Pancreas
Vol. 25, No. 1, pp. 45–48

Suppression of Human Pancreatic Carcinoma Cell Growth and Invasion by Epigallocatechin-3-Gallate

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Introduction: The consumption of green tea is associated with a lower risk of several types of human carcinomas. A number of studies have focused on the possible mechanisms of cancer prevention by tea extracts, especially polyphenols such as epigallocatechin-3-gallate (EGCG). **Aims and Methodology:** Green tea–derived EGCG was tested in human pancreatic carcinoma cells. The cells (PANC-1, MIA PaCa-2, and BxPC-3) were treated with different doses of EGCG (0, 25, 50, 100, and 200 μmol/L) for 48 hours in culture medium. Proliferation of pancreatic carcinoma cells was measured by means of the WST-1 colorimetric assay. For the study of cell invasion, the cells were incubated with 100 μmol/L EGCG for 2 hours. Then, the cells were added into the cell insert, coated with Matrigel basement membrane matrix. After incubation at 37°C for 24 hours, the cells that had invaded through the Matrigel were counted visually under the microscope. **Results:** The growth of all three pancreatic carcinoma cells was significantly suppressed by EGCG treatment in a dose-dependent manner. EGCG treatment caused significant suppression of the invasive ability of pancreatic carcinoma cells PANC-1, MIA PaCa-2, and BxPC-3 but did not affect the cell cycle protein cyclin D1. **Conclusion:** EGCG may be a potent biologic inhibitor of human pancreatic carcinomas, reducing their proliferative and invasive activities. **Key Words:** Pancreas—Cancer—Epigallocatechin-3-gallate—Polyphenol.

Tea is the most popular beverage worldwide. It has been shown that the consumption of green tea provides protection against a variety of cancer types and may also inhibit the conversion of premalignant lesions to malignancy (1). Although only little has been discovered about the effect of tea on pancreatic diseases, reduction of human pancreatic carcinomas in association with tea consumption has been reported (2). There is increasing evidence of the possible mechanisms of cancer prevention by tea extracts, especially green tea polyphenols such as epigallocatechin-3-gallate (EGCG) (3,4). In addition, it has been shown that EGCG may exert its growth-inhibitory effects through modulation of G1 regulatory enzymes such as cyclin-dependent kinases (CDKs) (4,5).

Growth-regulatory proteins of the transforming growth factor-β (TGF-β) are some of a few potent endogenous inhibitors of cell growth (6). TGF-β inhibits cell growth by interacting with protein kinases that control cell cycle progression. A number of studies have shown evidence of the relationship between TGF-β and pancreatic cancers. Therefore, we postulated that the possible growth-inhibitory effects of TGF-β on pancreatic cancers could be extrapolated to EGCG. In the current study, we determined whether EGCG affects proliferative and invasive activities of human pancreatic carcinoma cells.

Manuscript received January 19, 2001; Revised manuscript accepted September 19, 2001.

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MATERIALS AND METHODS

EGCG

A purified preparation of EGCG (>98% pure) that was isolated from green tea by means of reverse-phase high-performance liquid chromatography (HPLC) was kindly provided by Dr. Yukihiko Hara, of Food Research Laboratories (Mitsui Norin, Shizuoka, Japan). EGCG dissolved in phosphate buffered saline (PBS, 50 mmol/L; pH, 7.4) was used for the treatment of cells.

Human pancreatic carcinoma cell culture

Human pancreatic carcinoma cells (Panc-1, MIA PaCA-2, and BxPC-3) were obtained from American Type Culture Collection (ATCC; Dainippon-Seiyaku Laboratory Products, Tokyo, Japan).

