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Green tea catechin inhibits ephrin-A1-mediated cell migration and angiogenesis of human umbilical vein endothelial cells☆

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Abstract

Angiogenesis, the formation of new blood vessels from preexisting capillaries, is essential for tumor progression and metastasis. During tumor neovascularization, vascular endothelial growth factor and ephrin (Eph) families emerge as critical mediators of angiogenesis.

The green tea catechin epigallocatechin gallate (EGCG), a tyrosine kinase inhibitor, has been demonstrated in previous studies to be an effective antiangiogenesis agent. However, the inhibitory effect of green tea catechins on ephrin-A1-mediated tumor angiogenesis has not been demonstrated yet. Thus, in this study, we investigated the molecular mechanism of ephrin-A1-mediated cell migration and angiogenesis, as well as the inhibitory effects of EGCG.

Here we show that ephrin-A1 mediates endothelial cell migration and regulates vascular remodeling in tumor neovascularization in vitro. We also demonstrated that ephrin-A1-mediated cell migration required the activation of extracellular-regulated kinase (ERK-1/2) but not of phosphatidylinositol-3-kinase. The green tea catechin EGCG inhibited ephrin-A1-mediated endothelial cell migration, as well as tumor angiogenesis, in a dose-dependent manner. Furthermore, EGCG inhibited the ephrin-A1-mediated phosphorylation of EphA2 and ERK-1/2.

Taken together, these data indicated that activation of ERK-1/2 plays an essential role in ephrin-A1-mediated cell migration. EGCG inhibited ephrin-A1-mediated endothelial migration and angiogenesis. It suggests a novel antiangiogenesis application of EGCG in cancer chemoprevention.

Keywords: Epigallocatechin gallate; Ephrin-A1; EphA2; PI-3K; ERK-1/2; Cell migration; Angiogenesis

1. Introduction

Angiogenesis, the formation of new blood vessels from preexisting capillaries, is essential for tumor progression and metastasis, and consists of a multistep process involving a diverse array of molecular signals [1–3]. During tumor neovascularization, multiple processes (including the stimulation of endothelial cell proliferation, migration and assembly; the recruitment of perivascular cells; and extracellular matrix modeling) are involved. Three families of receptor tyrosine kinases (RTKs) — vascular endothelial growth factor (VEGF), angiopoietin and ephrin (Eph) — emerge as critical mediators of angiogenesis [4,5].

The Eph family of RTKs and their ligands play crucial roles in neuronal targeting, embryonic patterning and vascular development [6,7]. With the disruption of EphB2, EphB4 results in defects in primary capillary network remodeling and subsequent patterning defects in the embryonic vasculature [8–10]. These results suggested that Eph RTKs and their ligands are crucial for vascular development during embryogenesis. The A-class ligand ephrin-A1 has also been implicated in angiogenesis. Ephrin-A1 has been identified as an inducible gene of several growth factors, such as tumor necrosis factor α (TNF-α) and VEGF, in human umbilical vein endothelial cells (HUVECs) and is expressed in the developing vasculature during embryogenesis [11].

Moreover, ephrin-A1 induces endothelial cell migration and capillary assembly in vitro and angiogenesis in a corneal pocket assay in vivo, suggesting a role in the neovascularization of adult tissues [12,13]. Blocking EphA

☆ Any opinions, findings, conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Council.

