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Green tea (-)-epigallocatechin gallate induced growth inhibition of human placental choriocarcinoma cells

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ABSTRACT

This study investigated the pathways involved in the effect of green tea epigallocatechin gallate (EGCG) on mitogenesis in BeWo, JEG-3, and JAR placental choriocarcinoma cells. EGCG inhibited cell proliferation in dose-dependent and time-dependent manners, as indicated by the number of cells and incorporation of bromodeoxyuridine (BrdU). A catechin-specific effect of green tea was evident; EGCG was more effective than epicatechin, epicatechin gallate, and epigallocatechin in suppressing cell growth. When all three of the mitogen-activated protein kinase (MAPK) subfamilies, i.e., ERK, p38, and JNK, were examined, EGCG significantly increased levels of phospho-ERK1/2 (pERK1/2) and phospho-p38 (pp38) and did not alter the total protein levels of ERK1/2, p38 MAPK, JNK, and phospho-JNK. EGCG-induced increases in the levels of pERK1/2 and pp38 proteins were prevented by pre-treatment with specific inhibitors of ERK1/2 MAPK and p38 MAPK, respectively. These inhibitors also suppressed EGCG-induced decreases in both cell number and BrdU incorporation. Moreover, pre-treatment with an AMP-activated protein kinase (AMPK) inhibitor prevented the actions of EGCG on proliferation and AMPK phosphorylation. These data suggest that EGCG mediates choriocarcinoma cell growth via the AMPK, ERK, and p38 pathways, but not JNK pathway.

1. Introduction

Choriocarcinoma of the placenta is a malignant trophoblastic tumor comprised of cytotrophoblasts, intermediate trophoblasts, and syncytiotrophoblasts without formation of chorionic villi [1]. Trophoblasts develop into tumor cells due to their uncontrollable proliferation [2], and the proliferation of placental choriocarcinoma cells is regulated by endocrine and nutritional factors [3,4].

Green tea catechins (GTCs), especially (−)-epigallocatechin gallate (EGCG), have been proposed to be modulators of cancer cell growth [5–7] and been reported to circulate in the placenta of animals and blood of humans after consumption [5–9]. In particular, the highest levels of EGCG in pregnant rats fed GTCs are found in the placenta and fetus [8]. These studies suggest a possible effect of EGCG on placenta-associated cancer cells. Despite further *in vitro* demonstrations that EGCG protects placental hematopoietic lineages from radiation damage [10] and suppresses the uptake of glucose by trophoblasts after insulin stimulation [11], few studies have detailed whether EGCG signaling acts on mitogenesis in placental choriocarcinoma cells. Notably, other polyphenols (e.g., xanthohumol) decrease the cell viability and proliferation of trophoblasts [12].

Green tea EGCG has been described extensively in cancer cells and other cells [5–7,10–16]. EGCG could regulate the activity of non-choriocarcinoma cells through at least two pathways, the MAPK-mediated pathway and the AMPK-regulated pathway [5–7,13–16]. In cancer cells, the MAPK-mediated pathway regulates cell growth; for example, decreased ERK phosphorylation and JNK activity favors EGCG inhibiting the proliferation of H-*ras*-transformed cells and transformation of JB6 epidermal cells [6], whereas increased activities of ERK and JNK proteins are responsible for EGCG-mediated growth of oral cancer cells and CL1-5 lung cancer cells, respectively [14,15]. Furthermore, the AMPK-regulated pathway controls EGCG-suppressed growth of hepatoma cells [16]. In 3T3-L1 pre-adipocytes, decreased ERK favors EGCG inhibiting pre-adipocyte proliferation [17], whereas the AMPK-regulated pathway is not responsible for the anti-IGF signal of EGCG in pre-adipocyte growth [18]. Taken together, these findings suggest

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kinase family- and cell type-dependent effects of EGCG on cell growth.

The MAPK and AMPK proteins have been reported to regulate trophoblast growth [3,4], but whether any of these kinases are necessary for EGCG to control trophoblast mitogenesis is unknown. The present study was designed to understand the mechanism underlying the actions of EGCG in the regulation of BeWo, JEG-3, and JAR placental choriocarcinoma cell numbers as they grow. Specifically, we investigated whether EGCG regulates the phosphorylation of MAPK and AMPK proteins in choriocarcinoma cells.

2. Materials and methods

Chemical reagents. We isolated EGCG and other catechins (>98% pure) from green tea (*Camellia sinensis*) [17]. Other materials (i.e., PD98059) were purchased from Sigma (St. Louis, MO) unless otherwise mentioned. We purchased penicillin-streptomycin, Ham's F12K, RPMI-1640, MEM-EBSS and FBS from GIBCO-BRL Life Technologies (New York, NY) and the pre-stained protein ladder from Invitrogen Life Science Technologies (Carlsbad, CA). The primary and secondary antibodies were purchased from Cell Signaling (Beverly, MA) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively, except the actin antibody, which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (Supplementary Table 1).

Cell culture. BeWo cells (American Type Culture Collection, Rockville, MD; ATCC-CCL-98™) were maintained in Ham's F12K medium supplemented with 15% FBS. JEG-3 cells (ATCC-HTB-36™) and JAR cells (ATCC-HTB-144™) were grown in MEM/EBSS medium and RPMI-1640 medium, respectively, with 10% FBS, 0.5% L-glutamine, 0.5% sodium pyruvate, and 0.5% MEM nonessential amino acids [3,4]. All culture media contained 0.5% penicillin/streptomycin. Cells at a density of 1700 cells/cm^2 were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were starved with serum-free medium for the following experiments because it allowed the cells to be kept under basal conditions before the addition of the tea catechin to be tested.

