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Epigallocatechin-3-gallate inhibits the invasion of human oral cancer cells and decreases the productions of matrix metalloproteinases and urokinase-plasminogen activator

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BACKGROUND: Green tea polyphenols are considered beneficial to human health, especially as cancer chemopreventive agents in recent years. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, has been proven to suppress colonic tumorigenesis in animal and epidemiological studies, whereas its role in the oral carcinogenesis remains to be elucidated.

METHODS: Cytotoxicity, invasion, and migration assays were used to investigate the effects of human oral cancer cell line OC2 cells exposed to EGCG. To look at the precise involvement of EGCG in cancer metastasis, gelatin zymography and casein zymography were performed to evaluate the impacts of EGCG on matrix metalloproteinase (MMP)-2, MMP-9, and urokinase plasminogen activator (uPA) secretion in OC2 cells.

RESULTS: EGCG exhibited a dose-dependent inhibitory effect on the invasion and migration of OC2 cells in the absence of cytotoxicity ($P < 0.05$). EGCG was also found to decrease the expressions of MMP-2, MMP-9, and uPA in a concentration-dependent manner ($P < 0.05$).

CONCLUSION: Taken together, these results suggest that EGCG could inhibit the invasion and migration of human oral cancer cells and that the effects may partially because of the decreased productions of MMP-2, MMP-9, and uPA.

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Keywords: epigallocatechin-3-gallate; invasion; migration; matrix metalloproteinases; urokinase plasminogen activator

Introduction

Oral cancer is prevalent in Taiwan, India, and southwest Asia where areca quid chewing is wide spread (1). In Taiwan, about 80% of all oral cancer deaths are associated with this habit (2). Various ingredients in natural plants, such as silibinin, peonidin 3-glucoside, and cyanidin 3-glucoside etc., have been tested for potential anticarcinogenicity (3, 4).

Green tea is one of the most popular beverages in the world and it has received considerable attention because of its many scientifically proven beneficial effects on human health. Epidemiological studies have suggested that the ingestion of green tea may decrease the risk of cancer (5, 6). In addition, many pre-clinical studies have provided the convincing evidence that some substances of green tea may afford protection against cancer (7, 8). The anticarcinogenic effects of green tea have been attributed to the biological activities of its polyphenol components. Green tea extract contains catechins such as epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin gallate, and epicatechin (9). EGCG, the most abundant polyphenol in green tea, has been shown to inhibit proliferation and induce apoptosis of tumor cells (10, 11). In addition, EGCG was also found to inhibit cell growth on many oral cancer cell lines (12–14). Moreover, tea was reported to provide some direct evidences on the protection of oral pre-cancerous mucosa lesions (15). However, little is known about the precise mechanisms of EGCG on oral cancers.

Metastasis of cancer cells involves multiple processes and various cytophysiological changes, including changed adhesion capability between cells and extracellular matrix (ECM) and damaged intercellular interaction. Degradation of ECM by cancer cells *via* protease, such as urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), may lead to the separation of intercellular matrix to promote the mobility of cancer cells and eventually lead to metastasis (16, 17). In this study, the effects of EGCG on the invasion and migration was investigated by looking at the impact of EGCG on several relevant proteases, including MMP-2, MMP-9, and uPA, in OC2 cells (18), a human oral cancer cell line derived from buccal mucosa cancer. Understanding the basic principles of the inhibitory effects of EGCG on tumor invasion and migration may lead to development of new therapeutic strategies, in

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addition to supporting the role of green tea as an oral cancer chemopreventive agent.

Materials and methods

Reagents

EGCG, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). Matrigel was obtained from Collaborative Biomedical Products (Bedford, MA, USA) Tissue culture biologicals were purchased from Gibco Laboratories (Grand Island, NY, USA). The final concentrations of EGCG used in this study were 0–60 μM, respectively.

Cell cultures

OC2 cells derived from human buccal mucosa cancer were retrieved from frozen stock and cultured to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 μg⁄ml of streptomycin, 100 mg⁄ml of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every 3 days (18).

