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Original Contribution

Inhibition of sphingosine-1-phosphate- and vascular endothelial growth factor-induced endothelial cell chemotaxis by red grape skin polyphenols correlates with a decrease in early platelet-activating factor synthesis ☆

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Abstract

Vascular endothelial growth factor (VEGF) and platelet-derived lipid sphingosine-1-phosphate (S1P) are two proinflammatory mediators which contribute to angiogenesis, in part through the synthesis of platelet-activating factor (PAF). The red grape skin polyphenolic extract (SGE) both prevents and inhibits angiogenesis in the Matrigel model, decreases the basal motility of endothelial and cancer cells, and reverses the chemotactic effect of S1P and VEGF on bovine aortic endothelial cells (BAECs) as well as the chemotactic effect of conditioned medium on human HT-1080 fibrosarcoma, human U-87 glioblastoma, and human DAOY medulloblastoma cells. Inhibition of VEGF- and S1P-mediated chemotaxis by SGE is associated with a down-regulation of ERK and p38/MAPK phosphorylation and a decreased in acute PAF synthesis. Notably, as do extracellular inhibitors of PAF receptor, SGE prevents S1P-induced PAF synthesis and the resulting activation of the S1P/endothelial differentiation gene-1 cascade. Given the key role of VEGF and S1P in inflammation, angiogenesis, and tumor invasion, SGE may therefore contribute to prevent (or to delay) the development of diseases associated with angiogenesis dysregulation, including cancer. The dual inhibition of S1P- and VEGF-mediated migration of endothelial cell and of serum-stimulated migration of U-87 cells suggests a usefulness of SGE against highly invasive human glioblastoma.

Keywords: Angiogenesis; Cancer; Grape skin polyphenols; Sphingosine-1-phosphate; Vascular endothelial growth factor; Cell migration; Platelet-activating factor; Free radical

Introduction

Vascular endothelial growth factor (VEGF) has first been identified as a platelet-derived growth factor [1]. It is already established that free radicals markedly increase the expression and the release of VEGF in a variety of cell types [2,3]. VEGF contributes to inflammation through the synthesis by endothelial cells (EC) of platelet-activating factor (PAF), a potent inflammatory mediator capable of promoting EC migration [4]. Compelling evidence has revealed that VEGF expressed by cancer cells deprived from oxygen or nutrients is capable of inducing neovascularization (neoangiogenesis), the process by which tumors grow and invade surrounding host tissues [1,5,6]. Thereby, in addition to its proinflammatory effects, VEGF, through binding with high affinity on VEGF receptor-2

Abbreviations: pI3K, phosphatidylinositol 3-kinase; BAEC, bovine aortic endothelial cells; BCS, bovine calf serum; EC, endothelial cell; ECM, extracellular matrix; EDG, endothelial gene; S1P/EDG, endothelial gene signaling cascade activated by S1P; EBM-2, endothelial cell basal medium-2; FBS, fetal bovine calf serum; MAPK, mitogen-activated protein kinases; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; MSC, bone marrow stromal cell; MT1-MMP, membrane type 1-matrix metalloproteinase; PAF, platelet-activating factor; PBS, phosphate-buffered saline; p38/MAPK, p38 mitogen-activated kinase; S1P, sphingosine-1-phosphate; SGE, grape skin extract; VEGF, vascular endothelial growth factor.

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(VEGFR-2, Flk-1/KDR) at the endothelial cell surface, contributes to switch tumors from a dormant toward an invasive state [5]. Of note, VEGF is the proangiogenic cytotokine most closely associated with aggressive tumor proliferation [5–9]. As a result, inhibition of VEGF-mediated signaling in EC has become a primary target for antiangiogenic strategies [8]. Interestingly, in clinical practice, VEGF signaling may serve both as a prognosis factor of tumor aggressivity and as a marker for the effectiveness of antiangiogenic inhibitors used in cancer therapy [9,10].