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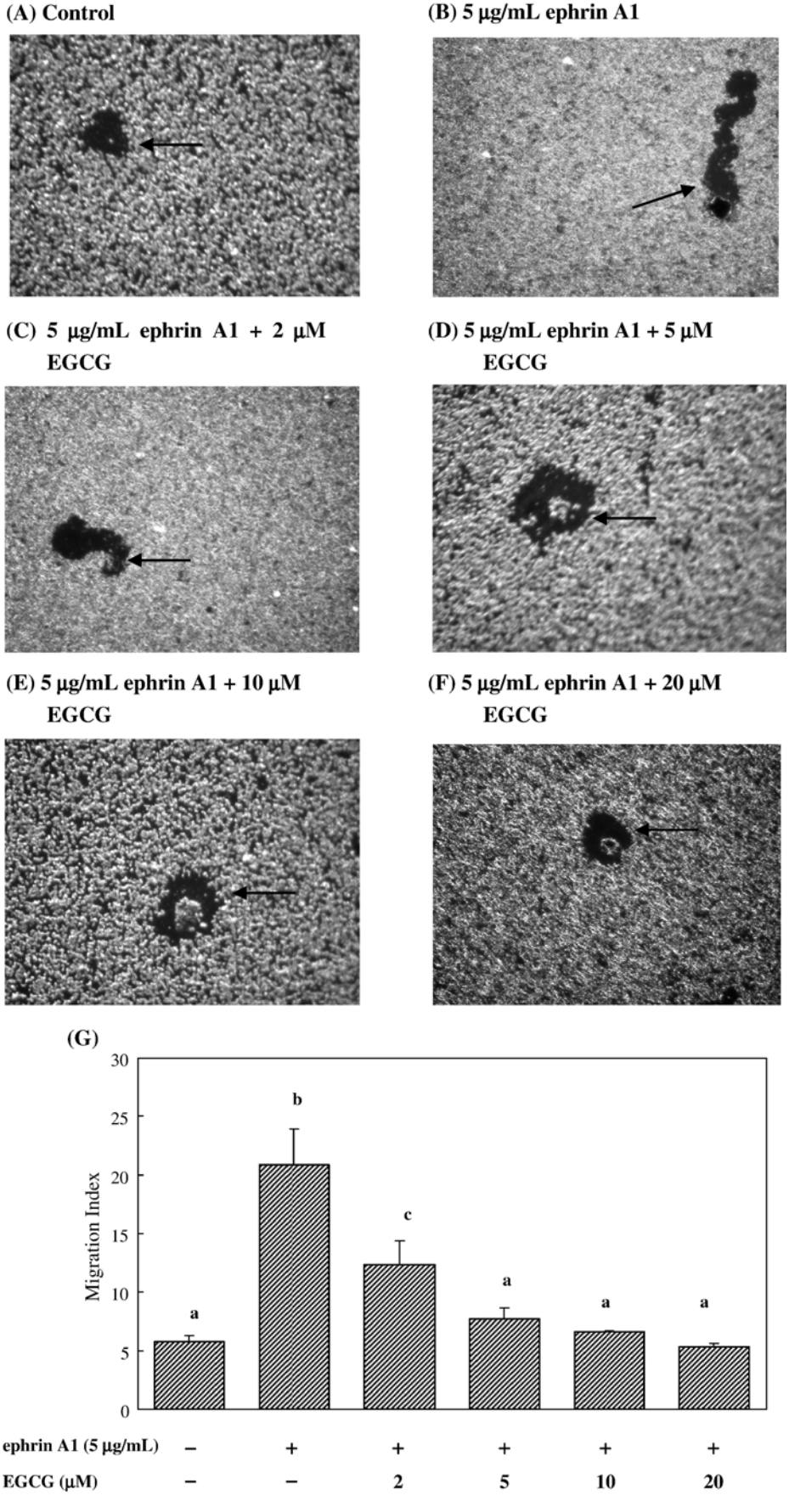
(A) Control
(B) 5 μg/mL ephrin A1
(C) 5 μg/mL ephrin A1 + 2 μM EGCG
(D) 5 μg/mL ephrin A1 + 5 μM EGCG
(E) 5 μg/mL ephrin A1 + 10 μM EGCG
(F) 5 μg/mL ephrin A1 + 20 μM EGCG
(G)
Migration Index
0
5
10
15
20
25
30
a
b
c
a
a
a
ephrin A1 (5 μg/mL) – + + + + +
EGCG (μM) – – 2 5 10 20

receptor activation could impair tumor angiogenesis [14]. These studies indicate that Eph signaling is critical for normal blood vessel development, as well as for pathogenic angiogenesis. Recent studies showed that VEGF could up-regulate the expression of ephrin-A1 in activated endothelial cells [15]. Upon stimulation by angiogenic factors such as ephrin-A1 proteins, the ephrin-A1-mediated activation of EphA2 enhances the proliferation of endothelial cells. It has been suggested that activation of EphA2 might be required for angiogenesis.

Many RTKs are involved in endothelial cell proliferation and migration during tumor angiogenesis. Targeting angiogenesis represents a new strategy for the development of anticancer therapies. Endothelial cell response to anti-angiogenesis therapies such as the green tea catechin epigallocatechin gallate (EGCG), a tyrosine kinase inhibitor that targets several growth factors, including VEGF receptor and VE cadherin, has been demonstrated in previous studies [16]. However, the effect of green tea catechins on ephrin-A1/EphA2-mediated angiogenesis has not been demonstrated yet.

Here we show that ephrin-A1 mediated the migration and angiogenesis of human umbilical endothelial cells. EGCG inhibited ephrin-A1-mediated endothelial migration and angiogenesis, and played a novel role in antiangiogenesis application. It represents a potential new target for therapeutic intervention in pathogenic angiogenesis.

2. Materials and methods

2.1. Reagents and antibodies

The recombinant ephrin-A1 protein and anti-extracellular-regulated kinase (ERK) 1/2 monoclonal antibody, anti-EphA2 monoclonal antibody, was purchased from R&D Systems (Minneapolis, MN). Antiphosphorylation monoclonal antibody (PY20) was purchased from Santa Cruz Biotech (Santa Cruz, CA). The primary endothelial cells, HUVECs, were purchased from Cambrex BioScience Walkersville (Walkersville, MD). A MEK-specific inhibitor (PD098059) and a phosphatidylinositol-3-kinase (PI-3K)-specific inhibitor (wortmannin) were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Briefly, HUVECs (Passages 6–9) were grown to confluency for 5–7 days using an endothelial basal medium (EBM)-supplemented medium (Cambrex BioScience Walkersville).

2.3. Supplementation with green tea catechins

HUVEC monolayers were incubated with different concentrations (0, 2, 5, 10 and 20 μM) of EGCG (Sigma) for 18 h. For an efficient uptake of EGCG by HUVECs, EGCG was incorporated into fetal bovine serum (FBS) for 30 min and mixed with phenol-red-free M-199 medium. After incubation, the cells were washed with phosphate-buffered saline (PBS) prior to the induction of cell migration by ephrin-A1.

