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The tea polyphenol, (−)-epigallocatechin gallate effects on growth, apoptosis, and telomerase activity in cervical cell lines

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Abstract

Objective. To investigate the effect of the major tea polyphenol, (−)-epigallocatechin gallate (EGCG) in cervical carcinogenesis.

Methods. Cell growth rate was examined after treatment for 4, 7, and 10 days with 0–100 μM EGCG in primary human endocervical cells (HEN), human papillomavirus type 18 (HPV 18)-immortalized endocervical cell (HEN-18), ectocervical cell (HEC-18), serum-adapted HEN-18 (HEN-18S), transformed HEC-18 (HEN-18T), and four cervical cancer cell lines. The effect of EGCG treatment was examined on dysplastic epithelium formation in organotypic culture, induction of apoptosis by DNA ladder assay and telomerase activity by PCR telomere extension assay.

Results. EGCG inhibited growth more than 90% in HEN-18 and HEC-18, whereas growth inhibition was less in ME180, TMCC-1, HeLa, SiHa, HEC-18T, and HEN-18S. In organotypic culture, thickness of epithelial multilayers was decreased in all EGCG-treated cells. EGCG resulted in apoptosis of HEN-18 or HEC-18, but not HEN-18S nor HEC-18T and inhibited telomerase activity in HEN-18 and HEC-18, as well as HEN-18S and HEC-18T.

Conclusion. Our data suggest that EGCG prevents the carcinogenesis of cervical cancer, induces apoptosis and inhibited telomerase activity. The effect by EGCG treatment may be associated with the induction of apoptosis and telomerase inhibition in early cervical lesions.

Keywords: Tea polyhenol; EGCG; cervical carcinogenesis

Introduction

Prevention of carcinogenesis is one of the major strategies for cancer control. Chemoprevention is the term for cancer prevention and cancer control by use of naturally occurring and/or synthetic compounds [1,2]. Cervical cancer is a major health problem worldwide [3]. Cervical cancer develops through a multistep process in which increasingly severe premalignant dysplastic lesions called cervical intraepithelial neoplasia (CIN) I, II, and III progress to invasive cancer [4]. Therefore, the patients with CIN are potential candidates for chemopreventive intervention.

In cervical cancer, some clinical, as well as basic research, studies have focused on chemoprevention with retinoids and/or interferon [5]. Previously, we reported that the treatments with retinoic acid and/or interferon-α may be effective for preventing or treating premalignant cervical lesions [5]. However, retinoids and interferon-α have several negative aspects including severe toxicity, which could result in low compliance [6]. In comparison with these agents, green tea, mainly through its major constituent epigallocatechin gallate (EGCG), has limited toxicity [7,8]. EGCG appears to be potentially an ideal agent for chemoprevention.

Green tea is one of the most common beverages consumed worldwide, and the possible beneficial health effects have received much attention. A number of epidemiological studies have shown that the consumption of green tea may protect against many cancer types [1,9–12] and may inhibit the conversion of pre-malignant lesions to malignancy [13,14]. The inhibitory effects of green tea against experimental carcinogenesis also have been demonstrated in many animal models [15–18]. Green tea contains a variety

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of polyphenols known as cathechins. (−)-Epigallocatechin gallate (EGCG) is a major component of polyphenols in green tea [19]. The antitumor effect of EGCG has been demonstrated [16–21]. However, the mechanisms responsible for these cancer-preventive effects have not been clearly demonstrated. In recent years, some studies suggest that EGCG protect against cancer by causing cell cycle arrest and inducing apoptosis [7,22–25]. It is also reported that telomerase inhibition could be one of the major mechanisms underlying the anticancer effects of EGCG [26,27]. However, little is known regarding the effects and pathways of EGCG in cervical oncogenesis. In present study, we used an in vitro cervical oncogenesis model composed of various HPV 18-immortalized ectocervical, endocervical, and cancerous cervical cells to examine the ability of EGCG to prevent cervical carcinogenesis. Furthermore, we investigated whether the mechanism involves EGCG induction of apoptosis and inhibition of telomerase activity.