In man, various agents present in serum contribute to amplify VEGF signaling and, consequently, aggressive cancer growth [11]. One of the most effective is the sphingolipid derivative: sphingosine 1-phosphate (S1P), which has recently been identified as the major chemoattractant of serum [12]. This bioactive lipid is released by stimulated platelets during activation of clotting cascade and by several cell types in response to various extracellular stimuli [13]. It is involved in a variety of physiological processes, including thrombosis and hemostasis [12], and plays an important role in the regulation of EC migration and proliferation which are prerequisite of the angiogenic process [12,14]. It is already established that in vivo, S1P contributes to neovascularization [12], to inflammation [14], and to the metastastic cascade [15]. Recently, S1P-driven EC inflammation has been associated with the synthesis of platelet-activating factor, a potent inflammatory mediator which takes part in the S1P chemotactic effect, and S1P-induced EC migration has been demonstrated to be PAF dependent [16].

All these data support a close link between increased S1P and VEGF signaling, inflammation, neoangiogenesis, and cancer invasion and metastasis. By inhibiting events sensitive to redox regulations, such as the activity of key enzymes involved in inflammation and angiogenesis, radical scavenging agents may thereby positively affect cancer proliferation. Until the discovery of the "French paradox" [17], much attention has been focused on the health properties of polyphenols from wine and grape berries (see [18,19] as reviews). These properties depend in large part to their antioxidant and free radical scavenging properties (OK). Because these are dose dependent and structure related [20], small qualitative and quantitative differences in the polyphenolic pattern of grape extracts are likely to have profound repercussions on the antiangiogenic, anti-inflammatory, and anticancer activity of these extracts. Agarwal and coworkers have reported that grape seed anthocyanins inhibit angiogenesis in vitro [21,22] while another team has reported the opposite effects [23,24]. It cannot be excluded that these different results are due to differences in the chemical composition of the extracts tested by each team. The polyphenolic composition of red grape skin polyphenols significantly differs from that of grape seeds [25]. To our knowledge, neither the antiangiogenic activity of red grape skin extract nor, more generally, the effects of grape polyphenols on S1P-induced EC migration have been examined. In the present study, we show for the first time that in vitro, red grape skin polyphenols issued from Cabernet Sauvignon berries (SGE) decreases the basal motility of endothelial and cancer cells, and antagonizes the chemoattractant effect of VEGF and S1P on BAEC, in part by down-regulating PAF synthesis. We demonstrated that SGE not only inhibits and prevents angiogenesis in a dose-dependent manner but also reduces the migration of cancer cells under basal and stimulated conditions. Taken together, the present findings suggest a chemopreventive and a therapeutic value of SGE against cancer and diseases characterized by alterations in the physiological mechanisms controlling angiogenesis. Considering its capacity to antagonize both S1P- and VEGF-induced EC motility, and the motility of U-87 human glioblastoma cells induced by conditioned medium, SGE may, in particular, be helpful against highly invasive glioblastoma.

Material and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, Ontario, Canada) and serum (FBS, fetal bovine calf serum, and BCS, bovine calf serum) was purchased from Hyclone Laboratories (Logan, UT). Matrigel was purchased from Becton Dickinson Labware (Bedford, MA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). Anti-phospho-ERK and anti-phospho-p38 polyclonal antibodies were respectively purchased from Cell Signaling Technology (Beverly, MA) and Calbiochem (San Diego, CA). Anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Human recombinant VEGF was obtained from R and D Systems (Minneapolis, MN) and S1P from Sigma (St. Louis, MO). Micro-bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). [^{3}H]Acetate and ^{3}H-PAF were from New England Nuclear (Boston, MA). All other reagents were from Sigma-Aldrich Canada. Methanol, acetonitrile, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). All these solvents were liquid chromatography grade.

