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# Effects of Epigallocatechin-3-gallate (EGCG) on A549 Lung Cancer Tumor Growth and Angiogenesis

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**Epigallocatechin 3-gallate (EGCG) has cytotoxic effects in many cancer cells. It has been reported that A549 lung cancer cells are markedly resistant to cell death induced by EGCG. In the present study, the effects of EGCG on A549 lung cancer cell growth and angiogenesis were studied. We found that EGCG dose-dependently suppressed A549 cell growth, while A549 cells were markedly resistant to cell death *in vitro*. Next we found that EGCG increased endostatin expression and suppressed vascular endothelial growth factor (VEGF) expression. We further studied to determine whether EGCG would suppress A549 tumor growth in nude mouse and angiogenesis. EGCG in drinking water significantly suppressed A549 tumor growth in nude mice. Histological analysis revealed that the number of CD34 positive vessels had a tendency to decrease in the tumor. In sum, EGCG had anti-proliferative effects of A549 on tumor growth and showed a tendency to suppress angiogenesis.**

**Key words:** epigallocatechin 3-gallate (EGCG); endostatin; angiogenesis; vascular endothelial growth factor (VEGF); nude mouse

Angiogenesis is necessary for tumors to grow. To suppress angiogenesis is one of the clinical cures to prevent or delay tumor growth.[1,2] Much information is available concerning its mechanism, and several proteins related to angiogenesis, such as VEGF and endostatin are well known.[3–5] VEGF binds to endothelial cell surface receptors and activates various functions of the cell, which causes angiogenesis.[6] Thus anti-cancer treatment targeting tumor-induced angiogenesis is expected. It is of great interest to apply the concept of antiangiogenic treatment to the prevention of cancer. Tea catechin (epigallocatechin 3-gallate, EGCG) is one of the key components, because EGCG has suppressive effects on human cancer growth in epidemiological studies.[7,8]

EGCG, one of the major polyphenols in green tea, has been reported to cause inhibition of tumor initiation, tumor promotion, and tumor cell growth.[9–11] Drinking tea can inhibit VEGF-induced angiogenesis *in vivo*, and EGCG can reduce the binding of VEGF to its receptors and thus affect downstream signaling.[12–14] But, the role of endostatin has not been documented. We have reported that in ornithine decarboxylase (ODC)-over-expressing cancer cells, endostatin expression was suppressed and endothelial proliferation and neovascularization promoted.[15] Angiogenesis in tumors is regulated not only by angiogenesis stimulators such as VEGF, but also by angiogenesis inhibitors such as endostatin, and might be the result of a net balance between angiogenesis stimulators and angiogenesis inhibitors.[16]

It has been reported that A549 lung cancer cells show resistance to EGCG-induced apoptosis.[17] Therefore, we hypothesized that anti-proliferative effect of EGCG may not be sufficient to inhibit tumor growth *in vivo*, and anti-angiogenesis effect of EGCG may play a pivotal role in EGCG-induced A549 cell growth inhibition. In addition, a limited number of papers have reported effects of EGCG on lung cancer tumor growth *in vivo*.[18–20] Only one paper has reported an effect of EGCG on A549 tumor growth *in vivo*.[20]

## Materials and Methods

*Cell culture.* Human non-small-cell lung carcinoma cell line A549 and cervical carcinoma cell line HeLa were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Irvine, UK), and retinoblastoma cell line Y79 and human leukemic cell line U937 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, and antibiotic-antimycotic solution (Wako Pure Chemicals, Osaka, Japan) at 37 °C, 5% $CO_2$. All cell lines were purchased from JCRB Cell Bank (Osaka, Japan). EGCG (purity >95%) was from Sigma-Aldrich Chemical (Tokyo).

*Effects of EGCG on cell growth and viability.* Cells ($1 \times 10^5$ cells per well for 35 mm plate) were seeded into plates, cultured overnight, and treated with EGCG (0 μM, 50 μM and 100 μM) for 24, 48 and 72 h. Then, the viability of cells was determined by counting the numbers of living and dead cells by trypan blue exclusion method.