Materials and methods

Cells, cell culture, and cell growth assays

Primary human endocervical cells (HEN) were prepared from uterine cervix from hysterectomy performed for benign conditions as described previously [28]. Cervical cancer model cells are summarized in Table 1. Keratinocyte serum-free medium (K-SFM, GIBCO, Grand Island, NY) was used for growth of HEN [28,29], HPV-18-immortalized HEN [28], and HPV-18-immortalized HEC [28]. Non-transformed serum-adapted HPV-18-immortalized human endocervical cells [30], HPV-18-immortalized human ectocervical cells transformed by treatment with cigarette smoke condensate [30], and other cancer cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (GIBCO). The uterine cervix adenocarcinoma cell line, TMCC-1 was kindly obtained from M. Sakamoto (Tokyo Medical College, Tokyo, Japan). For the inhibition assay of cell proliferation, EGCG (Wako Pure Chemical Industries, Osaka, Japan) was diluted 100% ethanol and stored at −20 C before use. Cells seeded at 2×10^5 cells per 5-cm plate were incubated with 0–100 μM EGCG in medium, which was changed every second day. Cell growth was determined by counting the number of cells with Coulter counter and expressed with the following formula:

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

Table 1
Origin and HPV status of cervical cells and cell lines

Cell line	Origin	HPV DNA type	Tumorigenicity
HEN	Endocervix	None	(−)
HEN-18	Endocervix	HPV-18	(−)
HEN-18-S[a]	Endocervix	HPV-18	(−)
HeLa	Cx, adenocarcinoma	HPV-18	(+)
TMCC-1	Cx, adenocarcinoma	HPV-18	(+)
HEC-18	Ectocervix	HPV-18	(−)
HEC-18-T[b]	Ectocervix	HPV-18	(+)
SiHa	Cx, squamous cell carcinoma	HPV-16	(+)
ME180	Cx, squamous cell carcinoma	HPV-68	(+)

HEN = human endocervical cells; − = negative; + = positive.
[a] Nontumorigenic, adapted to growth in serum.
[b] Tumorigenic, cigarette smoke condensate-transformed.

Organotypic raft culture

Raft epithelia formed from cells were examined using histopathology. Rafts were prepared, as described previously [5,31]. Briefly, cells were seeded on a collagen matrix support. When the cells reached confluence, the gel was raised to the air–liquid interface. The rafts were incubated with 100 μM EGCG or without EGCG (control) and the reconstructed epithelia were recovered after 12 days, embedded in paraffin and stained with hematoxilin-eosin for histopathology.

DNA ladder apoptosis assay

Cells were cultured in medium with 0 μM (control) or 100 μM EGCG for 4 days. High molecular weight cellular DNA was extracted, resolved by 1.5% agarose gel electrophoresis, and stained using an Apoptosis Ladder Detection Kit (Wako Pure Chemicals Industries).

Telomerase assay

Telomerase activity was quantified with the overlap extension PCR assay method [32] using TeloChaser (Toyobo Co., Osaka, Japan). Briefly, 2×10^4 cells grown in 0 or 100 μM EGCG for 4 days were obtained and suspended in Lysis solution. Cell extracts were assayed in extension mixture. After a 30-min incubation at 30°C for telomerase extension, the telomerase products were purified by Clean-Up solution, followed by isopropyl alcohol precipitation. Recovered pellets were mixed with 30 μl of PCR mixture, heated at 95°C for 150 s, and then subjected to 30 PCR cycles of 95°C for 30 s, 68°C for 30 s, and 72°C for 45 s. PCR products were resolved by electrophoresis in a 7% polyacrylamide gel and visualized with SYBR Green I nucleic acid stain (Molecular Probes, Inc., Eugene, USA).

Assay of growth was performed in replicates of three. The mean and standard deviation of all samples were calculated and compared with untreated controls. For statistical analysis, all results were compared with two-tailed Student *t* test. All experiments were repeated, and the results were reproducible.