The grape skin anthocyanins batch CB-2002/11/9 (SGE) was prepared in the laboratory from red grape skins issued from Cabernet Sauvignon variety. High-pressure liquid chromatography/electrospray ionization–mass spectrometry (HPLC/ESI-MS) analysis has revealed that SGE has a *trans*-resveratrol content of 1800 ppm (data not shown).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and maintained in endothelial cell basal medium-2 (EBM-2) supplemented with EGM-2 MV growth factor mixture (Clonetics, San Diego, CA). Bovine aortic endothelial cells (BAECs; Clonetics) were cultured in Dulbecco's modified Eagle's medium (DMEM) with low glucose, containing 10% BCS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). DAOY (human medulloblastoma) and U-87 MG (human glioblastoma) cell lines were obtained from ATCC (American Tissue Culture Collection, Manassas, VA) and HT1080 (human fibrosarcoma)

cells were kindly provided by ConjuChem (Montréal, Québec, Canada). All these cells were cultured in minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate and containing 10% BCS and antibiotics. The cells were cultured at 37°C under a humidified 95%–5% (v/v) mixture of air and CO_2.

Matrigel endothelial cell tube formation assay

Matrigel (12.5 mg/ml) was thawed at 4°C, and 50 ml was 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 HUVECs (20,000 cells/well) with or without 1% FBS. In some treatments, HUVECs were added after an 18-h treatment with the indicated concentrations of SGE (pre-treatment). In other treatments, HUVECs were treated for 18 h with SGE after adhesion to Matrigel. The medium was then removed and replaced by fresh medium EBM-2 supplemented (or not) with natural compound extracts (treatment). The formation of capillary-like structures was examined microscopically and pictures (50×) were taken using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope. The extent of 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).

Migration assays

Transwells (8-mm pore size; Costar, Cambridge, MA) were precoated with 0.5% gelatin–phosphate-buffered saline (PBS) by adding 600/100 μl in the lower/upper chambers for 24 h at 4°C. The transwells were then washed with PBS and assembled in 24-well plates. The upper chamber of each transwell was filled with 1.0×10^6 cells (BAEC or U87 or HT 1080 or DAOY) in 100 μl of medium, and the cells were allowed to adhere for 30 min. Cells were then treated for 2 h by adding 100 μl of 2-fold concentrated natural compound extracts prepared in serum-free medium into the upper chamber and 600 μl of serum-free medium into the lower chamber. Migration was initiated by adding VEGF (10 ng/ml) or S1P (1 μM) to the lower chamber (BAECs) or by replacing the medium with 600 μl of U87-conditioned medium containing drugs (tumor cells). The plate was incubated at 37°C in 5% CO_2–95% air for 3 h. Cells that have migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% Crystal Violet–20% methanol (v/v). The migration was quantified using computer-assisted imaging and data were expressed as the average density of migrated cells per four fields (50× magnification).

ERK and p38 phosphorylation

Cells were plated in 100-mm plastic dishes at 5000 cells/cm^2 and were grown to confluence before overnight serum starvation (18 h) without supplements. Cells were treated with vehicle or with natural compound extracts and stimulated with 50 ng/ml VEGF or 1 μM S1P for 10 min. After treatment, cells were washed once with PBS containing 1 mM NaF and 1 mM Na_3VO_4 and were incubated in the same medium for 1 h at 4°C. The cells were lysed 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, 1% (v/v) Triton X-100) containing NaF/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 (Pierce).

Immunoblotting procedures

Lysates (20 μg) were solubilized in Laemmli sample buffer, boiled for 4 min, and resolved by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked overnight at 4°C with TBS–Tween 20 (147 mM NaCl, 20 mM Tris–HCl, pH 7.5, and 0.1% Tween 20) containing 2% BSA and incubated 1 h at room temperature with primary antibody. Immunoreactive bands were revealed after 1 h incubation with HRP-conjugated anti-rabbit antibodies and the signals were visualized with an ECL detection system.

Measurement of PAF synthesis

PAF production by BAEC was measured by incorporation of [^{3}H]acetate into lyso-PAF as described previously [4]. Briefly, confluent BAECs (6-well tissue plate) were rinsed with Hank's balanced salt solution (HBSS) + Hepes (10 mM, pH 7.4) and then stimulated for 10 min with 1 ml of HBSS–Hepes buffer (10^{-2} M, pH 7.4) containing $CaCl_2$ (10^{-2} M), [^{3}H]acetate (2.5×10^{-5} Ci) and PBS with or without S1P (1 μM). The reaction was stopped by the addition of acidified methanol. Polar lipids were isolated according to Bligh and Dyer. After solvent evaporation under N_2, ^{3}H-PAF was purified by HPLC on silica gel vs an authentic ^{3}H-PAF sample, and quantified by counting radioactivity with a β-counter.

