Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound

Lyne Labrecque1, Sylvie Lamy1, Amélie Chapus1, Samira Mihoubi1, Yves Durocher2, Brian Cass2, Michel W. Bojanowski3, Denis Gingras1 and Richard Béliveau1,∗

1Laboratoire de Médecine Moléculaire, Hôpital Ste-Justine-Université du Québec à Montréal, Centre de Cancérologie Charles-Bruneau, 3175 Chemin Côte-Ste-Catherine, Montréal, Québec, Canada H3T 1C5, 2Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2 and 3Département de Neurochirurgie, Hôpital Notre-Dame, 1560 rue Sherbrooke Est Montréal, Québec, Canada H2L 4M1

The vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptors play essential and complementary roles in angiogenesis and combined inhibition of these receptors has been shown to result in potent antitumor activity in vivo. In this study, we report that ellagic acid (EA), a natural polyphenol found in fruits and nuts, inhibits VEGF-induced phosphorylation of VEGFR-2 in endothelial cell (EC) as well as PDGF-induced phosphorylation of PDGFR in smooth muscle cells, leading to the inhibition of downstream signaling triggered by these receptors. EA also specifically inhibited VEGF-induced migration of ECs as well as their differentiation into capillary-like tubular structures and abolished PDGF-dependent smooth muscle cell migration. Interestingly, EA presents a greater selectivity for normal cells than for tumor cells since the migration of the U87 and HT1080 cell lines were much less affected by this molecule. The identification of EA as a naturally occurring dual inhibitor of VEGF and PDGF receptors suggests that this molecule possesses important antiangiogenic properties that may be helpful for the prevention and treatment of cancer.

Introduction

Angiogenesis, the growth of novel capillaries from pre-existing vessels, plays a crucial role in tumor growth and metastasis (1). When deprived of oxygen or nutrients, tumor cells promote neovascularization by inducing the expression of angiogenic cytokines such as the vascular endothelial growth factor (VEGF). VEGF is an endothelial cell (EC) specific angiogenic cytokine that, through high affinity binding to its receptors such as VEGFR-2 (Flk-1/KDR), has been shown to induce EC proliferation and migration, vascular permeability, and to act as a crucial survival factor for EC (2). As a result, inhibition of VEGF-mediated signaling in EC has become a primary target for antiangiogenic strategies, and inhibitors directed against either VEGF or its receptor VEGFR-2 have been demonstrated to prevent vascularization and growth of a large number of experimental tumor types (3).

Although interference with VEGF-mediated signaling events is effective in preventing the early growth of neovessels, mature vessels from more established tumors are largely resistant to these inhibitors (4). These vessels are surrounded by periendothelial cells, such as vascular smooth muscle cells (vSMC) and pericytes, that stabilize the newly formed vasculature and promote endothelial cell survival (5). There is increasing evidence that secretion of platelet-derived growth factor-B (PDGF-B) by the EC and stimulation of the platelet-derived growth factor receptor-B (PDGFR-B) associated with vSMC/pericytes are crucial events in this process, leading to the recruitment and interaction of these cells with EC and resulting in the stabilization of the capillary wall (6). The importance of the PDGFR expressed by perivascular cells for the maintenance of tumor blood vessels was also illustrated by the observation that the combined inhibition of VEGF and PDGF receptors, by either simultaneous exposure to receptor-specific tyrosine kinase inhibitors or by an inhibitor with broad kinase specificity (SU6668), caused regression of established tumors (4,7). These studies highlight the need to identify novel molecules that inhibit both VEGF and PDGF receptor activities.

Ellagic acid (EA) is a naturally occurring phenolic constituent present in fruits and nuts, the highest levels of which are found in raspberries (8). Several research studies have identified EA as a potent anticarcinogenic and antiinflammatory compound. The mechanisms by which EA elicits these effects remain poorly understood, although it has been found to cause apoptosis in cancer cells (9), to prevent the binding of carcinogens to DNA (10), to inhibit tumor cell proliferation (11) and to interfere with some angiogenesis-dependent pathologies such as diabetic retinopathies (12). In this latter case, previous work from our laboratory on polyphenolic compounds, such as green tea catechins (13), has shown that some of these compounds act as potent inhibitors of VEGF receptor activity and that this effect is likely to have profound repercussions on tumor progression in vivo (13,14). In this study, we present evidence that EA exhibits antiangiogenic properties by specifically inhibiting VEGFR-2 and PDGFR activities and the phosphorylation of their substrates, leading to an inhibition of VEGF-induced EC migration and PDGF-induced smooth muscle cell migration, as well as to an inhibition of the morphogenic differentiation of EC into capillary-like structures. These results suggest that pleiotropic inhibition of key receptors involved in angiogenesis may represent an important mechanism involved in the chemopreventive properties of dietary-derived molecules such as EA.

