materials and Chemicals HCT116 cells were purchased from Dansippon Sumitomo Pharma (Osaka, Japan). McCoy’s 5A medium was from Invitrogen (CA, U.S.A.). WST-1 and Hoechst 33342 were obtained from Doyindo Laboratories (Kumamoto, Japan). Propidium iodide was from Sigma (MI, U.S.A.). The confluent status of cells in the plates was calculated with Adbe Photoshop 7.0. Anti-Fas neutralizing antibody was supplied from R & D Systems (London, U.K.). Anti-PARP antibody was from Cell Signalling Technology (MA, U.S.A.), and the other antibodies were from Sigma (MI, U.S.A.). HRP-linked secondary antibody and ECL-Plus reagents were from GE Healthcare UK Ltd. (Buckingham, U.K.). The Cyteltest Plus DNA reagent Kit was purchased from Becton Dickinson and Company (NJ, U.S.A.). The TUNEL assay reagent, DeadEnd® TUNEL Kit (DeadEnd® Fluorometric TUNEL System), was obtained from Promega (WI, U.S.A.). Results of flow cytometry were recorded with FACScan (Becton Dickinson and Company). Apoptosis was observed with a Nikon ECLIPSE TE-2000U.

Cell Culture HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% fetal calf serum (FCS), 50 μg/ml penicillin G, and 50 μg/ml streptomycin sulfate in a 5% CO2 and 95% air atmosphere at 37 °C.

Cell Viability Assay Aliquots (135 μl) of HCT116 cells (5.0 × 103—5.0 × 104 cells/ml) were seeded into 96-well flat-bottomed plates (6.4 mm diameter) and allowed to grow for 24 h. The cells were then incubated with various concentrations (0—1000 μM) of EGCG or EGC for 24 h. After calculation of the cell confluence in each well, the number of viable cells was counted using a WST-1 cell counting Kit with Sjeia Autoreader III (Sanko Junyaku, Japan). The IC50 values were obtained from cell viability experiments conducted in triplicate.
HCT116 cells were plated onto 60 mm diameter dishes (1.0×10^6 cells/dish) and allowed to grow for 24 h. The cells at about 50% confluence were incubated with H_2O_2 (300 μM), EGCG or EGC (50, 100, 200 μM) for 24 h. They were fixed in ethanol (−20 °C) for 5 min and washed twice with PBS before being stained with 1 μg/ml Hoechst 33342 for 30 min at room temperature in the dark. The cells were then washed twice with PBS and mounted with coverslips. Apoptosis, with condensed and fragmented nuclei, was observed with a fluorescence microscope.

DNA Fragmentation Assay HCT116 cells were plated onto 100 mm diameter dishes (1.2×10^6 cells/dish). After incubating for 40 h, the cells at about 50% confluence were incubated with H_2O_2 (300 μM), EGCG or EGC (50, 100, 200 μM) for 24 h. Floating and adhered cells were collected and were lysed in a buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, and 20 mM Na_2PO_4 for 1 h on ice. Lysates were cleared by centrifugation at 12000×g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, pH 7.9), and the water layer was diluted in an appropriate volume of a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, pH 7.9), and then precipitated with ethanol to be 70% ethanol. After salting out with alcohol (25 : 24 : 1, pH 7.9), and the water layer was diluted into a 100 μl of lysis buffer (0.5 mM NaH_2PO_4 (pH 7.0), 0.1 mM EDTA (pH 8.0), 0.1% SDS, 10% glycerol, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 0.022 TIU/ml aprotinin, and 1 mM PMSF). The lysates were homogenized and were centrifuged at 14000×g for 30 min at 4°C. An equal amount of protein was then resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was incubated successively with primary antibody and horseradish peroxidase-conjugated secondary antibody in the conventional way. Final detection was performed with ECL Plus reagents.

Western Blot Assay to Detect PARP, Cleaved Caspase-3, and Cyclin D1 The cells at about 80% confluence obtained in the same way as above were incubated with EGCG or EGC (50, 100, 200 μM) for 24 h. Adhered cells were collected, washed twice with PBS, and resuspended in 400 μl of lysis buffer (0.5 mM NaH_2PO_4 (pH 7.0), 0.1 mM EDTA (pH 8.0), 0.1% SDS, 10% glycerol, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 0.022 TIU/ml aprotinin, and 1 mM PMSF). The lysates were homogenized and were centrifuged at 14000×g for 30 min at 4°C. An equal amount of protein was then resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was incubated successively with primary antibody and horseradish peroxidase-conjugated secondary antibody in the conventional way. Final detection was performed with ECL Plus reagents.

Test of the Inhibition of the Catechin-Triggered Apoptosis by Anti-Fas Neutralizing Antibody HCT116 cells were plated onto 60 mm diameter dishes (1.0×10^6 cells/dish). After incubating for 24 h, the cells at about 50% confluence were incubated with 100 ng/ml of anti-Fas neutralizing antibody 1 h before being incubated with EGCG or EGC (100, 200 μM). After 24 h incubation, the cells were treated as described above and detected with ECL Plus reagents.

