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Delphinidin, a dietary anthocyanidin, inhibits platelet-derived growth factor ligand/receptor (PDGF/PDGFR) signaling

Sylvie Lamy[1], Édith Beaulieu[1], David Labbé[1], Valérie Bédard[1], 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 H3T 1C5, Canada

*To whom correspondence should be addressed. Tel: +514 345 4931 2366; Fax: +514 345 2359;
Email: molmed@recherche-ste-justine.qc.ca

Most cancers are dependent on the growth of tumor blood vessels and inhibition of tumor angiogenesis may thus provide an efficient strategy to retard or block tumor growth. Recently, tumor vascular targeting has expanded to include not only endothelial cells (ECs) but also smooth muscle cells (SMCs), which contribute to a mature and functional vasculature. We have reported previously that delphinidin, a major biologically active constituent of berries, inhibits the vascular endothelial growth factor-induced phosphorylation of vascular endothelial growth factor receptor-2 and blocks angiogenesis *in vitro* and *in vivo*. In the present study, we show that delphinidin also inhibits activation of the platelet-derived growth factor (PDGF)-BB receptor-β [platelet-derived growth factor receptor-β (PDGFR-β)] in SMC and that this inhibition may contribute to its antitumor effect. The inhibitory effect of delphinidin on PDGFR-β was very rapid and led to the inhibition of PDGF-BB-induced activation of extracellular signal-regulated kinase (ERK)-1/2 signaling and of the chemotactic motility of SMC, as well as the differentiation and stabilization of EC and SMC into capillary-like tubular structures in a three-dimensional coculture system. Using an anthocyan-rich extract of berries, we show that berry extracts were able to suppress the synergistic induction of vessel formation by basic fibroblast growth factor-2 and PDGF-BB in the mouse Matrigel plug assay. Oral administration of the berry extract also significantly retarded tumor growth in a lung carcinoma xenograft model. Taken together, these results provide new insight into the molecular mechanisms underlying the antiangiogenic activity of delphinidin that will be helpful for the development of dietary-based chemopreventive strategies.

Introduction

The concept of treating tumors by interfering with their vascularization is based on the premise that tumor growth is dependent on angiogenesis and that tumor progression is suppressed if neovascularization is prevented (1). When first conceived, antiangiogenic therapy for cancer offered the possibility of universal efficacy, low toxicity and little possibility of resistance (2). Vascular endothelial growth factor (VEGF) has been shown to be the central positive regulator of tumor angiogenesis and, therefore, has become the primary target when exploiting antiangiogenic strategies. Blockade of the VEGF pathway has yielded promising results both in animal models and in patients (3). Although interference with VEGF-mediated signaling events is effective in preventing the early growth of neovessels, mature vessels from established tumors are largely resistant to inhibitors directed against either VEGF or its receptor vascular endothelial growth factor receptor-2 (VEGFR-2) (4). These mature vessels are surrounded by periendothelial cells, such as pericytes and smooth muscle cells (SMCs), and the contact between these cells stabilizes new blood vessels and promotes endothelial cell (EC) survival (5). This resistance to VEGF blockade is associated with increased expression of angiogenic factors, such as platelet-derived growth factor (PDGF)-BB and angiopoietin-1, which may contribute to vessel stabilization (6). Recent advances in analysis of the mechanisms of vessel maturation indicate that interference with PDGF-BB/platelet-derived growth factor receptor-β (PDGFR-β) signaling results in disruption of already established endothelial–periendothelial associations and leads to vessel destabilization (7). SMCs are recruited by PDGF-BB-expressing EC to remodel, stabilize and mature new vessels (8,9). One way to reduce SMC coverage of vessels is to block the signaling pathways involved in recruiting SMC to EC, and the PDGF-BB/PDGFR-β system has been shown to be a crucial player in this recruitment (8). Accordingly, blocking PDGFR signaling in a transgenic mouse model of pancreatic islet carcinogenesis (Rip1Tag2) with the receptor tyrosine kinase inhibitor SU6668 caused regression of blood vessels, which was due to the detachment of SMC from tumor vessels, and thereby restricted tumor growth (4). Moreover, PDGF-BB and PDGFR-β knockout experiments have suggested a critical role for PDGF signaling in the establishment of functional blood vessels by recruiting stabilizing vascular mural cells to the developing blood vessel (10). In addition, SMCs that surround tumor vessels produce VEGF (11,12), and tumor vessels lacking SMC are more dependent on VEGF for their survival than are vessels associated with SMC (13). The importance of this system for the maintenance of tumor vessels was also illustrated by the observation that the combined inhibition of VEGF and PDGF receptors by kinase inhibitors caused the regression of established tumors (4,14,15). Taken together, these studies highlight the need to identify novel molecules that inhibit both VEGF and PDGF receptor activities.

There is currently increasing interest in the anticancer properties of naturally occurring molecules found in the diet (16). Epidemiologic studies have shown that a diet rich in fruits and vegetables has a beneficial, preventive effect against cancer (17,18). The mechanisms responsible for these beneficial effects seem to be related to the high content, in several fruits and vegetables, of phytochemicals such as isothyocyanates, allyl sulfides and polyphenols (19–21). During the last decade, the polyphenols found in grapes, wine, green tea and various fruits have received significant attention for their strong antioxidant properties, but there is now convincing evidence that these molecules also inhibit key processes associated with both tumor growth and angiogenesis (22). We and others have shown previously that polyphenols such as resveratrol, ellagic acid and green tea polyphenol epigallocatechin-3-gallate inhibit PDGFR autophosphorylation (23–31). Moreover, recent studies have shown that anthocyans (i.e. anthocyanins and their aglycones, the anthocyanidins), a class of polyphenols present at high levels in fruits, also act as potential cancer chemopreventive agents (21,32–34). In this respect, we have shown that delphinidin is a strong inhibitor of *in vitro* and *in vivo* angiogenesis through inhibition of VEGF receptor activities (35) and also by inhibiting the invasiveness of glioblastoma through interference with the plasminolytic system (36).

In this study, we present evidence that the antiangiogenic and antitumor properties of delphinidin may also involve inhibition of PDGFR activities, leading to an inhibition of PDGF-induced SMC migration as well as to an inhibition of the morphogenic differentiation of EC and SMC into capillary-like structures in a coculture

Abbreviations: EC, endothelial cell; ERK, extracellular signal-regulated kinase; FGF-2, fibroblast growth factor-2; HUVEC, human umbilical vein endothelial cell; PASMC, pulmonary aortic smooth muscle cell; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PDGFR-β, platelet-derived growth factor receptor-β; s.c., subcutaneous; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2.

model. Moreover, an anthocyan-rich extract markedly inhibited the effects of a combination of fibroblast growth factor-2 (FGF-2) and PDGF-BB in the induction of new blood vessel formation in *in vivo* Matrigel plug assays and inhibited human lung cancer cell growth in NCI-H460 xenograft model. These results suggest that delphinidin seems to act as a multifunctional anticancer agent through its inhibitory effects on several aspects of both tumor growth and tumor angiogenesis.