Results

Inhibition of cell growth was assayed following treatment for 4, 7, and 10 days with 0–100 μM EGCG in various cell types representing a cervical cancer model (Table 1). Cell growth inhibition assays demonstrated that HEN, normal endocervical cells, counterparts of immortalized cells, were less sensitive to EGCG treatment, whereas EGCG treatment resulted in a dose-dependent inhibition of cell growth in immortalized cell lines (90% growth inhibition in HPV 18-immortalized human ectocervical and endocervical cells by 10 μM EGCG) (Table 2). Fifty micromolars EGCG inhibited growth, but 10 μM EGCG treatment caused a lower inhibitory effect in transformed HPV 18-immortalized human ectocervical cells and non-transformed serum-adapted HPV 18-immortalized human endocervical cells. For cervical carcinoma cell lines, growth inhibition was less in ME180, TMCC-1, HeLa, and SiHa (Table 3). The inhibitory effect in cells derived from adenocarcinoma cells was less than that from squamous carcinoma cells.

The effect on EGCG treatment on epithelial dysplastic morphology was examined using organotypic raft culture. Untreated HPV 18-immortalized human ectocervical cells displayed low-grade SIL containing stratified and well-formed cornified layers (Fig. 1a). The reconstructed epithelium of the transformed HPV 18-immortalized human ectocervical cells was composed of highly dysplastic cells (high-grade SIL), although most of the thickness was without a cornified layer (Fig. 1c). Figs. 1b and d show the response of HPV 18-immortalized human ectocervical cells and the transformed HPV 18-immortalized human ectocervical cells to 10-day exposure to EGCG, as assessed using hematoxylin and eosin staining. The thickness of the multilayer preparation was decreased in EGCG-treated culture, suggesting an antiproliferative effect. In contrast, HPV 18-immortalized human endocervical cell morphology resembled CIN III (Fig. 2a). The rafts also showed sporadic cells with vacuolated cytoplasm, suggesting glandular cell differentiation. After adaptation to serum, HPV 18-immortalized human endocervical cells showed a more apparent glandular reconstruction with severely dysplastic cells (Fig. 2c). EGCG treatment of HPV 18-immortalized human endocervical cells and serum-adapted HPV 18-immortalized human endocervical cells showed similar results to those of HPV 18-immortalized human ectocervical cells and transformed HPV 18-immortalized human ectocervical cells (Figs. 2b and d, respectively).

To evaluate factors that may be involved in the growth inhibition and dysplastic growth of the cervical cell model, we studied whether EGCG induces apoptosis in cervical carcinogenesis. Cells treated with 0 and 100 μM EGCG for 4 days showed that EGCG treatment resulted in the formation of DNA fragments in HPV 18-immortalized human endocervical and ectocervical cells (Fig. 3, Lanes 2 and 6, respectively). In comparison with these cell lines, EGCG did not induce the formation of DNA fragments in transformed HPV 18-immortalized human ectocervical cells and non-transformed serum-adapted HPV-18 immortalized human endocervical cells (Fig. 3, Lanes 4 and 8), as well as normal endocervical cells (HEN) (data not shown). These results are consistent with those of the antiproliferative effect of EGCG between each cell line (Table 2).

Table 2
Effect of epigallocatechin gallate (EGCG) on cell growth rate of HPV-18 immortalized cell lines