2.4. Immunoprecipitation and Western blot analysis

Serum-starved HUVECs were stimulated with ephrin-A1 (5 μg/ml) in the presence or in the absence of EGCG for 30 min. Cells were lysed in a buffer containing: 1× PBS, 1% Ipegal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with 100 μM phenylmethylsulfonyl fluoride, aprotinin and the specific phosphatase inhibitor sodium orthovanadate. Cell lysates were cleared by centrifugation. Supernatants were transferred to fresh tubes for immunoprecipitation. To preclean nonspecific binding proteins, we first cleaned the supernatant with protein A Sepharose beads in the absence of antibodies. Total cell lysates were immunoprecipitated for 12 h at 4°C with anti-EphA2 monoclonal primary antibodies. Immune complexes were purified with protein A Sepharose beads and washed thrice in a lysis buffer. Immunoprecipitates were dissolved in Laemmli's sample buffer and boiled for 5 min before being transferred to 10% SDS polyacrylamide gel electrophoresis (PAGE) for Western blot analysis. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked using 5% nonfat milk in PBS and 0.01% Tween-20, and blotted with antiphosphorylation antibodies, according to the manufacturer's instructions. Blots were stripped and reprobed with anti-EphA2 monoclonal antibody as loading control. The phosphorylation of ERK-1/2 was measured using anti-ERK-1/2 monoclonal antibodies and the same procedure described above.

2.5. Cell motility colloidal gold phagokinetic assays

Cell migration on fibronectin was examined using migration track assay, as previously described for computer-assisted analysis [17]. Briefly, approximately 1500 cells were plated onto coverslips coated with 10 μg of fibronectin and treated with 5 μg/ml ephrin-A1 in the presence of 0, 2, 5, 10 and 20 μM EGCG. HUVECs were allowed to migrate for 12 h. At the end of the assay, cells were fixed, and migration was examined under dark fields optics and then photographed.

Fig. 1. Effect of EGCG on ephrin-A1-mediated endothelial cell migration. HUVECs, cultured in EGM with 2% FBS in a tissue culture dish, were lifted off by trypsinization, pelleted by centrifugation and resuspended in an EGM medium. Approximately 1500 cells were seeded on each prepared fibronectin-coated gold salt-covered coverslips. HUVECs were then stimulated with ephrin-A1 (5 μg/ml) in the EGM medium with or without 2, 5, 10 and 20 μM EGCG for 12 h until the measurement of cell migration. Incubation was stopped after 12 h by fixing the cells with 0.1% formaldehyde in PBS. The analysis of cell migration was described in Materials and Methods. (A–F) Representative photographic images. (G) MI of HUVECs under the stimulation of ephrin-A1 with or without the presence of EGCG. MIs were measured as described under Materials and Methods. The values are presented as the mean±S.E.M. of MI in 20 randomly selected fields in each culture dish. Similar results were observed from three independent experiments. Different letters represent statistically significant differences ($P<.05$).

