Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation

Lamy, Sylvie; Blanchette, Mélanie; Michaud-Levesque, Jonathan; Lafleur, René; Durocher, Yves; Moghrabi, Albert; Barrette, Stéphane; Gingras, Denis; Beliveau, Richard

Publisher's version / Version de l'éditeur:
https://doi.org/10.1093/carcin/bgi279
Carcinogenesis, 27, 5, pp. 989-996, 2006-05

Carcinogenesis vol.27 no.5 pp.989–996, 2006
doi:10.1093/carcin/bgi279
Advance Access publication November 23, 2005

Delphinidin, a dietary anthocyanidin, inhibits vascular endothelial growth factor receptor-2 phosphorylation

Sylvie Lamy[1], Mélanie Blanchette[1], Jonathan Michaud-Levesque[1], René Lafleur[1], Yves Durocher[3], Albert Moghrabi[2], Stéphane Barrette[2], Denis Gingras[1] and Richard Béliveau[1,2,*]

[1]Laboratoire de Médecine Moléculaire, Hôpital Ste-Justine-Université du Québec à Montréal and [2]Service d'hématologie–oncologie, Centre de Cancérologie Charles-Bruneau, Hôpital Ste-Justine, 3175 Côte-Ste-Catherine, Montréal, Québec, Canada, H3T 1C5 and [3]Animal Cell Technology Group, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada H4P 2R2

[*]To whom correspondence should be addressed at: Laboratoire de Médecine Moléculaire, Centre de Cancérologie Charles-Bruneau, Hôpital Ste-Justine, 3175 Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5; Email: molmed@recherche-ste-justine.qc.ca

Epidemiological studies have shown that a diet rich in fruits and vegetables has a beneficial preventive effect on cardiovascular diseases and cancer by mechanisms that have not yet been elucidated. In this work, we investigated the antiangiogenic activities of anthocyanidins, a class of polyphenols present at high levels in fruits. Among the tested anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin), delphinidin was the most potent angiogenic inhibitor. *In vitro*, low concentrations of delphinidin inhibited vascular endothelial growth factor (VEGF)-induced tyrosine phosphorylation of VEGF receptor (VEGFR)-2, leading to the inhibition of downstream signaling triggered by VEGFR-2. Inhibition of VEGFR-2 by delphinidin inhibited the VEGF-induced activation of ERK-1/2 signaling and the chemotactic motility of human EC as well as their differentiation into capillary-like tubular structures in Matrigel and within fibrin gels. *In vivo*, delphinidin was able to suppress basic fibroblast growth factor-induced vessel formation in the mouse Matrigel plug assay. The identification of delphinidin as a naturally occurring inhibitor of VEGF receptors suggests that this molecule possesses important antiangiogenic properties that may be helpful for the prevention and treatment of cancer.

Introduction

Angiogenesis, the process of new blood vessel growth from pre-existing capillaries, is involved in physiological and pathological processes such as embryonic development, wound healing, reproductive cycles, diabetic retinopathy, chronic inflammation, tumor growth and metastasis (1). Thus, suppression of abnormal angiogenesis may provide therapeutic strategies for the treatment of angiogenesis-dependent disorders. Vascular endothelial growth factor (VEGF), which has been shown to be a major factor secreted by tumor cells, plays an important role in the expansion of the microvascular network needed to supply oxygen and nutrients for the rapid growth of tumor masses (2). VEGF was initially defined, characterized and purified for its ability to induce vascular leak and permeability as well as for its ability to promote vascular endothelial cell proliferation and migration and to act as a crucial survival factor for endothelial cells (EC) (3). It is a specific EC mitogen that binds with high affinity to the EC receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), the latter being responsible for most of the mitogenic and chemotactic effects of VEGF (4). Therefore, the development of antiangiogenesis therapies directed against the VEGF–VEGFR-2 kinase axis has become an important approach for the treatment of angiogenesis-dependent diseases (5).

There is increasing evidence that polyphenols found in natural products may interfere with several processes involved in cancer and metastasis. In this respect, we have shown that several green tea catechins inhibit matrix metalloproteinase (MMP)-dependent activities *in vitro* (6) as well as tumor cell migration and invasion (7,8), and that these molecules are also potent inhibitors of *in vitro* angiogenesis (9,10), operating through inhibition of VEGF receptors (9). Furthermore, we have reported recently that ellagic acid, a polyphenol present in fruits and nuts, inhibits both VEGF and PDGF receptors (11). Among polyphenols, the anthocyanidins and their glycosylated forms (anthocyanins) appear to possess potentially beneficial effects against various diseases (12). These phenolic compounds are widely found in many berries, red grapes, purple sweet potatoes, red cabbages, and other pigmented foods, plants and vegetables (13). While the strong antioxidant potential of these molecules is well characterized (12), information about the antitumor and the antiangiogenic activities of anthocyanidins remains largely unknown. In the present study, we investigated whether anthocyanidins (cyanidin (Cy), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), peonidin (Pn), petunidin, (Pt)) and a glycosylated form of delphinidin [delphinidin 3-O-beta-glucopyranoside (Dp 3-glu)] modulate angiogenesis in both *in vitro* and *in vivo* models. We present evidence that delphinidin acts as a potent inhibitor of both VEGFR-2 activities and of phosphorylation of the p42/44 mitogen-activated protein (MAP) kinase pathway (also known as ERK-1/2, extracellular-signal-regulated kinase 1 and 2), leading to an inhibition of VEGF-induced EC migration as well as to an inhibition of the morphogenic differentiation of EC into capillary-like structures *in vitro*. Moreover, the compound markedly inhibited basic fibroblast growth factor (bFGF)-induced new blood vessel formation in *in vivo* Matrigel plug assays. All these data suggest that delphinidin exerts antiangiogenic effects via its inhibitory effect on VEGFR-2 activity.

Abbreviations: bFGF, basic fibroblast growth factor; Cy, cyanidin; Dp, delphinidin; Dp 3-glu, delphinidin 3-*O*-beta-glucopyranoside; EC, endothelial cell; ERK, extracellular-signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; Mv, malvidin; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; STS, staurosporine; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2.

Materials and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, ON, Canada) and serum was purchased from Hyclone Laboratories (Logan, UT). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Anthocyanidins (Cy, Dp, Mv, Pg, Pn and Pt) and anthocyanin (Dp 3-glu) were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, Canada). Protein A-Sepharose and Protein G-Sepharose were obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Qc, Canada). The anti-VEGFR-2 (C-1158) and anti-ERK-1/2 (K-23) polyclonal antibodies and the anti-phosphotyrosine (PY99) monoclonal antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-p44/42 MAPK (Thr 202/Tyr 204) 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 PerkinElmer Life Sciences (Boston, MA). Human recombinant bFGF was obtained from Upstate (Lake Placid, NY). Human recombinant VEGF (isoform 165) was produced and purified as described (11). Staurosporine (STS) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). All other reagents were from Sigma-Aldrich (Oakville, ON, Canada).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and maintained in EC basal medium-2 (EBM-2) supplemented with EGM-2 Mv growth factor mixture (Clonetics). The cells were cultured at 37°C under a humidified 95%/5% (v/v) mixture of air and CO_2. For experimental purposes, cells were plated in 100 mm plastic dishes at 5000 cells/cm^2 and were grown to confluence before overnight serum starvation without supplements. Cells were treated with vehicle or with anthocyanidins diluted in 100% EtOH, and stimulated with VEGF.