Materials and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, Ontario, Canada) and serum was purchased from HyClone Laboratories (Logan, UT). Cyanidin, delphinidin, pelargonidin and petunidin were obtained from Polyphenols Laboratories AS (Sandnes, Norway). The anthocyan-rich combination of berry extract, OptiBerry BX-600 (Lot no. 0502005, InterHealth Nutraceuticals, Benicia, CA), used in this study is a standardized blend of various berries, including wild blueberry (*Vaccinium angustifolium*), strawberry (*Fragaria chiloensis*), wild bilberry (*V.myrtillus*), elderberry (*Sambucus nigra*), cranberry (*V.macrocarpon*) and raspberry (*Rubus idaeus*). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). Protein A-Sepharose and Protein G-Sepharose were obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). The anti-PDGFR-β (958), anti-VEGFR-2 (C-1158) and anti-extracellular signal-regulated kinase (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 mitogen-activated protein kinase (Thr 202/Tyr 204) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence reagents were from PerkinElmer Life Sciences (Boston, MA). Human recombinant FGF-2 was obtained from Upstate Biotechnology (Lake Placid, NY). Human recombinant PDGF-BB was obtained from R&D Systems (Minneapolis, MN). Human recombinant VEGF (isoform 165) was produced and purified as described (25). Gleevec (Imatinib mesylate, STI571) was from Novartis Pharmaceutical (Basel, Switzerland). Plasminogen-depleted human plasma fibrinogen and human plasma thrombin were obtained from Calbiochem (La Jolla, CA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma–Aldrich, Oakville, ON Canada.

Cell culture

Human umbilical vein endothelial cells (HUVECs) and pulmonary aortic smooth muscle cells (PASMCs) were purchased from Clonetics (San Diego, CA) and maintained in EC basal medium-2 supplemented with endothelial cell growth medium-2 growth factor mixture (Clonetics) and smooth muscle medium-2 (Clonetics), respectively. Human large lung carcinoma cell line (NCI-H460) was purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 2.5 g/l D-glucose and 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid and containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured at 37°C under a humidified 95%/5% (vol/vol) mixture of air and CO_2. For experimental purposes, HUVEC and PASMC were grown to confluence before overnight serum starvation without supplements. Cells were treated with vehicle or with anthocyanidin diluted in 100% EtOH and stimulated with VEGF or PDGF-BB.

Immunoprecipitation and immunoblotting procedures

After treatment with PDGF-BB (50 ng/ml, 5 min), PASMCs were washed once with ice-cold phosphate-buffered saline (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 ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-O, O′-bis(2-aminoethyl)-N, N, N′, N′-tetraacetic acid, 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100] containing 1 mM Na_3VO_4. 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 bicinchoninic acid method. For immunoprecipitation studies, lysates were clarified by a 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 1 μg/ml of anti-PDGFR-β. Immunocomplexes were collected by incubating the mixture with 25 μl (50% suspension) of Protein A (rabbit primary antibody) -Sepharose beads for 2 h. 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 [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol and 0.00125% bromphenol blue], boiled for 4 min and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5% gel). The proteins were transferred onto polyvinylidene difluoride 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% (wt/vol) bovine serum albumin and probed with primary antibodies (PY99 or PDGFR-β) for 2 h at room temperature. Immunoreactive bands were revealed after a 1 h incubation with horseradish peroxidase-conjugated anti-mouse IgG, and the signals were visualized by enhanced chemiluminescence. The immunoreactive bands were quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA). VEGF-dependent phosphorylation of VEGFR-2 was monitored as described (35).

Analysis of ERK-1/2 phosphorylation

PASMCs treated with vehicle or with delphinidin for 18 h were stimulated with 50 ng/ml PDGF-BB for 5 min and then washed, incubated in PBS containing 1 mM each of NaF and Na_3VO_4 and solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100] as described previously. Lysates (20 μg) were solubilized in Laemmli sample buffer, boiled for 4 min and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% gel). The proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight at 4°C with Tris-buffered saline–Tween 20 containing 2% bovine serum albumin and incubated 1 h at room temperature with primary antibodies (pERK or ERK). Immunoreactive bands were revealed after 1 h incubation with horseradish peroxidase-conjugated anti-rabbit antibodies and the signals were visualized with an enhanced chemiluminescence detection system.

Migration assays

Transwells (8 μm pore size; Costar, Cambridge, MA) were precoated with 0.15% gelatin in 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 into 24-well plates. The upper chamber of each Transwell was filled with 50 μl of cells (1.0×10^6 cells/ml) and PASMCs were allowed to adhere for 30 min. The monolayers were then treated for 2 h by adding 50 μl of 2-fold concentrated inhibitor (delphinidin, Gleevec or berry extract) solution prepared in serum-free medium into the upper chamber and 600 μl of the compound solution (1×) into the lower chamber. After 2 h, PDGF-BB (10 ng/ml) was added to the lower chamber as a chemoattractant. The plate was 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% methanol (vol/vol). The migration was quantitated using computer-assisted imaging (Northern Eclipse software; Empix Imaging, Mississauga, Ontario, Canada) and data are expressed as the average density of migrated cells per four fields (magnification ×50).

Cell proliferation assays

Cells were plated in 96-well plates at 2500–5000 cells per well in 200 μl complete medium and incubated at 37°C under a humidified atmosphere containing 5% CO_2 for 24 h. The next day, the medium was removed and replaced by 100 μl of fresh medium containing 1% fetal bovine serum and the indicated concentrations of delphinidin or berry extract. Cell viability was determined by assaying the mitochondrial activity of treated cells after a 72 h incubation with the highly sensitive WST-1 assay. Briefly, 10 μl of tetrazolium salt WST-1 reagent (Roche, Laval, QC) was added to each well and the soluble formazan dye produced by metabolically active cells was monitored every minute for 30 min at 37°C on a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA).

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 smooth muscle medium-2 for 18 h. After cell detachment using trypsin–EDTA, an equal volume of 0.4% Trypan Blue Stain (Gibco BRL, Burlington, Ontario, Canada) was added to the cell suspension and the proportion of viable cells was evaluated under the field microscope. About 100 cells were counted and cells stained dark blue were not considered viable.

