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Inhibitory effect of the tea polyphenol, (−)-epigallocatechin gallate, on growth of cervical adenocarcinoma cell lines

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Abstract

To investigate the effect of (−)-epigallocatechin gallate (EGCG) on cervical adenocarcinoma, we performed a cell proliferation assay. TRAP assay is used for telomerase activity, flow cytometry analysis and pKi-67 immunofluoroscein staining in cervical adenocarcinoma cell lines (OMC-4, TMCC-1). Our results showed that EGCG inhibited the proliferation assay, TRAP assay for telomerase activity of both cell lines. Although cell apoptosis was induced, we observed that the expression of pKi-67 was suppressed. Our data suggest that EGCG may be effective for the treatment of cervical adenocarcinoma. The mechanisms of the anti-tumor effects were revealed to be the inhibition of telomerase activity, the induction of apoptosis and cell cycle dysregulation.
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Keywords: Cervical adenocarcinoma; EGCG; Telomerase; Apoptosis; pKi-67

1. Introduction

Green tea is one of the most common beverages consumed worldwide, especially in Asian countries. Numerous studies have reported that green tea polyphenols have significant anti-carcinogenic and anti-oxidative activities [1,2]. Furthermore, it is reported that green tea polyphenols have limited toxicity [3]. Tea polyphenols account for up to 30% of the dry weight of green tea, and include some flavonols, known as catechin. (-)-Epigallocatechin gallate (EGCG) is a major constituent of catechin and has the strongest anti-oxidation effect. EGCG protects human cells against radiation-induced DNA damage manifested as chromatoid break, DNA scission and gene mutation [4,5]. Several studies have shown that EGCG possesses anti-carcinogenetic effects in rat skin [6], and may inhibit the conversion of pre-malignant lesions to malignancy [7].

Many studies investigating the activities of compounds as possible anti-cancer agents have indicated that EGCG suppresses the growth of cancer in vivo and in vitro, including cancers of the lung, stomach, prostate gland, brain, breast, skin, colon, liver, and blood [8–12]. Although the mechanisms for these

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cancer prevention effects have not been clearly demonstrated, it has been reported that telomerase inhibition and the induction of apoptosis may be involved [13,14]. Recently we also reported that EGCG prevents the carcinogenesis of cervical cancer, associated with the induction of apoptosis and telomerase inhibition in early cervical lesions [15]. However, little is known regarding the effects and pathways of EGCG in carcinogenesis of cervical cancer.

Adenocarcinoma of the uterine cervix represents only about 5–20% of all primary cervical carcinomas but this percentage is gradually increasing [16,17]. Furthermore, it is known that cervical adenocarcinoma is less sensitive to radiation and chemotherapy than squamous cell carcinoma [18,19]. Therefore, it is necessary to develop a new anti-cancer agent for treatment of adenocarcinoma. In the present study, we used cervical adenocarcinoma cell lines OMC-4 and TMCC-1 to examine the ability of EGCG to inhibit tumor growth, and investigated whether the mechanism involves EGCG induction of apoptosis, inhibition of telomerase and dysregulation of the cell cycle.

2. Materials and methods

2.1. Cells, cell culture, and cell growth assays

Human cervical adenocarcinoma cell lines, OMC-4 and TMCC-1, were used for this study. OMC-4 was established by Dr M. Ueda (Osaka Medical Collage, Osaka, Japan) [20]. TMCC-1 was a kind gift of M. Sakamoto (Tokyo Medical College, Tokyo, Japan) [21]. Both cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). OMC-4 was negative for HPV DNA, but on the other hand HPV 18 DNA was detected in TMCC-1. For the inhibition assay of cell proliferation, EGCG (Wako Pure Chemical Industries, Osaka) was diluted in 100% ethanol and stored at −20 °C before use. Cells seeded at 1×10^5 cells /5-cm plate were incubated with 0–100 μM EGCG in medium, which changed every third day. Cell growth was determined by counting the number of cells with a Coulter counter and expressed with the following formula:

$$\text{Growth rate (\%)} = \frac{\text{cell number (experiment)}}{\text{cell number (control)}} \times 100$$

