Green tea polyphenol epigallocatechin-3-gallate inhibits the endothelin axis and downstream signaling pathways in ovarian carcinoma

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Abstract

The polyphenol epigallocatechin-3-gallate (EGCG), the principal mediator of the green tea, has been known to possess antitumor effect. The endothelin A receptor (ET_AR)/endothelin-1 (ET-1) axis is overexpressed in ovarian carcinoma representing a novel therapeutic target. In this study, we examined the green tea and EGCG effects on two ovarian carcinoma cell lines, HEY and OVCA 433. EGCG inhibited ovarian cancer cell growth and induced apoptosis that was associated with a decrease in Bcl-X_L expression and activation of caspase-3. Treatment with green tea or EGCG inhibited ET_AR and ET-1 expression and reduced the basal and ET-1-induced cell proliferation and invasion. The EGCG-induced inhibitory effects were associated with a decrease of ET_AR-dependent activation of the p42/p44 and p38 mitogen-activated protein kinases and phosphatidylinositol 3-kinase pathway. Remarkably, EGCG treatment resulted in a lowering of basal and ET-1-induced angiogenesis and invasiveness mediators, such as vascular endothelial growth factor and tumor proteinase activation. Finally, in HEY ovarian carcinoma xenografts, tumor growth was significantly inhibited by oral administration of green tea. This effect was associated with a reduction in ET-1, ET_AR, and vascular endothelial growth factor expression, microvessel density, and proliferation index. These results provide a novel insight into the mechanism by which EGCG, affecting multiple ET_AR-dependent pathways, may inhibit ovarian carcinoma growth, suggesting that EGCG may be useful in preventing and treating ovarian carcinoma in which ET_AR activation by ET-1 plays a critical role in tumor growth and progression. [Mol Cancer Ther 2006;5(6):1483–92]

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Introduction

Ovarian cancer is the leading cause of death from gynecologic cancer. Without clearly definable symptoms, ovarian cancer often remains undetected until an advanced stage, when prognosis is poor. Despite recent advances in cytoreductive surgery and combination chemotherapy, improvement in long-term survival of these patients has been slight (1). Development of new treatment options strongly relays on improved knowledge of the molecular mechanisms underlying ovarian carcinoma initiation and progression. The endothelin-1 (ET-1) and its selective receptor ET_A (ET_AR) have a key role in the development and progression of ovarian carcinoma by promoting tumor cell proliferation (2, 3), apoptosis protection (4), epithelial-to-mesenchymal transition (5), invasiveness (6), and vascular endothelial growth factor (VEGF)–mediated neovascularization through ET_AR-driven autocrine pathways (7–10). ET-1/ET_AR interaction results in activation of a pertussis toxin–insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca^{2+} levels, activation of protein kinase C, and mitogen-activated protein kinase (MAPK; ref. 11). Among downstream events after ET_AR activation, ET-1 causes phosphatidylinositol 3-kinase (PI3K)–dependent AKT activation, the epidermal growth factor receptor transactivation, which is partly responsible for MAPK phosphorylation (12), and activation of p125 focal adhesion kinase and paxillin, which transduce signals involved in tumor cell invasion (3, 6). Thus, ET-1/ET_AR signaling enhances the expression and activity of matrix metalloproteinase (MMP) and urokinase-type plasminogen activator (uPA) system and is a regulator of VEGF synthesis and production (6, 9). In human ovarian primary tumors, the elevated expression of ET-1 and ET_AR was significantly associated with microvessel density and VEGF expression. High levels of ET-1 were detected in the majority of human ovarian cancer ascites and were significantly correlated with VEGF ascitic concentrations (8). ET_AR blockade by the selective receptor antagonist, ABT-627, has been shown to inhibit the growth of ovarian carcinoma xenografts concomitantly with a reduction of microvessel density, MMP-2, VEGF, and cyclooxygenase-2 enzyme expression, indicating that the blocking of ET-1/ET_AR axis may represent a potential therapeutic target in ovarian carcinoma management (13–15).

Several studies on human and animal models have shown that green tea may be effective in preventing and treating cancer of the breast, prostate, esophagus, pancreas, and colon (16–20). Green tea consumption is associated with a reduced risk (21) and can enhance the survival of patients with epithelial ovarian cancer (22). The anticancer properties of green tea, and of the bioactive polyphenol, (−)-epigallocatechin-3-gallate (EGCG), are a result of induction of G_1 arrest and apoptosis as well as regulation of cell cycle–related proteins in ovarian cancer cell lines (19). A recent study shows that EGCG treatment led to enhanced intracellular hydrogen peroxide and enhances the sensitivity to cisplatin in ovarian cancer cell lines (23).

Novel insight into the molecular mechanism of green tea polyphenol-mediated inhibition of ovarian tumor growth is essential in devising preventive and therapeutic approach. In the present study, we show that green tea polyphenol-induced tumor cell growth suppression and apoptosis may be mediated through inhibition of ET-1/ET_AR axis and its related signaling cascade leading to cell proliferation, invasion, and neoangiogenesis in human ovarian carcinoma cells. These results provide evidence that ET-1/ET_AR-pathway is a critical target for green tea and EGCG in ovarian cancer growth and identify a potential role of green tea polyphenol in the prevention and treatment of this malignancy.

Materials and Methods

Cells and Cell Culture Conditions

Human ovarian carcinoma cell lines, HEY and OVCA 433, characterized previously for ET-1 receptor expression and for ET-1 production (2, 7), were cultured in DMEM containing 10% FCS and 1% penicillin-streptomycin at 37°C under 5% CO_2-95% air. The cells were serum starved by incubation for 24 hours in serum-free DMEM. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). ET-1 (Peninsula Laboratories, Belmont, CA) was used at 100 nmol/L and incubated with the cells for the indicated times. Pretreatment of cells with 1 μmol/L BQ 123 (Peninsula Laboratories) or with the indicated concentrations of EGCG (Sigma, St. Louis, MO) or green tea infusion was done for 30 minutes before the addition of ET-1. Green tea infusion was prepared from commercially available (China or Sri Lanka) green tea leaves steeped in 1 liter (12.4 g/L) of boiled distilled water for 1 to 2 minutes.