Capillary tube formation by EC/SMC in fibrin gels

HUVEC and PASMC were embedded within fibrin gels by the following procedure. Two hundred and fifty microliters of 2.5 mg/ml human fibrinogen solution (made in serum-free medium) was mixed with of human thrombin (0.5 U/ml) and the solution 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. HUVEC (2.5×10^5 cells/ml) and PASMC (1.0×10^5 cells/ml),

suspended in serum-free EC basal medium containing vehicle or delphinidin, were seeded onto each fibrin gel. After 24 h, cells had spread to form a confluent monolayer. 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 30 min at 37°C and 1 ml aliquots of fresh serum-free EC basal medium supplemented with FGF-2/VEGF or PDGF-BB/VEGF (50 ng/ml) and containing (or lacking) delphinidin were then added to each well. After 24 h, the cultures were photographed (×50) using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope, and the length of the capillary network was quantified with a map scale calculator.

Capillary tube stabilization by EC/SMC in fibrin gels

HUVEC and PASMC were embedded within fibrin gels as described above and formation of tubular structures was induced using FGF-2/VEGF (50 ng/ml) for 4 days. After 2 days of treatment with growth factors, delphinidin or Gleevec was added daily for 2 days. The cultures were photographed (×50) at days 4, 5 and 6 using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope, and the length of the capillary network was quantified with a map scale calculator.

Animals

Female nude mice (Crl:CD-1®-Foxn1^{nu}; 20–25 g, 6 weeks of age) (Charles River Laboratories, St Constant, Quebec, Canada) were housed in cover-top filter cages and placed in a high efficiency particulate air filtered horizontal laminar flow station. Caging equipment with which mice had contact was autoclaved prior to use and mice had *ad libitum* access to food and autoclaved water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Ste-Justine Hospital prior to animal use and mice were kept in accordance with the guidelines of the Canadian Council on Animal Care. All procedures were performed under sterile conditions in a vertical laminar flow hood. For Matrigel and cell injections, the mice were anesthetized by inhalation with isoflurane (2%/1 l O_2).

In vivo Matrigel plug assay

The Matrigel plug assay is based on the method of Passaniti *et al.* (37) with modifications. Briefly, immunodeficient mice 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, Ontario, Canada) containing berry extracts (500–1000 μg/ml), FGF-2 (250 ng/ml), PDGF-BB (250 ng/ml) and heparin (0.0025 U/ml). Control mice were injected with Matrigel without FGF-2/PDGF-BB. Matrigel containing ethanol alone was injected into some animals to serve as baseline controls. At the end of the study (day 7), some mice were injected through the tail vein with 200 μl of high-molecular weight (2 000 000 MW) fluorescein isothiocyanate-labeled dextran to visualize blood vessels within the plugs. Mice were killed 10 min after injection, and Matrigel plugs were harvested, washed with PBS and photographed with a digital Nikon Coolpix^{TM} 5000 camera (Nikon Canada, Mississauga, Ontario, Canada). Matrigel plugs were then fixed in 10% formalin and photographs of blood vessels within Matrigel implants were obtained using a microscope with a fluorescent light source (Zeiss Axiovert 100).

NCI-H460 tumor xenograft model

Mice were s.c. inoculated with NCI-H460 lung carcinoma (2.5×10^6 cells in 0.1 ml of 1% methylcellulose/modified Eagle's medium/1 mM sodium pyruvate) into the right flank of the mice. Two days before cell inoculation, the animals were treated orally by gavage with berry extracts diluted in autoclaved water (5 mg per mouse per day). Tumor volumes were measured daily with calipers and tumor volumes were calculated using the following formula: tumor volume (mm^3) = $a \times b^2 \times 0.52$, where a is the longest diameter, b is the shortest diameter and 0.52 is a constant to calculate the volume of an ellipsoid. All mice were killed when the first mouse of the control group reached an average volume of 1000 mm^3. Experiments were performed on six animals for each group.

Statistical analysis

The data are presented as means ± SEMs and statistical analyses were performed with Student's *t*-test when one group was compared with the control group. Statistical comparisons between groups were performed using one-way analysis of variance followed by Tukey's test. In the xenograft study, statistical significance of the difference between control and berry extracts group was determined by one-way analysis of variance followed by Bonferroni's *t*-test.

Results

Delphinidin inhibits PDGF-induced tyrosine phosphorylation of PDGFR-β

As we demonstrated previously (35), delphinidin inhibited VEGF-induced tyrosine phosphorylation of VEGFR-2 in HUVEC (Figure 1A). To evaluate whether delphinidin could also inhibit the phosphorylation of another tyrosine receptor involved in angiogenesis, PDGFR-β, we examined the effect of delphinidin on PDGF-BB-induced phosphorylation of this receptor. Quiescent PASMCs were incubated for 18 h in serum-free medium in the presence or absence of 25 μM delphinidin. The cells were then stimulated with 50 ng/ml PDGF-BB for 5 min and the phosphorylation state of PDGFR-β was assessed by immunoprecipitation of the receptor, followed by Tyr(P) immunodetection. Under these experimental conditions, we