*RT-PCR.* The cells ($3 \times 10^6$) in 100 mm plastic dishes were cultured with DMEM containing 10% FBS in the presence or absence of 50 μM EGCG for 0, 3, and 6 h. Total RNA was extracted with Quick Prep™ Total RNA Extraction Kit (GE Healthcare, Tokyo) following the manufacturer's instructions. Reverse transcription with oligo (dT) primers was used to generate cDNA from total RNA (1 μg). The synthesized cDNAs for endostatin, VEGF, and GAPDH were amplified with specific primers. The endostatin forward primer was 5′ ACG CAT CTT CTC CTT TGA CG 3′, and the reverse primer was 5′ TGA TTT

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*Abbreviations*: EGCG, epigallocatechin 3-gallate; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay

TGG AGG GAT CTC 3′. The VEGF forward primer was 5′ ACC AAA CAA GGA GCT GGA TG 3′, and the reverse primer was 5′ TCG TAC ATG GCC GTC TGT AA 3′. The GAPDH primer was 5′ TCC ACC ACC CTG TTG CTG TA 3′, and the reverse primer was 5′ ACC ACA GTC CAT GCC ATC AC 3′. Each PCR mixture contained an appropriate set of forward and reverse primers (0.2 pmol/L), each of the dNTPs at 0.25 mmol/L, 1.25 U of *Taq* polymerase, and 2.5 mmol/L $MgCl_2$ in a PCR buffer. The PCR procedure consisted of 29 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, with initial denaturation of sample cDNA at 94 °C for 1 min and an additional extension period of 10 min after the last cycle. The PCR products were subjected to 1.5% agarose gel electrophoresis, staining with ethidium bromide, and quantitation by densitometry by Scion Image (Boston, MA).

*Assay for endostatin and VEGF concentrations by ELISA.* A549 cells were cultured in the presence and the absence of EGCG at the indicated concentrations for 0, 3, and 6 h. The supernatants of the cell cultures were harvested and stored at −20 °C until use. The levels of endostatin and VEGF in the culture media were analyzed by enzyme immunoassay technique (ELISA) (Cytoimmune, College Park, MD; genzyme Techne, Minneapolis, MN), following the manufacturers' instructions. The detection sensitivity of the assay was 1.953 ng/mL for endostatin and 3.0 pg/mL for VEGF.

In vivo *experiment.* The animal study was approved by the animal committee of the University of Tokyo, and all procedures involving animal care were in accord with institutional guidelines in compliance with national laws. BALB/c nude (nu/nu) male mice were purchased from SLC (Hamamatu, Japan), and were maintained according to institutional guidelines. A549 cells ($1 \times 10^7$ cells in 0.1 mL of PBS) were inoculated subcutaneously into the right sided backs of mice (three mice for each treatment group). Tumor growth was monitored with calipers every 2–3 d. Tumor size was calculated by the formula $l \times w^2/2$ [length (l) and width (w)]. Three d after inoculation, drinking water bottles were replaced with EGCG or dimethyl-sulfoxide (DMSO) 0.05% solutions. EGCG powder (Sigma) was dissolved in DMSO. The solutions to be administered were replaced every other day. On the final day of treatment, the mice were sacrificed. Tumors were removed, fixed in formalin, and paraffin embedded.

*Immunohistochemical study.* Tissues were fixed immediately in 10% buffered formalin, embedded in paraffin, and cut into 3 mm thick sections. The sections were deparaffinized and hydrated by passage through xylene and a graded ethanol series (100% to 70%). Antigen retrieval was done on the sections by incubation in 100 mM sodium citrate (pH 6.0) at 100 °C for 40 min. The sections were then treated with 3% $H_2O_2$ for 5 min to eliminate endogenous peroxidase activity, and stained by the immunoperoxidase technique. Anti-CD34 polyclonal goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:90 in phophosphate-buffered saline solution (PBS) was applied as primary antibody for 1 h at room temperature. Phophosphate-buffered saline solution with goat serum (Dako, Tokyo) was applied for the negative control. The sections were then incubated with the Histofine simple stain MAX-PO(G) (Nichirei Co., Tokyo) for 60 min at room temperature. The stain was visualized with 3,3′-diaminobenzidine (DAB) as chromogen.