Matrigel endothelial cell tube formation assays

Matrigel (12.5 mg/ml) was thawed at 4°C, and 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 6 h at 37°C with HUVEC (20 000 cells/well), which had previously been treated for 18 h with the indicated concentrations of anthocyanidins. The formation of capillary-like structures was examined microscopically and photographs (50×) were taken using a Retiga 1300 camera and a Zeiss Axiovert 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, Mississauga, ON, Canada).

Capillary tube formation by EC in fibrin gels

HUVECs were embedded within fibrin gels at a concentration of 2.5×10^5 cells/ml by the following technique. 250 μl of 2.5 mg/ml human fibrinogen solution (made in serum-free medium) was placed into a tube and human thrombin (0.5 U/ml) was added to the fibrinogen. This was quickly pipetted into the wells of 24-well plates (covering the entire surface of the wells) and allowed to clot at 37°C for 30 min. One milliliter aliquots of EC suspended in serum-free EC basal medium were seeded onto each fibrin gel. After 24 h, cells had spread to form a confluent monolayer. Cells were then pretreated with vehicle or with delphinidin (25 μM) for 1 h at 37°C. The same procedure was used to generate a second fibrin gel overlying the apical surface of the cells. This fibrin gel was allowed to polymerize for 10 min at 37°C and then, after the pretreatment of cells, 1 ml aliquots of fresh serum-free EC basal medium, supplemented with VEGF (75 ng/ml) and containing (or lacking) delphinidin (25 μM), were added to each well. After 6 h, the cultures were photographed (100×).

Assessment of cell viability

Cell viability was determined by the trypan blue exclusion assay. Briefly, cells were treated with 25 μM delphinidin and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C in EBM-2 medium for 18 h. After detaching with trypsin–EDTA, an equal volume of 0.4% trypan blue reagent (Gibco BRL, Burlington, ON, Canada) was added to the cell suspension and the proportions of viable cells were evaluated under the field microscope. About 100 cells were counted and cells stained dark blue were not considered viable.

Fluorimetric caspase 3 assay

Cells were grown to ~85% confluence and treated for 18 h with delphinidin (25 μM) or with a well-characterized inducer of EC apoptosis, staurosporine (10 nM), as a positive control. Cells were collected, washed in cold phosphate-buffered saline (PBS) and lysed in Apo-alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4°C. The lysates were clarified by centrifugation at 16 000 *g* for 20 min and caspase 3 activity was determined as described previously (14) using DEVD-AFC as the substrate.

Immunoprecipitation and immunoblotting procedures

After treatment with VEGF (100 ng/ml) as described previously (15), cells were washed once with ice-cold PBS (pH 7.4) containing 1 mM Na_3VO_4 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% (v/v) Nonidet P-40 and 1% (v/v) Triton X-100] containing 1 mM Na_3VO_4. Lysates were clarified by centrifugation at 10 000 *g* for 10 min, and the resulting supernatants were used to immunoprecipitate VEGFR-2 overnight with 1 μg/ml of antibody at 4°C followed by the addition of protein A-(rabbit primary antibody) Sepharose beads at 4°C for 2 h, as described previously (11). Non-specifically bound proteins were removed by washing the beads three times with lysis buffer and once with PBS containing 1 mM Na_3VO_4. The proteins were extracted with 2-fold concentrated Laemmli sample buffer, boiled 4 min, and resolved by SDS–PAGE (7.5% gel). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked overnight at 4°C in Tris-buffered saline/Tween-20 [147 mM NaCl, 20 mM Tris–HCl (pH 7.5) and 0.1% Tween-20] containing 2% (w/v) bovine serum albumin (BSA), and probed with primary antibodies (PY99 or VEGFR-2) for 2 h at room temperature. Immunoreactive bands were revealed after a 1 h incubation with HRP-conjugated anti-mouse IgG, and the signals were visualized by enhanced chemiluminescence. The immunoreactive bands were quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

Analysis of ERK-1/2 phosphorylation

Cells treated with vehicle or with anthocyanidins for 18 h were stimulated with 50 ng/ml VEGF for 10 min and then washed, incubated in PBS containing 1 mM of each NaF and Na_3VO_4, and solubilized on ice in lysis buffer. Lysates (20 μg) were solubilized in Laemmli sample buffer, boiled for 4 min, separated on 10% SDS–PAGE and analyzed by western blotting.

Migration assays

Chemotactic motility of HUVEC was assayed using Transwells (8 μm pore size; Costar, Cambridge, MA) as described previously (11). Briefly, HUVECs were seeded at a density of 1.0×10^6 cells/ml in serum-free medium and were allowed to adhere to gelatin-coated transwells for 30 min. Then, cells were incubated for 2 h with various concentrations of delphinidin before the addition of VEGF (50 ng/ml) to the lower chamber as a chemoattractant and placed at 37°C in 5% CO_2/95% air for another 3 h. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet/20% (v/v) methanol. The migration was quantitated using computer-assisted imaging and data are expressed as the average density of migrated cells per four fields (magnification ×50).

In vivo Matrigel plug assay

The Matrigel plug assay is based on the method of Passaniti (16), with modifications. Briefly, Crl:CD-1® -nuBR nude mice (Charles River Laboratories, St Constant, Qc, Canada) were treated by injection subcutaneously (s.c.) into the ventral midline region of the right flank with 0.5 ml of phenol red-free Matrigel (BD Bioscience, Mississauga, ON, Canada) containing delphinidin (300 μM), bFGF (250 ng/ml) and heparin (0.0025 U/ml) to allow the formation of solid gel plugs. Matrigel containing ethanol alone was injected into some animals to serve as baseline controls. After 7 days, the mice were killed and the Matrigel implant harvested, washed with PBS, immediately frozen and lyophilized overnight. After resolubilization in 0.1% Triton X-100, the concentration of hemoglobin was determined directly by absorbance at 405 nm and compared with a standard curve of purified hemoglobin (Sigma-Aldrich, Oakville, ON, Canada), as described previously (17).

Statistical analysis

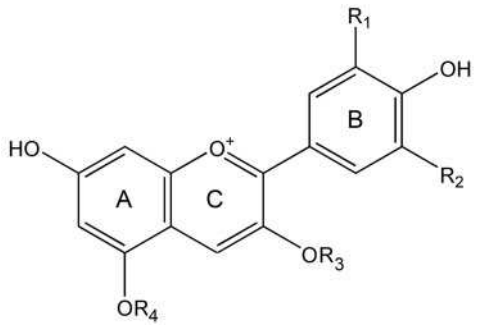
The data are presented as mean ± SEM and statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t*-test.