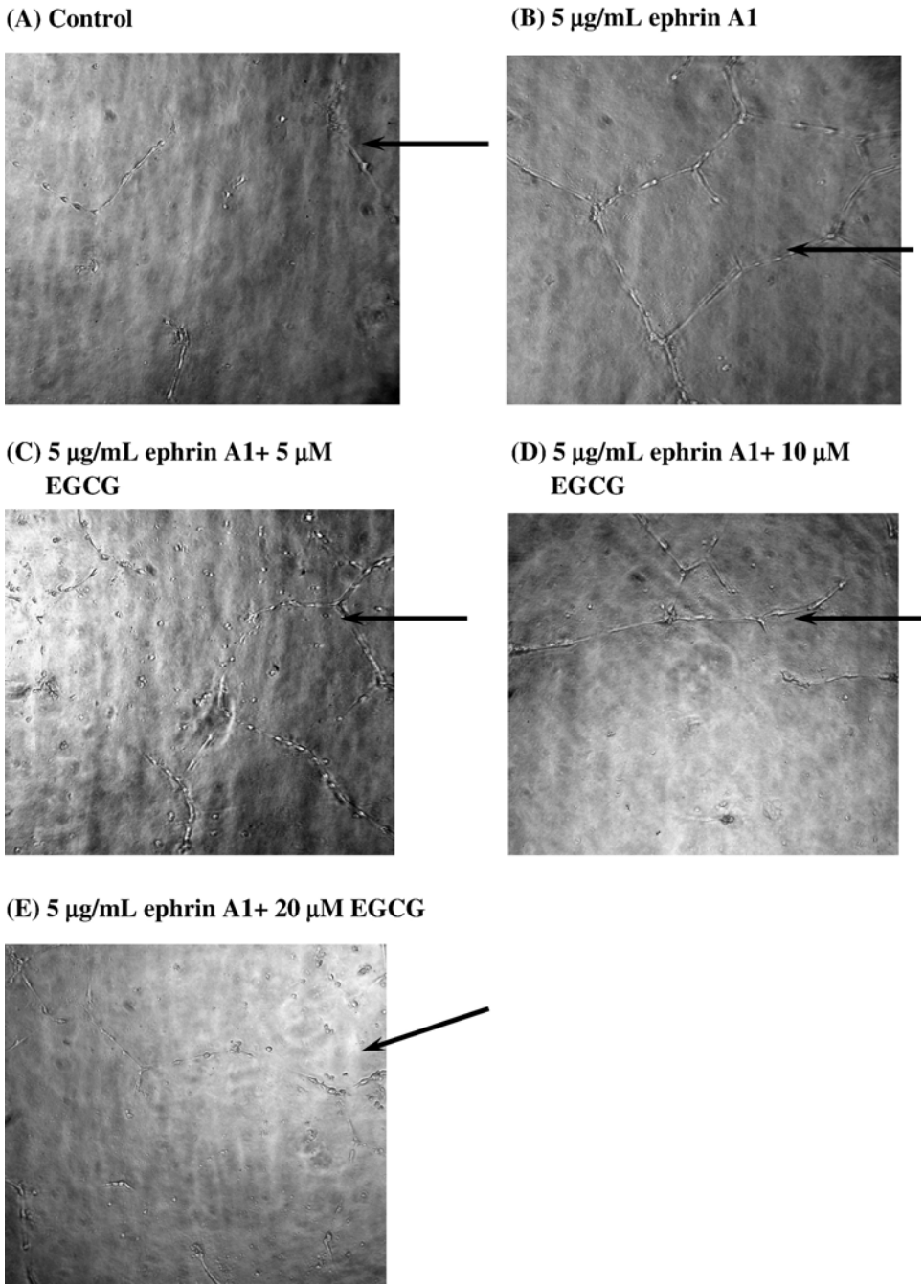

Fig. 2. Effect of EGCG on ephrin-A1-mediated angiogenesis. HUVECs, cultured in EGM with 10% FBS in a tissue culture dish, were lifted off by trypsinization, pelleted by centrifugation and resuspended in the EGM medium. Approximately 1×10^4 cells were seeded on 3-D Matrigel. HUVECs were then stimulated with ephrin-A1 (5 μg/ml) in the EGM medium with 0, 5, 10 and 20 μM EGCG for 48 h until the measurement of tubular formation. HUVECs were fixed with 0.5 ml of a glutaraldehyde/paraformaldehyde mixture (2.5%) and stained with modified May–Gruenwald's solution (0.25%). Tubular structure formations on 3-D Matrigel were visualized under an inverted phase-contrast microscope (×200), and photomicrographs were documented by a Nikon CoolPix digital camera. (A–E) Representative photographic images. Tubular structure formations were indicated with arrows.

Twenty randomly selected and nonoverlapping fields under each experimental condition were analyzed with an attached camera and a computer using the NIH Image 1.6 analyzer program (Scion, Frederick, MD). On a fibronectin-coated colloidal gold substratum, each black gold particle-free track represents a migration track made by a single cell. The system calculated the percentage of the total field area viewed by a camera that was consumed with linear cell migration tracks.

2.6. Angiogenesis assay

HUVECs were cultured in an EBM-supplemented medium that was changed every 2 days. For tube formation

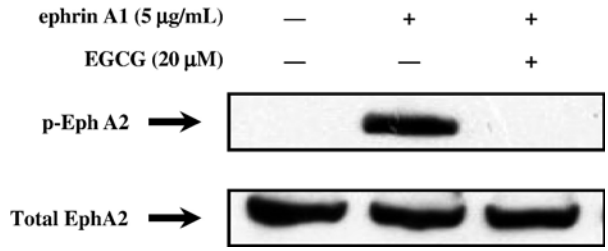

Fig. 3. Effect of EGCG on ephrin-A1-mediated EphA2 phosphorylation. Postconfluent HUVECs cultured on T-75 flasks were incubated in M-199 plus 0.1% FBS with 20 μM EGCG at 37°C for 18 h. After washing out the medium, HUVECs were incubated in M-199 plus 1% FBS with 20 μM EGCG and were stimulated by ephrin-A1 (5 μg/ml) at 37°C for 30 min. Total cell lysates were immunoprecipitated with anti-EphA2 antibody and blotted with antityrosine antibody, as described in Materials and Methods. The levels of detection in coimmunoprecipitated products represent the amount of tyrosine-phosphorylated EphA2 in HUVECs. The blots were stripped and reprobed with anti-EphA2 polyclonal antibody as loading control. The results presented are representative of two different experiments. Immunoreactive bands are noted with arrows.

assay, a confluent monolayer of HUVECs from Passages 6–9 was cultured in phenol-red-free M-199 medium on 3-D Matrigel. The Matrigel was diluted to a concentration of 4 μg/μL, and the pH was neutralized by adding phenol-red-free M-199. Aliquots of 100 μL were added to 24-well culture plates and incubated at 37 °C until gelatinization occurred. HUVECs were seeded on Matrigel-coated 96-well plates (1×10^4 cells/well) to form confluent monolayers using phenol-red-free M-199 (pH 7.4) containing 10% FBS, 9 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. After the stimulation of monolayers in 96-well plates with ephrin-A1 for 48 h (see below), HUVECs were fixed with 0.5 ml of glutaraldehyde/paraformaldehyde mixture (2.5%) and stained with modified May–Gruenwald's solution (0.25%). Tubular structure formations on 3-D Matrigel were visualized under an inverted phase-contrast microscope (×200), and photomicrographs were documented by a Nikon CoolPix digital camera (Tokyo, Japan). Tube formation was defined as straight cellular extensions joining two cell masses or at branch points.