Abbreviations: BAEC, bovine aortic endothelial cell; EA, ellagic acid; EC, endothelial cell; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HUVEC, human umbilical vein endothelial cell; PASMC, pulmonary aortic smooth muscle cell; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SIP, sphingosine-1-phosphate; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; vSMC, vascular smooth muscle cells.
Materials and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, Ontario, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT). Electroporation reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). EA was obtained from LKT (St Paul, MN). The polyclonal (C-1158) and monoclonal (A3) antibodies, used for precipitation and detection, respectively, of VEGFR-2 and the anti-PhosphoERK pAb (958) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phosphotyrosine mAb PY99 was also purchased from Santa Cruz Biotechnologies. Anti-phosphocaveolin mAbs (P-Tyr 14) and EGFR P10016) were from Transduction Laboratories (Lexington, KY) and a mAb against PLCγ-1 (clone D-7-3) was from Upstate (Charlottesville, VA). Anti-phosphoERK polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Bucks, U.K., Quebec, Canada). Human recombinant PDGF was obtained from R&D Systems (Minneapolis, MN). Micro bichinonic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich Canada.

VEGF production

Vascular endothelial growth factor (isofrom 165) was PCR-amplified from a pHlast/VEGF plasmid (Invivogen, San Diego, CA) and cloned into the pTT vector (15). VEGF was produced following large-scale transient transfection of human 293SFE cells in serum-free medium, as described previously (16). The recombinant protein was expressed by the transiently transfected cells and secreted into the medium. The culture was harvested 5 days after transfection, the medium was clarified by centrifugation at 10,000 rpm for 10 min and filtered through a 0.22 μm membrane. The clarified culture medium was loaded onto a heparin-Sepharose column and the bound VEGF was then eluted using an NaCl gradient in phosphate-buffered saline (PBS). A buffer exchange for final purification was performed using the micro bichinonic acid method (Pierce). A buffer exchange was performed using the micro bichinonic acid method (Pierce). For immunoprecipitation and Western blotting procedures, respectively. Human glioblastoma cells (U87) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and human fibrosarcoma cells (HT1080) were kindly provided by ConjuChem (Montréal, Québec, Canada); both cell lines were maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS and antibiotics. All cells were cultured at 37°C under a humidified atmosphere containing 5% CO2. For experimental purposes, cells were plated in 100-mm plastic dishes at 5000 cells/cm² and were grown to confluence before overnight serum starvation. Cells were treated with vehicle or with EA diluted in 0.1 N NaOH and stimulated with 50 ng/ml VEGF, PDGF or EGF, or with 1 μM S1P. U87-conditioned medium was obtained by collecting media from confluent U87 cells following 48 h serum starvation.

Immunoprecipitation and immunoblotting procedures

After treatment with cytokines, cells were washed once with PBS containing 1 mM sodium orthovanadate and were incubated in the same medium for 1 h at 4°C. The cells were solubilized on ice in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40 and 1% Triton X-100) containing 1 mM sodium orthovanadate. The cells were then scraped from the culture dishes and the resulting lysates were clarified by centrifugation at 10,000 g for 10 min. Protein concentrations were determined using the micro bichinonic acid method (Pierce). For immunoprecipitation studies, lysates were clarified by 1 h incubation at 4°C with a mixture of Protein A/Protein G-Sepharose beads. After removal of the Sepharose beads by low-speed centrifugation, identical amounts of protein (200 μg) from each sample were transferred to fresh tubes and incubated in lysis buffer overnight at 4°C in the presence of 2 μg/ml of specific antibodies. Immunocomplexes were collected by incubating the mixture with 25 μl (50% suspension) of Protein A-Sepharose beads (rabbit primary antibody) or Protein G-Sepharose (mouse primary antibody) beads, for 2 h. Nonspecifically-bound material was removed by washing the beads three times in 1 ml of lysis buffer containing 1 mM sodium orthovanadate and bound material was solubilized in 25 μl of 2-fold concentrated Laemmli sample buffer, boiled for 5 min and resolved by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked for 1 h at room temperature with TBS-Tween-20 (147 mM NaCl, 20 mM Tris-HCl, pH 7.5 and 0.1% Tween-20) containing 2% BSA and incubated overnight at 4°C with primary antibody. Immunoreactive bands were revealed after a 1 h incubation with HRP-conjugated anti-mouse or anti-rabbit antibodies and the signals were visualized with an ECL detection system.