Results

Delphinidin inhibits tube formation of endothelial cells

The effect of six kinds of anthocyanidins (Cy, Dp, Mv, Pg, Pn and Pt) (Table I) and of a glycosylated form, Dp 3-glu, on the morphological differentiation of EC was investigated using a 2D model with Matrigel, a laminin-rich reconstituted basement membrane matrix. Treatment of HUVEC with 15 μM of the compounds for 18 h, before adhesion to Matrigel,

Table I. Structure of anthocyanidins



Anthocyanidin	R_1	R_2
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Malvidin (Mv)	OCH_3	OCH_3
Pelargonidin (Pg)	H	H
Peonidin (Pn)	OCH_3	H
Petunidin (Pt)	OCH_3	OH

R_3, R_4 = H or sugar moiety.

affected tube formation compared to the control (Figure 1A); this process was nearly abolished by delphinidin (~76% inhibition) (Figure 1B). As shown in Figure 1C, delphinidin abrogated the width and the length of endothelial tubular structures in a concentration-dependent manner (IC_{50} = 9.5 μM; Figure 1D). This process was completely abolished by delphinidin at 25 μM. These results show that delphinidin was the anthocyanidin most effective at inhibiting EC differentiation.

Delphinidin inhibits VEGF-induced tube formation in 3D fibrin gels

There is now considerable evidence that 3D cell models represent attractive systems for closely mimicking the *in vivo* cellular microenvironment (18). Growth of EC within fibrin gels is a suitable model for studying angiogenesis (19), as fibrin is a common component of the extracellular matrix at sites of wounding, chronic inflammation and tumor stroma (20). When VEGF was added to the HUVEC embedded in 3D fibrin gels, VEGF rapidly led to the formation of elongated and robust tube-like structures, compared to the control. Using this model, we observed that delphinidin also potently inhibited VEGF-induced capillary-like structure formation by HUVEC (Figure 2).

The antiangiogenic activity of delphinidin is not related to cell death

In order to verify whether the inhibitory effect of 25 μM delphinidin was due to cytotoxicity, we measured HUVEC viability by trypan blue exclusion and caspase 3 activity. Induction of caspase 3 was used to monitor potential apoptotic actions of delphinidin in EC, based on the well-described role of this executioner caspase in apoptosis (21). Figure 3A shows that delphinidin did not induce significant cell death compared to control cells after 18 h of treatment. Furthermore, no caspase 3 activity was detected in cells treated with delphinidin, unlike cells treated with staurosporine, an apoptotic inducer (Figure 3B), confirming the specific antiangiogenic effect of delphinidin on EC tube formation.

Delphinidin inhibits VEGF-induced tyrosine phosphorylation of VEGFR-2

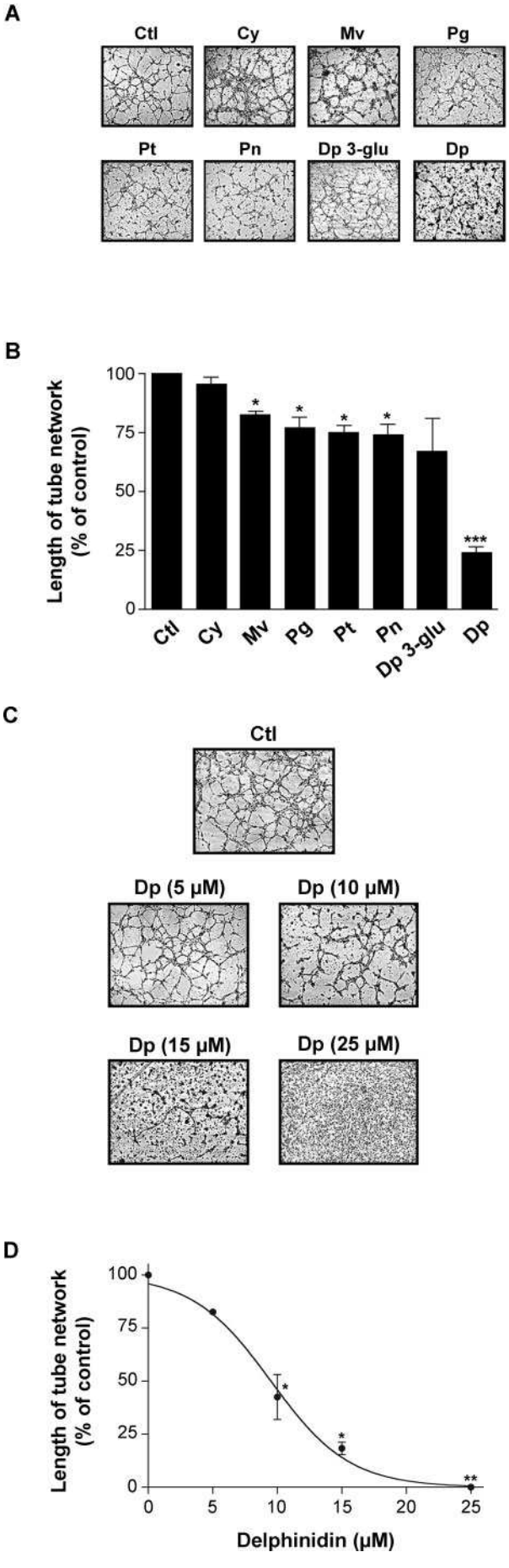
To better understand the inhibitory action of delphinidin on the induction of *in vitro* angiogenesis, we examined its effect on the function of VEGF. Since VEGF has been shown to be the central positive regulator of the early growth of neovessels (22), and inhibition of VEGFR-2 activity limits the ability of most tumors to stimulate the formation of blood vessels (23), we examined whether delphinidin could have an effect on VEGF-induced tyrosine phosphorylation of VEGFR-2. Quiescent HUVECs were incubated for 18 h in serum-free medium in the presence or in the absence of 25 μM delphinidin or Dp 3-glu. The medium was then replaced with fresh serum-free medium without delphinidin and the cells were stimulated with 100 ng/ml of recombinant VEGF for 1 min. The phosphorylation state of the VEGFR-2 was assessed by immunoprecipitation of the receptor followed by Tyr(P) immunodetection. Under these experimental conditions, we observed a complete inhibition of VEGF-induced phosphorylation of VEGFR-2 by delphinidin, whereas Dp 3-glu was less potent, confirming that delphinidin is the most potent anthocyanidin inhibitor (Figure 4A, top panel). Blotting of membranes with an antibody directed against VEGFR-2 showed that delphinidin did not affect the amount of VEGFR-2 in the immunoprecipitate (Figure 4A, *bottom panel*). Interestingly, the tyrosine phosphorylation of VEGFR-2 was rapidly inhibited by delphinidin after as little as 15 min and this inhibitory effect gradually increased at longer incubation times (Figure 4B). The fact that the inhibition by delphinidin was already complete at 25 μM for 18 h prompted us to more precisely examine the effect of lower concentrations of this molecule on tyrosine phosphorylation of VEGFR-2. As shown in Figure 4C, delphinidin caused a dose-dependent inhibition of VEGF-induced tyrosine phosphorylation of VEGFR-2 with an IC_{50} of 2 μM. Overall, these results strongly support the concept that delphinidin interferes with the activity of VEGFR-2 at the EC level.