*Evaluation of CD34 staining.* CD34-positive cells were evaluated by the method described by El-Assal *et al.*[21] Briefly, CD34-stained tumors were studied by two independent observers, with no knowledge of the data, using a double-headed light microscope. Cells were counted at high power (200 × magnification) in 10 areas. The mean value for the 10 areas counted by the two observers was considered the CD34-positive cell count of an individual tumor tissue.

*Statistical analysis.* Results were expressed as mean ± SD for at least three separate experiments. Results were analyzed by one-way analysis of variance (ANOVA). Differences with $p$ values of 0.05 were considered significant.

## Results

*Effects of EGCG on A549 cell growth and viability*

The effects of EGCG (50 and 100 μM) on A549 cell viability were studied. Preliminary experiment revealed that 5, 10, and 25 μM EGCG showed no anti-proliferative effects on A549 cell growth (data not shown). Hence, we chose 50 and 100 μM EGCG in the present study. After 24 h, 48 h, and 72 h the viability of the A549 cells was analyzed by the trypan blue exclusion method. The viability of the cells treated with 50 μM and 100 μM EGCG was not significantly decreased, as compared with control (Fig. 1, upper panel). Cell numbers were counted, and the results are shown in the lower panel of Fig. 1. EGCG (50 and 100 μM) suppressed cell numbers significantly, as compared to control ($^*p < 0.05$). The results confirmed a previous report that A549 lung cancer cells showed resistance to cell death induced by EGCG.[17]

*Effects of EGCG on mRNA expression of endostatin and VEGF*

The effects of 50 μM EGCG on the expression of endostatin and VEGF mRNA was studied by semi-quantitative RT-PCR. As shown in Fig. 2A, the expression of endostatin mRNA increased in a time-dependent manner ($p < 0.05$ at 6 h), while that of VEGF mRNA was not significantly decreased after 6 h. But after 12 h and 24 h, its expression level significantly decreased (Fig. 2B, $p < 0.05$).

*Endostatin and VEGF levels in culture media as analyzed by ELISA*

Effects of EGCG on endostatin and VEGF levels in A549, HeLa, U937, and Y79 cell culture media were analyzed using ELISA. EGCG (50 μM) significantly increased endostatin level in A549 cell culture media after 12 h and 24 h of treatment (Fig. 3A, $^*p < 0.05$),

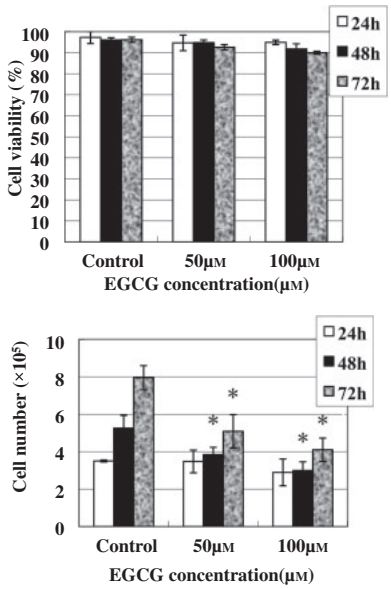


**Fig. 1.** Effects of EGCG on the Viability of A549 Cells.

A549 cells were treated with 50 μM or 100 μM EGCG for 24 h, 48 h, and 72 h, and then, the numbers of living and dead cells was counted by trypan blue exclusion method. Figure 1 upper panel shows cell viability, and lower panel shows cell numbers. Data are expressed as means ± SD, n = 3. $^*p < 0.05$.