Cell line	Treatment (μM)	Growth rate (% of untreated control)		
		Day 4	Day 7	Day 10
HEN	100	58.9 ± 4.9**	35.9 ± 4.2**	33.1 ± 4.8**
HEN-18	100	13.8 ± 3.1*	9.8 ± 0.7*	9.4 ± 0.6*
	50	37.3 ± 1.5*	10.5 ± 0.5*	6.8 ± 1.4*
	10	50.4 ± 3.9*	9.1 ± 4.6*	7.2 ± 1.7*
	5	58.6 ± 3.2**	39.3 ± 1.5*	79.7 ± 6.1***
HEN-18S	100	40.8 ± 0.6*	12.6 ± 0.8*	7.6 ± 0.7*
	50	95.2 ± 1.6***	66.0 ± 6.4***	68.6 ± 2.7**
	10	123 ± 13.6****	87.3 ± 1.2***	92.1 ± 4.0****
HEC-18	100	28.4 ± 2.6*	8.9 ± 0.4*	3.9 ± 2.1*
	50	35.6 ± 5.2**	18.6 ± 2.0*	8.3 ± 0.9*
	10	39.8 ± 10.9***	41.6 ± 2.5*	8.2 ± 1.3*
	5	51.6 ± 2.5*	40.8 ± 2.3*	34.4 ± 1.7*
HEC-18T	100	45.2 ± 3.6**	19.9 ± 1.4*	10.0 ± 1.1*
	50	60.9 ± 1.9*	77.5 ± 4.8***	90.9 ± 2.8***
	10	69.4 ± 3.2**	77.2 ± 1.8**	87.7 ± 2.9***

HEN = human endocervical cell; HEN-18 = human papillomavirus type18-immortalized human endocervical cell; HEN-18S = nontransformed, serum adapted HEN-18; HEC-18 = human papillomavirus type18-immortalized human ectocervical cell; HEC-18T = transformed HEC-18; The results represent the mean ± the standard deviation of percent. P is the statistical significance of difference in cell growth rate between each day treated and untreated control cells.
* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.05$.
**** Not significant.

Table 3
Effect of epigallocatechin gallate (EGCG) on cell growth rate of cervical cancer cell lines

Cell line	Treatment (μM)	Growth rate (% of untreated control)		
		Day 4	Day 7	Day 10
SiHa	100	90.2 ± 13.2****	49.7 ± 3.2**	30.7 ± 1.0*
	50	92.3 ± 2.3***	98.9 ± 3.2****	127.9 ± 1.5***
ME180	100	60.3 ± 7.2*	17.9 ± 5.4*	16.5 ± 6.5*
	50	105.8 ± 5.3****	72.4 ± 2.7**	81.4 ± 2.1**
HeLa	100	75.3 ± 5.1***	94.2 ± 1.3***	62.6 ± 0.9*
	50	88.1 ± 3.0***	102 ± 1.0****	101.6 ± 2.0****
TMCC-1	100	70.8 ± 5.1***	90.7 ± 0.7**	106 ± 1.1***
	50	90.8 ± 4.0****	108.2 ± 1.0**	97.5 ± 2.8****

The results represent the mean ± the standard deviation of percent. P is the statistical significance of difference in cell growth rate between each day treated and untreated control cells.
* $P < 0.001$.
** $P < 0.01$.
*** $P < 0.05$.
**** Not significant.