Results

Treatment of endothelial cells by SGE inhibits the formation of capillary-like structures in the Matrigel model

As a first approach in the evaluation of the antiangiogenic activity of SGE, we tested its capacity to inhibit the formation of capillary-like structures in the Matrigel model, commonly used to identify antiangiogenic drugs. Matrigel is a reconstituted basement membrane containing various endogenous growth factors, including transforming growth factors (TGF)-β, EGF, b-FGF, and PDGF that stimulate angiogenesis in vitro and in vivo [26].

In a first series of experiments, human umbilical veinous endothelial cells were treated by SGE (0, 5, 10, or 25 μg/ml) after adhesion to Matrigel. In the absence of SGE, HUVECs migrate, proliferate, and differentiate into an extensive network of thin tubules which mimic capillaries. The network formation was decreased by 11.1% with 10 μg/ml SGE, and was

completely abolished when SGE was used at 25 μg/ml (Fig. 1). No effect was observed when the cells were treated with SGE at 5 μg/ml (data not shown).

The antiangiogenic effect of SGE is enhanced when HUVECs are preincubated with SGE before adhesion to Matrigel

In a second series of assays, HUVECs were exposed for 18 h to SGE (0, 5, 10, and 15 μg/ml) in serum-free medium or in the presence of 1% serum, before adhesion to Matrigel. The medium was then removed, and the cells were allowed to form capillary-like structures. Under these conditions, a dose-dependent decrease of network length was observed in the dose range 5–15 μg/ml (Fig. 2). When SGE was used at a concentration of 15 μg/ml, tubulogenesis was suppressed as well in the absence (Fig. 2A) as in the presence (Fig. 2B) of serum. The antiangiogenic effect of SGE was maximal when the cells were preincubated with SGE in the absence of serum. In the absence of serum, this effect was observed, but at a lower intensity. Indeed, SGE at 5, 10, and 15 μg/ml decreased the tube network formation by 28.5, 71.4, and 98% in serum-free medium (Fig. 2A) instead of 3.6, 38.6, and 97% in the presence of 1% serum (Fig. 2B).

Pretreatment with SGE inhibits the basal motility of BAEC and antagonizes the chemotactic effect of VEGF and S1P

Endothelial cell migration in response to proangiogenic stimuli is a prerequisite of angiogenesis and a hallmark of neoangiogenesis. Because previous reports have revealed that VEGF and S1P increase BAEC migration [16], we used BAEC and Boyden-derived chambers to examine whether a 2-h preincubation with SGE affected EC migration in the absence, and in the presence, of these two proinflammatory and proangiogenic agents. As seen in Fig. 3, addition of VEGF or S1P to the lower compartment of the Boyden chamber strongly increased BAEC motility. The migration was increased 4.3-fold with 10 ng/ml VEGF and 5.6-fold with 1 μM S1P. A preexposure of BAEC to SGE, dose dependently decreased the migration of BAEC under basal conditions, and antagonized the chemotactic effect of VEGF and S1P. When SGE was used at 25 μg/ml, the basal migration was reduced by 73%, the migration induced by VEGF was almost completely suppressed (−93%), and the migration induced by S1P was decreased by 40%. Of note, this latter value was similar to that (−41%) observed by Bernatchez et al. [16] after pretreatment of BAEC with a selective antagonist of extracellular PAF receptor [BN52021 (10 μM)].