2.7. Statistical analysis

The quantitative migration methodology was used to determine differences in migration capability between experimental sets of migrating endothelial cells and control sets of endothelial cells. In brief, statistical analyses of differences in migration capability among triplicate sets of experimental conditions were performed using Biostatistics software. The confirmation of differences in migration as being statistically significant requires the rejection of the null hypothesis, which states that there is no difference between the mean migration indices (MIs) obtained from replicate sets at the $P=.05$ level (Student's t test).

3. Results

3.1. Effect of EGCG on ephrin-A1-mediated endothelial cell migration

In the current study, we investigated the role of the ephrin-A1 pathway in the regulation of HUVEC migration and angiogenesis. To investigate intracellular signal transduction following attachment and migration in fibronectin, we examined the inhibitory effects of EGCG on the migration of HUVECs.

Here we showed that ephrin-A1 significantly enhanced vascular endothelial migration. At a concentration of 2 μM, EGCG suppressed ephrin-A1-induced migration by about 40%. At a concentration of 5 μM, EGCG suppressed ephrin-A1-induced migration by over 80%; at 20 μM, it completely abolished the effect of ephrin-A1 on HUVECs (Fig. 1F). We also found that EGCG blocked cell migration in fibronectin without compromising cell viability (data not shown). As shown in Fig. 1, on a fibronectin-coated colloidal gold substratum, unstimulated cells made few migration tracks (Fig. 1A). However, the cells made markedly linear migration tracks, leaving behind black gold particle-free tracks under the stimulation of ephrin-A1 (5 μg/ml) (Fig. 1B) (location of the cell inside each track is indicated by an arrow). In contrast, parallel cells incubated in a medium containing different concentrations of EGCG lost their ability to migrate to fibronectin in a concentration-dependent manner and completely stopped migrating in the presence of 2–20 μM EGCG (Fig. 1C–F). As mentioned above, the inhibition of cell migration was not due to a toxic effect of EGCG on cells under these conditions, as tested by trypan blue exclusion and cell proliferation assays (data not shown). To measure the level of cellular migration, MIs were analyzed as described under Materials and Methods. As shown in Fig. 1G, in the absence of EGCG, HUVECs migrated and produced MIs over 20. However, in the presence of EGCG, MIs were reduced dramatically in a concentration-dependent manner. These results suggested that EGCG-sensitive cellular targets are involved in ephrin-A1-driven signal transduction leading to HUVEC migration.

3.2. Effect of EGCG on ephrin-A1-mediated angiogenesis

To investigate subsequent actions following ephrin-A1-mediated endothelial cell migration, we further investigated the effect of ephrin-A1 on tubular formation and examined the inhibitory effects of EGCG.

Here we showed that ephrin-A1 induced the tubular structure formation of HUVECs in 3-D Matrigel (Fig. 2B) compared to unstimulated endothelial cells (Fig. 2A). We also found that ephrin-A1-induced angiogenesis was significantly inhibited in a dose-dependent manner when HUVECs were supplemented with increasing doses (5, 10 and 20 μM) of EGCG (Fig. 2C–E). Ephrin-A1-induced tubular structure formation was significantly inhibited at concentrations of 5 μM (Fig. 2C) and 10 μM (Fig. 2D), and was almost abolished at a concentration of 20 μM (Fig. 2E).