2.2. Telomerase activity

Telomerase activity was measured by the TRAP assay using an end-labeled telomerase substrate (TS) primer as described [22]. We used the TRAPeze Telomerase Detection Kit (Intergen Company, USA). Briefly, 1×10^5 cells cultured in medium with 0 (control) 50, or 100 μM EGCG for 4 days and stored at −80 °C were lysed in CHAPS buffer on ice for 30 min and centrifuged at $12{,}000\times g$ for 20 min at 4 °C. The telomerase reaction was carried out in 48 μl of Master Mix containing TRAP Reaction buffer, dNTP mix, TS primer, TRAP primer mix, 2 U of Taq polymerase (Takara, Japan) and 2 μl of the cell extract. Reaction tubes were placed in a thermocycler (Perkin/Elmer, USA) for 30 min at 30 °C, followed by 33 cycles of PCR at 94 °C for 30 s, 59 °C for 30 s and 74 °C for 45 s. PCR products were resolved by electrophoresis in a 12% polyacrylamide gel and visualized with SYBR Green I nucleic acid stain (Molecular Probes, Inc., Eugene, USA).

2.3. Flow cytometry analysis

To detect early apoptosis and late apoptosis induced by EGCG, 3×10^5 cells were treated with 0 or 100 μM EGCG for 36 h, stained for 10 min at room temperature with FITC-conjugated Annexin V and PI in a Ca^{2+}-enriched binding buffer (Annexin V-FITC kit; Bender Medsystems, Vienna, Austria), and analyzed by flow cytometry. Annexin V and PI emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), using emission filters of 525 and 575 nm, respectively. Representative data from one experiment ($n=3$) are shown.

2.4. pKi-67 immunofluorescein staining

Cells (1×10^5) incubated on slides with EGCG for 48 h were washed in PBS, fixed for 15 min with 4% paraformaldehyde, followed by 20 min with 0.1% Triton-X. Anti-pKi-67 antibody

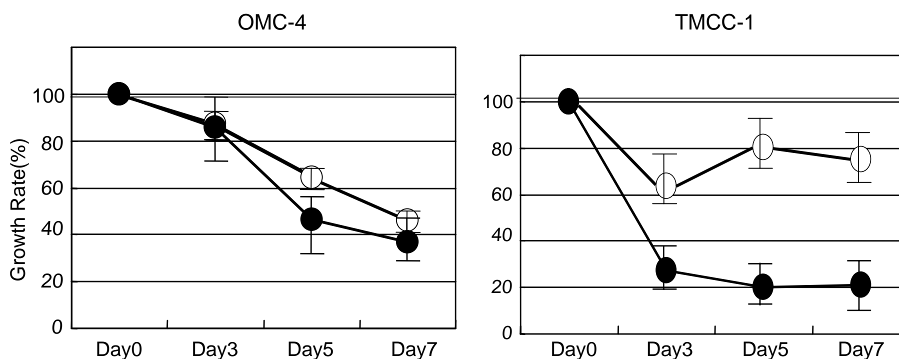

Fig. 1. Effect of epigallocatechin gallate (EGCG) on growth rate of OMC-4 and TMCC-1 cells at 50 and 100 μM EGCG treatment (○ 50 μM, ● 100 μM). After treatment, cells were cultured for 7 days. The mean values of cell counts in triplicate were plotted. Bars represent standard deviation.

(Dako, France) was applied (60 min, at room temperature, 1/50 dilution). A secondary antibody, rabbit–anti-mouse conjugated to fluorescein isocyanate (FITC), was used and applied (60 min, at room temperature, 1/10 dilution). The cells were counterstained with propidium iodide (15 min, 5 μg/ml). Immunofluorescence-labeling observations were performed with an Axioplan2 fluorescence microscope (Carl Zeiss, Germany), and analysis of images was performed using the software, Axiovision, (Carl Zeiss, Germany).