Cytotoxicity assay

To evaluate the cytotoxicity of EGCG, MTT colorimetric assay was performed to determine the cell viability as described previously (19). Briefly, 2×10^4 cells per well were seeded to 96 well plate and left overnight to attach. Serial dilutions of EGCG (0–60 μM) in 100 μl volumes were added, and cells were treated for 24 h. After treatment, 50 μl of MTT solution (1 mg⁄ml in PBS) was added to each well and incubated for another 4 h at 37°C. To each well, 150 μl of dimethyl sulfoxide was added. Optical density was determined by eluting the dye with dimethyl sulfoxide and the spectrophotometric absorbance measured at 550 nm by using a spectrophotometer (Hitachi, Tokyo, Japan).

Invasion and migration assays

For cell invasion assay, OC2 cells were treated with different concentrations of EGCG. After 24-h incubation period, cells were removed by trypsinizing, and their *in vitro* invasiveness was tested by the Boyden chamber invasion assay (20). Matrigel was diluted to 25 mg⁄50 ml with cold filtered distilled water, and applied to 8-mm pore size polycarbonate membrane filters. Treated cells were seeded to Boyden chamber (Neuro Probe, Cabin John, MD, USA) at the upper part at a density of 1.5×10^4 cells⁄well in 50 ml of serum free medium and then incubated for 12 h at 37°C. The bottom chamber also contained standard medium with 20% FCS. The cells that had invaded to the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted under a light microscope. To determine the effect of EGCG on cell migration, cells were seeded into Boyden chamber on membrane filters which were not coated with Matrigel. Migration of cells treated or untreated with EGCG was measured as described in the invasion assay.

Zymography

The activities of MMP-2 and -9 of the condition medium were measured by gelatin-zymogram protease assays as described previously (21). Confluent cells were trypsinized, counted, and plated at a concentration of 1×10^5 cells in 60 mm culture dish and allowed to achieve confluence. Cells were cultured for 24 h, at which time the medium was changed to a medium containing 0.5% FCS and various concentrations of EGCG. The conditioned medium samples were collected after 24-h incubation period. Conditioned media were prepared with standard SDS-gel loading buffer containing 0.01% SDS without β-mercaptoethanol and not boiled before loading. Then, prepared samples were subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 150 V for 3 h in an OWL P-1 apparatus (Thermo Fisher Scientific, Inc. Portsmouth, NH, USA). After electrophoresis, gels were washed twice with 100 ml distilled water containing 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove SDS. The gel was then incubated in 100 ml reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 0.02% NaN_3) for 12 h at 37°C, stained with Coomassie brilliant blue R-250 and destained with methanol-acetic acidwater. The gelatin cleavage rate was analyzed from the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean SD.

Visualization of uPA activity was performed as described as Huang et al. (22). Briefly, 2% w⁄v casein and 20 mg⁄ml plasminogen were added to 8% SDS-PAGE gels. Samples with a total protein of about 20 mg were then loaded onto the gels. The uPA activity of cells treated or untreated with EGCG was measured as described in the gelatin zymography. The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Statistical analysis

Triplicate experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. The significance of the results obtained from control and treated groups were statistically analyzed by paired Student's *t*-test.

Results

The cytotoxicity of various concentrations (0–60 μM) of EGCG on OC2 cells for 24 h by MTT colorometric assay was shown in Fig. 1. Compared with that of controls, the remaining cell viability was not significantly altered by EGCG, even at a concentration up to 60 μM ($P > 0.05$). Therefore, it was clear that the

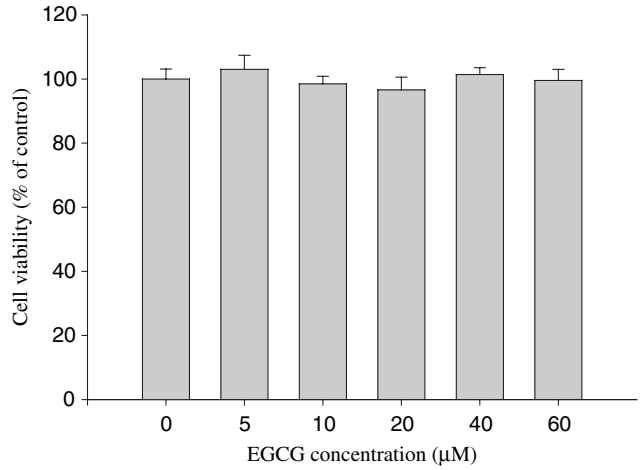

Figure 1 Effect of epigallocatechin-3-gallate (EGCG) on the cell viability of OC2 cells. Cells were treated with 0–60 μM EGCG for 24 h before subjected to a MTT assay for cell viability. Each point and bar represent a mean SD.