Migration assays

Transwells (8-μm pore size; Costar, Cambridge, MA) were precoted with 0.5% gelatin-PBS by adding 600/100 μl in the lower/upper chambers for 24 h at 4°C. The transwells were then washed with PBS and assembled in 24-well plates. The upper chamber of each transwell was filled with 100 μl of cells (1.0 × 105 cells/ml) and the cells were allowed to adhere for 1 h. Cells were then treated for 2 h by adding 100 μl of 2-fold concentrated drug solution prepared in serum-free medium into the upper chamber and 600 μl of the drug solution into the lower chamber. Migration was initiated by adding VEGF (10 ng/ml), PDGF (10 ng/ml) or S1P (1 μM) to the lower chamber (EC and PASMC) or by replacing the medium with 600 μl of U87-conditioned medium containing drugs (tumor cells). The plate was placed at 37°C in 5% CO2–95% air for 4 h. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin and stained with 0.1% Crystal Violet–20% methanol (v/v). The migration was quantitated using computer-assisted imaging and data were expressed as the average density of migrated cells per four fields (50× magnification).

Matrigel endothelial cell tube formation assays

Matrigel (12.5 mg/ml) was thawed at 4°C and aliquots of 50 μl were quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37°C. The wells were then incubated for 18 h at 37°C with HUVEC (25,000 cells/well), which had previously been treated for 6 h with the indicated concentrations of EA. The formation of capillary-like structures was examined microscopically and pictures (50×) were taken using a Retiga 1300 camera and a Zeiss Axiosvert S100 microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse).

Results

EA inhibits VEGF-induced signaling in endothelial cells

Following oxygen deprivation, tumor cells induce the secretion of VEGF, which specifically binds to VEGFR-2 at the EC surface. We first investigated the effects of EA on VEGFR-2 activity. Quiescent BAECs were incubated for 18 h with different concentrations of EA, followed by a 1 min stimulation with 50 ng/ml of VEGF. Under these experimental conditions, we observed a significant inhibition (48%) of VEGF-induced phosphorylation of VEGFR-2 by 5 μM EA, which was nearly complete (83%) at a concentration of 10 μM (Figure 1A, top panel). Blotting of membranes with an antibody directed against VEGFR-2 showed that EA did not affect the amount of VEGFR-2 in the immunoprecipitates (Figure 1A, bottom panel).

We next investigated whether the inhibitory effect of EA on VEGFR-2 activity could be observed following shorter durations of treatment. BAECs were incubated with EA, at a concentration of 10 μM, for a period ranging from 15 min to 24 h. We observed that the inhibition of VEGFR-2 appeared after as little as 60 min of treatment with EA and that VEGFR-2 phosphorylation was completely inhibited after 120 min of incubation with the compound (Figure 1B).

Following VEGF-induced autophosphorylation of VEGFR-2, the receptor phosphorylates and interacts with several substrates, such as PLCγ-1 (17), resulting in the activation of intracellular signaling events, including Src family kinase-dependent tyrosine phosphorylation of caveolin-1 (18). We observed that, in accordance with VEGFR-2 inhibition, VEGF-induced phosphorylation of both proteins is inhibited.
by EA (Figure 1C), further suggesting inhibition of VEGFR-2 tyrosine phosphorylation by EA interfering with the signaling cascades triggered by the binding of VEGF to EC.

EA preferentially inhibits VEGF- and PDGF-mediated signaling pathways

We then investigated the effect of EA on the p42/44 MAPK pathway (ERK). ERK is involved in cell proliferation and is activated by several stimuli such as VEGF, PDGF, epidermal growth factor (EGF) and sphingosine-1 phosphate (S1P), a sphingolipid metabolite important in cell proliferation and cell survival. As shown in Figure 2, all these stimuli induce phosphorylation of ERK in control conditions. Following an 18 h treatment of BAEC with 10 μM EA, we observed a marked diminution of VEGF- and PDGF-induced phosphorylation of ERK, with 66 and 61% inhibition, respectively. Interestingly, no inhibition was observed for phosphorylation of ERK induced by EGF and S1P. These results suggest that EA preferentially interferes with signaling triggered by VEGF and PDGF.