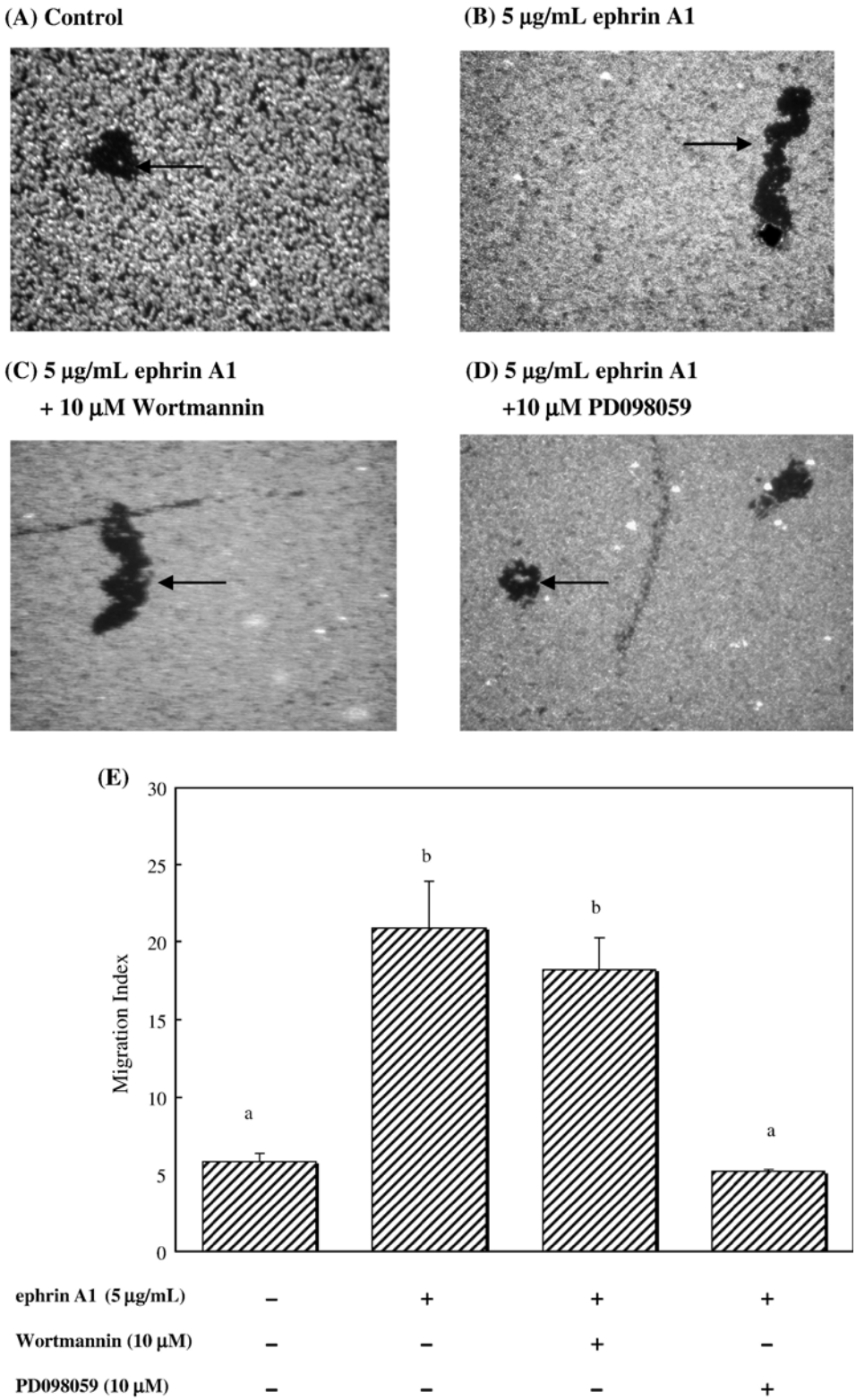

Fig. 4. ERK-1/2, but not PI-3K, is required for ephrin-A1-mediated HUVEC migration. HUVECs, cultured in EGM with 2% FBS in a tissue culture dish, were lifted off by trypsinization, pelleted by centrifugation and resuspended in the EGM medium. Approximately 1500 cells were seeded on each pre-prepared fibronectin-coated gold salt-covered coverslips. HUVECs were then stimulated with ephrin-A1 (5 μg/ml) in the EGM medium with or without 10 μM specific PI-3K inhibitor (wortmannin) and 10 μM specific MEK inhibitor (PD098059) for 12 h until the measurement of cell migration. The incubation was stopped after 12 h by fixing the cells with 0.1% formaldehyde in PBS. The analysis of cell migration was described in Materials and Methods. (A–D) Representative photographic images. (E) MIs were measured as described under Materials and Methods. The values are presented as the mean ± S.E.M. of MI in 20 randomly selected fields in each culture dish. Similar results were observed from three independent experiments. Different letters represent statistically significant differences ($P < .05$).

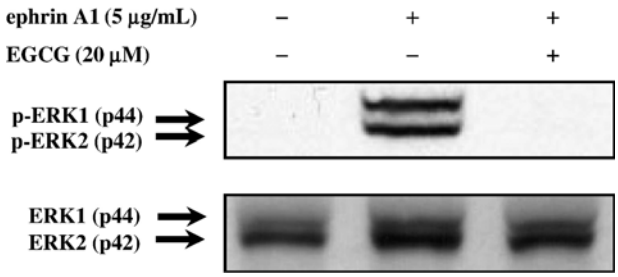

Fig. 5. Effects of EGCG on the ephrin-A1-mediated activation of ERK-1/2 pathways. Confluent HUVECs were incubated with 20 μM EGCG at 37°C for 18 h. After washing out the medium, HUVECs were then stimulated by ephrin-A1 (5 μg/ml) for 30 min. Total cell lysates were immunoprecipitated with anti-ERK-1/2 antibody and blotted with antiphosphorylation antibody as described in Materials and Methods. The values represent the amount of ERK-1/2 phosphorylation in HUVECs. The blots were stripped and reprobed with ERK-1/2 antibody as loading control. Each experiment was repeated twice. The photographs of immunoreactive bands are noted with arrows.

These results suggest that EGCG could inhibit ephrin-A1-mediated HUVEC cell migration and tumor angiogenesis.