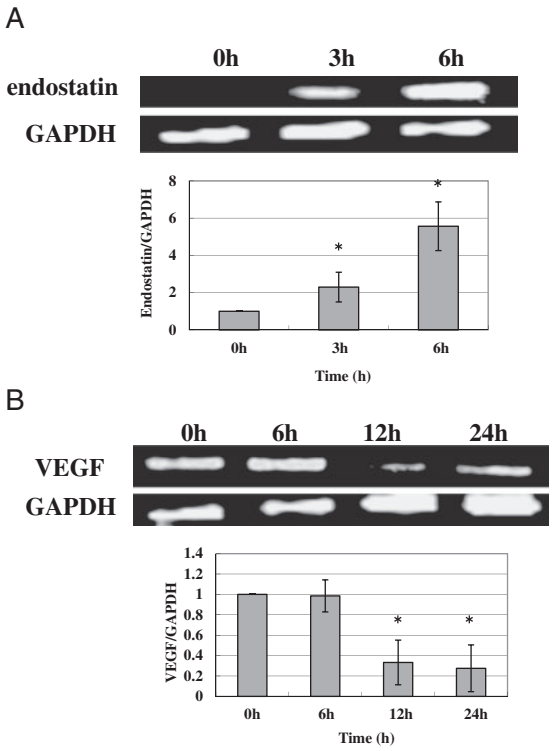


**Fig. 2.** Effect of EGCG on the mRNA Expression of Endostatin and VEGF.

The effect of 50 μM EGCG on the expression of endostatin and VEGF mRNA in A549 cells were studied by semi-quantitative RT-PCR as described in Materials and Method. The expression of endostatin mRNA at 3 h and 6 h had increased in a time-dependent manner ($^*p < 0.05$ at 6 h) (Fig. 2A). VEGF mRNA was not significantly decreased after 6 h. But after 12 h and 24 h, its expression level significantly decreased (Fig. 2B, $^*p < 0.05$). The endostatin and VEGF expression levels were corrected by the GAPDH expression levels. The data are expressed as mean ± SD (n = 3).

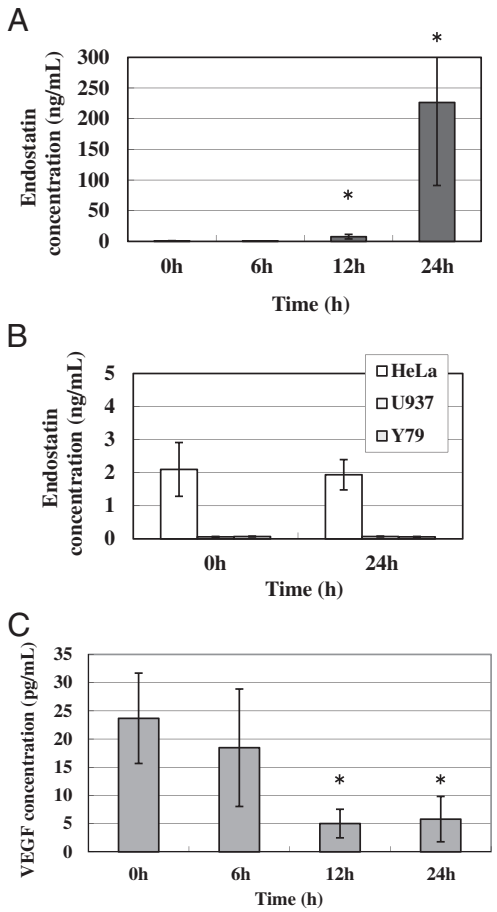


**Fig. 3.** Endostatin and VEGF Levels in Culture Media Analyzed by ELISA.

Effects of EGCG on endostatin and VEGF levels in A549, HeLa, U937, and Y79 cell culture media were analyzed by ELISA. EGCG significantly increased the endostatin level in A549 cell culture media after 12 h and 24 h treatment (Fig. 3A, $^*p < 0.05$), whereas, endostatin levels in HeLa cells were not significantly changed (Fig. 3B). Endostatin levels in U937 and Y79 cell culture media were hardly detectable (Fig. 3B). As shown in Fig. 3C, the VEGF levels in A549 cell culture media were significantly decreased in a time-dependent manner (at 12 h and 24 h; $^*p < 0.05$). The data are expressed as mean ± SD (n = 3).

whereas, the endostatin levels in the HeLa cells were not significantly changed (Fig. 3B). The endostatin levels in U937 and Y79 cell culture media were hardly detectable (Fig. 3B). As shown in Fig. 3C, the VEGF levels in the A549 cell culture media were decreased significantly in a time-dependent manner ($^*p < 0.05$). These results indicate that endostatin upregulation by EGCG is specific to A549 cells.