Experimental treatments. To study the effect of GTCs on growth, we plated BeWo, JEG-3, and JAR cells (2×10^4 cells/well) in triplicate wells of 12-well plates [17]. To evaluate the presence of dose-dependent effects of EGCG on cell growth, serum-starved cells were treated with 0–100 μM EGCG in 1% FBS-supplemented medium for 24–72 h. After incubation, cells were counted on a hemocytometer using the 0.4% trypan blue exclusion method.

Cell proliferation was measured using a bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay kit (Roche Applied Science, Mannheim, Germany) [19]. Briefly, BeWo, JEG-3, and JAR cells (2000 cells/well) were plated into a 96-well microplate containing medium. After allowing 24 h for attachment, cells were starved with serum-free medium for 24 h. The medium was then replaced with fresh medium containing EGCG and 1% FBS for 8 h and incubated with BrdU (10 μM) for 16 h. After incubation, cells were washed with 10 mM PBS and then collected by centrifugation at 1500 rpm for 5 min. Cell pellets were dried at 60 °C for 1 h. Cell pellets were fixed with FixDenat solution at 25 °C for 30 min, probed with mouse-anti-BrdU-POD for 1 h, and visualized by the addition of 3,3,5,5-tetramethylbenzidine substrate for 5 min. An aliquot of 100 μL of H_2SO_4 was added to stop the reaction. The absorbance was read at 450 nm. Culture medium alone and cells incubated with anti-BrdU-POD in the absence of BrdU were used as blank controls for nonspecific binding.

To compare the effects of different catechins on changes in cell number, serum-starved cells were treated with epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), or EGCG at 0–100 μM for 24–72 h. After incubation, the number of cells was counted.

To compare the effects of different catechins on changes in cell proliferation, we treated choriocarcinoma cells with 20 μM of EC, ECG, EGC, or EGCG for 8 h in 1% FBS-supplemented medium. After 16 h, the BrdU incorporation was measured.

To study the effect of kinase inhibitors on the EGCG-suppressed growth of choriocarcinoma cells, serum-starved cells at a density of 17,000 cells/cm^2 were pre-treated for 1 h with PD98059 (50 μM) [20], U0126 (10 μM) [21], SB230580 (10 μM) [22], or compound C (1 μM) [23]. After 0.5, 8, and 24 h of treatment with 20-μM EGCG, we measured protein kinase levels, BrdU incorporation, and the number of cells, respectively.

Western blot analysis. Immunoblot analysis was performed according to a slightly modified method [24]. Briefly, 50–75 μg of protein was separated by 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis with 2× gel-loading buffer (100 mM Tris–HCl (pH 6.8), 0.2% bromophenol blue, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol). Electrophoresis-separated proteins were blotted onto polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA), which were then incubated with primary antibody (e.g., anti-ERK) at a dilution of 1:1000 (~0.2 μg/mL), followed by secondary antibody (e.g., goat anti-rabbit IgG, or donkey anti-goat IgG conjugated with horseradish peroxidase) at a dilution of 1:2000 (~0.2 μg/mL). The immunoblots were visualized by adding Western Lightning™ chemiluminescence

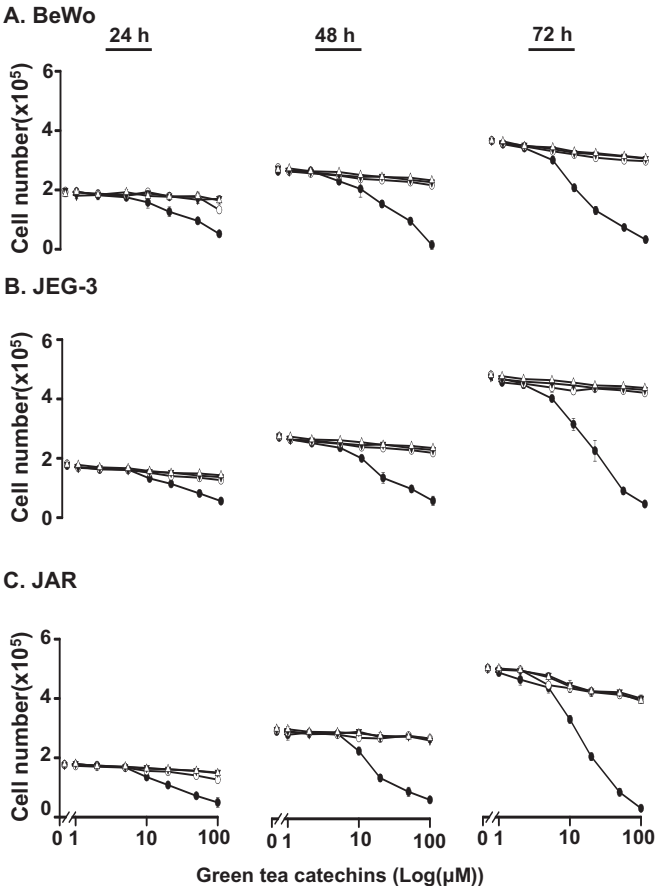

Fig. 1. Reductive effect of green tea catechins on the number of human choriocarcinoma BeWo (A), JEG-3 (B), and JAR cells (C) depending on the dosage (0–100 μM) and duration of treatment. Cells were counted using the trypan blue exclusion method. △, EC; ▼, ECG; ○, EGC; ●, EGCG. Data are expressed as the mean ± SE of triplicates in three experiments. For clarity, SE bars and significance values are not shown. Some symbols (e.g., △ and ○) may be shown with overlapping positions in parts of the figure panel due to the close values.