3. Results

3.1. Effects of EGCG on cell proliferation

To determine the anti-tumor effects of EGCG on uterine adenocarcinoma cell lines OMC-4 and TMCC-1, we treated cells with doses of EGCG ranging from 50 to 100 μM. As shown in Fig. 1, we observed that EGCG treatment for 48 h inhibited cell growth after day 3. The growth rate of OMC-4 cells in response to 50 μM was 64 and 46% at days 5 and 7, and 46 and 36% in response to 100 μM at days 5 and 7, respectively. TMCC-1 was less sensitive than OMC-4 at 50 μM, but at 100 μM, the growth rate was 20 and 21% at days 5 and 7, respectively. EGCG treatment resulted in a dose-dependent inhibition of cell growth in both cell lines.

3.2. Inhibition of telomerase activity

Telomerase has been proposed to represent a novel and potentially selective target for cancer therapy. The effect of EGCG treatment on telomerase activity was examined by the TRAP assay method. Telomerase activity is usually found to be high in both cervical adenocarcinoma cell lines. As shown in Fig. 2,

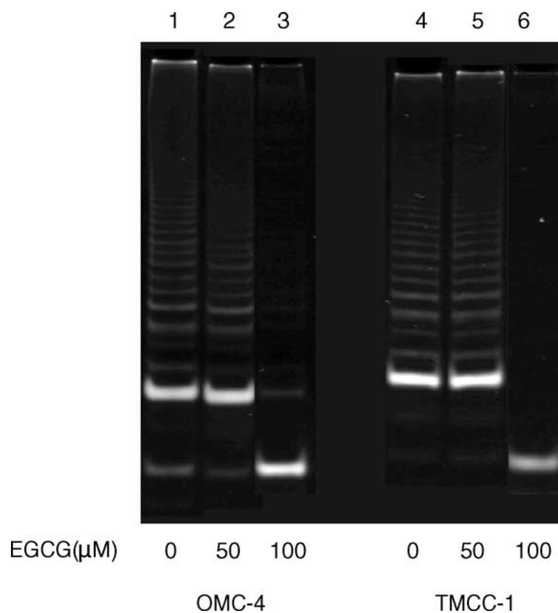

Fig. 2. Effect of epigallocatechin gallate (EGCG) on telomerase activity in OMC-4 (lanes 1–3) and TMCC-1 (lanes 4–6) cell lines by TRAP assay. Lanes 1 and 4, no treatment; lanes 2 and 5, 50 μM EGCG; lanes 3 and 6, 100 μM EGCG.