Pretreatment with SGE inhibits the motility of various cancer cells under basal and stimulated conditions

To determine whether inhibition of cell motility by SGE was specific to endothelial cells, complementary assays were further conducted in Boyden-derived chambers on U-87 MG (human glioblastoma), DAOY (human medulloblastoma), and HT1080 (human fibrosarcoma) cells. In our assays, the three human cancer cell lines were preincubated for 2 h in the absence or the presence of SGE (25 μg/ml) and then allowed

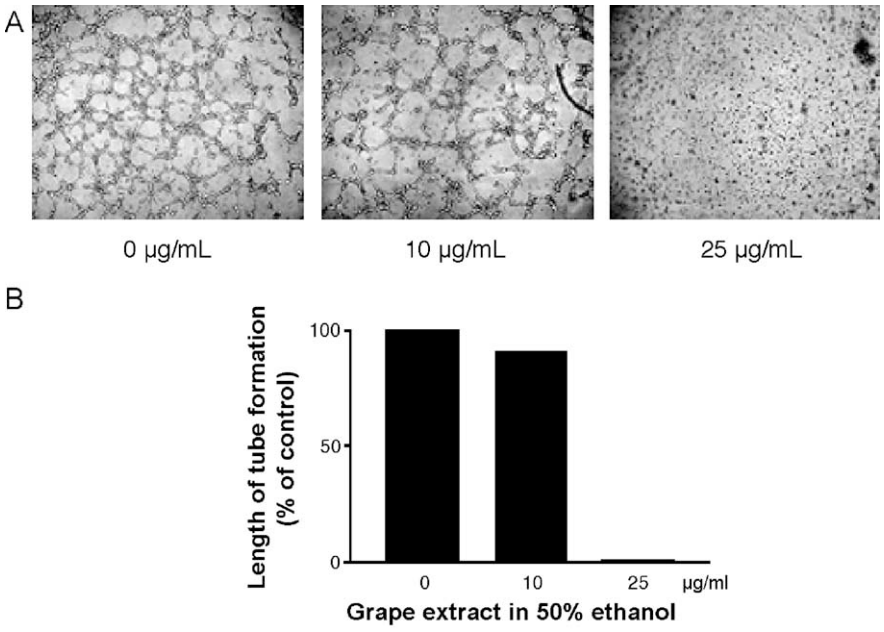

Fig. 1. SGE inhibits capillary-like structure formation on a 2D-Matrigel basement model. HUVECs (20,000 cells/well) were seeded on Matrigel and allowed to adhere for 6 h at 37°C. The medium was removed and replaced by fresh EBM-2 medium supplemented with 0, 5, 10, or 25 μg/ml SGE, and incubated for 18 h at 37°C as described under Materials and methods. (A) The formation of capillary-like structures was examined microscopically and pictures (50× magnification) were taken using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope. (B) The total length of capillary-like structures was quantified by analysis of digitized images with the Northern ECLIPSE software program. For each test, five randomly chosen areas were measured and averaged. The result was the average of two independent tests.