Delphinidin inhibits VEGF-induced ERK phosphorylation

Following VEGF-induced autophosphorylation of VEGFR-2, the receptor induces activation and/or phosphorylation of several substrates such as ERK-1/2 (4,24). As shown in Figure 5A (top panel), VEGF caused a marked increase in the tyrosine phosphorylation state of ERK-1/2 in untreated HUVEC. The increase in tyrosine-phosphorylated ERK-1/2 was completely inhibited by delphinidin at 25 μM compared to Dp 3-glu, which had no effect (Figure 5A, top panel). The total amount of ERK-1/2 was unaffected by these treatments (Figure 5A, bottom panel). As shown in Figure 5B, delphinidin significantly inhibited VEGF-induced phosphorylation of ERK-1/2 in a dose-dependent manner, achieving a half maximal effect at 11.8 μM. These results demonstrate that the inhibitory effect of delphinidin on VEGFR-2 tyrosine phosphorylation alters the signaling cascade triggered by VEGF.

Delphinidin inhibits VEGF-induced migration of endothelial cells

Since migration of EC represents a critical step in angiogenesis, allowing cells to disseminate from pre-existing vessels and to form new vessels, the effect of delphinidin on migration was also investigated. We studied the effect of delphinidin on



HUVEC migration only after VEGF stimulation since, under control conditions, HUVEC migration is very low (data not shown). HUVECs were allowed to adhere to gelatin-coated transwells and were incubated for 2 h with different concentrations of delphinidin before the addition of VEGF (50 ng/ml) to the lower chamber. Under these conditions, delphinidin inhibited VEGF-induced migration of HUVEC in a dose-dependent manner (Figure 6). Overall these results demonstrate that delphinidin has the ability to block VEGF-induced *in vitro* angiogenesis.

Delphinidin inhibits bFGF-induced angiogenesis in vivo

In order to extend the observations made *in vitro* and to study the efficacy of delphinidin *in vivo*, an established *in vivo* angiogenesis model, the mouse Matrigel plus assay (16), was performed. In this assay, angiogenesis-inducing compounds such as bFGF are introduced into cold liquid Matrigel which, following subcutaneous injection, solidifies and permits subsequent penetration by host cells that induce vascularization. Assessment of angiogenic reactions in the Matrigel plug is achieved either by examination of plugs or by determination of the hemoglobin content. Matrigel containing bFGF (250 ng/ml) with or without delphinidin (300 μM) was s.c. injected into Crl:CD-1® -nuBR nude mice and, 7 days later, the formed Matrigel plugs in mice were excised and photographed. Matrigel plugs with bFGF alone appeared red in color. In contrast, Matrigel plugs that contained no bFGF or bFGF plus delphinidin were pale in color, indicating no or less blood vessel formation (Figure 7A). The hemoglobin content inside the Matrigel plugs was measured to quantify the inhibition of angiogenesis induced by delphinidin. Delphinidin significantly inhibited the hemoglobin quantity (to ∼1.6 μg/ml) compared with the bFGF control (∼3.7 μg/ml) (Figure 7B), indicating that delphinidin is capable of inhibiting angiogenesis formation *in vivo*.

Discussion

Anthocyanidins, aglycons of anthocyanins, are abundant in fruits and vegetables, and make a major contribution to the total polyphenol dietary intake (25). They have been associated with a broad spectrum of beneficial health effects ranging from the treatment of diabetic retinopathy (26), atherosclerosis (27) and various microvascular diseases to potential anti-inflammatory (28), antitumoral (29), antimutagenic and anti-carcinogenic (30), and chemoprotective properties (31). Only a few studies have described the antiangiogenic activities of anthocyanidins (32–38). One study reported that delphinidin induces a downregulation of cyclin A and D1 expression and an upregulation of $p27^{kip1}$ expression in HUVEC (36). These two proteins are essential for cell-cycle progression, and these changes in their expression resulted in the inhibition of

Fig. 1. Effect of anthocyanidins on the Matrigel-induced tube formation of HUVEC. (**A**) HUVECs were pretreated for 18 h with 15 μM anthocyanidins before adhesion on Matrigel. Then, cells were collected and plated on Matrigel-coated 96-well plates at 20 000 cells/plates. After 6 h, pictures were taken (50×). Results are representative of three independent experiments. (**B**) The length of the tube network was quantitated using Northern Eclipse software. (**C**) HUVECs were pretreated for 18 h with various concentrations (1, 5, 10, 15 or 25 μM) of delphinidin before adhesion on Matrigel and then cultured for 6 h on Matrigel. (**D**) The length of the tube network was quantified. Values are means of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control alone); bars, ±SEM.

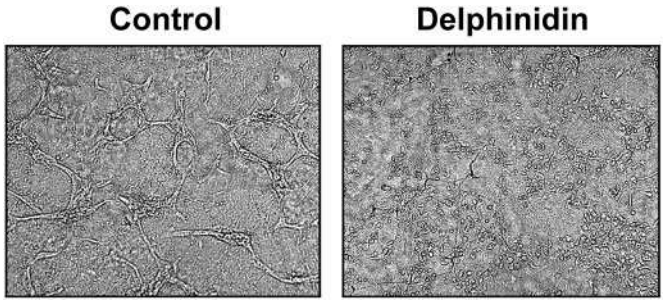


Fig. 2. Effect of delphinidin on VEGF-induced tube formation of HUVEC in 3D fibrin gels. HUVECs were cultured within fibrin gels in serum-free medium containing (or lacking) delphinidin (25 μM) and stimulated with VEGF (75 ng/ml) as described in Materials and methods. After 6 h, cells were photographed (×100). The pictures shown are representative of three independent experiments.

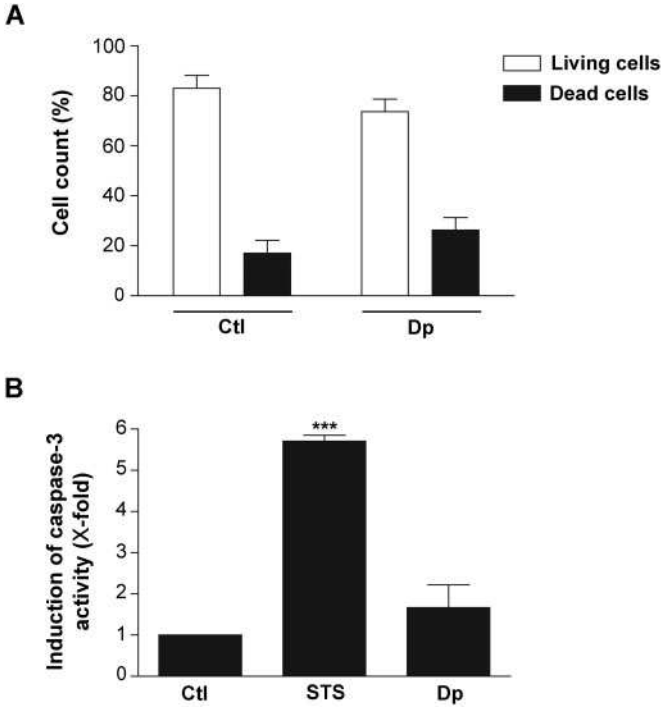


Fig. 3. Effect of delphinidin on viability of HUVEC. (**A**) Subconfluent HUVECs were treated with or without 25 μM delphinidin. Adherent and non-adherent cells were collected, and viability was assessed by trypan blue exclusion. Cell death is expressed as the percentage of cells incorporating the dye relative to the total amount of cells. (**B**) Subconfluent HUVECs were either left untreated or treated with 25 μM delphinidin or with 10 nM staurosporine for 18 h. Extracts from control and treated cells were used to determine DEVDase (caspase 3) activity, as described in Materials and methods. Values are means of three independent experiments (***$P < 0.001$ versus control alone); bars, ±SEM.