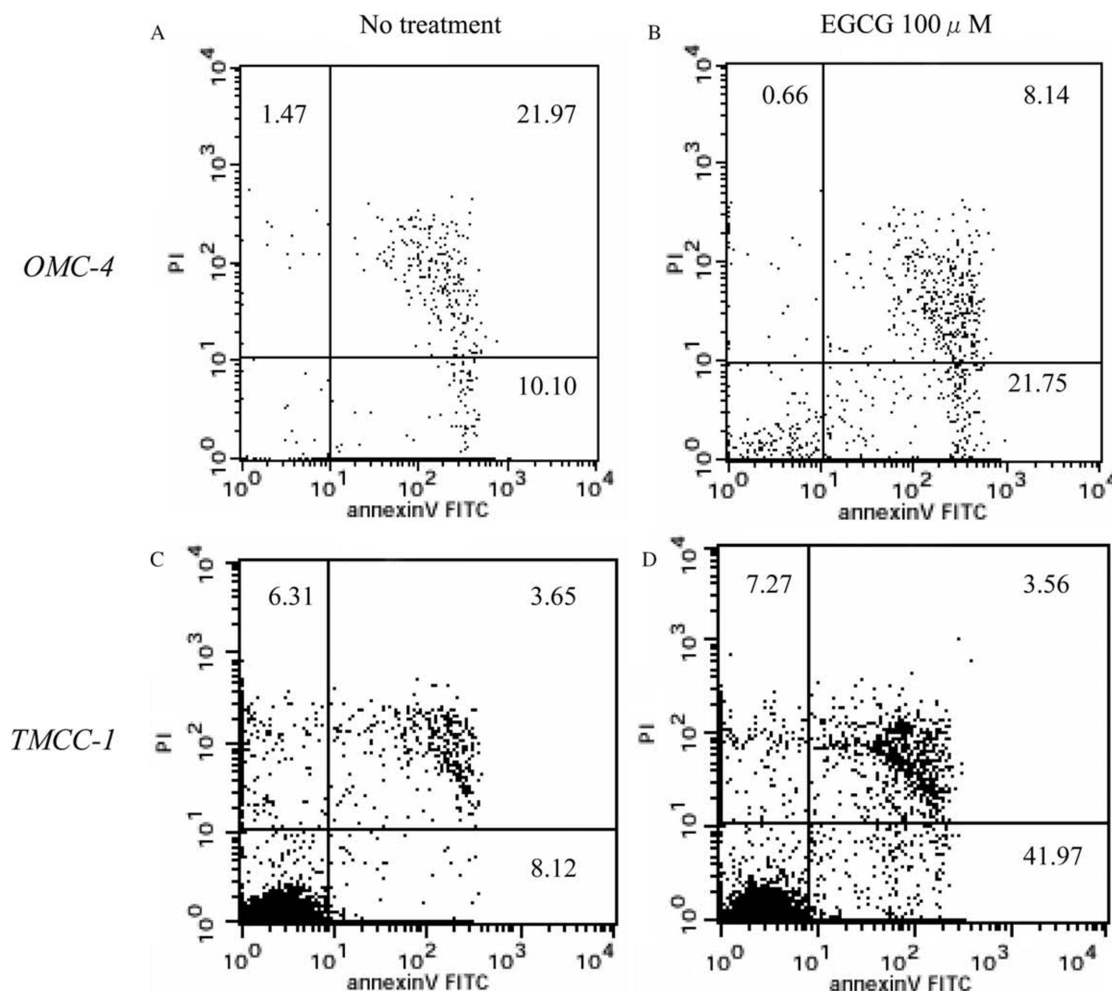

Fig. 3. FACS analysis of OMC-4 and TMCC-1 after staining with annexin V and PI. (A) and (C) No treatment; (B) and (D) cells treated with 100 μM EGCG in OMC-4 and TMCC-1, respectively. The lower right indicates the percentage of cells binding annexin V only as an apoptosis population. The same results were obtained from three separate experiments.

telomerase product signal was significantly decreased in the two cell lines treated with 100 μM EGCG (lanes 3 and 6).

3.3. Induction of apoptosis by EGCG

It has been reported that EGCG can induce apoptosis in some tumor cells. We studied whether EGCG induced apoptosis in cervical adenocarcinoma cell lines. We checked for apoptotic cells in the two cell lines by a double-staining method using FITC-conjugated annexin-V and PI after treatment with 100 μM EGCG using a FACSCalibur (Fig. 3). A significant induction of apoptotic (PI negative/ annexin-V positive) cells in both OMC-4 and TMCC-1 was observed 36 h after treatment with EGCG, with increases from 10.1 to 21.75% and from 8.12 to 41.97%, respectively. The population of necrotic (PI positive/annexin-V positive) cells did not change in TMCC-1, on the other hand, in OMC-4, it decreased. The population of apoptotic and necrotic cells in OMC-4 was