Cell Proliferation Assay

HEY and OVCA 433 cells were seeded at 5×10^4 per well in 12-well plates, cultured for 24 hours, and incubated in serum-free medium in the presence of different concentrations of EGCG. After 24 hours, cells were assayed for cell viability (trypan blue exclusion test) and counted.

Thymidine Incorporation Assay

Cells were seeded in 96-well plates at ~80% confluence (2×10^6 per well) and incubated in serum-free medium for 24 hours to induce quiescence. Indicated concentrations of EGCG and 100 nmol/L ET-1 were added. After 24 hours, when cells were confluent, 1 μCi [methyl-^{3}H]thymidine (5.0 Ci/mmol) was added to each well. Six hours later, culture medium was removed, and cells were washed twice with PBS, treated with 10% trichloroacetic acid for 15 minutes, washed twice with 100% ethanol, and solubilized in 0.4 N NaOH. The cell-associated radioactivity was then determined by liquid scintillation counting. Responses to all treatments were assayed in sextuplicate, and results were expressed as the means of three separate experiments.

Apoptosis Assay

Cells floating in the culture supernatants were collected by centrifugation and pooled with adherent cells recovered from the plates. Cells (1×10^6) were double stained with FITC-conjugated Annexin V and propidium iodide using the Vybrant Apoptosis kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR) and were immediately analyzed by cytofluorometric analysis.

DNA Fragmentation

Fragmented DNA was extracted from cells deprived of serum in the absence or presence of different concentrations of EGCG as described previously (4) and separated by electrophoresis.

Western Blot Analysis

For protein detection, whole-cell lysates were subjected to SDS-PAGE (10%) and revealed by Western blotting using antibody to ET-1 (clone TR.ET.48.5; Affinity Bioreagents, Golden, CO), ET_AR (Abbott Laboratories, IL), caspase-3, p42/p44 and p38 MAPKs, AKT (Cell Signaling, Beverly, MA), membrane type 1 MMP (MT1-MMP; Chemicon International, Temecula, CA), uPA, uPA receptor, and Bcl-X_L (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and VEGF (Sigma). Blots were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and the relative intensity of signals was quantified using NIH image (Scion Corp., Frederick, MD). The membranes were reprobed with anti-β-actin to assure the equal amount of protein (Oncogene, CN Biosciences, Inc., Darmstadt, Germany).

Gelatin Zymography

The ovarian tumor cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/mL gelatin as described previously (6). Briefly, the cells were washed for 30 minutes at 22°C in 2.5% Triton X-100 and then incubated in 50 mmol/L Tris (pH 7.6), 1 mmol/L $ZnCl_2$, and 5 mmol/L $CaCl_2$ for 18 hours at 37°C. After incubation, the gels were stained with 0.2% Coomassie blue. Enzyme-digested regions were identified as white bands on a blue background. Molecular sizes were determined from the mobility, using gelatin zymography standards (Bio-Rad Laboratories, Richmond, CA).

Reverse Transcription-PCR

Total RNA from HEY and OVCA 433 cells was extracted using TRIzol (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using AccessQuick RT-PCR System (Promega, Madison, WI) according to the manufacturer's instructions. The primers sets were as follows: ET-1 5′-TGCTCCTGCTCGTCCCTGATGGATAAAGAG-3′ and

5′-GGTCACATAACGCTCTCTGGAGGGCTT-3′, ET_AR 5′-CACTGGTTGGATGTGTAATC-3′ and 5′-GGAGATCAAT-GACCACATAG-3′, VEGF 5′-GGCTCTAGATCGGGCCT-CCGAAACCAT-3′ and 5′-GGCTCTAGAGCGCAGAGTC-TCCTCTTC-3′, and glyceraldehyde-3-phosphate dehydrogenase 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. Thirty-five cycles of amplification were done under the following conditions: melting at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 60 seconds. The PCR products were analyzed by electrophoresis on a 2% agarose gel and the relative intensity of signals was quantified using NIH image.

Northern Blotting

RNA samples (30 μg/lane) were separated by electrophoresis on 2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. The membranes were UV cross-linked and hybridized in the QuickHyb hybridization Solution (Stratagene, La Jolla, CA). The cDNA probe used for analysis of the ET_AR mRNA was labeled with [α-^{32}P]dCTP using a random primer Oligolabeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To ensure RNA integrity and to confirm equal loading between lanes, the filters were stripped and rehybridized with a probe for 18S rRNA.

ELISA

Subconfluent HEY cells were serum starved for 24 hours and incubated for the indicated times. The conditioned medium was then collected, centrifuged, and stored in aliquots at −20°C. ET-1 release was measured in triplicate on microtiter plates by an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. ET-1 could be measured in the range 0 to 120 pg/mL. The sensitivity is $<$1.0 pg/mL. The VEGF protein levels were determined in triplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems). The sensitivity of the assay is $<$5.0 pg/mL. Intra-assay variation is 5.4% and interassay variation is 7.3%. Gelatinase activities in conditioned medium were determined by a MMP Gelatinase Activity Assay kit (Chemicon) according to the manufacturer's instructions. The sensitivity of the assay is $<$5 ng/mL MMP in the range 10 to 200 ng/mL.

Chemoinvasion Assay

Chemoinvasion was assessed using a 48-well modified Boyden chamber (NeuroProbe, Pleasanton, CA) and 8-μm pore polyvinyl pyrrolidone–free polycarbonate Nucleopore filters (Costar, New York, NY) as described previously (6). The filters were coated with an even layer of 0.5 mg/mL Matrigel (Becton Dickinson, Bedford, MA). The lower compartment of chamber was filled with chemoattractants (ET-1 100 nmol/L and EGCG at the indicated concentrations) and/or antagonists (27 μL/well). Serum-starved HEY cells (0.5×10^6/mL) were harvested and placed in the upper compartment (55 μL/well). Where specified, the cells were preincubated for 30 minutes at 37°C with the indicated concentrations of EGCG. After 6 hours of incubation at 37°C, the filters were removed and stained with DiffQuick (Merz-Dade, Dudingen, Switzerland) and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