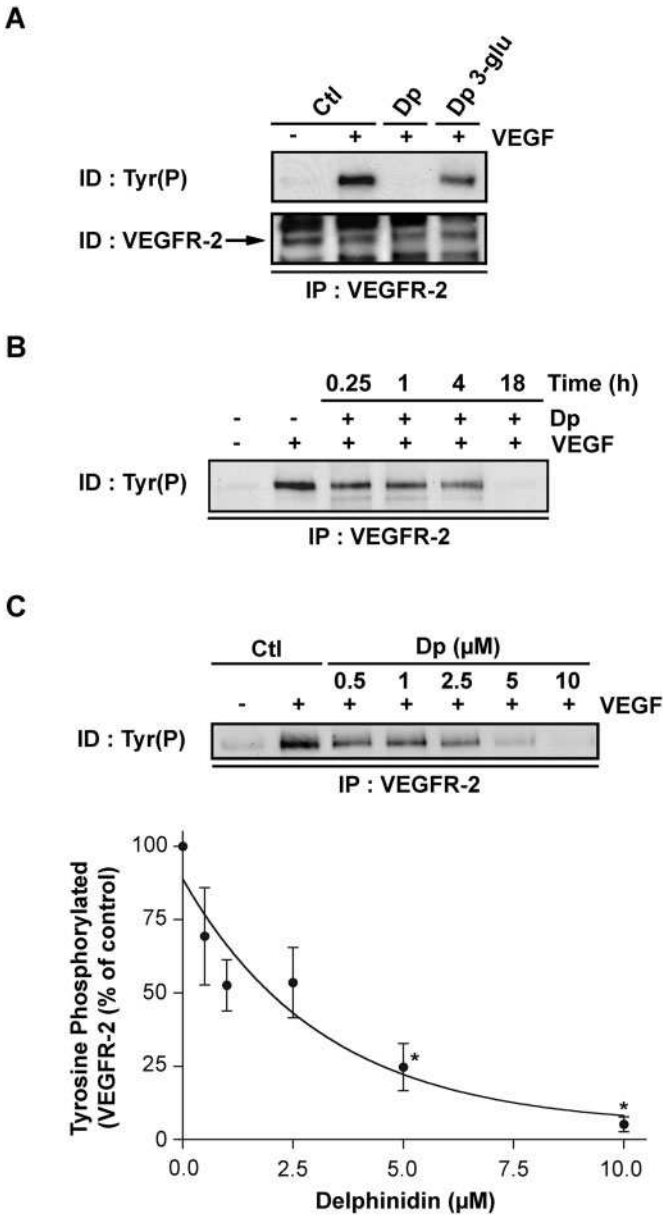


Fig. 4. Effect of delphinidin on VEGF-induced tyrosine phosphorylation of VEGFR-2 in HUVEC. (**A**) Quiescent HUVECs were incubated in serum-free medium in the presence or in the absence of delphinidin or Dp 3-glu at 25 μM for 18 h, (**B**) with delphinidin (25 μM) for 0.25, 1, 4 and 18 h or (**C**) with delphinidin at the indicated concentrations for 18 h. The medium was replaced with serum-free medium without delphinidin or Dp 3-glu, and HUVECs were stimulated with 100 ng/ml recombinant VEGF for 1 min. Cells were lysed and the levels of tyrosine-phosphorylated VEGFR-2 were monitored by immunoprecipitation with anti-VEGFR-2 and immunoblotting with anti-Tyr(P) monoclonal antibody (PY99). (C) Results were analyzed by densitometry and values are means of three independent experiments. (*$P < 0.05$ versus VEGF alone); bars, ±SEM.

VEGF-induced HUVEC migration and proliferation, and in the inhibition of neovascularization of developing embryo in the chick chorioallantoic membrane (CAM) assay (36). Here, we demonstrate for the first time that the inhibitory effect of delphinidin on angiogenesis may involve its effect on the predominant VEGF receptor, VEGFR-2. Delphinidin inhibited VEGF-dependent tyrosine phosphorylation of VEGFR-2 in a dose- and time-dependent manner and this inhibitory effect is associated with the inhibition of the downstream signaling pathways triggered by the receptor, such as the ERK-1/2 pathway in HUVEC. The inhibitory effect of delphinidin on these processes resulted in the inhibition of two key events underlying angiogenesis, i.e. EC migration and morphogenic differentiation into capillary-like structures, in both *in vitro* and *in vivo* models.

VEGF is a strong activator of ERK-1/2 via VEGFR-2 and the assumption that this pathway plays a central role in angiogenesis is supported by the finding that specific inhibitors of MEK-1/2 (mitogen-activated protein kinase/ERK

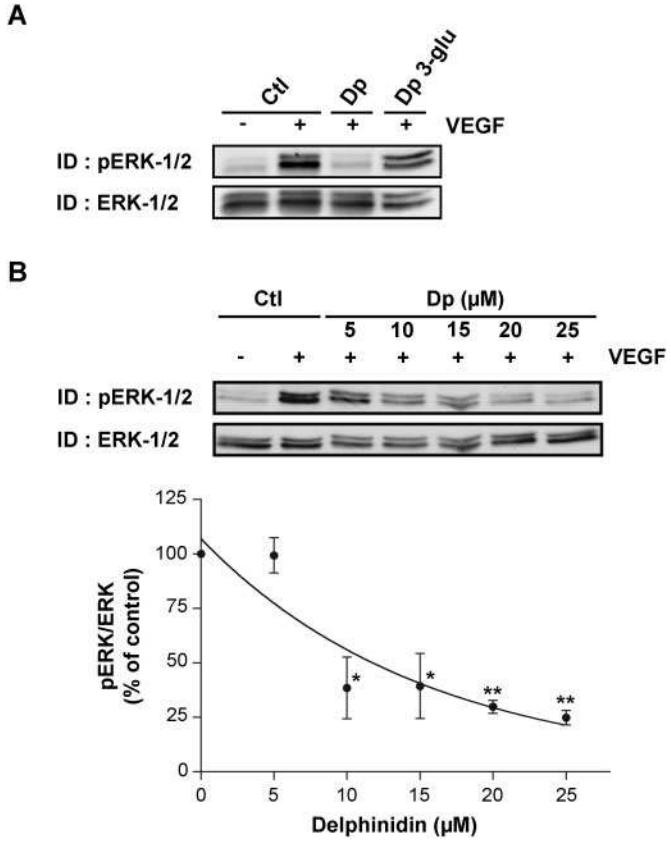


Fig. 5. Effect of delphinidin on phosphorylation of ERK-1/2 induced by VEGF in HUVEC. (**A**) Quiescent HUVECs were incubated in serum-free medium in the presence or in the absence of delphinidin or Dp 3-glu at 25 μM or (**B**) with delphinidin at the indicated concentrations for 18 h and stimulated with 100 ng/ml recombinant VEGF for 10 min. Cells were lysed and equal amounts of protein were separated by SDS–PAGE electrophoresis. The phosphorylated forms of ERK-1/2 (top panel) and the effects of the treatments on the amount of ERK-1/2 (bottom panel) were visualized by immunoblotting using specific antibodies. (B) Results were analyzed by densitometry and values are means of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$ versus VEGF alone); bars, ±SEM.