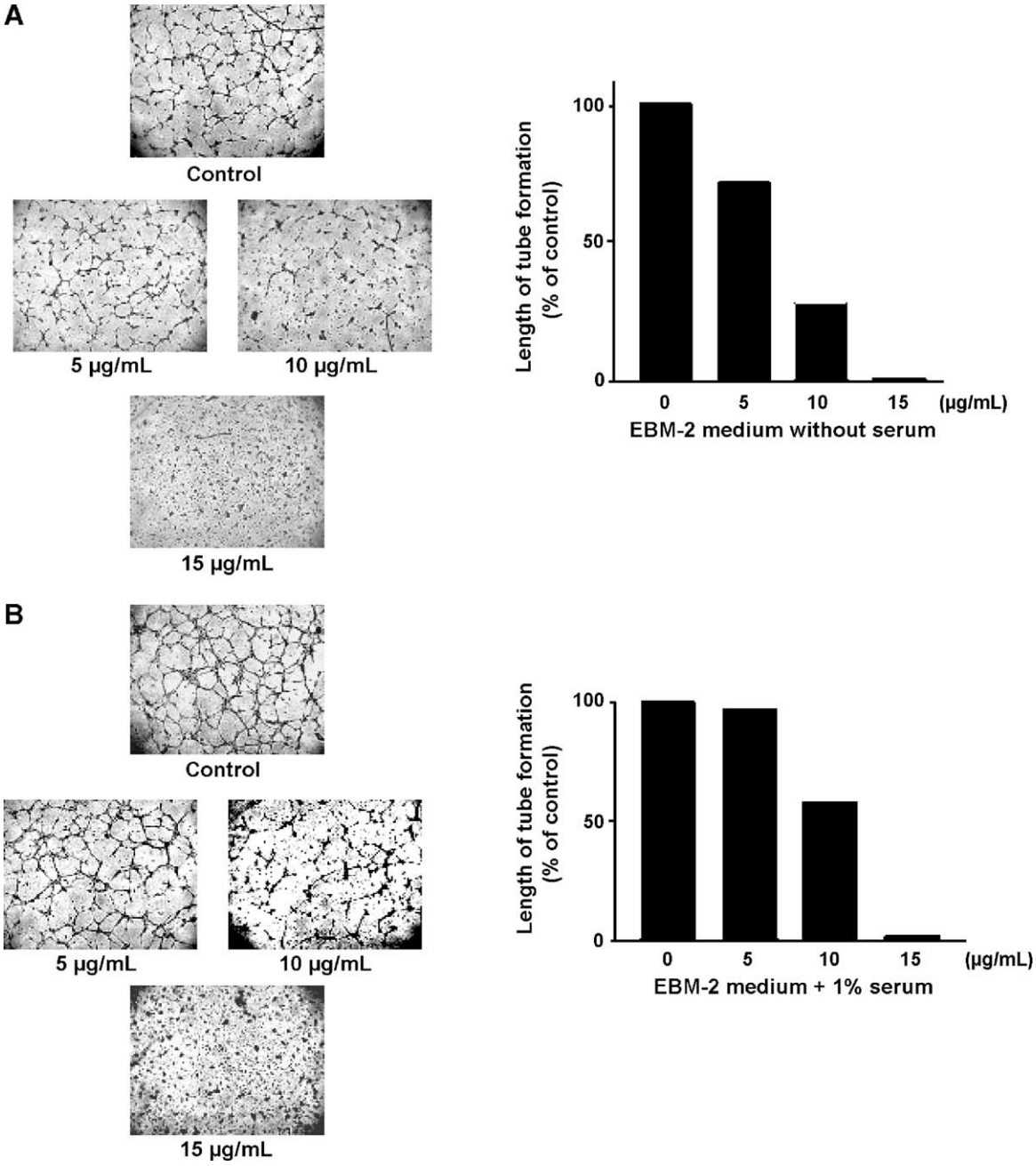

Fig. 2. Pretreatment with SGE prevents capillary-like structure formation on a 2D-Matrigel basement model. HUVECs were incubated for 18 h at 37°C with 0, 5, 10, and 15 µg/ml SGE in (A) serum-free media or (B) medium supplemented with 1% serum and then seeded on Matrigel. The medium was removed and replaced by fresh EBM-2 medium (OK) without SGE. The cells were incubated at 37°C as described under Materials and methods. The examination and quantification of the tube network length (50× magnification) were done as previously. The result was the average of at least two independent tests.

to migrate for 3 h at 37°C in the presence or absence of U-87-conditioned medium. In the presence of conditioned medium, the cell motility was increased in the order HT-1080 > U-87 > DAOY from 1.8 (H-1080) to 13.7 times (DAOY) (Fig. 4). A 2-h preincubation of BAEC with 25 µg/ml SGE decreased the migration of cancer cells under both the basal and the stimulated conditions. Under basal conditions (absence of conditioned medium), the reduction of cell migration varied from 45% (HT-1080) to 87% (U-87 cells). In the presence of conditioned medium, the migration of U-87 and DAOY cells was almost completely suppressed while the migration of HT-1080 was decreased by 85% and maintained around the control value.