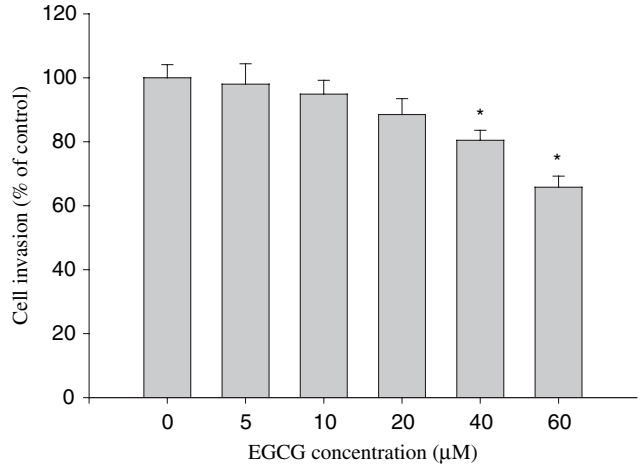

Figure 2 Effects of epigallocatechin-3-gallate (EGCG) on the invasion of OC2 cells through Matrigel. An invasive rate of 100% is defined by control experiments of invasive OC2 cells tested in the absence of EGCG. Each point and bar represent a mean ± SD. *Denotes significant differences from control values with $P < 0.05$.

treatment of EGCG, at concentrations ranging from 0 to 60 μM, has no cytotoxic effects to OC2 cells during 24-h incubation period. Thus, this concentration range was then applied in all subsequent experiments.

As shown in Fig. 2, the invasive capacity through a reconstituted basement membrane (Matrigel) toward a chemoattractant was dose-response restrained by EGCG higher than 40 μM ($P < 0.05$). At a concentration of 60 μM, EGCG was found to reduced the invasive capacity of OC2 cell about 34% ($P < 0.05$). Such inhibitory effect was also observed for the cell migration of the EGCG-treated cells. The inhibitory effect was also in a concentration-dependent manner ($P < 0.05$) (Fig. 3). At a concentration of 60 μM, EGCG was found to reduced the migration of OC2 cells about 40% ($P < 0.05$).

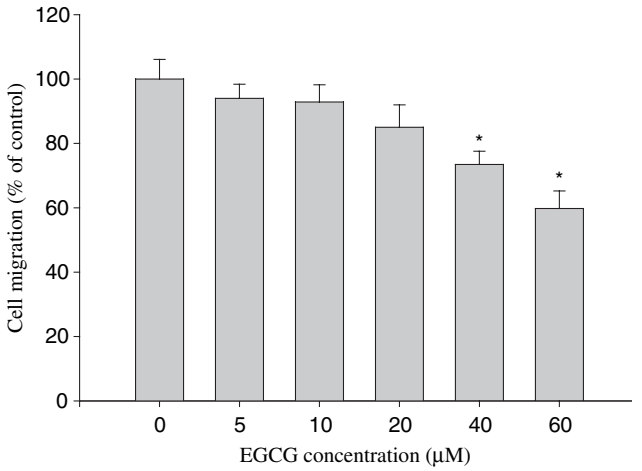

Figure 3 Effects of epigallocatechin-3-gallate (EGCG) on the migration of OC2 cells. A migration rate of 100% is defined by control experiments of migrative OC2 cells tested in the absence of EGCG. Each point and bar represent a mean SD. *Denotes significant differences from control values with $P < 0.05$.