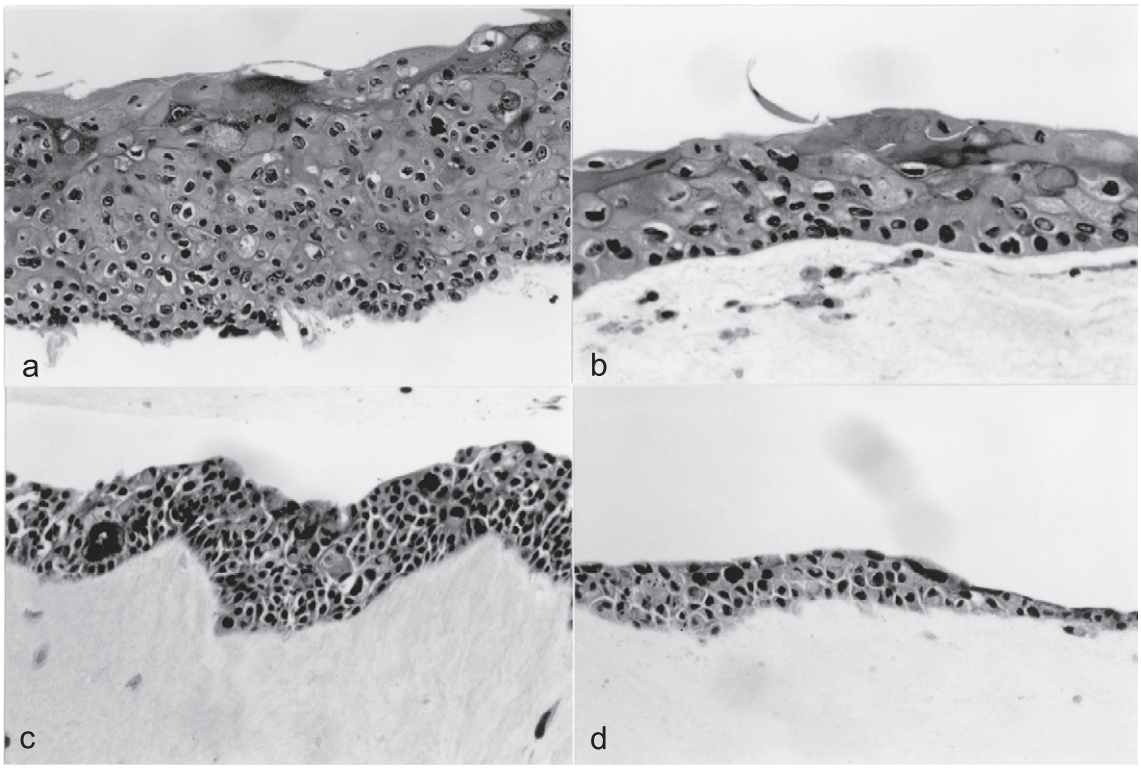

Fig. 1. Effect of epigallocatechin gallate (EGCG) on morphology of epithelium formed from HPV 18-immortalized ectocervical cells (HEC-18) and transformed HPV 18-immortalized human ectocervical cells (HEC-18T). Histology of organotypic culture (rafts) stained by hematoxylin-eosin is shown for HEC-18(a and b) and HEC-18T (c and d) that were untreated (a and c) or treated with 100 μM of EGCG (b and d). (Original magnifications ×200).