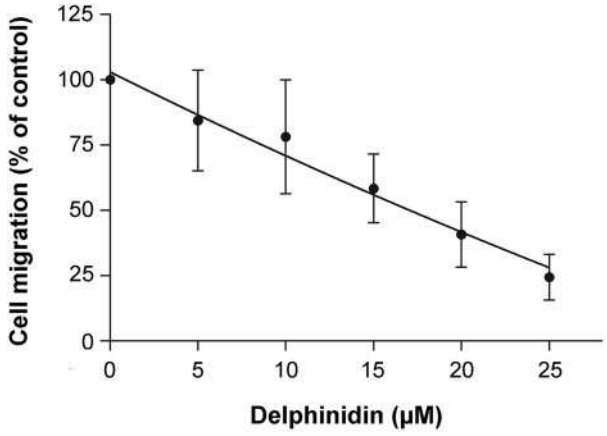


Fig. 6. Effect of delphinidin on VEGF-induced migration of endothelial cells. HUVECs were pretreated for 2 h with various concentrations (5, 10, 15, 20 or 25 μM) of delphinidin before treatment with VEGF. After 3 h incubation, migration was quantified by counting the cells that crossed the membrane to the lower side of the filter with optical microscopy at ×50 magnification. The number of cells that migrated was compared to that observed with VEGF-treated cells. Values are means of five independent experiments; bars, ±SEM.

kinase 1/2), the kinase responsible for ERK-1/2 activation, reduce EC tubulogenesis *in vitro* (39). Furthermore, ERK-1/2 plays an important role in regulating cell motility (40). Therefore, the inhibition of VEGF-induced EC migration by delphinidin may be related to its inhibitory action on VEGFR-2 activity, resulting in the inhibition of ERK-1/2. We observed that delphinidin also potently inhibited capillary-like structure formation by HUVEC embedded in 3D fibrin gels in response to VEGF. Moreover, delphinidin remarkably suppressed induction of new blood vessel formation (∼66%), in Matrigel plugs implanted in mice, in response to bFGF. Although, in this model, bFGF is a stronger inducer of new blood vessels than is VEGF (data not shown), an intimate cross-talk exists among bFGF and VEGF during angiogenesis. Several experimental evidences point to the possibility that bFGF induces neovascularization indirectly by the activation of the VEGF/VEGFR system. Indeed, it has been shown that the quiescent endothelium of vessels of mouse cornea express both VEGF mRNA and protein only after bFGF treatment, and systemic administration of anti-VEGF neutralizing antibodies dramatically reduces bFGF-induced vascularization (41). Thus, bFGF requires the activation of the VEGF/VEGFR system for promoting angiogenesis and conversely, VEGF may require bFGF for exerting its angiogenic potential. Since the formation of neovessels by angiogenesis *in vivo* is a very complex process involving the interplay of several cytokines and their receptors, it is possible that the antiangiogenic activities of delphinidin observed in our *in vivo* Matrigel plug assay (which already contains several endogenous angiogenic factors including VEGF and bFGF) may be related to its inhibitory effect on VEGFR-2 activity via bFGF and VEGF actions. The fact that the potent inhibitory effect of delphinidin on neovascularization has also been demonstrated in the CAM model, in which VEGF receptors are expressed and VEGF is endogenously produced (36), strongly supports the concept that *in vivo*, delphinidin may be a potent angiogenesis inhibitor.

Delphinidin is found in high amounts in berries as compared to other fruits (42). Lowbush 'wild' blueberries (*Vaccinium angustifolium*) are one of the richest fruit sources of anthocyanins (43) and exhibit one of the highest hydrophilic $ORAC_{FL}$ (oxygen radical absorbance capacity fluorescein) values for fruits tested so far (44). The possible health benefits of anthocyanins in preventing chronic and degradative diseases such as heart disease and cancer have been partly attributed to their antioxidant capacity (12,25). However, since the other anthocyanidins tested in this work have an antioxidant capacity very similar to that of delphinidin but did not inhibit tube formation by HUVEC, the existence of additional mechanisms responsible for the inhibitory effect of delphinidin on VEGFR-2 is likely. Although the mechanisms by which delphinidin elicits this inhibitory effect is not yet known, structure–function analysis suggests that the inhibitory effects of delphinidin were enhanced by the presence of three hydroxyl groups at the B-ring as compared to other anthocyanidins (Table I). Furthermore, a free hydroxyl group at Position 3 also seems to be essential for potent inhibition since the presence of sugar residues at this position in delphinidin 3-*O*-beta-glucopyranoside eliminated the inhibitory properties of delphinidin. Thus, delphinidin, a compound with four hydroxyl groups (3, 3′, 4′ and 5′), clearly exhibited the highest inhibitory potency. Besides VEGFR-2, the presence of vicinal hydroxyl

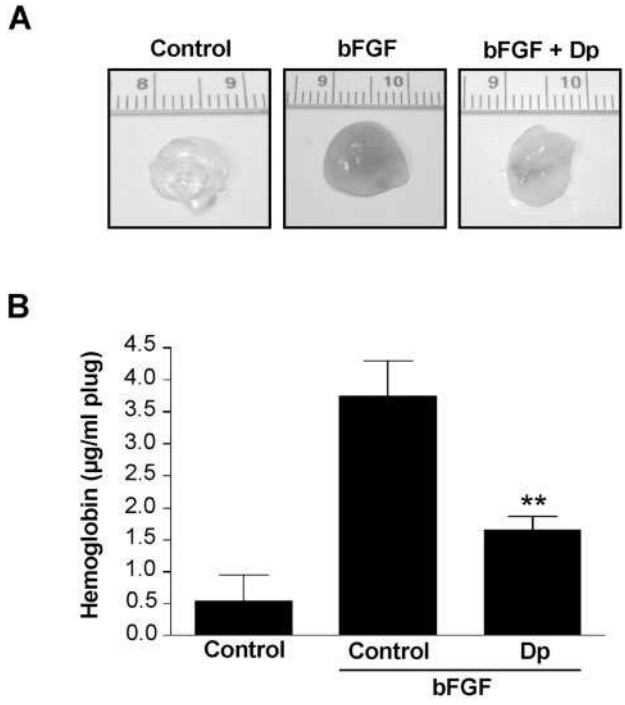


Fig. 7. Effect of delphinidin on bFGF-induced angiogenesis *in vivo*. Crl:CD-1®-nuBR nude mice were injected s.c. with 0.5 ml of Matrigel containing delphinidin (300 μM), bFGF (250 ng/ml), and heparin (0.0025 units/ml) to allow the formation of solid gel plugs. After 7 days, the mice were sacrified and Matrigel plugs were excised. (A) representative Matrigel plugs that contained no bFGF (*Control*), bFGF alone, or bFGF plus delphinidin were photographed. (B) Quantification of neovessel formation by measurement of hemoglobin in the Matrigel plugs. Five mice were used in each group. The data are presented as means (**, $P < 0.01$ *versus* bFGF alone); bars, ±SEM.