HEY Xenografts in Nude Mice

Female athymic (*nu*$^+$/*nu*$^+$) mice, ages 4 to 6 weeks, were purchased from Charles River Laboratories (Milan, Italy). The treatment protocol followed the guidelines of animal experimentation adopted by the Regina Elena Cancer Institute under the control of the Ministry of Public Health. Mice were given injections s.c. into one flank with 1.5×10^6 viable HEY cells, as determined by trypan blue staining, resuspended in 200 μL PBS. After 7 days, when tumors of ~0.2 to 0.3 cm^3 in volume were detectable, mice were randomized in two groups (n = 10). One group received green tea, prepared from commercially available (China or Sri Lanka) green tea leaves (12.4 g/L), as source of drinking fluid for 60 days. The other group (control) was provided with water *ad libitum*. One experiment was terminated after 40 days to allow the harvesting of tumor xenografts for immunohistochemical and Western blot analysis. Tumor size was measured with calipers and was calculated using the formula: π / 6 × larger diameter × (smaller diameter)^2.

Immunohistochemical Analysis

Indirect immunoperoxidase staining was carried out on acetone-fixed 4-μm frozen tissue sections stored at −20°C as described previously (15). Briefly, sections were incubated with monoclonal rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule-1; generously donated by Dr. A. Mantovani, Mario Negri Institute, Milan, Italy) and anti-Ki-67 monoclonal antibody (clone MIB1; Ylem, Rome, Italy). The avidin-biotin assays were done using the Vectastain Elite kit (for nonmurine primary antibodies) and the mouse on mouse kit (for murine primary antibodies) obtained from Vector Laboratories (Burlingame, CA). Mayer's hematoxylin was used as nuclear counterstain. Negative control stain was represented by sections in which the incubation with the primary antibody was either omitted or substituted by isotype-matched immunoglobulins. The evaluation of microvessel density was done by two independent observers on a ×20 field. Areas containing the highest numbers of capillaries and small venules were identified by scanning at lower power. Ki-67 positivities were expressed as tumor cells with nuclear staining counted in three separate ×40 microscopic fields (at least 400 cells per field were counted).

Statistical Analysis

Results are representative of at least three independent experiments each done in triplicate. All statistical analysis was assessed using a two-tailed Student's t test and by two-way ANOVA as appropriate.

Results

Effect of EGCG on Cell Growth and Apoptosis

EGCG, the green tea polyphenol, is known to reduce the growth of various epithelial cancer cells, including those of

breast and prostate cancer (16). The effects of EGCG on ovarian carcinoma cell growth inhibition was examined in HEY and OVCA 433 cells treated with EGCG for 24 hours. As is evident from Fig. 1, increasing concentration of EGCG resulted in increased inhibition of cell viability in both cell lines. Treatment with 10 μmol/L EGCG did not produce any significant effect; however, treatment of higher concentrations of EGCG (20-40 μmol/L) resulted in significant dose-dependent reduction in cell viability with an IC_{50} of 20 μmol/L.

To determine whether the growth inhibition that we observed was associated with apoptotic changes, HEY cells were analyzed for the presence of early apoptotic events on Annexin V staining. The number of apoptotic cells was determined as early apoptotic cells shown in lower right (LR) quadrant of the fluorescence-activated cell sorting histograms (Fig. 1B). After 24 hours of serum starvation, treatment of HEY cells with 10 and 20 μmol/L EGCG for 24 hours increased the number of early apoptotic cells from 10% to 18.6%, respectively. As expected, the induction of apoptosis was higher when cells were treated with EGCG for 48 hours (Fig. 1B). The number of early apoptotic cells was increased from 12% to 23% in cells treated with 10 and 20 μmol/L EGCG, respectively. Furthermore, analysis of DNA fragmentation after 20 and 40 μmol/L EGCG treatment in serum-free HEY cells showed a marked enhancement of nucleosomal ladder formation (data not shown). In view of the above-described effects of EGCG on apoptosis induction, we examined the effects of EGCG on the levels of apoptosis-related protein expression. As determined by Western blot analysis, treatment of HEY cells (Fig. 1C) with EGCG resulted in a dose-dependent reduction of the antiapoptotic protein Bcl-X_L expression after 24 hours of treatment. Moreover, treatment with EGCG caused a marked (2.0-fold increase with respect to untreated control cells) activation or cleavage of caspase-3 (19-17 kDa) in a dose-dependent manner. These observation further support the involvement of both Bcl-X_L and mitochondrial pathway in EGCG-induced apoptosis in ovarian carcinoma cells.

EGCG Inhibits ET_AR and ET-1 Expression

Sustained ET_AR signaling caused by an autocrine ET-1/ET_AR loop has been implicated in ovarian tumor growth and progression (2, 7). Previous studies with EGCG in other cell types indicated that it can inhibit activation of tumor growth-promoting receptors (24). Therefore, it was of interest to examine in the two ovarian cancer cell lines the effect of green tea or EGCG on cellular levels of ET_AR and ET-1. HEY cells, characterized previously to secrete high level of ET-1 in the range required for activation of ET_AR in an autocrine fashion, were treated with different concentrations of EGCG and total RNA, and whole-cell lysates were collected after 6 and 24 hours, respectively. Treatment with EGCG resulted in a significant dose-dependent inhibition of ET_AR (Fig. 2A-C) and ET-1 expression at mRNA (Fig. 2A and B) and protein (Fig. 2C) levels as shown by RT-PCR, Northern blot, and Western blot analyses, respectively. The inhibitory effects were evident at 5 μmol/L EGCG and reached the maximum at 40 μmol/L EGCG. The reduction of ET-1 expression was concomitantly with a decrease of ET-1 secretion in conditioned medium in both cell lines (Fig. 2D, *right*).