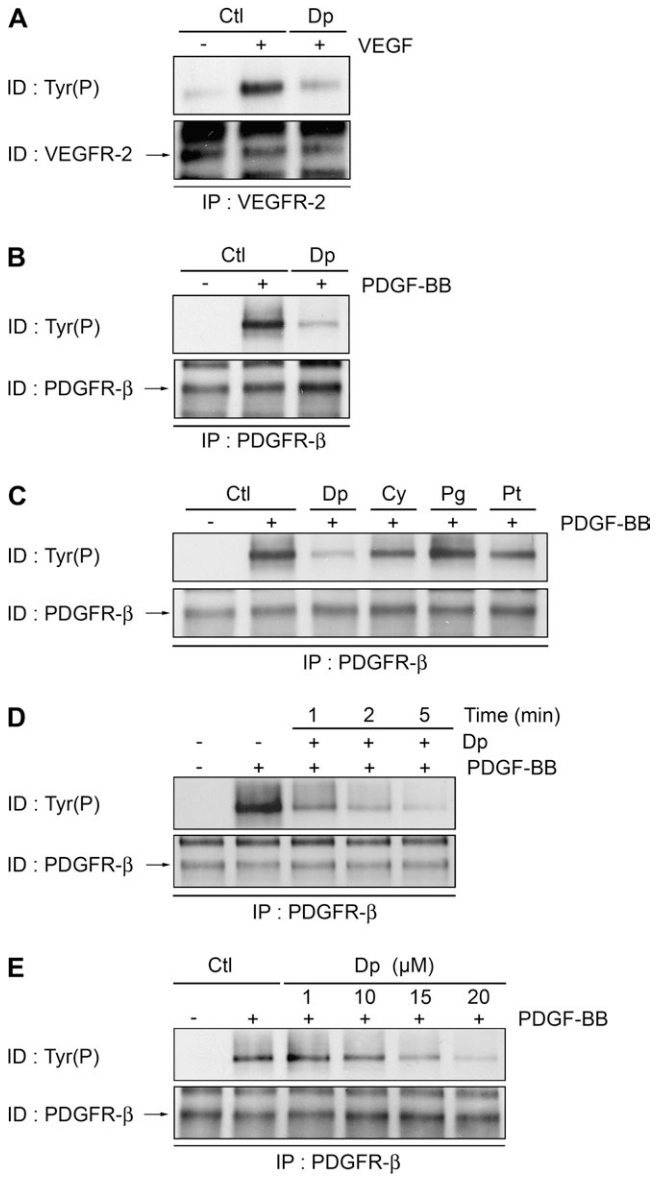

Fig. 1. Effect of delphinidin on phosphorylation of VEGFR-2 and PDGFR-β. (**A**) Quiescent HUVECs or (**B**) PASMCs were incubated in serum-free medium in the presence or absence of 25 μM delphinidin (Dp) for 18 h. (**C**) PASMCs were incubated with cyanidin (Cy), delphinidin, Pelargonidin (Pg) and petunidin (Pt) at 25 μM for 18 h. (**D**) PASMCs were incubated with delphinidin (25 μM) for 1, 2 and 5 min or (**E**) with delphinidin at the indicated concentrations for 18 h. The medium was replaced with serum-free medium without delphinidin, and HUVECs or PASMCs were stimulated with 100 ng/ml recombinant VEGF for 1 min or 50 ng/ml recombinant PDGF-BB for 5 min. Cells were lysed and the levels of tyrosine-phosphorylated receptors were monitored by immunoprecipitation with anti-VEGFR-2 or anti-PDGFR-β and immunoblotting with anti-Tyr(P) monoclonal antibody (PY99). Results are representative of three independent experiments.

observed that delphinidin dramatically reduced the phosphorylation of PDFGR-β induced by PDGF-BB (Figure 1B, top panel), an effect similar to the inhibition of the tyrosine phosphorylation of VEGFR-2 (Figure 1A, top panel). Blotting of the membranes with an antibody directed against PDGFR-β showed that delphinidin did not affect the amount of this receptor in the immunoprecipitate (Figure 1B, bottom panel). Moreover, among the anthocyanidins tested, we observed that delphinidin is the most potent inhibitor (Figure 1C, top panel). Interestingly, the inhibition of tyrosine phosphorylation of PDGFR-β by 25 μM delphinidin was very rapid, being observed as early as 1 min following the addition of the molecule and was already complete at 5 min (Figure 1D, top panel). As shown in Figure 1E (top panel), the inhibition of PDGF-induced tyrosine phosphorylation of PDGFR-β by delphinidin was dose dependent, with an median inhibition concentration (concentration that reduces the effect by 50%) around 10 μM.

Delphinidin inhibits PDGF-induced ERK phosphorylation

In order to investigate whether the inhibition of PDGFR-β tyrosine phosphorylation by delphinidin also results in the inhibition of downstream intracellular signaling events, we examined the effect of delphinidin on the PDGF-BB-induced ERK-1/2 phosphorylation, which is involved in signals induced by phosphorylation of PDGFR-β. PASMCs were pretreated with delphinidin for 18 h before the addition of 50 ng/ml PDGF-BB for 5 min. As shown in Figure 2A (top panel), delphinidin inhibited the tyrosine phosphorylation of ERK-1/2 in a concentration-dependent manner, with complete inhibition at 20 μM. Moreover, tyrosine phosphorylation of ERK-1/2 was rapidly inhibited by delphinidin after as little as 5 min of incubation and was completely abolished at 30 min (Figure 2B, top panel).

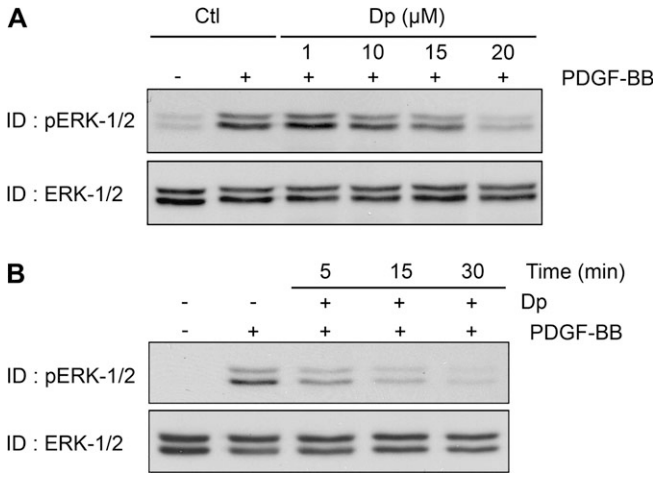

Fig. 2. Effect of delphinidin on phosphorylation of ERK-1/2 induced by PDGF-BB in PASMC. (**A**) Quiescent PASMCs were incubated in serum-free medium in the presence or absence of delphinidin (Dp) at the indicated concentrations for 18 h or (**B**) with 25 μM delphinidin for 1, 10, 15 and 20 min and stimulated with 50 ng/ml recombinant PDGF-BB for 5 min. Cells were lysed and equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel 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. Results are representative of three independent experiments.