RESULTS

EGCG and EGC Inhibited the Growth of HCT116 Cells to the Same Extent at Low Cell Confluence The HCT116 cells were exposed to EGCG or EGC and cell viability and IC_{50} values were obtained by conducting a conventional WST-1 assay.\(^{13}\) Interestingly, cell growth was promoted by both catechins at concentrations less than 50 μM (data not shown). Furthermore, these two catechins had nearly the same IC_{50} values at a cell confluence ranging from 1.8 to 28.1% (Figs. 1A, B). On the other hand, at more than 28.1% confluency, the cell growth inhibition by the catechins diminished dramatically with increasing cell confluency. This tendency was more remarkable with EGCG than EGC.

Apoptosis Was Induced by Both EGCG and EGC In order to identify the apoptosis caused by the catechins, HCT116 cells at about 50% confluence were exposed to each catechin at concentrations ranging from 50 to 200 μM for 24 h, and stained with Hoechst 33342. Apoptosis with condensed and fragmented nuclei began to be observed at 50 μM and was marked at 200 μM (Fig. 2A). This finding was also supported by the DNA fragmentation assay which showed DNA laddering patterns at 50—200 μM (Fig. 2B). Furthermore, in the TUNEL assay, DNA strands labeled with fluorescein-12-dUTP were stained with propidium iodide to be analyzed by flow cytometry.

Examination of Cell Cycle Regulation by Flow Cytometry HCT116 cells were plated onto 60 mm diameter dishes (1.0×10^6 cells/dish) and allowed to grow for 24 h. The cells at about 50% confluence were incubated with EGCG or EGC (50, 100, 200 μM) for 24 h. Floating and adhered cells were collected and were lysed in a buffer containing 10 mM Tris (pH 8.0), 0.1% SDS, 10% glycerol, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 0.022 TIU/ml aprotinin, and 1 mM PMSF). The lysates were centrifuged at 14000×g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of a mixture of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, pH 7.9), and then diluted into a 100 μl of lysis buffer (0.5 mM NaH_2PO_4 (pH 7.0), 0.1 mM EDTA (pH 8.0), 0.1% SDS, 10% glycerol, 5 μg/ml leupeptin, 0.1 μg/ml pepstatin, 0.022 TIU/ml aprotinin, and 1 mM PMSF). The lysates were homogenized and were centrifuged at 14000×g for 30 min at 4°C. An equal amount of protein was then resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was incubated successively with primary antibody and horseradish peroxidase-conjugated secondary antibody in the conventional way. Final detection was performed with ECL Plus reagents.

Western Blot Assay of the Inhibition of the Catechin-Triggered Apoptosis by Anti-Fas Neutralizing Antibody HCT116 cells were plated onto 60 mm diameter dishes (1.0×10^6 cells/dish). After incubating for 24 h, the cells at about 50% confluence were incubated with 100 ng/ml of anti-Fas neutralizing antibody 1 h before being incubated with EGCG or EGC (100, 200 μM). After 24 h incubation, the cells were treated as described above and detected with ECL Plus reagents.

Test of the Inhibition of the Catechin-Triggered Apoptosis by Anti-Fas Neutralizing Antibody HCT116 cells were plated onto 60 mm diameter dishes (1.0×10^6 cells/dish). After incubating for 24 h, the cells at about 50% confluence were incubated with 100 ng/ml of anti-Fas neutralizing antibody 1 h before being incubated with EGCG or EGC (100, 200 μM). After 24 h incubation, the cells were treated as described above and detected with ECL Plus reagents.
The use of WST-1. The IC50 values were obtained from cell viability experiments conducted by EGCG and EGC at the low concentration (10 μM), in spite that EGC in-duced cell death to a similar extent as EGCG in the DNA fragmentation and TUNEL assays. In the Western blot assay, both EGCG and EG activated the phosphorylation of the major MAPKs, ERK1/ERK2, JNK1/JNK2, and p38, which respond to extracellular stress are activated by EGCG in many human cancer cells.14-19 Therefore, the phosphorylation levels of these signaling molecules by EGCG or EGC (at 10, 50, 100 μM) were assessed using Western blotting. As shown in Fig. 4A, at 10 μM, EGCG activated the phosphorylation of JNK1/JNK2, ERK1/ERK2, and p38, whereas EGC did not. The latter started activating these proteins at 50 μM. These phosphorylation levels peaked at 1-2 h. Subsequently, the activation of caspase-3 was measured, since it is a point where different caspase-dependent apoptotic pathways converge and thus plays an important role in the cell death. The levels of cleaved caspase-3 and the cleaved protein of PARP increased dose-dependently after the incubation of EGCG and EGC with the cells for 24 h, respectively (Fig. 4B).

Test of the Inhibition of the Catechin-Triggered Apoptosis by Anti-Fas Neutralizing Antibody

It was reported that anti-Fas neutralizing antibodies as well as inhibitors of caspases such as caspase-3, -8, and -10 efficiently inhibited the EGC-induced apoptosis in the human breast cancer cell line MCF-7, thus suggesting the involvement of Fas signaling in EGC-triggered apoptosis.20 We thus tried to determine whether the apoptosis triggered by EGCG or EGC is attributable to Fas signaling through the use of an anti-Fas neutralizing antibody added prior to the exposure of the cells to EGCG or EGC. The Fas-induced apoptosis was evaluated with a DNA fragmentation assay which is not necessarily appropriate for quantitative measurements of apoptosis. The result obtained led us to conclude that the anti-Fas neutralizing antibody did not disrupt the EGCG- or EGC-triggered apoptosis as shown in Fig. 5.