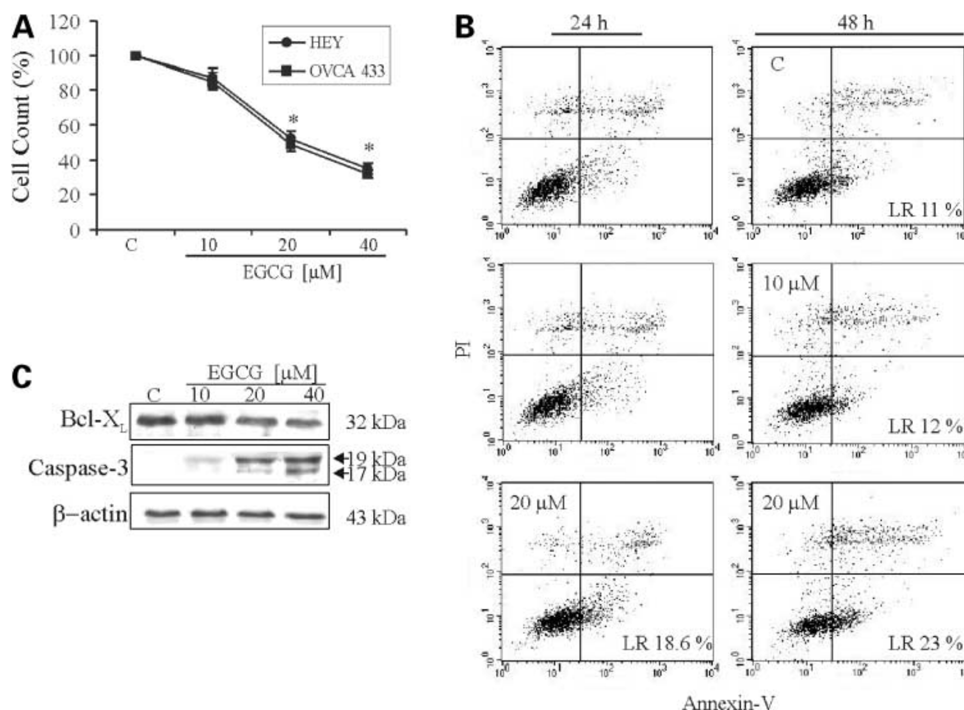


Figure 1. EGCG inhibits cell growth and induces apoptosis in ovarian carcinoma cells HEY and OVCA 433 cells treated with the indicated concentrations of EGCG for 24 h. Cell viability was assessed by trypan blue dye exclusion. Results are percentage of growth, with 100% representing control cells. Representative of at least three independent experiments each done in triplicate. *Bars,* SD. *, $P < 0.005$, compared with the control (**A**). HEY cells were treated with different concentrations of EGCG for 24 or 48 h and detection of apoptotic cells was evaluated by Annexin V and propidium iodide double staining and analyzed by flow cytometry (**B**). Expression of apoptotic proteins was analyzed with anti-Bcl-X_L and anti-caspase-3 by Western blot. Anti-β-actin was used as control (**C**).

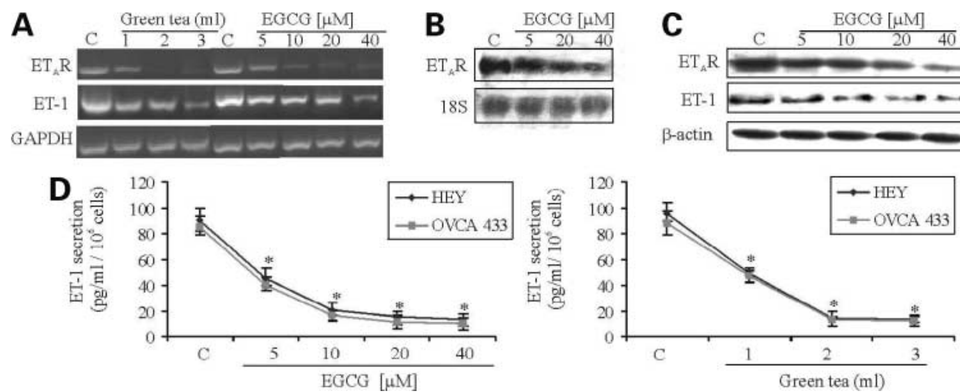


Figure 2. ET_AR and ET-1 expression after EGCG treatment. Serum-starved HEY cells were treated with the indicated concentrations of green tea or EGCG for 6 and 24 h and total RNA or proteins were collected, respectively. ET_AR and ET-1 mRNA expression was evaluated by RT-PCR (**A**), Northern blot (**B**), and Western blot (**C**). ET-1 protein secretion was measured by ELISA (**D**) in the conditioned medium of HEY and OVCA 433 cells treated for 24 h with the indicated concentrations of EGCG or green tea. Primers for the amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were used as controls for RT-PCR experiments and anti-β-actin was used as control for Western blot analysis. Representative of at least three independent experiments each done in triplicate. *Bars,* SD. *, $P < 0.005$, compared with the control.

Moreover, we found that increased doses of green tea infusion resulted in a dose-dependent reduction of ET_AR and ET-1 mRNA and ET-1 production in both HEY and OVCA 433 cells as shown by RT-PCR and ELISA, respectively (Fig. 2A and D, *left*). These results show that EGCG and green tea are able to reduce the expression of ET_AR and ET-1 in ovarian carcinoma cells.

EGCG Inhibits the Basal and the ET-1-Induced Ovarian Carcinoma Cell Proliferation

To determine whether the reduction of ET_AR and ET-1 expression resulted in an inhibition of ET-1/ET_AR-induced mitogenic activity, the EGCG effect was examined on the basal and ET-1-induced cell proliferation. HEY (Fig. 3A) and OVCA 433 (Fig. 3B) cells displayed a characteristic ET-1-dependent induction of cell proliferation as measured by [^{3}H]thymidine incorporation. As shown in Fig. 3A and B, treatment with EGCG resulted in a concentration-dependent reduction in the basal and ET-1-induced HEY and OVCA 433 cell proliferation, suggesting that the susceptibility of ovarian carcinoma cell lines to EGCG may involve the blocking of ET_AR/ET-1 autocrine signaling pathway.