substituents on the B-ring of delphinidin also seems to be crucial for interaction with another tyrosine kinase receptor, EGFR (epidermal growth factor receptor) in the human vulva carcinoma cell line A431 (45,46). This observation suggests that the tyrosine kinase activity associated with these two cell surface receptors is indeed inhibited by delphinidin. Thus, this molecule seems to act as a multifunctional anticancer agent through its inhibitory effect on several aspects of both tumor growth and tumor angiogenesis.

Depending on nutrition customs, the daily intake of anthocyans (i.e. anthocyanins and anthocyanidins) in humans is estimated to be a few hundred milligrams per day (47). They are absorbed from the stomach (48) and intestinal cells (49) and are rapidly detected in plasma *in vivo* (50), suggesting that they are bioavailable to exert their biological effects. Studies of the pharmacokinetics of these compounds after their consumption as single agents, anthocyans mixtures or berry extracts suggest that anthocyans reach levels of 10^{-8}–10^{-7} M in human blood (51). In our study, we observed that low concentrations of delphinidin were sufficient to significantly inhibit VEGFR-2 activity. Indeed, a 50% inhibition of VEGF-dependent tyrosine phosphorylation of VEGFR-2 could be observed at a concentration of 2 μM delphinidin. It is thus tempting to speculate that the inclusion of berries in the diet may have chemopreventive effects through the inhibition of angiogenesis.

In conclusion, this study provides the first evidence that VEGF stimulation of the tyrosine phosphorylation of VEGFR-2 is inhibited in a dose- and time-dependent manner by one of the most abundant anthocyanidins, delphinidin. We have shown recently that a number of dietary-derived polyphenols, including the green tea catechin EGCG and ellagic acid, a phenolic acid present in some fruits and nuts (9,11), act as potent VEGFR inhibitors, suggesting that efficient pharmacological impairment of this important signaling cascade can be achieved by dietary intervention strategies (52). The observation that delphinidin also acts as a potent inhibitor of VEGFR-2, shutting off downstream signaling cascades crucial for EC tube formation, further support this hypothesis and highlights the potency of dietary-derived polyphenols as crucial determinants of the chemopreventive properties of fruits and vegetables.

Acknowledgements

R.B. is holder of a Research Chair in Cancer Prevention from UQÀM. This work was supported by a grant from the Cancer Research Society to R.B.

Conflict of Interest Statement: None declared.

References

1. Folkman,J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, **1**, 27–31.
2. Folkman,J. and Shing,Y. (1992) Angiogenesis. *J. Biol. Chem.*, **267**, 10931–10934.
3. Zachary,I. (2003) VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem. Soc. Trans.*, **31**, 1171–1177.
4. Cross,M.J., Dixelius,J., Matsumoto,T. and Claesson-Welsh,L. (2003) VEGF-receptor signal transduction. *Trends Biochem. Sci.*, **28**, 488–494.
5. Underiner,T.L., Ruggeri,B. and Gingrich,D.E. (2004) Development of vascular endothelial growth factor receptor (VEGFR) kinase inhibitors as anti-angiogenic agents in cancer therapy. *Curr. Med. Chem.*, **11**, 731–745.
6. Demeule,M., Brossard,M., Page,M., Gingras,D. and Beliveau,R. (2000) Matrix metalloproteinase inhibition by green tea catechins. *Biochim. Biophys. Acta*, **1478**, 51–60.
7. Annabi,B., Lachambre,M.P., Bousquet-Gagnon,N., Page,M., Gingras,D. and Beliveau,R. (2002) Green tea polyphenol (−)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. *Biochim. Biophys. Acta*, **1542**, 209–220.
8. Pilorget,A., Berthet,V., Luis,J., Moghrabi,A., Annabi,B. and Beliveau,R. (2003) Medulloblastoma cell invasion is inhibited by green tea (−)epigallocatechin-3-gallate. *J. Cell. Biochem.*, **90**, 745–755.
9. Lamy,S., Gingras,D. and Beliveau,R. (2002) Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res.*, **62**, 381–385.
10. Annabi,B., Lee,Y.T., Martel,C., Pilorget,A., Bahary,J.P. and Beliveau,R. (2003) Radiation induced-tubulogenesis in endothelial cells is antagonized by the antiangiogenic properties of green tea polyphenol (−) epigallocatechin-3-gallate. *Cancer Biol. Ther.*, **2**, 642–649.
11. Labrecque,L., Lamy,S., Chapus,A., Mihoubi,S., Durocher,Y., Cass,B., Bojanowski,M.W., Gingras,D. and Beliveau,R. (2005) Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. *Carcinogenesis*, **26**, 821–826.
12. Hou,D.X. (2003) Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr. Mol. Med.*, **3**, 149–159.
13. Harborne,J.B. (1988) *The Flavonoids: Advances in Research Since 1980*. Chapman and Hall, London; New York.
14. Boivin,D., Gendron,S., Beaulieu,E., Gingras,D. and Beliveau,R. (2002) The antiangiogenic agent neovastat (AE-941) induces endothelial cell apoptosis. *Mol. Cancer Ther.*, **1**, 795–802.
15. Gingras,D., Lamy,S. and Beliveau,R. (2000) Tyrosine phosphorylation of the vascular endothelial-growth-factor receptor-2 (VEGFR-2) is modulated by Rho proteins. *Biochem. J.*, **348**, 273–280.
16. Passaniti,A., Taylor,R.M., Pili,R., Guo,Y., Long,P.V., Haney,J.A., Pauly,R.R., Grant,D.S. and Martin,G.R. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab. Invest.*, **67**, 519–528.