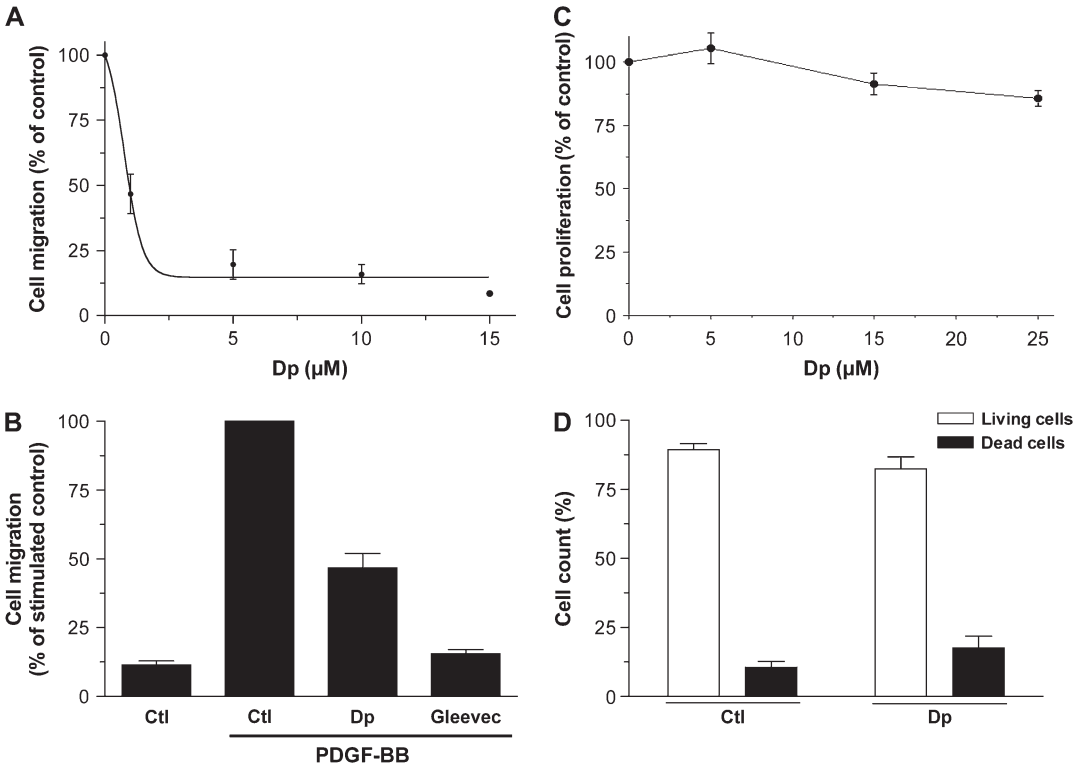

Fig. 3. Effect of delphinidin on PDGF-BB-induced proliferation and migration of SMCs. (**A**) PASMCs were pretreated for 2 h with various concentrations of delphinidin (Dp) or (**B**) with delphinidin or Gleevec at 5 μM before treatment with PDGF-BB. After 3 h incubation, migration was quantified by 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 with that observed with PDGF-BB-treated cells. Values are means of three independent experiments ($P < 0.001$ versus PDGF-BB alone); bars, ±SEM. (**C**) PASMCs were incubated for 48 h in the presence of 1% fetal bovine serum in the presence of the indicated concentrations of delphinidin and the extent of cell proliferation was measured using the WST-1 assay. (**D**) Subconfluent PASMCs were treated with or without 25 μM delphinidin for 18 h. 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. Results are means ± SEMs of three independent experiments.

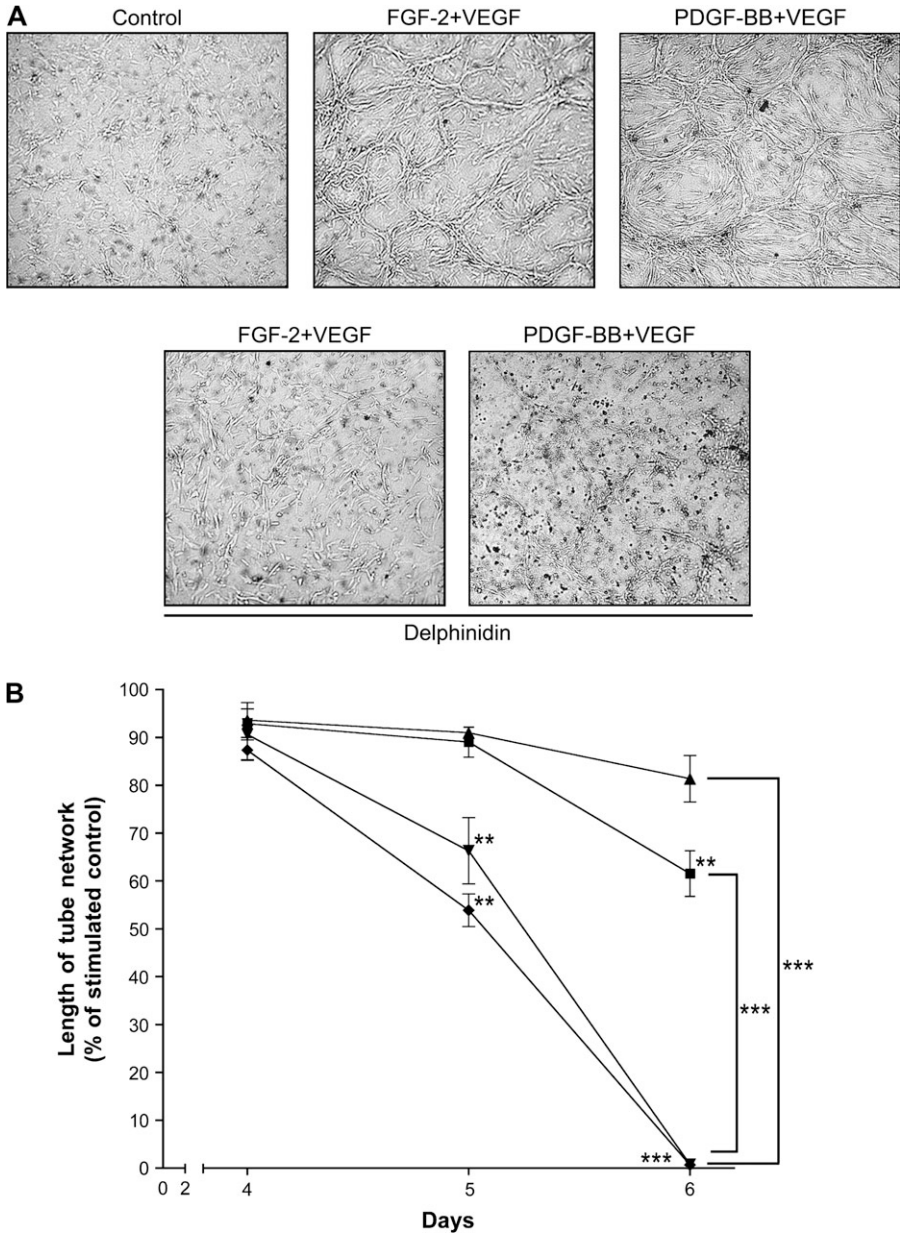

Fig. 4. Effect of delphinidin on growth factor-induced tube formation and stabilization of HUVEC/PASMC in three-dimensional fibrin gels. HUVECs were cocultured with PASMC within fibrin gels in serum-free medium containing (or lacking) delphinidin (Dp) and stimulated with growth factors as described in Materials and Methods. (**A**) Cocultured cells were pretreated with delphinidin at 12.5 μM and then stimulated with growth factors (FGF-2/VEGF or PDGF-BB/VEGF) before the formation of tubes. After 24 h, cells were photographed (×50). The pictures shown are representative of three independent experiments. (**B**) Cocultured cells were treated with delphinidin (filled triangle, 25 μM; filled square, 50 μM or inverted filled triangle, 75 μM) or Gleevec (filled diamond, 25 μM) when the tubes were formed with the addition of FGF-2 and VEGF. After 4, 5 and 6 days of culturing, the length of the tube network was quantified using a map scale calculator. Values are means of three independent experiments. Statistically significant differences, as compared with day 4 as well as between treatments at day 6, were calculated by one-way analysis of variance followed by Tukey's test ($^{**}P < 0.01$; $^{***}P < 0.001$); bars, ±SEM.