EGCG Reduces ET_AR-Dependent Signaling Pathways

Activation of ET_AR by ET-1 triggers multiple signal transduction pathways that include MAPK- and PI3K-dependent pathways (3, 4). We, therefore, evaluated whether the EGCG-dependent effects were associated with reduced ET_AR-driven downstream signaling pathway in HEY cells. As expected, ET-1 treatment induced a rapid phosphorylation of p38 and p42/p44 MAPKs and AKT as assessed by the use of specific antibodies that recognized the phosphorylated form of these kinases (Fig. 3C). When HEY cells were treated with increasing concentrations of EGCG, the basal and ET-1-induced activation of p38 and

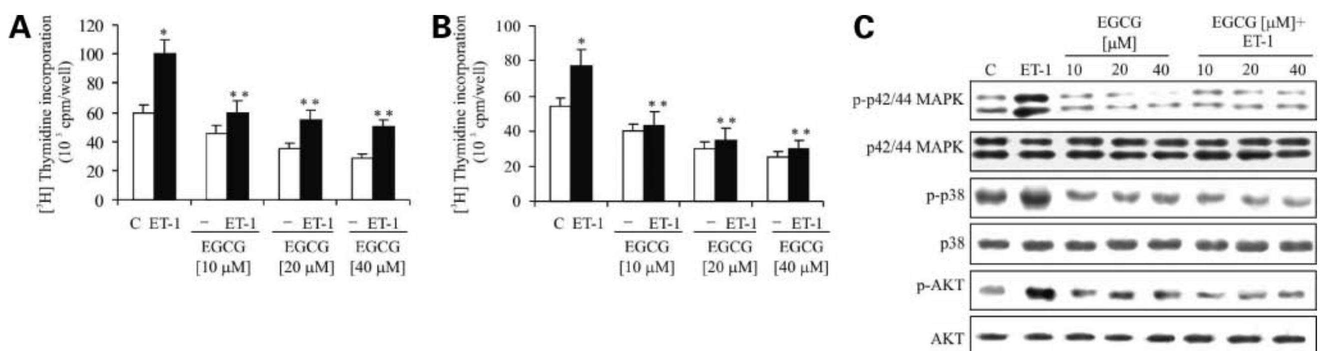


Figure 3. EGCG effects on ET-1-induced ovarian carcinoma cell proliferation. EGCG reduces ET_AR-dependent signaling pathways. Serum-starved HEY (**A**) and OVCA 433 (**B**) cells were treated with the indicated concentrations of EGCG alone or in combination with 100 nmol/L ET-1 for 24 h and cell proliferation was analyzed. *Bars,* SD. *, $P < 0.005$, compared with the control; **, $P < 0.001$, compared with ET-1. Serum-starved HEY cells were treated with the indicated concentrations of EGCG for 30 min before stimulation with 100 nmol/L ET-1. After 5 min, whole-cell lysates were blotted for detection of total and activated p42/p44 MAPK, p38 MAPK, and AKT by using specific antibodies (**C**).

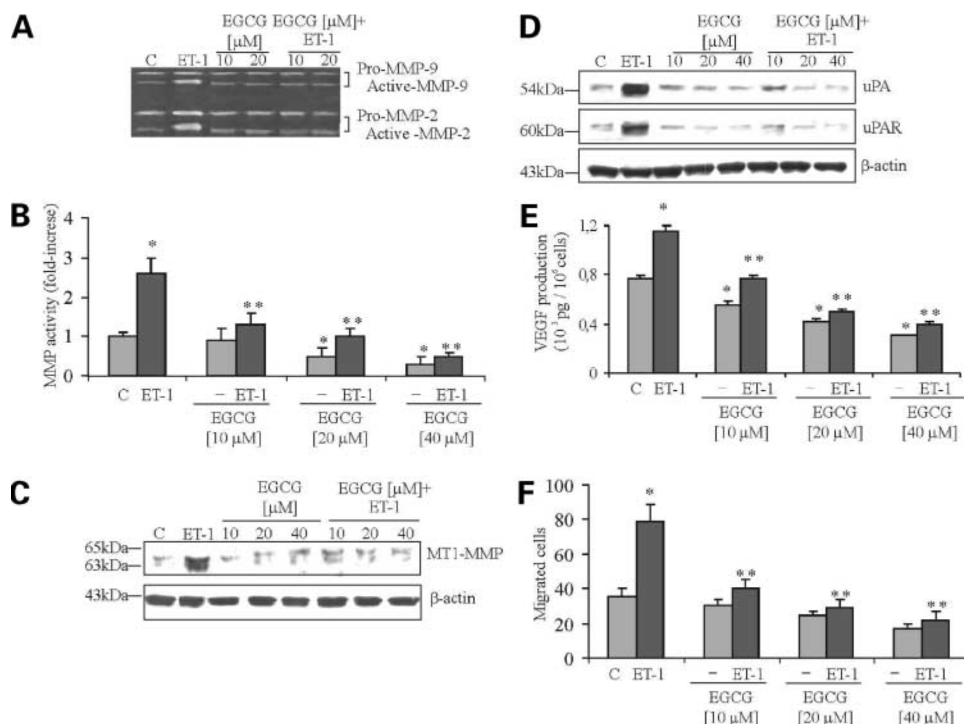


Figure 4. EGCG effects on ET-1-induced cell invasion. Serum-starved HEY cells were treated with different concentrations of EGCG alone or in combination with 100 nmol/L ET-1 for 24 h. Gelatin zymography was used to determine the activities of MMP-2 and MMP-9 (**A**). MMP activity was also measured by a MMP Gelatinase Activity Assay kit. *Bars,* SD. *, $P < 0.005$, compared with the control; **, $P < 0.001$, compared with ET-1 (**B**). MT1-MMP (65-kDa latent form and 63-kDa active form) expression and activation was measured by Western blot (**C**). Conditioned medium was tested for uPA and uPA receptor (*uPAR*) by Western blotting (**D**) or for VEGF secretion by ELISA (**E**). The relative amounts of protein were normalized with β-actin (**D**). *Bars,* SD. *, $P < 0.001$, compared with the control; **, $P < 0.005$, compared with ET-1 (**E**). Cell invasion was measured using a Boyden chamber invasion assay (**F**). *Bars,* SD. *, $P < 0.005$, compared with the control; **, $P < 0.001$, compared with ET-1.

p42/p44 MAPKs and AKT was dose-dependently inhibited. In contrast, ET-1 or EGCG treatment did not altered the total level of p38, p42/p44 MAPKs, and AKT proteins (Fig. 3C).