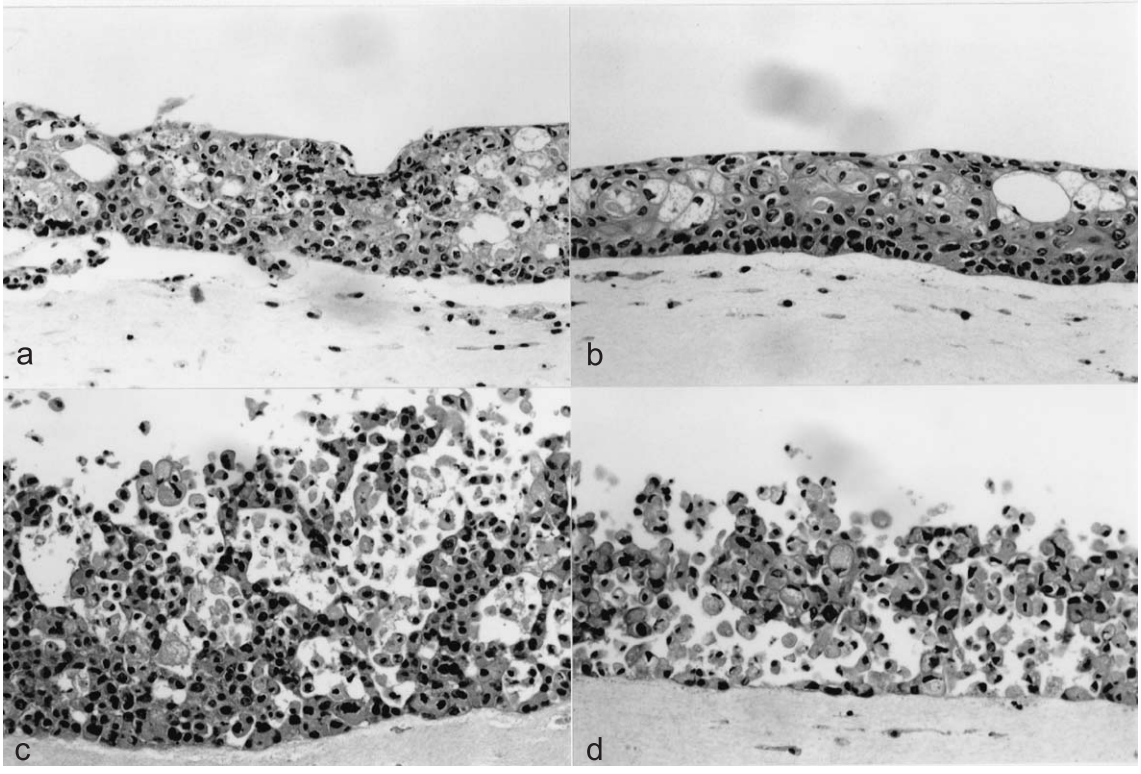

Fig. 2. Effect of epigallocatechin gallate (EGCG) on morphology of epithelium formed from HPV 18-immortalized endocervical cells (HEN-18) and serum-adapted HPV 18-immortalized human endocervical cells (HEN-18S). Histology of organotypic culture (rafts) stained by hematoxylin–eosin is shown for HEN-18(a and b) and HEN-18S (c and d) that were untreated(a and c) or treated with 100 μM of EGCG (b and d). (Original magnifications ×200).

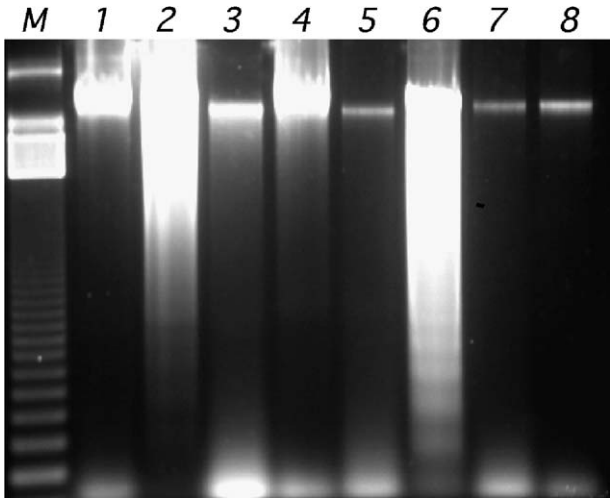

Fig. 3. Effect of epigallocatechin gallate (EGCG) on apoptosis in monolayers of HPV 18-immortalized ectocervical cells (HEC-18), transformed HPV 18-immortalized human ectocervical cells (HEC-18T), HPV 18-immortalized endocervical cells (HEN-18), and serum adapted HPV 18-immortalized human endocervical cells (HEN-18S). Apoptosis was assayed by DNA ladder formation analysis. HEC-18 (lane 1 and 2), HEC-18T (lane 3 and 4), HEN-18 (lane 5 and 6), and HEN-18S (lane 7 and 8) were cultured in medium with or without 100 μM of EGCG. M: 123 bp ladder marker; Lane 1,3,5,7: control medium; Lane 2,4,6,8: 100 μM of EGCG. DNA by ladder formation obtained from cells after EGCG treatment confirmed apoptosis.

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 stretch PCR assay method. Telomerase product signal was dramatically decreased in immortalized cell lines treated with EGCG (Fig. 4, Lanes 2 and 4), as well as transformed HPV 18-immortalized human ectocervical cells and non-transformed serum-adapted HPV 18-immortalized human endocervical cells (Fig. 4, Lanes 6 and 8). Telomerase activity was not detected in normal endocervical cell (HEN) (data not shown).