Delphinidin inhibits PDGF-induced migration of SMCs

Numerous studies have shown that activation of mitogen-activated protein kinase, such as ERK-1/2, is important for PDGF-induced cell migration (38–41). Since delphinidin inhibited PDGF-BB-induced PDGFR-β and ERK phosphorylation, we studied the effect of this molecule on PASMC migration. Cells were allowed to adhere to gelatin-coated Transwells and were incubated for 2 h with different concentrations of delphinidin before the addition of PDGF-BB (10 ng/ml) to the lower chamber. Under these conditions, delphinidin inhibited PDGF-BB-induced migration of PASMC in a dose-dependent manner (Figure 3A). We next compared the efficacy of delphinidin in inhibiting PASMC migration to that achieved with Gleevec (STI571), a drug used clinically for the treatment of chronic myeloid leukemia (42) and gastrointestinal stromal tumor (43) and whose mechanism of action includes inhibition of PDGFR. For both these molecules, significant inhibition occurred at 5 μM (Figure 3B). These results indicate that delphinidin, a naturally occurring molecule, is almost as effective as Gleevec at inhibiting PDGF-induced PASMC migration. In order to verify whether the inhibitory effect of delphinidin was not due to cytotoxicity, the impact of the molecule on cell proliferation and cell viability was measured. As shown in Figure 3C and D, incubation of the cells for 24 h in the presence of delphinidin induced neither significant decrease in cell proliferation nor increased cell death compared with control cells, confirming the specific

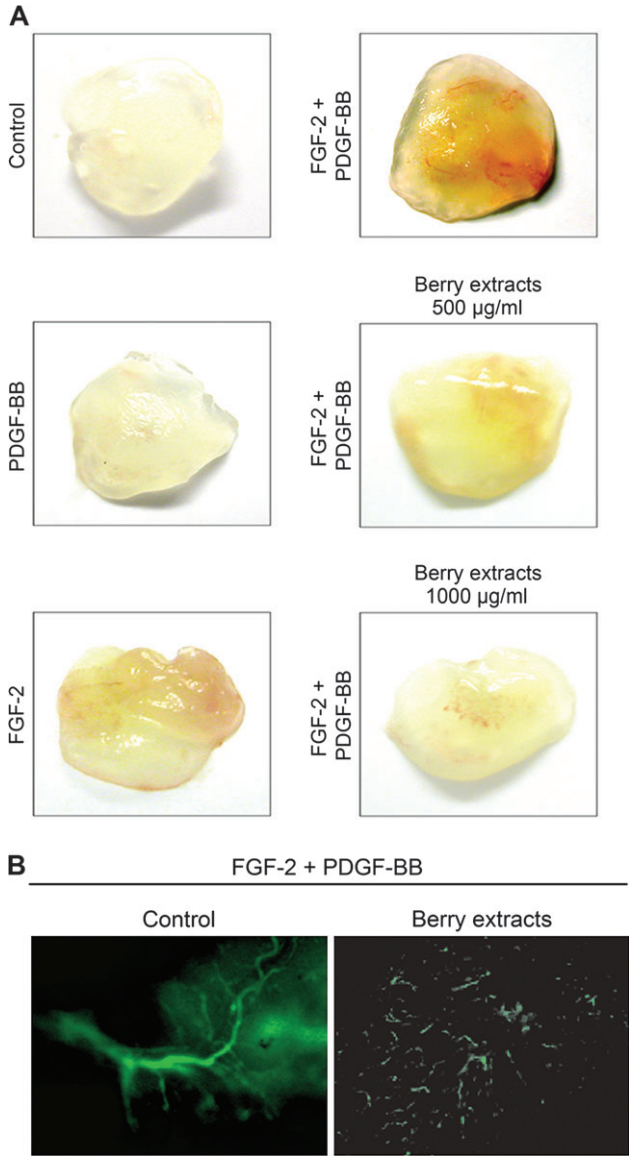

Fig. 5. Effect of berry extracts on synergistic angiogenesis *in vivo* induced by FGF-2 and PDGF-BB. Crl:CD-1®-Foxn1^{nu} nude mice were injected s.c. with 0.5 ml of Matrigel containing berry extracts (500 or 1000 µg/ml), FGF-2 (250 ng/ml), PDGF-BB (250 ng/ml) and heparin (0.0025 U/ml) to allow the formation of solid gel plugs. After 7 days, the mice were killed and Matrigel plugs were excised. Four mice were used in each group. (**A**) Representative Matrigel plugs that contained no growth factors (control), FGF-2 alone, PDGF-BB alone or growth factors plus berry extracts were photographed. (**B**) Matrigel plugs supplemented with FGF-2 and PDGF-BB containing berry extract or not were removed from mice after fluorescein isothiocyanate–dextran injection. The vasculature was visualized and photographed using a fluorescent microscope.

inhibitory effect of delphinidin on the phosphorylation of PDGFR-β and ERK-1/2.

Delphinidin inhibits growth factor-induced tube formation in three-dimensional fibrin gels

Communication between the EC and SMC compartments appears to be essential for normal blood vessel development (8,44). This type of cell–cell interaction has been explored in several static coculture models (44–48). Cocultures formed by culturing EC directly on SMC provide the best model since they replicate the spatial arrangement of cells in arteries and permit close interaction between the two cell types. Using this assay, we further investigated the antiangiogenic effects of delphinidin on vessel formation and stabilization. HUVEC and PASMC were grown together on three-dimensional fibrin gels and addition of either FGF-2/VEGF or PDGF-BB/VEGF rapidly led to the formation of elongated tube-like structures, compared with the control (Figure 4). We used a combination of these growth factors because it has been reported to be effective in inducing new vessels, as compared with growth factors alone (49,50). Before the capillary network was formed, treatment of the cocultured cells with 12.5 µM delphinidin resulted in a strong inhibition of the tube formation induced by both FGF-2/VEGF and PDGF-BB/VEGF compared with the stimulated controls (Figure 4A). Although the inhibitory effect of delphinidin toward VEGFR-2 (35) may contribute to this inhibition of tube formation, similar results were obtained when capillary structures were induced in the absence of VEGF (using either PDGF or PDGF + FGF-2) (data not shown), suggesting that the effect of delphinidin on PDGFR also plays a major role in the observed inhibition of tube formation.