Cell proliferation analysis

Proliferation of pancreatic carcinoma cells was measured with use of a WST-1 (Takara Biomedicals, Shiga, Japan) colorimetric assay. Cells (70–80% confluent) were treated with various doses (0, 25, 50, 100, and 200 μmol/L) of

EGCG and cultured in a 96-well microplate for 2 days. Then, 10 μL WST-1 was added to the cells and incubated for 2 hours, and the absorbance (450/630 nm) was measured with an enzyme-linked immunosorbent assay (ELISA) plate reader. Each data point was tripled and repeated at least three times.

Cell invasion assay

The relative abilities of cells to invade through reconstituted basement membrane were determined with use of an in vitro invasion assay system, as previously described (7). Cells were preincubated with 100 μmol/L EGCG for 2 hours. Then 500 μL/well of the serum-free medium was added to the plate well, and 200 μL/well of the cell suspension (5×10^4/mL) was added to the prepared Matrigel-coated cell insert (8-μm pore size; 25 μg/well coated; Becton Dickinson Labware, Tokyo, Japan). The chambers were incubated at 37°C in 5% CO_2 in air for 12 hours. After the incubation, the cells on the upper surface of the filter were completely wiped clean with a cotton swab and monitored visually under a microscope (AX80T; Olympus, Tokyo, Japan). Then the filters were fixed in methanol and stained with hematoxylin to enable identification of the cells invading through the Matrigel basement membrane matrix. Various areas of the lower surface were randomly scanned (Photograb-2500 for Macintosh SH-25/M; Fujix, Tokyo, Japan), and the cells were counted in hexaplicates with use of analytical software (Mac SCOPE; Mitani, Tokyo, Japan).

Preparation of cell lysates and western blot analysis

Cells were trypsinized, scraped, and then washed twice with cold PBS (10 m*M*; pH, 7.4). The cell suspension was centrifuged at 2,000*g* for 10 minutes at 4°C; the medium was aspirated and then stored at −70°C, followed by freezing in liquid nitrogen.

Ice-cold lysis buffer (50 m*M* Tris-HCl, 150 nm NaCl, 1 mL EGTA, 20 m*M* NaF, 100 m*M* Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin; pH, 7.4) was added to the cells, which then were placed over ice for 30 minutes. The lysates were passed through a 21-gauge needle to break up the cell aggregates. The lysates were cleared at 14,000*g* for 15 minutes at 4°C, and the supernatant (total cell lysates) was collected. The protein concentration was determined by DC assay (Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer's protocol.

The expression of cyclin D1 was examined by western blotting. In brief, 25–50 μg of protein was resolved over 12% polyacrylamide–sodium dodecylsulfate gels, and transferred protein was blocked in blocking buffer (5% nonfat dry milk and 1% Tween 20 in 20 m*M* triethanolamine-buffered saline; pH, 7.6) for 1 hour at room temperature, followed by incubation with antihuman cyclin D1 antibody. Affinity-purified mouse monoclonal antihuman cyclin D1 antibody (Fujisawa Pharmaceutical, Tokyo, Japan) was used in dilution (1:100). The gel transfer was incubated in blocking buffer overnight at 4°C and in secondary antibody horseradish peroxidase C conjugate (Amersham Life Science, Tokyo, Japan), and protein expression was detected by chemiluminescence with an ECL Plus detection kit (Amersham Life Science) and autoradiography with XAR-5 film (Eastern Kodak Japan, Tokyo, Japan).

Statistical analysis

The data are expressed as arithmetic mean ± SEM unless otherwise stated. The Student *t* test was used to determine statistical significance. The difference was considered to be significant at $p < 0.01$.

RESULTS

Suppression of pancreatic carcinoma cell proliferation by EGCG

Three different human pancreatic carcinoma cells, PANC-1, MIA PaCa-2, and BxPC-3, were employed to evaluate the effect of EGCG on cell proliferation. As seen in Figure 1, significant, dose-dependent suppression of proliferation was observed in all these cells. Treatment with EGCG (200 μmol/L) resulted in suppression of the growth of PANC-1 (15.4%), MIA PaCa-2 (26.0%), and BxPC-3 (44.6%). Those growth inhibitions were also confirmed with microscopic observation.