Discussion

Polyphenols derived from green tea, particularly EGCG, have been demonstrated to possess anticarcinogenic and chemopreventive effects both in vitro and in vivo [1,9–13,15–21]. Since most studies of the effects of EGCG have been performed in only cancer cells, it is not clear whether these pharmacological effects of EGCG are specific for cancer cells. Furthermore, little is known about the effects of EGCG in cervical cancer carcinogenesis. In the present study, we used an in vitro cervical oncogenesis model composed of normal endocervical cells, HPV 18-immortalized endocervical cells, HPV 18-immortalized ectocervical cells, non-transformed serum-adapted HPV 18-immortalized endocervical cells, transformed HPV 18-immortalized ectocervical cells [5,28], and various cervical cancer cell lines. Our study showed that EGCG possessed growth inhibitory activities against immortalized cell lines, which represent different CIN premalignant lesions in a cervical oncogenesis model. Immortalized cell lines were much more sensitive to EGCG before than after transformation and serum adaptation, which confer greater cervical

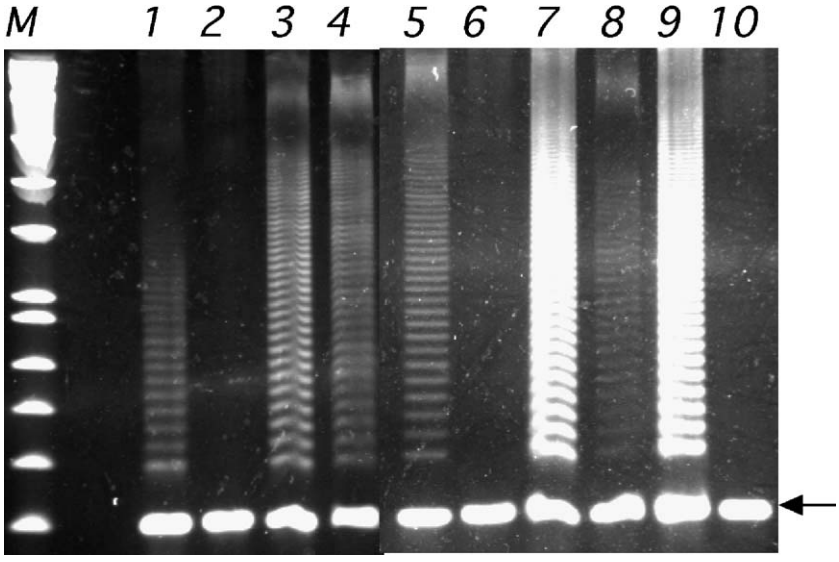

Fig. 4. Effect of epigallocatechin gallate (EGCG) on telomerase activity in HPV 18-immortalized ectocervical cells (HEC-18), transformed HPV 18-immortalized human ectocervical cells (HEC-18T), HPV 18-immortalized endocervical cells (HEN-18), and serum adapted HPV 18-immortalized human endocervical cells (HEN-18S). Semiquantitative telomerase activity was assayed by stretch PCR assay method. HEC-18(lane 1 and 2), HEC-18T (lane 3 and 4), HEN-18 (lane 5 and 6) , and HEN-18S (lane 7 and 8) were cultured in medium with or without 100 μM of EGCG. M:ϕ×174/Hinf I marker; Lane 1,3,5,7: control medium; Lane 2,4,6,8:100 μM of EGCG; Lane 9: positive control(HeLa cell); Lane10: negative control. The arrow indicated the internal standard DNA (65 bp).

cell growth potential and higher grade CIN. We have previously reported greater retinoic acid (RA) sensitivity of HPV 18-immortalized endocervical cells than non-transformed serum-adapted HPV 18-immortalized endocervical cells [5]. Combined with the present findings, these results suggest that the sensitivity to EGCG, as well as RA in cervical cell lines, decreases with the progression of the carcinogenic process. We have also observed here that normal endocervical cells, counterpart of immortalized cells, were less sensitive to EGCG treatment, although the analysis was limited to a single analysis at 100 μM. It is reported that EGCG showed growth inhibitory effect on colon and breast cancer cells but not on their normal counterparts [21]. In cancer cell lines, cell growth rate was inhibited by EGCG, 70–85% in squamous cell carcinoma cell lines, SiHa and ME180, whereas growth inhibition was less in adenocarcinoma cell lines, TMCC-1 and HeLa (30–38%). Thus, growth inhibition by treatment with EGCG may differentially depend on cellular origin in cervical cancer. The combined results support the hypothesis that the target sites of EGCG in premalignant and cancer cells may be different from those of normal cells. The differential effects of EGCG between premalignant and normal cells and the effect in the low concentration in premalignant cells may make EGCG a good model compound for the future design of specific chemoprevention reagents.