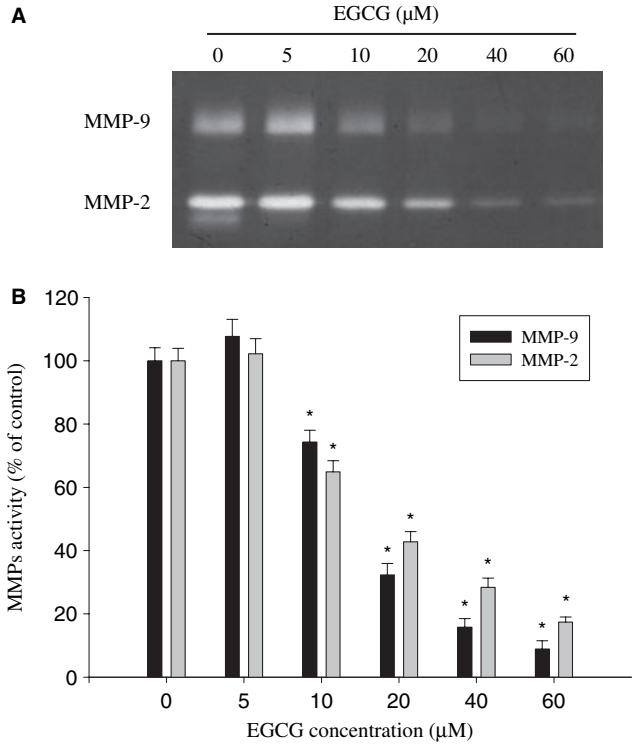

Figure 4 (A) Gelatin zymogram of conditioned medium from OC2 cells treated with the various concentrations of epigallocatechin-3-gallate (EGCG) during 24-h incubation period. (B) Levels of matrix metalloproteinase (MMP)-2 and -9 from conditioned medium treated with the EGCG were calculated from their gelatinolytic activity, as measured by AlphaImager 2000. Each point and bar represent a mean ± SD. *Denotes significant differences from control values with $P < 0.05$.

Figure 4 is a zymogram that contains conditioned medium samples from OC2 cells treated with EGCG. The gelatin zymograms revealed that the main gelatinase secreted by OC2 migrated at 72 kDa and represents

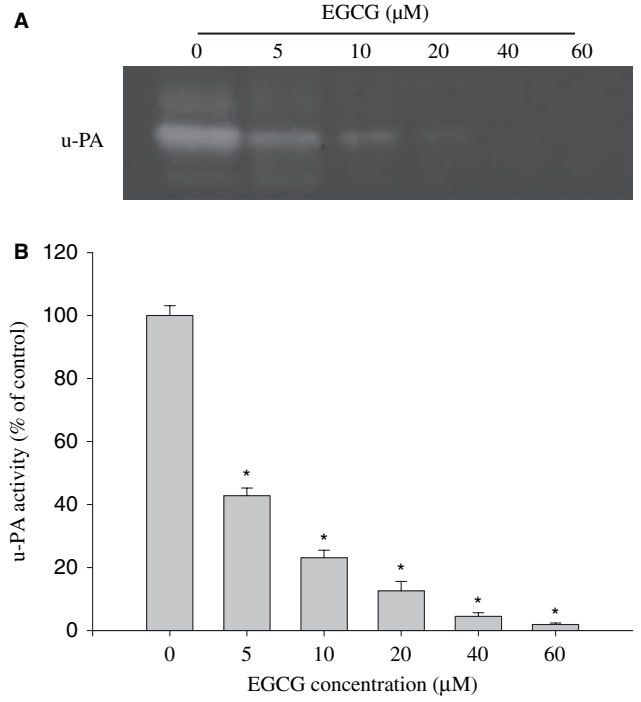

Figure 5 (A) Casein zymogram of conditioned medium from OC2 cells treated with the various concentrations of Epigallocatechin-3-gallate (EGCG) during 24 h incubation period. (B) Levels of urokinase-plasminogen activator (uPA) from conditioned medium treated with the EGCG were calculated from their caseinolytic activity, as measured by AlphaImager 2000. Each point and bar represent a mean SD. *Denotes significant differences from control values with $P < 0.05$.

MMP-2. Minor gelatinolytic bands were also observed at 92 kDa regions that correspond to MMP-9.

The conditioned medium samples from OC2 cells treated with the concentrations of EGCG higher than 10 μM showed the levels of MMP-2 and -9 to be significantly inhibited (Fig. 4A). The inhibitory pattern was demonstrated in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000, the amount of MMP-2 was 65%, 43%, 28%, and 17% at concentrations of 10, 20, 40, and 60 μM, respectively, as compared with control (Fig. 4B). The amount of MMP-9 was 74%, 32%, 16%, and 9% at concentrations of 10, 20, 40, and 60 μM, respectively, as compared with control (Fig. 4B).