EGCG Inhibits ET-1-Induced VEGF Production, Tumor Proteinase Activation, and Invasion in Ovarian Carcinoma Cells

MMPs and MT1-MMP, which hydrolyzes type I collagen and activates MMP-2, are highly relevant in angiogenesis as well as in tumor cell invasion and metastasis (6). Because it has been reported previously that EGCG inhibited invasion by directly decreasing MMP activity in different tumors (25), we investigated whether EGCG may inhibit ET-1-induced MMPs and MT1-MMP activity in ovarian carcinoma cells. Gelatin zymography revealed an increase in MMP-2 and MMP-9 activity after 24 hours in ET-1-stimulated HEY cell conditioned medium compared with that of untreated control cells. Pretreatment with EGCG reduced the basal and ET-1-induced MMP-2 and MMP-9 active forms (Fig. 4A). MMPs and MT1-MMP activity was also measured by MMP Gelatinase Activity Assay kit and by Western blot analysis, respectively (Fig. 4B and C). As shown in Fig. 4, concentrations of EGCG ranging from 10 to 40 μmol/L induced a dose-dependent inhibition of the basal and ET-1-induced MMP activity as well as MT1-MMP expression and activity. In addition, ET-1 induced over-expression of uPA and its receptor in ovarian carcinoma cells (6, 13). Treatment of HEY cells with EGCG prevented the stimulation of uPA and uPA receptor induced by ET-1 (Fig. 4D), confirming the inhibitory effect of green tea polyphenol on ET-1-mediated activation of tumor proteinases involved in ovarian tumor progression. In view that in ovarian carcinoma cells ET-1 induces expression of VEGF production and promotes cell invasion (13, 14), we examined the ability of green tea polyphenol to modulate a marker of angiogenesis and invasion, such as VEGF. The effects of EGCG on ET-1-induced VEGF were analyzed in HEY cells treated for 24 hours with different concentrations of EGCG. Treatment with EGCG resulted in a significant reduction of the basal level and ET-1-induced VEGF protein secretion as measured by ELISA. As shown in Fig. 4E, 10, 20, and 40 μmol/L EGCG treatment resulted in a 33%, 56%, and 65% reduction in ET-1-induced VEGF production, respectively. Because ET-1 has a key role in the process of invasiveness by promoting migration and invasion through extracellular matrix barriers (6, 13), chemoinvasion assay was used to measure the effects of EGCG on untreated cells and in response to ET-1. Treatment with EGCG significantly ($P < 0.001$) inhibited in a dose-dependent manner the basal and ET-1-induced invasion of HEY cells (Fig. 4F). These results show that EGCG is able to reduce the expression of invasive determinants, such as MMP, uPA, and VEGF, in ovarian carcinoma cells resulting in inhibition of cell invasion.

Green Tea Reduces HEY Xenograft Tumor Growth

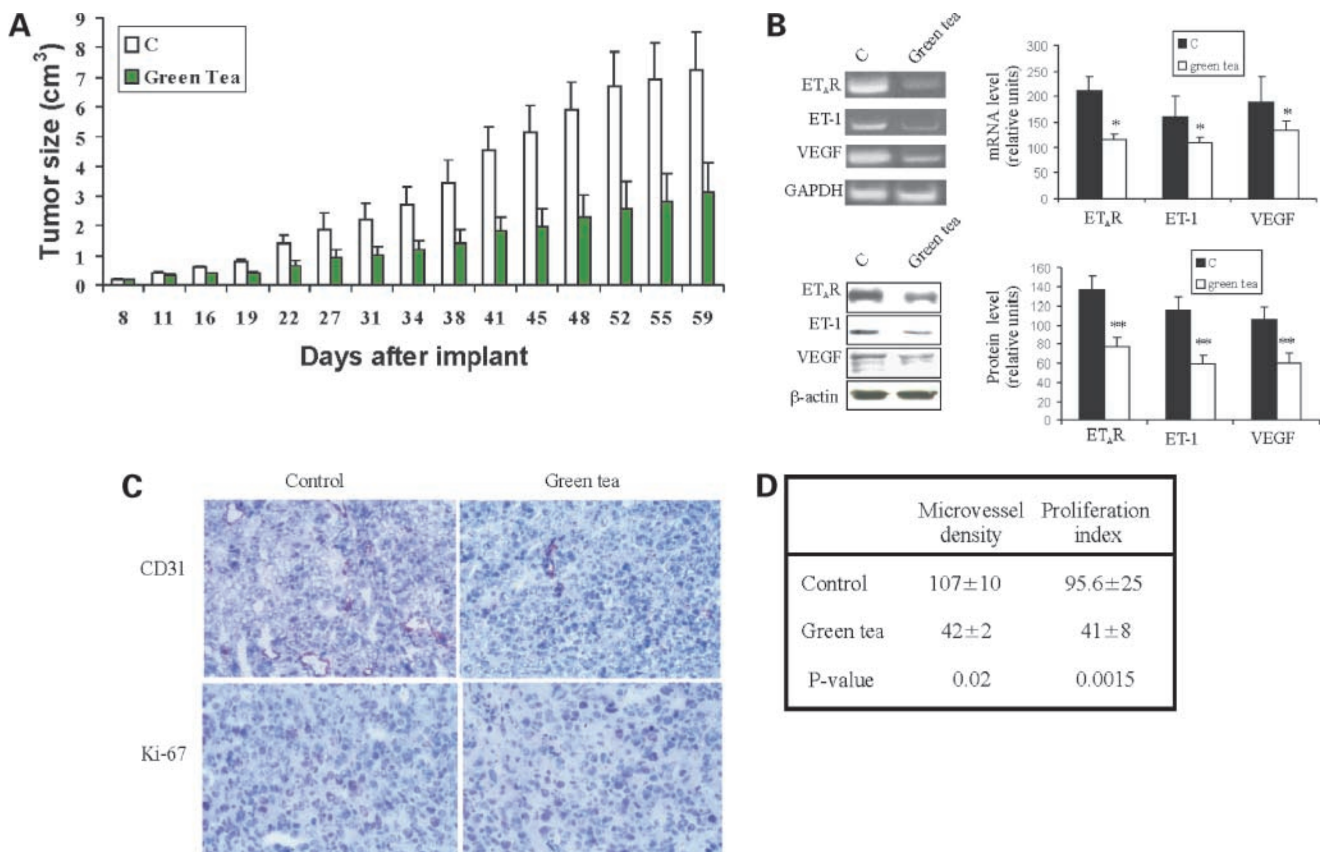
Given the ability of EGCG and green tea to suppress ET-1-induced ovarian cancer cell proliferation and proangiogenic factor production *in vitro*, we examined whether green tea could inhibit ovarian carcinoma growth *in vivo*. Continuous daily treatment in drinking water with green tea extract, starting 7 days after HEY cell injection, was analyzed on nude mice bearing HEY ovarian carcinoma xenografts. Oral administration of green tea significantly ($P < 0.001$) reduced the tumor growth compared with