We also investigated the effect of other naturally occurring plant phenols, structurally related to EA, on VEGF-induced phosphorylation of ERK. Ferulic and caffeic acids are phenolic compounds that are also found in red raspberry in association with EA, while polymerization of gallic acid leads to EA formation. We observed that, of the four studied phenolic compounds, EA alone inhibits ERK phosphorylation induced by VEGF (Figure 2B), suggesting that not all phenolic acids are as effective as EA at inhibiting growth factor-mediated signaling pathways.

EA inhibits PDGF-induced signaling in smooth muscle cells

As PDGF-induced phosphorylation of ERK is clearly inhibited by EA in ECs, we next investigated the effect of the polyphenol on perivascular cells using PASMC. Cells were serum-deprived for 24 h in the presence of various concentrations of EA, followed by PDGF stimulation. We observed that EA markedly inhibits PDGFR and ERK phosphorylation with an IC50 of ~2 μM (Figure 3A). Interestingly, this effect is very rapid since PDGFR phosphorylation is inhibited as early as 5 min following the addition of EA (Figure 3B).

We next compared the efficacy of EA in inhibiting PDGFR activation to that achieved with STI571 (Gleevec), a drug used...
clinically for the treatment of chronic myeloid leukemia and whose mechanism of action includes inhibition of PDGFR. PASMC were treated with various doses of STI571 and the extent of PDGFR phosphorylation was monitored by immunoprecipitation. As expected, STI571 inhibits PDGF-induced phosphorylation of PDGFR with an IC50 of ~0.3 μM (Figure 3C) (20). This indicates that EA, a naturally occurring molecule, is almost as effective as Gleevec at inhibiting the PDGFR receptor.

In order to determine whether EA could inhibit the activity of other growth factor receptors, we next investigated its effect on EGFR phosphorylation. Glioblastoma cells (U87), which contain high levels of this receptor, were incubated with EA, followed by EGF-stimulation and the extent of receptor stimulation was monitored by immunoprecipitation. No inhibition of EGFR or ERK phosphorylation was observed following treatment with EA (Figure 3D), again suggesting that EA preferentially inhibits VEGF and PDGF receptors.

EA specifically inhibits VEGF-induced and PDGF-induced migration of endothelial and smooth muscle cells

VEGF is known to be an important chemoattractant for ECs (21). To evaluate the effect of EA on EC migration, BAEC and HUVEC were allowed to adhere to gelatin-coated transwells and were incubated for 3 h with 10 μM EA before the addition of chemoattractant to the lower chamber. At this concentration of EA, no modification of the actin cytoskeleton architecture could be observed (data not shown). Under these conditions, we observed that both VEGF (Figure 4A) and S1P (Figure 4B) induce EC migration but that only VEGF-induced migration was inhibited by EA, both in BAEC and HUVEC. Similarly, we observed that EA markedly inhibited PDGF-induced migration of PASMC (Figure 4C), consistent with the strong inhibition of PDGFR phosphorylation in these cells.

We then investigated the effect of EA on tumor cell migration. U87 (glioblastoma) and HT1080 (fibrosarcoma) cells were treated for 3 h with 10 μM EA and subjected to migration induced by U87-conditioned medium. As shown in Figure 4D, we observed only a slight inhibition of HT1080 migration following EA treatment and a 40% inhibition of U87 migration, less than the inhibition observed for EC at this concentration. These results suggest that EA had a greater selectivity for normal than tumor cells.

EA inhibits the formation of capillary-like structures by ECs

To confirm the importance of EA in inhibiting angiogenesis, we examined its effect on Matrigel-induced morphogenic differentiation of ECs into capillary-like tubular structures, another widely used angiogenesis assay. As shown in Figure 5, HUVEC, in the absence of EA, formed well-defined tubular structures. Treatment of these cells with the indicated concentrations of EA for 6 h, before adhesion to Matrigel, affected tube formation. This process was inhibited by 38 and 46% following treatment with 5 and 10 μM of EA, respectively. These concentrations of EA have been shown to inhibit VEGF-induced phosphorylation of VEGFR-2 by 48 and 83%, respectively (Figure 1A). These results show that EA is effective at inhibiting EC differentiation, confirming the effect of EA on angiogenic events.