3.3. Effect of EGCG on ephrin-A1-mediated EphA2 phosphorylation

Due to significant antiangiogenic activity, we further evaluate the molecular mechanisms by which EGCG inhibits cell migration and tube formation. To determine the inhibitory effect of EGCG on the ephrin-A1 (5 μg/ml) stimulation of EphA2 proteins, the expression of tyrosine phosphorylation in EphA2 was examined by immunoprecipitation. Our data have indicated that 20 μM EGCG was effective for its inhibitory action. As shown in Fig. 3, we found that ephrin-A1 was strongly inducted in tyrosine-phosphorylated states of EphA2 proteins. The inducing effect of ephrin-A1 on tyrosine phosphorylation was visible within 30 min in ephrin-A1-stimulated HUVECs, but not in unstimulated cells. On the other hand, when cells were preincubated with EGCG for 18 h prior to ephrin-A1 stimulation, the level of tyrosine-phosphorylated EphA2 markedly decreased. Thus, EGCG could inhibit the phosphorylation of EphA2 in HUVECs.

3.4. ERK-1/2, but not PI-3K, is required for ephrin-A1-mediated HUVEC migration

Although PI-3K has been found to be an important mediator of cell migration, we tested the role of PI-3K in HUVEC migration under conditions of cell stimulation by ephrin-A1. To determine whether the inhibition of cell migration by EGCG in ephrin-A1-stimulated HUVECs could be further regulated by the downstream PI-3K signaling pathway, the level of HUVEC migration was assessed by using the specific PI-3K inhibitor wortmannin. As shown in Fig. 4, ephrin-A1 significantly enhanced HUVEC migration by more than threefold (Fig. 4B) compared to that seen in unstimulated HUVECs (Fig. 4A). However, the treatment of HUVECs with 10 μM wortmannin could not inhibit ephrin-A1-mediated HUVEC migration (Fig. 4C). This finding suggests that some major pathways other than the PI-3K pathway are involved in ephrin-A1-mediated HUVEC migration.

Since ERK-1/2 MAPK has also been found to be an important mediator of cell migration, we first tested the role of ERK-1/2 in HUVEC migration under conditions of cell stimulation by ephrin-A1. To determine whether the inhibition of cell migration by EGCG in ephrin-A1-stimulated HUVECs could be further regulated by the downstream ERK-1/2 signaling pathway, the level of HUVEC migration was assessed using the MEK1-specific inhibitor PD98059. As shown in Fig. 4D, the treatment of HUVECs with 10 μM PD98059 effectively inhibited ephrin-A1-mediated HUVEC migration. These results showed that blockade of ERK-1/2 signaling almost completely reduced HUVEC cell migration (Fig. 4E).

3.5. Effects of EGCG on ephrin-A1-mediated activation of ERK-1/2 pathways

To determine the inhibitory effect of EGCG on the ephrin-A1 stimulation of ERK-1/2 proteins, the expression of tyrosine phosphorylation in ERK-1/2 was examined by Western blot analysis. Our dose–response data have indicated that EGCG was maximally effective for inhibitory action at 20 μM; we therefore tested EGCG only at this working concentration. As shown in Fig. 5, we found that ephrin-A1 was strongly inducted in tyrosine-phosphorylated states of ERK-1/2. The inducing effect of ephrin-A1 on tyrosine phosphorylation was visible within 30 min in ephrin-A1-stimulated HUVECs, but not in unstimulated cells. On the other hand, when cells were preincubated with EGCG for 18 h prior to ephrin-A1 stimulation, the increasing level of tyrosine-phosphorylated ERK-1/2 was markedly decreased. Thus, EGCG could inhibit the phosphorylation of ERK-1/2 in the presence of ephrin-A1.

4. Discussion

Since solid tumors are angiogenesis-dependent, the suppression of endothelial cells could contribute to overall tumor inhibition. Protein tyrosine kinase genes comprise the largest family of oncogenes. This is not surprising since tyrosine kinases are important components of signal transduction pathways that control cell shape, proliferation, differentiation, migration and angiogenesis. With 14 distinct members, Eph kinases constitute the largest family of RTKs [18]. Increasing evidence implicates Eph family proteins in cancer [6]. Ephrin-A1, the first Eph receptor ligand to be identified, was cloned from HUVECs as a gene induced by TNF-α [19]. Angiogenic activity for ephrin-A1 was demonstrated in vivo in a rat corneal angiogenesis assay [13]. Ephrin-A1, which has been shown to be an inducible gene of VEGF, is required for a microvascular network that is needed to supply oxygen and nutrients for the rapid growth of a tumor mass [15].

In the present study, we further investigated the signaling pathways involved in HUVEC cell migration and tumor angiogenesis using an in vitro model of tubular structure formation. The antitumorigenic properties of green tea catechins have been studied in many other studies [20–26]. In our previous studies, we also demonstrated that EGCG effectively inhibited VEGF-induced angiogenesis via several intracellular signaling pathways [16]. However, the detailed mechanisms of EGCG on ephrin-A1-mediated endothelial cell migration and angiogenesis are still not well known yet. To simulate the physiological effects of green tea consumption, we modified several significant experimental designs in this study by using a low level of green tea catechins (2–20 μM). We found that 5 μg/ml ephrin-A1 increased cell migration in HUVECs. In the presence of ephrin-A1, each single HUVEC made a markedly linear migration track, leaving behind black gold particle-free tracks. However, cell migration was dose-dependently inhibited by EGCG treatment (Fig. 1C–F). At a concentration of 2 μM, EGCG suppressed ephrin-A1-induced migration by about 40%. At a concentration of 5 μM, EGCG suppressed ephrin-A1-induced migration by over 80%; at 20 μM, it completely abolished the effect of ephrin-A1 on HUVECs (Fig. 1F). By using the cell motility colloidal gold phagokinetic assay system, it allows us to quantitate the migration track and distance made by each single cell. It can also exclude the possibility of each migration track being made by multiple proliferating cells. We also tested the effect of ephrin-A1 on the proliferation of human vascular endothelial cells. It showed that ephrin-A1 did not influence cell proliferation. This result is consistent with previous findings [18]. It suggested that ephrin-A1 mediated cellular adhesion, attachment to extracellular matrices and migration.