The mechanisms of cancer inhibition by EGCG are unclear, although several hypotheses have been proposed. It is known that the regulation of apoptosis and the cell cycle could be important targets for cancer chemoprevention [33–37]. Some studies have shown that EGCG treatment results in an induction of apoptosis in several human carcinoma cells [7,22–24]. We investigated whether EGCG causes apoptosis in a cervical cancer oncogenesis model. Our results demonstrated that EGCG treatment results in the DNA ladder formation of immortalized cells, HPV 18-immortalized endocervical cells, and HPV 18-immortalized ectocervical cells. Non-transformed serum-adapted HPV-18 immortalized endocervical cells and transformed HPV-18 immortalized ectocervical cells, which are less sensitive to EGCG in growth inhibition, were not induced to undergo DNA ladder formation by EGCG. These results suggested that the induction of apoptosis by EGCG treatment is one of the important mechanisms for EGCG-mediated cancer prevention in cervical cancer oncogenesis. It is also reported that EGCG induced apoptosis in carcinoma cells but not in the normal cells [21]. Recently, EGCG was shown to increase apoptosis in skin tumor, but not non-tumor areas of the epidermis in an ultraviolet light-induced mice skin tumor model [38]. The difference between EGCG-sensitive cells and normal counterparts in response to EGCG-induced apoptosis may suggest that components involved in the apoptotic pathway could be the specific target for EGCG in precancerous and some cancer cells, but not normal cells.

The activation of telomerase has been proposed to be a critical event in the immortalization of human cells and is characteristic of most human cancer cell lines and tumors [39–42]. Previously, we reported that telomerase activity was not detected in normal human cervical cells, but HPV-immortalized human cervical cells and transformed cells showed telomerase activity [42]. These results suggest that telomerase activation is a relatively early-stage event in cervical carcinogenesis, and this activation is associated with the initiation and progression of cervical lesions [41,42]. In the present study, we demonstrated 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. Naasani et al. [26,27] reported that EGCG treatment reduced life span accompanied with shortening telomeres and inhibiting telomerase activity. These results suggested that telomerase inhibition could be one of the important mechanisms in EGCG treatment. Considering all the results, the targeting of telomerase could therefore be a promising strategy in treatment for cancer, including cervical cancer.

In studies with mice and rats in which inhibition of skin, lung, and esophageal tumorigenesis was found, the effective EGCG levels were lower than were those of in vitro models [43]. There is the disparity between the concentrations needed to achieve the various acute effects observed in vitro and the plasma levels at which significant anticancer and chemopreventive effects were observed in animal and epidemiological studies [44]. Several mechanisms of cancer inhibition by EGCG in vivo have been proposed. EGCG binds strongly to many biological molecules and affects a variety of enzyme activities and signal transduction pathways [45,46]. It was also reported that EGCG suppresses endothelial cell growth in vitro and the formation of new blood vessels in chick chorioallantoic membrane [47]. These effects were associated with vascular endothelial growth factor (VEGF). Angiogenesis is important in the growth of all solid tumors [48]. The inhibition of angiogenesis by EGCG may explain why drinking green tea prevents the growth of a variety of different tumor types at lower serum concentration than those used in vitro.

Our data suggest that EGCG may be effective for preventing or treating premalignant lesion. In vivo studies are ongoing to examine possibly applying EGCG in clinical trials testing its efficacy in interdicting cervical cancer carcinogenesis.

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