controls receiving drinking water alone (Fig. 5A). Mice treated with green tea showed a 60% reduction in tumor size with no signs of acute or delayed toxicity. As shown in Fig. 5B, green tea caused a significant reduction of ET_AR and ET-1 expression at both mRNA ($P < 0.005$) and protein ($P < 0.001$) levels as evaluated by RT-PCR and Western blot analysis on multiple samples ($n = 3$) of xenografts. We also observed a marked reduction of VEGF mRNA and protein expression in animals that received green tea (Fig. 5B).

To determine whether the reduction in tumor growth was associated with a reduction of angiogenesis and cell proliferation, tissue sections of HEY tumors on day 40 after tumor cell injection were analyzed by immunohistochemistry (Fig. 5C). As summarized in Fig. 5D, the Ki-67-positive tumor cells in untreated tumors ranged from 85.5 to 122.9 (median, 95.6) and were significantly ($P < 0.00015$) higher than in the corresponding green tea–treated tumors (range, 35.6-50.2; median, 41). Tumor-induced vascularization, which was quantified as microvessel density expression, in untreated xenografts ranged from 95 to 120 (median, 107) and were significantly ($P < 0.02$) higher than the corresponding values in treated xenograft (range, 50.2-34.1; median, 42). These results suggest that oral administration of green tea induces ovarian tumor growth and neovascularization inhibition that is associated with a reduced ET_AR and ET-1 expression *in vivo*.

Discussion

The limited treatment options of ovarian cancer have prompted the need for developing alternative strategies for the management of this disease. Chemoprevention and therapy by the use of green tea or green tea polyphenols have offered new approaches to block tumor growth and progression. Green tea extract, and especially its major polyphenolic component EGCG, is capable of inhibiting the growth of a variety of human cancer cells, including ovarian carcinoma, via induction of apoptosis *in vitro*



	Microvessel density	Proliferation index
Control	107±10	95.6±25
Green tea	42±2	41±8
P-value	0.02	0.0015

Figure 5. Antitumor activity of green tea on HEY human ovarian carcinoma xenografts. Mice were given injection of 1.5×10^6 HEY cells s.c. into the dorsal flank. After 7 d, mice received oral administration of green tea (12.4 g/L) for 60 d. Each group consisted of 10 mice. **A,** antitumor activity of green tea. *Bars,* SD. The comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.001$). The mice were sacrificed on day 40 after tumor injection and tumors were removed from control or green tea treated mice. The effects of the treatment with green tea on ET_AR, ET-1, and VEGF mRNA and protein expression were measured in multiple samples ($n = 3$) of xenografts by RT-PCR and Western blot, respectively. Densitometric analysis was evaluated by Scion image and results were expressed as relative units. *Bars,* SD. *, $P < 0.005$, compared with the control; **, $P < 0.001$, compared with the control (**B**). Immunohistochemical analysis of CD31 and Ki-67 expression in HEY tumor xenografts (**C**). Original magnification, ×20. **D,** quantitative assessment of immunohistochemical analysis for microvessel density and proliferation index.

(19, 23, 24, 26). The clinical relevance of this observation was underscored by the fact that green tea consumption is associated with a reduced risk of epithelial ovarian cancer in a dose-dependent manner and can enhance the survival of these patients (21, 22). In this study, we showed that EGCG rapidly induced apoptotic cell death in human ovarian carcinoma cells in association with the down-regulation of the antiapoptotic protein Bcl-X_L and the activation of caspase-3. Whereas cell cycle arrest and apoptosis have claimed to be responsible for the effect of EGCG and how the molecule targets the signal transduction pathway that inhibit growth and progression of the ovarian cancer cell remain to be further dissected.

The ET-1/ET_AR autocrine loop is overexpressed in primary and metastatic ovarian carcinomas compared with normal ovaries and contributes to ovarian cancer growth and progression (2–10, 13, 14), suggesting that the inhibition of ET_AR signaling pathway may improve ovarian cancer treatment (11). Thus, in human primary ovarian tumors, overexpression of the ET-1 axis and elevated ascites levels of ET-1 are associated with advanced-stage ovarian carcinoma (5, 8). ET-1 binds the ET_AR, a G-protein-coupled receptor that transduces signals to the nucleus primarily via the MAPK and PI3K/AKT pathway (2, 3). In addition, activation of ET_AR by ET-1 promotes PI3K-dependent integrin-linked kinase expression and activity. Dominant-negative integrin-linked kinase suppresses the ET-1-induced phosphorylation of glycogen synthase kinase-3β and AKT as well as *Snail* and β-catenin protein stability, transcriptional activity, and invasiveness, implicating for the first time integrin-linked kinase, and its downstream substrates glycogen synthase kinase-3β and AKT as checkpoints of finely tuned interconnected signals induced by ET-1/ET_AR to modulate EMT (5, 27). In this context, ET-1/ET_AR induces the disruption of normal host-tumor interactions regulating changes in cadherin, connexin, and MMP expression and activity, migration, and invasion (12–14). These findings complement and extend the recent analysis of a genome-wide expression profile of late-stage ovarian cancer, whereby ET-1 has been identified as a key gene that activates cell signaling controlling cell migration, spread, and invasion (28).