In order to study the effect of delphinidin on the stabilization of vessels, the capillary networks were treated with various concentrations of delphinidin and Gleevec. In our assay, we used costimulation of the capillary network with FGF-2 and VEGF because the vessels formed by these growth factors are more robust and stable than those induced by the combination of PDGF-BB and VEGF. Moreover, it was recently shown that costimulation by VEGF and FGF-2 enhances endothelial–mural interaction through PDGF-BB/PDGFR-β signaling in an *in vitro* model of mural cell recruitment: VEGF enhances PDGF-BB secretion by the endothelium and FGF-2 enhances PDGFR-β expression in SMC (50). Using this combination of growth factors, we observed that higher concentrations of delphinidin and Gleevec were needed to destabilize the vessels since complete destruction of tubular structures were observed following 6 days of treatment of the cocultures with 25 µM Gleevec or 75 µM delphinidin (Figure 4B). The observation that the destabilizating effect of Gleevec is more potent than that of delphinidin is not surprising since Gleevec is a more potent inhibitor of PDGFR as compared with delphinidin. Nevertheless, the destabilization of vessels by delphinidin after 2 days of the latest treatment with the compound (day 6) suggests that the inhibitory effect of this molecule on PDGFR signaling may lead to the regression of established vessels.

Anthocyan-rich extract inhibits FGF-2 and PDGF-BB-induced angiogenesis in vivo

Since it was reported that the combination of FGF-2 and PDGF-BB synergistically induces stable vascular networks in experimental animal models *in vivo* (51), we next examined the effect of anthocyans on vessel formation induced by these growth factors, using a Matrigel plug assay (Figure 5A). To this end, we used an anthocyan-rich berry extract, which is more representative of dietary intake of anthocyans and which has potent effects *in vitro* and *in vivo* (52,53). Matrigel plugs that contained no growth factors, FGF-2 alone, PDGF-BB alone or a combination of FGF-2 and PDGF-BB in the presence or absence of berry extracts were injected s.c. into Crl:CD-1®-Foxn1^{nu} mice, forming semisolid plugs. Plugs without growth factors or with PDGF-BB had virtually no vascularization or vessel structures after 7 days (Figure 5A) compared with FGF-2. Plugs supplemented with a combination of FGF-2 and PDGF-BB had extensive vascularization and vessels throughout the plug. However, plugs taken from mice treated with 500 or 1000 µg/ml of berry extract had markedly reduced vascularization within the plugs (Figure 5A). These results were confirmed by the fluorescein isothiocyanate–dextran assay, which shows that blood vessels in the control FGF-2 and PDGF-BB supplemented plugs had a well-defined branching structure indicative of a functioning capillary network (Figure 5B). In contrast, in plugs containing the berry extracts, the fluorescence associated with blood vessels was less intense with evidence of vascular disruption (Figure 5B). Since we reported previously that delphinidin impairs vessel formation in the Matrigel assay (35), this suggests that this molecule may contribute to the inhibition of neovascularization triggered by the berry extract. However, based on the reported amount

of delphinidin in OptiBerry (1% wt/wt), a synergistic action from the various anthocyanidins present in the extract is very likely since the concentration of delphinidin in the Matrigel plugs containing the berry extract is ~20 times lower than in our previous study.

Anthocyan-rich extract inhibits human tumor growth

The *in vivo* efficacy of anthocyan-rich berry extracts against human lung carcinoma was studied using tumor xenografts in athymic mice by s.c. implantation of NCI-H460 cells, a cell line shown previously to overexpress PDGFs and their receptors (3). This extract potently inhibited PDGF-dependent tyrosine phosphorylation of PDGFR-β as well as PDGF-dependent cell migration but had no significant effect on either PASMC or tumor cell proliferation (Figure 6A).

Oral gavage feeding of berry extracts at 200 mg/kg/day for 32 days did not induce any significant change in body weight (data not shown). Furthermore, we did not observe any adverse health effects