Discussion

The formation of new tumor-associated vessels by angiogenesis is a very complex process involving the interplay of several cytokines and their receptors. Although much effort has gone into the identification of compounds that specifically interfere with the effects triggered by a given cytokine...
there is now considerable evidence that effective inhibition of angiogenesis is likely to require the simultaneous inhibition of other pathways that contribute to neovascularization. Among these, the recruitment and stabilization of neo-vessels by perivascular cells, such as vSMC and pericytes, via PDGF-dependent signaling has emerged as an important event contributing to neovessel stability (22). Hence, the inhibition of both VEGF and PDGF receptors, by the combination of SU5416 (VEGFR-2) and STI571 (PDGFR), blocks further growth of end-stage and well-vascularized tumors, eliciting detachment of pericytes and disruption of tumor vascularity (4). Similarly, administration of SU6668, a kinase inhibitor possessing broad specificity, to mice bearing human tumor xenografts resulted in the regression of late-stage tumors (7).

The results presented in the current paper provide evidence that combined inhibition of these two important angiogenic receptors can also be achieved by dietary-derived molecules. We observed that EA, a polyphenol found in high quantity in fruits and nuts, interferes with EC-associated VEGFR-2 phosphorylation, resulting in the inhibition of the downstream signaling triggered by this receptor and in the inhibition of two key events underlying angiogenesis, i.e. EC migration and morphogenic differentiation into capillary-like structures. In parallel, EA showed potent inhibitory activity against perivascular cells through its inhibition of PDGFR activity and signaling, leading to an inhibition of vSMC migration.

Fig. 4. Effect of EA on cell migration. (A and B) Endothelial cells (BAEC, HUVEC), (C) smooth muscle cells (PASMC) and (D) tumor cells (U87, HT1080) were allowed to adhere to transwells coated with gelatin. Cells were treated in the presence or absence of 10 μM EA for 2 h and migration was initiated by adding (A and B) 10 ng/ml of VEGF or 1 μM of S1P, (C) 10 ng/ml of PDGF or (D) U87-conditioned medium to the lower chamber and the plate was placed at 37°C for 4 h. The number of cells that crossed the membrane was compared with that observed with untreated cells. Results are means ± SD of three independent experiments.

Fig. 5. Effect of EA on the Matrigel-induced capillary-like structure formation by HUVEC. HUVEC were treated for 6 h with the indicated concentrations of EA and cultured for 18 h on Matrigel. The length of the capillary network was quantified as described in Materials and methods. Results are representative of three independent experiments.
These appear relatively specific since EA had no effect on the potent induction of EC migration induced by S1P and was ineffective in inhibiting EGFR activity in tumor cells. Moreover, EA affected tumor cell migration to a much lower extent than perivascular cells since the concentration of EA leading to complete inhibition of VEGF-induced migration of ECs had no significant effect on that of tumor cells. These results thus suggest that ECs and smooth muscle cells had greater sensitivity to EA than did tumor cells and provide substantial evidence that interference of EA with both perivascular and vascular cell functions, through VEGF and PDGF receptor blockades, is likely to contribute to the antiangiogenic effects of this molecule.

EA inhibited VEGF- and PDGF-dependent receptor phosphorylation with IC_{50} values of 5 and 2 μM, respectively, which are in the same range as the values for SU6668, which inhibits VEGF- and PDGF-dependent signaling with IC_{50} of 0.5 and 1 μM, respectively (7). These results provide further support to our previous observation that some polyphenolic compounds, such as green tea catechins, are as potent as some drugs in clinical development at inhibiting key cytokine receptors involved in angiogenesis (13). The inhibitory effect of EA on PDGFR was somewhat lower than that of STI571 (20), but since STI571 has no effect on VEGFR-2, the inhibition of both receptors by EA may still confer an intrinsic advantage to the use of this molecule to inhibit angiogenesis.

EA is found in high amounts in certain berries, e.g. strawberry and raspberries, where it is present in either a free or a condensed (ellagitannin) form (8). Most in vitro studies on this compound have used concentrations varying from 5 to 50 μM (9–11,23). The bioavailability of this compound in humans remains unknown but plasma concentrations reaching 1 μM have been observed following oral administration of biological extracts rich in EA to rats (24). It is thus tempting to speculate that the low concentrations of EA shown here to inhibit VEGF and PDGF receptors are behaviorally achievable in humans and that the inclusion of berries in the diet may have chemopreventive effects through the inhibition of angiogenesis.

In summary, our results suggest that EA induces biological effects analogous to the pharmacological impairment of receptor tyrosine kinase functions that are currently achieved with drugs in clinical development. In the light of these results, it will be extremely interesting to further examine how the effects reported here could contribute to the chemopreventive and anticancer properties of this compound.

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References


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