17. McMahon,G.A., Petitclerc,E., Stefansson,S., Smith,E., Wong,M.K., Westrick,R.J., Ginsburg,D., Brooks,P.C. and Lawrence,D.A. (2001) Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J. Biol. Chem.*, **276**, 33964–33968.
18. Jacks,T. and Weinberg,R.A. (2002) Taking the study of cancer cell survival to a new dimension. *Cell*, **111**, 923–925.
19. Lafleur,M.A., Handsley,M.M., Knauper,V., Murphy,G. and Edwards,D.R. (2002) Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). *J. Cell Sci.*, **115**, 3427–38.
20. Dvorak,H.F., Nagy,J.A., Berse,B., Brown,L.F., Yeo,K.T., Yeo,T.K., Dvorak,A.M., van de Water,L., Sioussat,T.M. and Senger,D.R. (1992) Vascular permeability factor, fibrin, and the pathogenesis of tumor stroma formation. *Ann. NY Acad. Sci.*, **667**, 101–111.
21. Nicholson,D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.*, **6**, 1028–1042.
22. Tse,V., Xu,L., Yung,Y.C., Santarelli,J.G., Juan,D., Fabel,K., Silverberg,G. and Harsh,G.t. (2003) The temporal-spatial expression of VEGF, angiopoietins-1 and 2, and Tie-2 during tumor angiogenesis and their functional correlation with tumor neovascular architecture. *Neurol. Res.*, **25**, 729–738.
23. Shaheen,R.M., Davis,D.W., Liu,W., Zebrowski,B.K., Wilson,M.R., Bucana,C.D., McConkey,D.J., McMahon,G. and Ellis,L.M. (1999) Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res.*, **59**, 5412–5416.
24. Breslin,J.W., Pappas,P.J., Cerveira,J.J., Hobson,R.W. II and Duran,W.N. (2003) VEGF increases endothelial permeability by separate signaling pathways involving ERK-1/2 and nitric oxide. *Am. J. Physiol. Heart Circ. Physiol.*, **284**, H92–H100.
25. Prior,R.L. (2003) Fruits and vegetables in the prevention of cellular oxidative damage. *Am. J. Clin. Nutr.*, **78**, 570S–578S.
26. Boniface,R. and Robert,A.M. (1996) [Effect of anthocyanins on human connective tissue metabolism in the human]. *Klin. Monatsbl. Augenheilkd.*, **209**, 368–372.
27. Aviram,M. and Fuhrman,B. (2002) Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. NY Acad. Sci.*, **957**, 146–161.
28. Wang,H., Nair,M.G., Strasburg,G.M., Chang,Y.C., Booren,A.M., Gray,J.I. and DeWitt,D.L. (1999) Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.*, **62**, 294–246.
29. Koide,T., Hashimoto,Y., Kamei,H., Kojima,T., Hasegawa,M. and Terabe,K. (1997) Antitumor effect of anthocyanin fractions extracted from red soybeans and red beans *in vitro* and *in vivo*. *Cancer Biother. Radiopharm.*, **12**, 277–280.
30. Galvano,F., La Fauci,L., Lazzarino,G., Fogliano,V., Ritieni,A., Ciappellano,S., Battistini,N.C., Tavazzi,B. and Galvano,G. (2004) Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.*, **15**, 2–11.
31. Malik,M., Zhao,C., Schoene,N., Guisti,M.M., Moyer,M.P. and Magnuson,B.A. (2003) Anthocyanin-rich extract from *Aronia meloncarpa* E induces a cell cycle block in colon cancer but not normal colonic cells. *Nutr. Cancer*, **46**, 186–196.
32. Khanna,S., Roy,S., Bagchi,D., Bagchi,M. and Sen,C.K. (2001) Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. *Free Radic. Biol. Med.*, **31**, 38–42.
33. Khanna,S., Venojarvi,M., Roy,S., Sharma,N., Trikha,P., Bagchi,D., Bagchi,M. and Sen,C.K. (2002) Dermal wound healing properties of redox-active grape seed proanthocyanidins. *Free Radic. Biol. Med.*, **33**, 1089–1096.
34. Roy,S., Khanna,S., Alessio,H.M., Vider,J., Bagchi,D., Bagchi,M. and Sen,C.K. (2002) Anti-angiogenic property of edible berries. *Free Radic. Res.*, **36**, 1023–1031.
35. Atalay,M., Gordillo,G., Roy,S., Rovin,B., Bagchi,D., Bagchi,M. and Sen,C.K. (2003) Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett.*, **544**, 252–257.
36. Favot,L., Martin,S., Keravis,T., Andriantsitohaina,R. and Lugnier,C. (2003) Involvement of cyclin-dependent pathway in the inhibitory effect of delphinidin on angiogenesis. *Cardiovasc. Res.*, **59**, 479–487.
37. Martin,S., Favot,L., Matz,R., Lugnier,C. and Andriantsitohaina,R. (2003) Delphinidin inhibits endothelial cell proliferation and cell cycle progression through a transient activation of ERK-1/-2. *Biochem. Pharmacol.*, **65**, 669–675.
38. Bagchi,D., Sen,C.K., Bagchi,M. and Atalay,M. (2004) Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry (Mosc.)*, **69**, 75–80, 1 p. preceding 75.
39. Ilan,N., Mahooti,S. and Madri,J.A. (1998) Distinct signal transduction pathways are utilized during the tube formation and survival phases of *in vitro* angiogenesis. *J. Cell Sci.*, **111**, 3621–3631.
40. Howe,A.K., Aplin,A.E. and Juliano,R.L. (2002) Anchorage-dependent ERK signaling—mechanisms and consequences. *Curr. Opin. Genet. Dev.*, **12**, 30–35.
41. Seghezzi,G., Patel,S., Ren,C.J., Gualandris,A., Pintucci,G., Robbins,E.S., Shapiro,R.L., Galloway,A.C., Rifkin,D.B. and Mignatti,P. (1998) Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J. Cell. Biol.*, **141**, 1659–1673.
42. Wu,X. and Prior,R.L. (2005) Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries. *J. Agric. Food Chem.*, **53**, 2589–2599.
43. Francis,F.J. (1989) Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.*, **28**, 273–314.
44. Wu,X., Beecher,G.R., Holden,J.M., Haytowitz,D.B., Gebhardt,S.E. and Prior,R.L. (2004) Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.*, **52**, 4026–4037.
45. Meiers,S., Kemeny,M., Weyand,U., Gastpar,R., von Angerer,E. and Marko,D. (2001) The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J. Agric. Food Chem.*, **49**, 958–962.
46. Marko,D., Puppel,N., Tjaden,Z., Jakobs,S. and Pahlke,G. (2004) The substitution pattern of anthocyanidins affects different cellular signaling cascades regulating cell proliferation. *Mol. Nutr. Food Res.*, **48**, 318–325.
47. Hollman,P.C. and Katan,M.B. (1999) Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.*, **37**, 937–942.
48. Passamonti,S., Vrhovsek,U., Vanzo,A. and Mattivi,F. (2003) The stomach as a site for anthocyanins absorption from food. *FEBS Lett.*, **544**, 210–213.
49. Talavera,S., Felgines,C., Texier,O., Besson,C., Manach,C., Lamaison,J.L. and Remesy,C. (2004) Anthocyanins are efficiently absorbed from the small intestine in rats. *J. Nutr.*, **134**, 2275–2279.
50. Mazza,G., Kay,C.D., Cottrell,T. and Holub,B.J. (2002) Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.*, **50**, 7731–7737.
51. Cooke,D., Steward,W.P., Gescher,A.J. and Marczylo,T. (2005) Anthocyans from fruits and vegetables—does bright colour signal cancer chemopreventive activity? *Eur. J. Cancer*, **41**, 1931–1940.
52. Beliveau,R. and Gingras,D. (2004) Green tea: prevention and treatment of cancer by nutraceuticals. *Lancet*, **364**, 1021–1022.

Received May 31, 2005; revised November 16, 2005;
accepted November 20, 2005