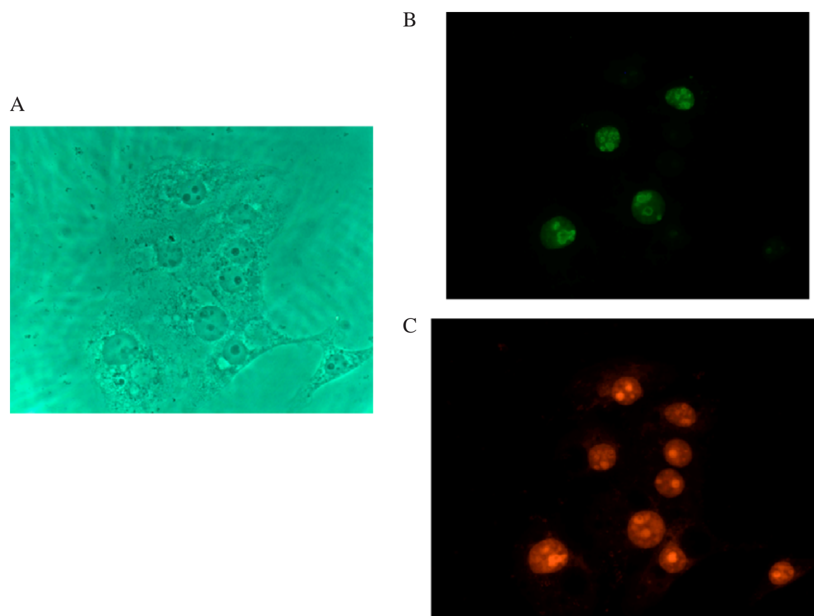

Fig. 4. A sample of cells treated with EGCG in the immunofluoroscein staining of pKi-67. (A) Phase contrast; (B) and (C) fluorescent contrast of pKi-67-FITC and PI, respectively.

different from that of TMCC-1. The final event numbers collected from the OMC-4 and TMCC-1 samples were 5000 and 10,000 events, respectively.

3.4. Suppression of pKi-67 expression

pKi-67 is mostly described as a proliferation marker that is expressed during all phases of the cell cycle except the G0 phase. We observed cells with phase contrast (Fig. 4A) and fluorescent contrast (Fig. 4B, staining by pKi-67-FITC; Fig. 4C, staining by PI). Using fluorescent contrast, we confirmed the nuclear uptake by PI staining and the expression of pKi-67 by FITC staining. Table 1 shows the ratio of pKi-67 positive cells to total PI positive cells. For OMC-4, the pKi-67 positivity rate was 50 and 28.7% in control and EGCG-treated cells, respectively. For TMCC-1, the pKi-67 positivity rate of control cells was 98% and that of cells treated with EGCG was 85%. The differences were significant for both cell lines.

4. Discussion

It is generally accepted that cervical adenocarcinoma is associated with relatively more aggressive biological activity, less favorable prognosis, earlier local extension and lymph node metastasis and less sensitivity to ionizing radiation and chemotherapy, as compared to its squamous cell counterpart. Previously, we reported that OMC-4, a well-differentiated adenocarcinoma cell line, had less sensitivity to chemotherapy and retinoic acid [23,24].

Recently, several in vitro and in vivo studies have demonstrated that dietary polyphenol not only has anti-oxidative effects, but it can also prevent cancer [13,25]. In particular, many reports have indicated that dietary polyphenols can control the proliferation

Table 1
Rate of pKi-67 positive cell of OMC-4 and TMCC-1

	control	EGCG 100 μM
OMC-4	50%	28.7%*
TMCC-1	98%	85%*

*$P<0.001$.

of various cancers through the action of EGCG [9,10]. Our previous study showed that EGCG possessed growth inhibitory activities against immortalized cell lines, which represent different CIN premalignant lesions in a cervical oncogenesis model [15]. Immortalized cell lines were more sensitive to EGCG than carcinoma cell lines. However, it was not clear whether EGCG possesses anti-cancer activities against cervical adenocarcinoma. In this study, we used two cervical adenocarcinoma cell lines, OMC-4 and TMCC-1, from well- and poorly differentiated adenocarcinoma, respectively. Our study showed that EGCG is capable of inhibiting cell growth. In our study, the effective IC50 dose of EGCG to adenocarcinoma cell line was about 100 μM. This dose is higher than the dose that we used on the immortalized cervical cancer cell line and squamous cell carcinoma cell lines.

We have previously reported that the sensitivity to EGCG in cervical cell lines originated from endocervical cells decreased with the progression of the carcinogenenic process [15]. It is also reported that cervical adenocarcinoma cell lines were less sensitive to EGCG than squamous cell carcinoma cell lines. Thus, in cervical cancer, cell growth inhibition by treatment with EGCG may differentially depend on cellular origin, although the mechanism is unclear.

Although the mechanism of the anti-cancer effect of EGCG has not been clarified, it has been reported that EGCG induced apoptosis, and G1 or G2-M arrest of the cell cycle [26,27]. Our results using flow cytometry also demonstrated that EGCG treatment results in the induction of apoptosis. After 36 h treatment with EGCG, the number of annexin-V positive cells increased.