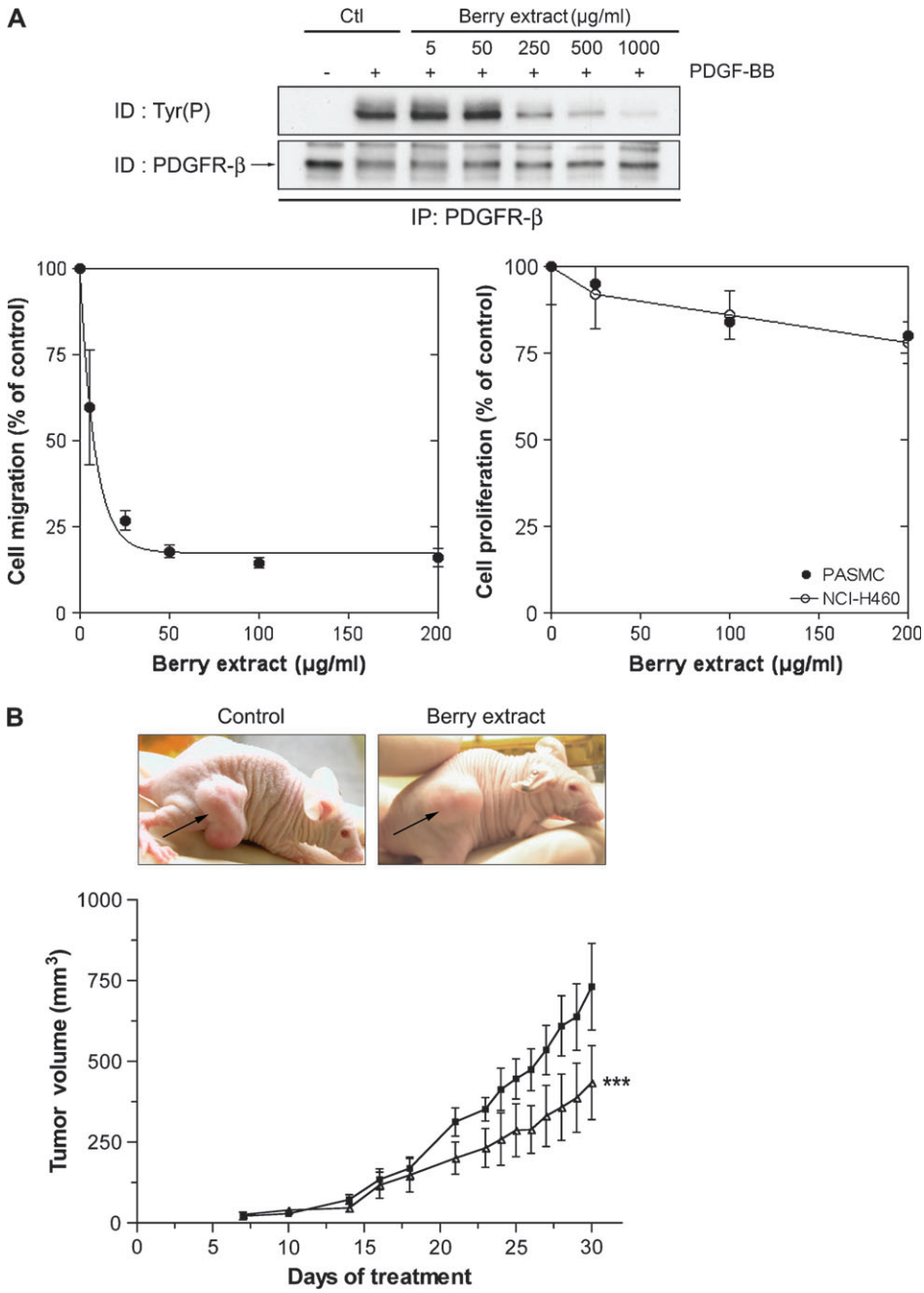

Fig. 6. Effect of berry extract feeding on NCI-H460 tumor xenograft growth in athymic nude mice. (**A**) Upper panel: effect of berry extracts on phosphorylation of PDGFR-β. PASMCs were incubated 18 h in serum-free medium in the presence or absence of the indicated concentrations of the berry extracts and stimulated with 50 ng/ml recombinant PDGF-BB for 5 min. Cells were lysed and the levels of tyrosine-phosphorylated receptors were monitored by immunoprecipitation with anti-PDGFR-β and immunoblotting with anti-Tyr(P) monoclonal antibody. Results are representative of two independent experiments. Lower panel: comparative effect of the berry extract on PDGF-BB-induced migration of PASMC (left) and on the proliferation of PASMC (filled circle) and NCIH-460 tumor cells (closed circle) (right). Results are means ± SEMs of three independent experiments. (**B**) Tumor volume (mm^3) as a function of berry extract treatment. Each mouse was s.c. implanted with 2.5×10^6 cells mixed with methylcellulose in the right flank. Mice were gavaged with 200 mg/kg dose of berry extract each day for the duration of the study, starting 2 days before cell inoculation. Statistical significance of differences between control and berry extract-fed groups was calculated by one-way analysis of variance followed by Bonferroni's *t*-test ($^{***}P < 0.001$ versus control alone); bars, ±SEM.

as monitored by activity and posture of mice. At the end of the experiment, the berry extracts reduced tumor volume per mouse by 40.6% (731.4 ± 299.4 mm^3 in control versus 434.1 ± 280.2 mm^3 in berry extracts-fed group; Figure 6B), although a time-dependent growth inhibitory effect of berry extracts on tumor growth was in evidence throughout the study. These results suggest an *in vivo* anticancer efficacy of anthocyan-rich extract in the nude mice NCI-H460 lung cancer xenograft model, without any toxicity, and that this inhibitory effect may be related, at least in part, to the inhibition of the PDGF-dependent signaling axis.

Discussion

The inhibition of angiogenesis as a means of preventing tumor growth has received considerable attention in recent years (54,55). Recently, tumor vascular targeting has been expanded to include not only EC but also periendothelial cells or vascular mural cells, such as pericytes and SMC, which provide both survival signals and structure support to EC and contribute to a mature, functional vasculature (56). Consequently, strategies aimed at the suppression of both VEGF- and PDGF-mediated neovessel growth are essential for efficient inhibition of tumor progression.

In this study, we show that delphinidin inhibits PDGF-BB-dependent tyrosine phosphorylation of PDGFR-β in a dose- and time-dependent manner and that this inhibitory effect is associated with the inhibition of downstream signaling events triggered by the receptor, such as PDGF-BB-dependent phosphorylation of the $p42^{MAPK}$ and $p44^{MAPK}$ isoforms (ERK-1/2). This inhibitory effect was very rapid, being observed after only 5 min of preincubation of the cells with the molecule. Such a rapid inhibition of PDGFR was also observed for ellagic acid (25), a polyphenol present in fruits and nuts, further emphasizing the sensitivity of PDGFR to dietary-derived polyphenols. Although the mechanims underlying the inhibitory effect of these dietary-derived polyphenols remain to be established, previous work suggested that some of these molecules, such as epigallocatechin gallate from green tea, inhibit activation of receptor tyrosine kinase by competing with adenosine triphosphate for binding to the kinase domain of this receptor, resulting in impaired activation of key signaling intermediates (57). Since similar competitive inhibition with respect to adenosine triphosphate has also been observed for other polyphenols (58), it is tempting to speculate that such a mechanism could also be involved in the inhibitory effect of delphinidin toward PDGFR.

Importantly, the inhibition of PDGFR by delphinidin was correlated with an inhibition of SMC migration, a crucial event in the stabilization of neovessels. Interestingly, under our experimental conditions, this inhibitory effect of delphinidin was similar to that of Gleevec, a PDGFR inhibitor, suggesting that low concentrations of delphinidin may have a significant impact on *in vivo* angiogenesis.

In this respect, we observed that delphinidin potently inhibited the capillary-like structure formation and stabilization by HUVEC and PASMC in response to VEGF, FGF-2 and PDGF-BB. The inhibitory effect of delphinidin *in vitro* is likely to be relevant *in vivo* since berry extracts containing anthocyanidins such as delphinidin remarkably suppressed new blood vessel formation in Matrigel plugs implanted in mice, possibly through their inhibitory effects on both VEGFR and PDGFR. This effect is likely to be of significant importance for tumor growth *in vivo* since oral administration of berry extracts markedly reduce the growth of NCI-H460, a highly invasive lung carcinoma cell line, with 40% reduction in tumor volume. This inhibition was completely devoid of toxicity, in agreement with studies showing a lack of adverse effects for single oral administration of as much as 5000 mg/kg of berry extracts in rats (59).

Since delphinidin targets epidermal growth factor receptor (60), VEGFR-2 (35) and also, in the present paper, PDGFR-β, it is tempting to speculate that the antitumor effect of the anthocyan-rich extract observed in our study may be related to its inhibitory effect on these pathways. In agreement with this hypothesis, combined inhibition of the VEGF and epidermal growth factor receptor pathways has been shown to enhance antitumor efficacy and to help overcome tumor resistance mechanisms in non-small-cell lung cancer (61,62). Furthermore, it was demonstrated that the use of Gleevec in non-small-cell lung cancer xenografts results in decreases in phosphorylated PDGFR-β and VEGF expression, affecting angiogenesis, intratumoral interstitial fluid pressure and tumor oxygenation (63).

In summary, we demonstrated for the first time that the inhibitory effect of delphinidin on angiogenesis could not only involve its inhibitory effect on VEGF (35) but also its inhibition of the PDGFR-β. The combined inhibition by delphinidin of these two important angiogenic receptors is likely to play a central role in the antiangiogenic and antitumor activity of this compound. These findings emphasize the chemopreventive possibilities of dietary-derived molecules and highlight the importance of nutrition in cancer prevention.

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