In this study, we showed that in ovarian carcinoma cell lines EGCG is able to inhibit ET-1 and ET_AR expression and downstream signaling pathways resulting in a reduction of cell proliferation, angiogenesis, and metastasis determinant expression. Remarkably, oral administration of green tea, in nude mice bearing ovarian tumor xenograft, induces a consistent reduction in tumor growth, concomitantly with an inhibition of ET-1, ET_AR, and VEGF expression and a reduction in vascular density.

Suppression of several tyrosine kinase receptors, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and VEGF, is now emerging as principal EGCG mechanism of action in several models (24, 26, 28–32). Our results indicate that in ovarian carcinoma cells EGCG is able to induce a dose-dependent reduction of the G-protein-coupled receptor ET_AR mRNA and protein expression. Moreover, treatment with green tea induces a dose-dependent reduction in ET_AR and ET-1 mRNA expression and ET-1 secretion comparable with that obtained after EGCG treatment. The major catechins in green tea are EGCG, epigallocatechin, and epicatechin. EGCG accounts for 50% to 80% of catechins representing 200 to 300 mg in a brewed cup of green tea. Recently, it has been shown that green tea or purified EGCG when given to mice restrained Kaposi's sarcoma tumor growth and angiogenesis. Green tea extract showed an equivalent, if not greater, effect in tumor growth inhibition *in vivo* than did purified EGCG, suggesting that EGCG may cooperate or synergize with other polyphenols green tea or that the other components of green tea may improve the stability or bioavailability of EGCG (25). Nevertheless, a study comparing the cellular and molecular effect of EGCG with the effect of polyphenon E, a standardized and well-characterized decaffeinated extract of green tea, shows that pure EGCG and polyphenon E (33), as well as caffeine (34), had similar potencies.

Several evidences indicate that EGCG may directly interact with kinases at multiple levels in cervical cancer cell lines (29) in breast carcinoma cell lines (17) and in head and neck squamous cell carcinoma cell lines (26). In ovarian carcinoma cells, we observed that the reduction of ET-1 and ET_AR expression by EGCG was associated with a dose-dependent inhibition of ET-1-induced downstream signaling cascade that involved both MAPK and PI3K/AKT pathways. Interestingly, EGCG reduces also the basal level of cell proliferation and p42/p44 and p38 MAPKs and AKT activation. These results are consistent with our previous reports showing that the interruption of ET-1/ET_AR autocrine loop, by selective ET_AR antagonist or ET_AR siRNA, blocks basal cell growth, survival, and invasion *in vitro* and *in vivo* (5, 15).

The natural history of most tumors is invariably characterized by the acquisition of migratory, invasive, and angiogenic phenotype (35). We reported previously that ET-1 was expressed in 84% of the ovarian carcinomas with a highly significant correlation with tumor grade, VEGF expression, and vascularization (8). Moreover, ET-1 contributes to the acquisition of migratory and invasive phenotype by inducing the disruption of host-tumor interactions (36) and by up-regulating protease (6) and VEGF expression (8, 9, 13, 14). Green tea polyphenols are becoming increasingly recognized for their antiangiogenic, antimetastatic (17, 18), and anti-invasive properties as potent inhibitors of VEGF production (32) and MMPs, such as MMP-2 and MMP-9, in different cell types (25, 37). Studies have shown that polyphenols from green tea possess antitumor and anti-metastatic activity in animal xenograft and allograft model, suggesting a possible therapeutic potential (17, 18). Here, we showed that EGCG induces apoptosis in human ovarian carcinoma cells influencing the signaling molecules involved in apoptotic pathway. Moreover, EGCG reduced the basal and ET-1-induced VEGF expression, MMPs and uPA/uPA receptor activity, and cell invasion, indicating that reduction of ET-1/ET_AR autocrine loop by EGCG may be responsible for the antiangiogenic and anti-invasive activity of EGCG in ovarian carcinoma.

Because all the molecular effectors involved in tumor invasiveness are triggered by ET_AR activation, blockade of ET_AR therefore represents a promising therapeutic target in ovarian carcinoma. Thus, we have shown previously that *in vivo* interruption of ET_AR autocrine pathway using an highly specific ET_AR antagonist results in growth reduction of tumor xenografts associated with a significant inhibition of microvessel density, expression of VEGF, MMP-2, and cyclooxygenase-2, increased tumor apoptosis, and modulation of EMT determinants (5, 14, 15).

In the present study, we show that the oral administration of green tea resulted in a significant inhibition of ovarian carcinoma growth. We observed that this treatment decreased the ET-1 and ET_AR expression that was associated with a substantial reduction of angiogenic and proliferative markers, such as VEGF expression, microvessel density, and proliferation index, in treated mice compared with controls. These findings indicate that green tea acts as an antitumor agent interfering with the ET-1/ET_AR autocrine signaling pathway that creates a tumor microenvironment more permissive to progression.

In regard to the EGCG bioavailability, pharmacokinetic studies in humans indicate the peak plasma concentration of EGCG is $\sim$0.2 to 1 μg/mL (38, 39), whereas our findings show that EGCG inhibits ET_AR activation and downstream signaling pathways at doses 10 to 40 μmol/L over a relatively short period of time. Nevertheless, doses of green tea or doses of EGCG equivalent to those consumed by heavy tea drinkers have been shown to exert antitumor effects in rodents (40). This may reflect the longer duration of exposure and/or tissue accumulation in the intact animal. Thus, during prolonged administration to patients, EGCG may accumulate in the plasma and/or tumor tissue.

All together, the present findings identify green tea polyphenol as a multifunctional agent that may be potentially useful in developing preventive and therapeutic protocols.

In conclusion, the present results suggest that EGCG inhibits the ET_AR-induced signaling, thereby inducing apoptosis and inhibiting the proliferation and invasion of ovarian cancer cells. We provide strong experimental ground for this concept by showing that green tea polyphenol suppresses tumor growth in ovarian carcinoma xenografts, thus validating ET-1/ET_AR axis as a critical target in ovarian cancer progression.

Because high levels of ET-1 axis are associated with several malignancies (11) the impairing of ET_AR signaling by green tea could be of broader therapeutic relevance.

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