Suppression of pancreatic carcinoma cell invasion by EGCG

The effects of EGCG on cell invasion through the Matrigel basement membrane matrix were studied (Fig. 2A). Preincubation with EGCG (100 μmol/L; 2 hours) resulted in the suppression of invasive ability of PANC-1 (12.0%), MIA PaCa-2 (8.7%), and BxPC-3 (9.5%). The cells that invaded through the Matrigel are indicated an arrow in Figure 2B.

Expression of cell cycle regulatory protein, cyclin D1

The expression of cyclin D1 was studied with use of western blotting (Fig. 3). There was no significant difference of cyclin D1 expression with and without EGCG, although BxPC-3 was of slightly weaker intensity with EGCG than without EGCG. Overall, addition of EGCG had no effect on cyclin D1 expression. The amount of protein was confirmed by the expression of β-actin.

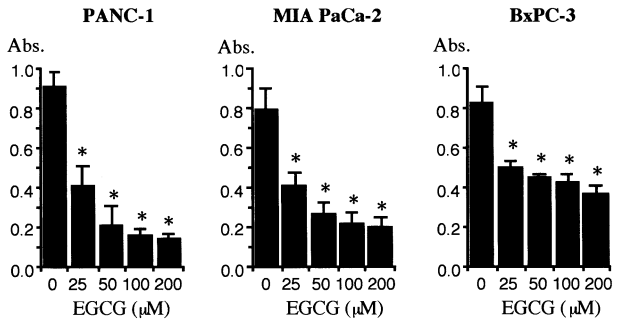

FIG. 1. Effect of EGCG on cell proliferation of human pancreatic carcinoma cells. EGCG treatment (25–200 μmol/L for 48 hours) causes dose-dependent inhibition of cell growth (*$P < 0.01$). Values are mean ± SEM in triplicate.

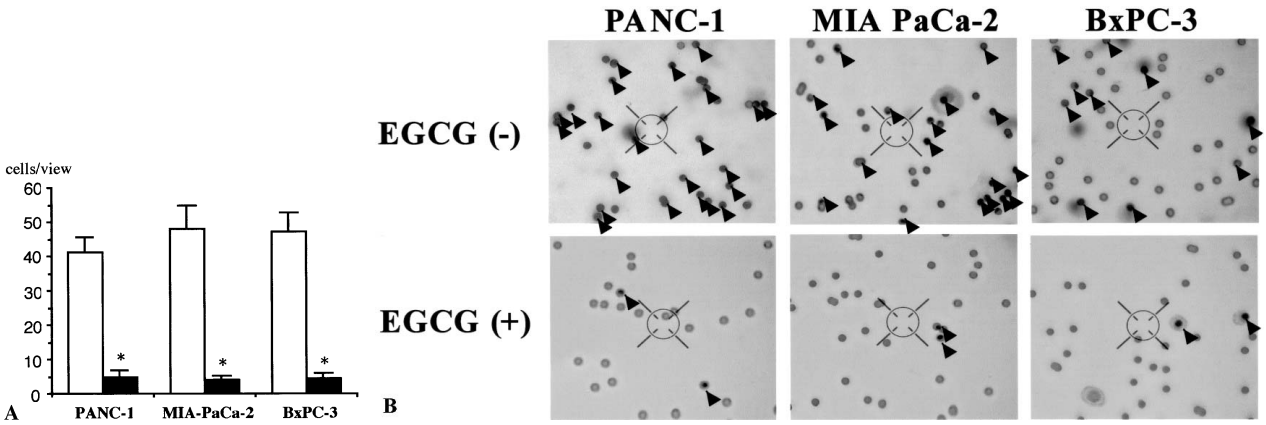

FIG. 2. Effect of EGCG on cell invasion of human pancreatic carcinoma cells. **(A)** Preincubation with EGCG (100 μmol/L for 2 hours) causes inhibition of cell invasion through Matrigel basement membrane (*$P < 0.01$). Values are the mean ± SEM for three samples, with six areas counted at random for each data point. **(B)** Morphology of invaded cells through Matrigel. Arrow indicates the cells invading through Matrigel coated on 8-μm-pore-size polyethylene terephthalate membrane chamber.