We also found that 5 μg/ml ephrin-A1 stimulation induced vascular angiogenesis in HUVECs, whereas tubular structure formation was also dose-dependently inhibited by EGCG treatment (Fig. 2C–E). At concentrations of 5 and 10 μM, EGCG significantly suppressed ephrin-A1-induced tubular formation. At a concentration of 20 μM, EGCG completely abolished the effect of ephrin-A1-induced angiogenesis on HUVECs. In mouse experiments, green tea extract suppressed xenograft size and decreased tumor vessel density [26]. Yang et al. [23] recently reviewed the anticancer properties of green tea constituents. Thus, it is plausible that a mixture of these catechins, which are present in green tea, might be more effective than a single catechin in suppressing angiogenesis.

To further investigate the importance of the ephrin-A1/EphA2 signaling pathway, we also examined the effect of EGCG on the activation of EphA2. In this study, it showed that ephrin-A1 induced the activation of EphA2 RTK and effectively inhibited EGCG treatment (Fig. 3). EphA RTKs acts like other RTK family proteins and transduce signaling pathways via intracellular molecules (such as ras oncoproteins) and ERK-1/2 MAPK pathways. It is plausible that ephrin-A1 may mediate cell migration and angiogenesis via the regulation of one of these signaling pathways.

Recent studies reported that ERK-1/2 molecules support the proliferation of endothelial cells and the development of new capillaries [27–29]. Although the exact roles of ERK-1/2 in tubular structure formation are not well known, we speculate that a specific inhibitor blocks the active site of ERK-1/2 and prevents its downstream signaling pathways, which are required for the sprouting of blood vessels. In this in vitro model of cell migration, we found that a specific MEK inhibitor (PD098059) effectively inhibited ephrin-A1-induced endothelial cell migration. This blocking effect was not observed when the specific PI-3K inhibitor wortmannin was applied to these cells (Fig. 4). These results indicated the importance of ERK-1/2 in ephrin-A1-mediated cell migration.

Furthermore, the activation of ERK-1/2 was stimulated by ephrin-A1 treatment, whereas EGCG dose-dependently inhibited ERK-1/2 activity (Fig. 5). These results indicated that the blockade of ERK-1/2 activation is the major modulator for their effect on ephrin-A1-mediated cell migration. Collectively, current evidence indicates that ERK-1/2 activation is an important component in ephrin-A1-mediated endothelial migration and in the assembly of new blood vessels.

In the present study, we demonstrated for the first time that green tea catechin can inhibit ephrin-A1-induced endothelial migration and angiogenesis in vitro and that ephrin-A1 may phosphorylate EphA2. We also demonstrated that stimulation of HUVECs with ephrin-A1 increased ERK-1/2 phosphorylation, whereas EGCG supplementation suppressed ephrin-A1-induced ERK-1/2 activation. These results suggested that EGCG inhibits ephrin-A1-induced cell migration in endothelial cells. EGCG may specifically target and inhibit ephrin-A1-induced tyrosine phosphorylation cascade during tumor angiogenesis.

Cheng et al. [15] have observed that signals of tyrosine phosphorylation in the EphA2 complex markedly increased in ephrin-A1-stimulated endothelial cells. Thus, it is conceivable that changes in the tyrosine phosphorylation of EphA2 receptors are associated with changes in cell migration. While ephrin-A1, through phosphorylation signaling, would induce a pleiotropic response allowing endothelial cells to migrate, assemble into tubes and increase their vessel formation, our data indicate that green tea catechins counter this process, leading to the inhibition of angiogenesis. EGCG has been reported to inhibit inducible expressions of tyrosine phosphorylation in receptors such as platelet-derived growth factor receptor and fibroblast growth factor receptor. Most recently, EGCG was reported to inhibit VEGF receptor phosphorylation when it was simultaneously added to a culture with VEGF.

In conclusion, one of the mechanisms by which green tea catechins may exert their antiangiogenic effect is, in part, through the inhibition of ephrin-A1-mediated migration, as needed for vascular tube formation during

angiogenesis. These findings provide a novel mechanistic insight into the potential effects of green tea catechins on the reduction of angiogenesis and tumor growth. Our in vitro results are also consistent with the concept that the consumption of green tea, in which catechins are the major polyphenols, is associated with a reduced risk of cancer development, as evident from population studies and animal experiments showing the suppression of angiogenesis and cancer development.

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