To examine whether EGCG could inhibit the uPA activity in OC2 cells, cells were treated with 0–60 μM EGCG and the intensity of caseinolytic activitiy were determined by casein zymography (Fig. 5A). EGCG was found to inhibited the caseinolytic activitiy in a dose-dependent manner ($P < 0.05$). The quantitative measurement by the AlphaImager 2000 was shown in Fig. 5B. The amount of uPA was 43%, 23%, 13%, 5%, and 2% at concentrations of 5, 10, 20, 40, and 60 μM, respectively, as compared with control.

Discussion

Studies suggest that the consumption of green tea may help prevent cancers from humans (5, 6, 23). EGCG, a major component in green tea polyphenols, is accounting for more than 40% of the total polyphenolic mixture (9). It was then reasonable to speculate that EGCG, the main bioactive component of green tea, may play a role for the prevention of cancer metastasis.

Metastasis has been found to be accompanied by various physiological alterations involved in the degradation of ECM, which allowed cancer cells to invade blood or lymphatic system to spread to another tissue or organ. In this study, we first demonstrated that EGCG could inhibit the migration and invasive ability of OC2 cells without affecting cell viability. Our results are in agreement with Maeda-Yamamoto et al. (24), who reported that tea polyphenols were found to inhibit invasion on human fibrosarcoma HT1080 cells, and Garbisa et al. (25), who demonstrated that EGCG inhibited the cell proliferation and invasion on SK-N-BE human neuroblastoma cells and HT1080 cells. Recently, Kim et al. (26) have found that the invasive ability was found to be inhibited by EGCG in HT1080 cells. These results suggest that the cancer invasive inhibitory effect of EGCG is not caused by the cytotoxicity. In addition, EGCG may be useful as an effector for the prevention of cancer metastasis.

It has been shown that the cell and matrix interactions promote cell migration, proliferation, and ECM degradation (27) while inhibitory agents for cell adhesion may reduce the invasiveness and metastatic potential of tumor cells (28). Furthermore, the interactions between cells with ECM, which promote adhesion and migration, are believed to be essential for invasion, migration, and metastasis of tumors (29). Therefore, the impacts of EGCG on several proteases involved in ECM degradation were investigated in this study. MMPs play a crucial role in the process of cancer invasion and metastasis. In the present study, we demonstrated that the expressions of MMP-2 and -9 of OC2 cells were reduced by EGCG treatment in a dose-dependent manner. Similar results were found that EGCG is a potent inhibitor for the expression and activity of MMP-2 and -9 in HT1080 cells (24, 25, 30) and oral cancer cell line OEC-M1 cells (31). Therefore, the inhibition of the migration or invasion mediated by MMP-2 or -9 may be a key feature for the prevention of cancer invasion or metastasis.

In this study, we also found that the uPA activity was inhibited by EGCG in a dose-dependent manner. Consistently, it was proposed that anti-invasive activity of EGCG is associated with the inhibition of the caseinolytic activitiy (32). Recently, Kim et al. (26) who have found that EGCG can potently suppress the uPA expression in HT1080 cells. The plasmin-dependent pathway is understood to be a significant alternative pathway for the initiation of ECM degradation by MMPs (17). In addition to MMP-2 and -9, the inhibition of uPA expression by EGCG is also important for the anti-invasive function of EGCG.

The present results suggest that EGCG inhibits the migration and invasion of OC2 cells by inhibiting the activation of MMP-2, MMP-9, and uPA enzymes. Previously, the concentrations of EGCG in saliva and

plasma have been reported as up to 4.8 μg/ml–77.9 ng/ml after drinking green tea (33, 34). In addition, 10 cups of green tea daily supplemented with green tea tablets have been recommended to the general population for the prevention of cancer (23). However, it is very difficult, if not impossible, to determine how much effective concentrations of EGCG were present in human oral tissues from patients who drink tea. In this study, it was found that the concentrations of EGCG could easily reach the effective level on OC2 cells *in vitro*. The dietary supplements of tea may serve the purpose *in vivo*.

In conclusion, EGCG was considered to be the agent with the most potential anti-metastasis activity because it can inhibit the migration and invasion in the absence of cytotoxicity. Except for the anti-metastasis activity of EGCG presented in this study, further studies of other ingredients of green tea are significant to investigate their roles in oral carcinogenesis.

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