*Effects of EGCG on A549 tumors* in vivo

Since EGCG inhibited VEGF and increased endostatin expression in A549 cells *in vitro*, we examined to determine whether EGCG would inhibit tumor growth *in vivo*. A549 cells form tumors when injected subcutaneously into nude mice. Treatment with drinking water with EGCG (0.025% and 0.05%) started 3 d before A549 cell inoculation. EGCG (0.05%) significantly ($p < 0.05$) reduced tumor growth after 13–21 d, as compared to controls receiving water alone (Fig. 4).

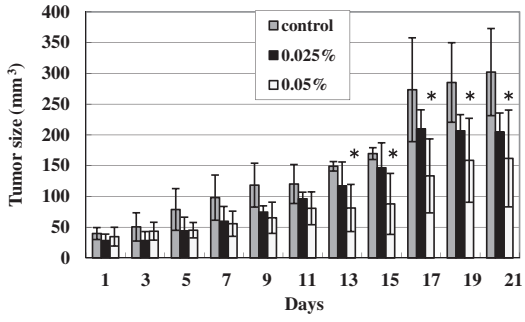


**Fig. 4.** Effects of EGCG on A549 Tumor *in Vivo*.

The effects of EGCG on A549 tumor growth in nude mice were analyzed. A549 cells form tumors when injected subcutaneously into nude mice. Treatment of drinking water with EGCG (0.025% and 0.05%) started 3 d before A549 cell inoculation. EGCG (0.05%) significantly ($^*p < 0.05$) reduced the tumor growth after 13 d, as compared with controls receiving water alone. Tumor size was calculated by the formula $l \times w^2/2$ [length (l), and width (w)]. Data are expressed as mean ± SD (n = 3).

*Effects of EGCG on angiogenesis* in vivo

The xenograft tumors were further analyzed for angiogenesis. We counted CD34-positive endothelial cells in the control tumors (Fig. 5A) and in the EGCG-treated tumors (Fig. 5B) under a microscope. As shown in Fig. 5C, EGCG (0.025% and 0.05%) decreased vascular endothelial cell numbers in a dose-dependent