DISCUSSION

Pancreatic cancer involves several disorders in TGF-β inhibitory pathways, TGF-β and its receptors (6, 8–10), and their signal transduction proteins Smad2 (10) and Smad4 (11–14). Normalization of all these disorders in pancreatic cancer is extremely difficult, but it is possible to compensate for the endogenous inhibitory signals by inducing the CDK inhibitors to cause cell-cycle arrest (15,16). For that purpose EGCG is a good candidate; it has significant bioactivity against cancer, mimicking the TGF-β signal against the target, inducing CDK inhibitors p21 and p27, and directly inhibiting CDK2 and CDK4 (4,5).

To evaluate the effect of EGCG on cell proliferation, we used three different human pancreatic carcinoma cells: PANC-1, MIA PaCa-2, and BxPC-3. Treatment with EGCG (200 μmol/L) resulted in significant suppression of cell growth dose-dependently. Those growth inhibitions were also confirmed with microscopic observation. Laio et al. (17) reported that cell growth in human prostate and breast tumors was inhibited by EGCG but not by other catechins. A recent study showed that EGCG modulated nuclear transcriptional factor (NF)–κB in human epidermoid carcinoma cells (A431), followed by G0/G1-phase cell arrest as well as growth inhibition, but not in normal human epidermal keratinocytes (18). Another study also showed that EGCG did not affect the serum-induced *c-fos* and *c-myc* gene expression in normal human fibroblast (WI38) cells but significantly enhanced their expression in virally transformed cells (19). The inhibitory effect of EGCG is distinct in cancer cells but not in normal cells.

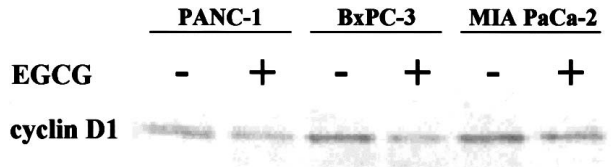

FIG. 3. Representative western blot of cyclin D1 of pancreatic carcinoma cells. Incubation with EGCG (100 μmol/L for 48 hours) shows no apparent effect on cyclin D1 expression. The amount of protein was confirmed by the expression of β-actin. Data represent one of three different experiments.

In addition to its effect on cell proliferation, the effects of EGCG on cell invasion were studied with use of Matrigel basement membrane matrix. Preincubation with EGCG resulted in significant suppression of invasiveness. Our results are similar to those reported by Zhang et al. (20), whose study involved a rat hepatoma cell line.

We then examined the effect of EGCG on a representative molecule of the cell cycle, cyclin D1. In spite of the significant suppressive effects on cell proliferation and cell invasion, there was almost no effect on cyclin D1 expression. Incubation with EGCG resulted in a significant decrease in cell number; the remaining cells seemed to grow at the same extent to which the key cell cycle molecule, cyclin D1, was expressed. Although we cannot deny the possible involvement of other factors, it is rational to conclude that the cell cycle itself is not affected by EGCG. It has been reported that antioxidant potential is a possible factor in the inhibitory effect of EGCG, which is far greater than those of vitamin E and vitamin C (21). Upregulation of transcription via antioxidant response elements may induce Fra1/Fra2, thereby antagonizing the transactivation of AP-1 complexes (22).

The National Cancer Institute plans to develop tea compounds as cancer chemopreventive drugs for humans (23,24). Further studies of EGCG may provide important data about molecular mechanisms of cancer chemoprevention, and it is possible that a new biologic-response modifier may be useful in the management of pancreatic cancers.

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