**DISCUSSION**

The experiment on the viability of HCT116 cells showed that EGCG and EGC inhibited cell growth at approximately the same IC50 values at cell confluence levels ranging from 1.8 to 28.1% in the 96-well plates. However, the IC50 values became greater as cell confluence increased, and the inhibition rates diminished more markedly with EGC than EGCG. This finding could be explained in terms of the resistance of confluent cells to the catechins, similar to the resistance of confluent colon cancer cells to anthracyclines reported by Pelletier, et al.21 i.e., changes of passive diffusion induced by the increase in confluence could reduce the intake of EGCG or EGC, and this tendency was more conspicuous with EGC.

EGCG- or EG-induced apoptosis was observed in the Hoechst staining analysis. It initiated at 50 μM and was marked at 200 μM. This result was also supported by the DNA fragmentation and TUNEL assays. In the Western blot assay, both EGCG and EGC activated the phosphorylation of the major MAPKs, ERK1/ERK2, JNK1/JNK2, and p38, though to different extents. Furthermore, only a small fluctuation in the distribution of cells in Go-G1, S, and G2-M was observed even at 200 μM of EGCG and EGC. In support of this result, neither EGCG nor EGC modified the expression level of cyclin D1 inducing G1 arrest. The findings obtained so far suggest that MAPK-mediated apoptosis, not cell cycle regulation, is a major pathway for the inhibition of HCT116 cell growth by EGCG and EGC. It is well established that EGCG activates simultaneously the phosphorylation of JNK1/JNK2, p38, and ERK1/ERK2 in various cancer cells, macrophages, and keratinocytes,14-19 sometimes having an opposite biological effect since ERK1/ERK2 is related with cell proliferation and survival, whereas JNK1/JNK2 and p38 are involved in stress-activated apoptosis. The simultaneous activation of these proteins would be associated with the regulation of apoptosis or survival signaling in cells.14,22,23

In the Western blot assay, EGCG enhanced the phosphorylation of the stress-activated MAPK proteins more than did EGC at the low concentration (10 μM), in spite that EGC induced cell death to a similar extent as EGCG in the DNA fragmentation and TUNEL assays. We therefore examined the possibility of the involvement of Fas-signaling as another pathway of apoptosis induced by EGC. This idea, however, was not supported by the result of the experiment using the...
Fig. 2. Induction of Apoptosis by EGCG or EGC in HCT116 Cells

(A) Nuclear condensation and fragmentation: HCT116 cells (1.0×10^6) were incubated for 24 h and treated with test compounds for 24 h, and then processed for Hoechst 33342 staining. Nuclear staining was observed under a fluorescence microscope. Data shown are those for the control (no sample) and for treatment with H_2O_2 (300 μM), EGCG or EGC (50, 200 μM). (B) DNA laddering: HCT116 cells (1.2×10^6) were incubated for 40 h and then treated with test compounds for 24 h. Cellular DNA was analyzed by agarose gel electrophoresis. Data shown are those for the control (no sample) and for treatment with H_2O_2 (300 μM), EGCG or EGC (50, 100, 200 μM). (C) TUNEL assay by flow cytometry: The cells (1.0×10^6) were incubated and then treated with test compounds for 24 h. Cells were treated with fluorescein-12-dUTP and rTdT Enzyme as well as with propidium iodide. Changes in the population of viable cells and apoptotic cells among HCT116 cells were examined by flow cytometry. FL1-H represents DNA content, whereas FL2-H corresponds to fluorescein-12-dUTP-labeled DNA fragments. Data shown are those for the control (no sample) and for treatment with H_2O_2 (300 μM), EGCG (200 μM), and EGC (200 μM). All data are representative of three independent experiments.

Fig. 3. Effects of EGCG or EGC on the Cell Cycle in HCT116 Cells

HCT116 cells (1.0×10^6) were incubated for 24 h and treated with test compounds for 24 h. The cells were then treated with a BD Cycletest Plus DNA reagent Kit. Data shown are those for the control (no sample) and for treatment with EGCG or EGC (200 μM) and are representative of three independent experiments.
anti-Fas neutralizing antibody, which did not disrupt the EGCG- or EGC-triggered apoptosis as shown in Fig. 5. Such a discrepancy might have been caused by the difference in the confluence of cells in the plates between the Western blot assay (ca. 80% confluence) and the two apoptosis assays (ca. 50% confluence).

In conclusion, we demonstrated that EGC inhibited the growth of the HCT116 colon cancer cell line by inducing apoptosis to nearly the same extent as EGCG at low cell confluency. The cancer cell growth inhibition by the catechins at low cell confluency would be greater in vivo. It is therefore worth investigating further the role of EGC in cancer chemoprevention.

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REFERENCES AND NOTES