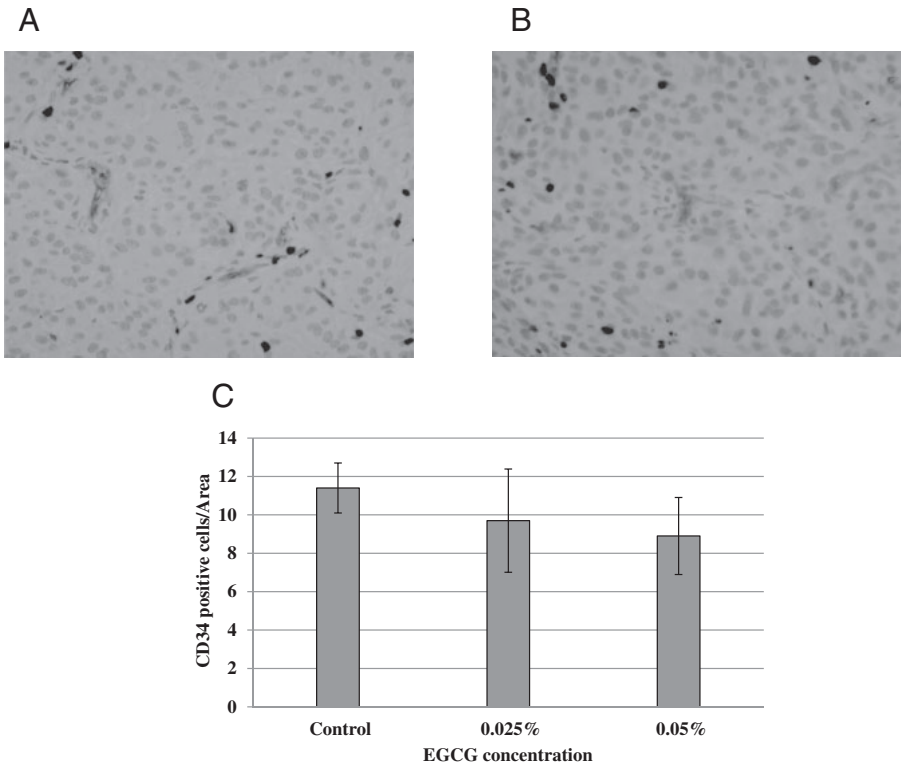


**Fig. 5.** Effects of EGCG on Angiogenesis *in Vivo*.

Xenograft tumors were analyzed for angiogenesis. CD34-positive endothelial cells were counted in control tumors and in EGCG-treated tumors under a microscope. EGCG (0.025% and 0.05%) decreased vascular endothelial cell number in a dose-dependent manner, but it did not reach a significant level. Panels A and B show CD34-positive vessels stained with anti-CD34 antibody in control group and EGCG 0.05%-treated one, respectively. The original magnification was ×400. Panel C shows quantitative data of the number of CD34-positive vessels of control, and EGCG-treated groups (0.025% and 0.05%). Data are expressed as mean ± SD (n = 3).

manner. But it did not reach a significant level. These results indicate that EGCG can decrease angiogenesis.

## Discussion

Different mechanisms of action of EGCG in diverse physiological and pathological situations have been proposed.[22] Inhibition of tumor cell proliferation is generally considered to be mediated through modulation of the activities of cell-cycle regulatory proteins or apoptosis-related proteins. It has been reported that EGCG repressed A549 growth through cyclin-dependant kinase Cdk2 and Cdk4 and through induction of cyclin-dependant kinase inhibitors p21 and p27.[23] EGCG also induces apoptosis.[24] Growth inhibition in a variety of tumor cell lines were achieved at doses from 20 to 100 μM.[13,24–26] Several studies have indicated that EGCG at higher concentrations also perturbs downstream signaling, repressing the mitogen-activated protein kinase pathway, activator protein-1, and nuclear factor-B,[27,28] although other studies have indicated that EGCG increases mitogen-activated protein kinase and activator protein-1 activity.[25,29]

Green tea polyphenols are increasingly recognized for their antiangiogenic properties. Relatively high levels of EGCG (80–90 μM) have been found to reduce vascular endothelial growth factor (VEGF) production in breast carcinoma cell lines.[30] It has been reported that EGCG inhibited angiogenesis *in vivo*,[31] but the effect of EGCG on endostatin expression was not studied. Our results indicate that EGCG not only inhibited VEGF expression but also increased endostatin expression. To our knowledge, this is first to report that EGCG increased endostatin. In this study we studied the effects of EGCG on endostatin secretion in other three cell lines (Hela, U937, and Y79 cells). Endostatin in U937 and Y79 cell culture media were hardly detectable, and EGCG failed to increase endostatin secretion. Hela cells secrete about 2 ng/mL of endostatin in culture media, but EGCG failed to increase endostatin secretion. Taken together, the upregulation of endostatin induced by EGCG is considered to be specific only to A549 cells. The mechanism of this effect of EGCG remains to be clarified.

In the present study, we found that oral administration of EGCG significantly inhibited tumor growth. This correlated with a tendency towards reduction in the vascularization of the tumors in the animals treated with EGCG as compared with the controls. Our results suggest that the antiangiogenic potential of EGCG might be partially responsible for growth suppression of tumor. The mechanism of endostatin induction by EGCG is mediated at both the mRNA and the protein level, but the exact mechanism remains to be clarified.

In the present study, we found that EGCG significantly inhibited tumor growth *in vivo*, but did not induce tumor regression. These results are consistent with previous studies.[18–20] in which EGCG showed antiproliferative effects on lung cancer tumor *in vivo*, but failed to induce tumor regression.

We found that 0.05% EGCG inhibited tumor growth *in vivo*. This dose was not associated with any side effects either in the present study or in other ones.[32] Human volunteers have been given 10-fold higher doses

of EGCG with little or no adverse effects,[33] Suggesting that EGCG could be useful for therapeutic clinical application. Our results suggest that EGCG may be applicable in lung cancer therapy.

In conclusion, EGCG has anti-proliferative effects on A549 tumor growth and has a tendency to suppress angiogenesis.

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