Recently, it has been reported that EGCG inhibits the activity of topoisomerase I, which plays a role in DNA replication, transcription, and chromosome condensation [28,29]. Therefore, it has been hypothesized that EGCG inhibits cancer cell growth through many different regulatory pathways, along with apoptosis cell cycle arrests.

It is reported that telomerase inhibition could be one of the major mechanisms underlying the anti-cancer effects of EGCG [14]. We also observed that telomerase activity was suppressed by the treatment of EGCG. Telomerase activity was mostly inhibited with 100 μM EGCG treatment. Telomerase activity was also weakened by 50 μM EGCG treatment but the difference was not significant by digitalized densitometry (data not shown). But, in spite of weak inhibitory effect to telomerase activity, 50 μM EGCG inhibited growth in OMC-4. Previously, we reported that EGCG treatment inhibits telomerase activity in immortalized cervical cell lines, as well as non-transformed, serum-adapted HPV-18 immortalized endocervical cells and transformed HPV-18 immortalized ectocervical cells [15]. The targeting of telomerase by EGCG could be a promising strategy in treatment for cervical cancer, including cervical adenocarcinoma, although other mechanisms responsible for these anti-cancer effects should be considered.

The Ki-67 protein, pKi-67 is well known in tumor biology and cancer research [30]. Cell cycle analyses have revealed that the Ki-67 protein is a proliferative-associated antigen, which is expressed in all active parts of the cell cycle (G1, S, G2, and mitosis), but not in the resting cell (G0) [31,32]. This feature made pKi-67 a valuable tool in histopathology for use in estimating the growth fraction of a given cell population. Therefore, MIB1, antibody against pKi-67, has been used in numerous studies on the prognostic importance of cell proliferation measurement in clinical samples of neoplasms. In this study, we used immunofluoroscein staining pKi-67 for the purpose of observing how pKi-67 expression changes morphologically in the nucleus of the cell treated with EGCG. There are some reports about immunofluoroscein staining of pKi-67 that was suppressed during cell cycle arrest under treatment with an anti-cancer drug [33]. In our study, EGCG decreased the expression of pKi-67 in OMC-4 and TMCC-1 cells. Between the two cell lines, the positive rate of pKi-67 labeling differed, because pKi-67 associates the doubling time with cell proliferation. In TMCC-1, pKi-67 was translocated in the cytoplasm. It is reported that pKi-67 labeling was translocated in the cytoplasm in the treatment of the topoisomerase I inhibitor [33]. EGCG inhibits the activity of topoisomerase I [28,29]. The relocation of pKi-67 in the cytoplasm may be associated with anti-proliferative effect of EGCG through the inhibition of topoisomerase I, but the mechanism is unknown. Although the role of pKi-67 is not clear, it is particularly important to investigate the effect on pKi-67 in the cell cycle and

cell structures by anti-cancer drugs. With regard to our results, the suppression of pKi-67 by EGCG might be associated with one of the mechanisms of cancer inhibition of EGCG. The mechanisms may be involved in the change of intercellular functions of cytoplasm, DNA synthesis and checkpoint of cell cycle arrest and dysregulation, although further investigation is needed.

In vivo studies with mice and rats, the effective EGCG levels were lower than were those of in vitro models. In general, the concentration used in many in vitro studies was 50–300 μM as well as in this study. There is disparity between the concentrations needed to achieve the effects observed in vitro and the plasma levels at which significant anti-cancer and chemopreventive effects were observed in animal and epidemiological studies [34]. In addition to the induction of apoptosis and the inhibition of telomerase activity, several mechanisms of cancer inhibition by EGCG in vivo have been proposed. It is reported that the inhibition of angiogenesis by EGCG may explain why drinking green tea prevents the growth of a variety of different tumor type at lower serum concentration than those used in vitro.

In consideration of the results, our data suggest that EGCG might be effective for preventing and treating not only premalignant lesions, but also cancer moreover, adenocarcinoma of the cervix, which is known to be less-responsive to radiation and anti-cancer drugs. In vivo studies are currently underway to examine possible use of EGCG in clinical trials to test its efficacy in interdicting cervical cancer carcinogenesis and cancer growth in cervical adenocarcinoma.

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