Pretreatment with SGE prevents the phosphorylation of p42/44 and p38 MAPK

The p42/44 and p38 mitogen-activated protein kinases (ERK) and the phosphatidylinositol 3-kinase (PI3K) are downstream targets of VEGF. ERK are implicated in EC proliferation and migration. They are activated in response to multiple stimuli including PDGF, epidermal growth factor (EGF), and S1P

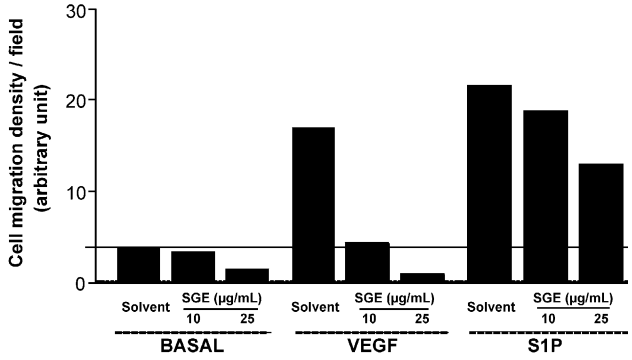

Fig. 3. SGE inhibits the basal migration of bovine aortic endothelial cells (BAEC) and antagonizes the chemoattractant effect of vascular endothelial growth factor (VEGF) and sphingosine-1-phosphate (S1P). BAECs (1.0×10^6 cells) were seeded on gelatin-coated filters, allowed to adhere for 30 min, treated with SGE at 0 and 25 µg/ml for 2 h, and allowed to migrate for 3 h, at 37°C in the absence or the presence of 10 ng/ml VEGF or 1 µM S1P. Cells that have migrated were fixed, stained with 0.1% Crystal Violet–20% methanol (v/v), and counted using computer-assisted imaging. Data are expressed as the average density of migrated cells per four fields (50× magnification).

[11,13]. The p38/MAPK are activated by various cellular stresses, inflammatory cytokines, and growth factors. As PI3K, p38/MAPK also are activated by S1P [4,11]. The use of selective inhibitors of each type of kinase has revealed that p38/MAPK and, to a lesser extent, ERK are essential regulators of VEGF- and S1P-driven PAF synthesis in EC [16,27]. As a first approach to evaluate whether inhibition of VEGF- and S1P-induced chemotactic activity by SGE was related to inhibition of PAF synthesis, we examined the effect of a 2-h pretreatment with 25 µg/ml SGE on ERK and p38/MAPK phosphorylation in control, and under VEGF- and S1P-stimulated conditions. As seen in Fig. 5, these two stimuli markedly increased p38/MAPK phosphorylation. As expected, ERK phosphorylation was also strongly increased by VEGF. A preexposure to SGE decreased by 66% the VEGF-mediated ERK phosphorylation, and reduced by 87 and 39% the phosphorylation of p38/MAPK mediated by VEGF- and S1P, respectively. Bernatchez et al. [16] have demonstrated that PAF synthesis is significantly reduced when BAEC are pretreated with a selective inhibitor of p38 MAPK [SB203580 (10 µM)] before stimulation by S1P (1 µM), and that this pretreatment also reduced by 42% the S1P-induced migration of BAEC. In our study, a 2-h pretreatment of BAEC with 25 µg/ml SGE reduced by 40% the migration of BAEC induced by S1P, and by 39% the phosphorylation of p38/MAPK mediated by this bioactive lipid. The used amount of S1P was the same (1 µM) in the two studies. Our results thus evidenced that SGE decreased both VEGF- and S1P-induced signaling They also supported the hypothesis that inhibition of EC migration by SGE was in part due to a decreased PAF synthesis.

Pretreatment by SGE inhibited S1P-induced PAF synthesis

To ascertain this hypothesis, we measured the effect of a preexposure to 25 µg/ml SGE on S1P-induced PAF synthesis. The synthesis of PAF by S1P and VEGF involves the conversion of EC membrane phospholipids into lyso-PAF (the precursor of PAF) by two types of phospholipase A2 [the cytosolic phospholipase A_2 ($cPLA_2$) and the group V secreted phospholipase A_2 ($sPLA_2$-V)phospholipase A2 isoforms]. This is followed by the conversion of lyso-PAF into PAF by lyso-PAF acetyltransferase [4,16]. In our experiment, PAF production was quantified by incorporation of [^{3}H]acetate into lyso-PAF. Results in Fig. 6 show that 10 min after addition of 1 µM S1P, PAF expression was increased 3.1-fold and that a 2-h exposure to 25 µg/ml SGE reduced this

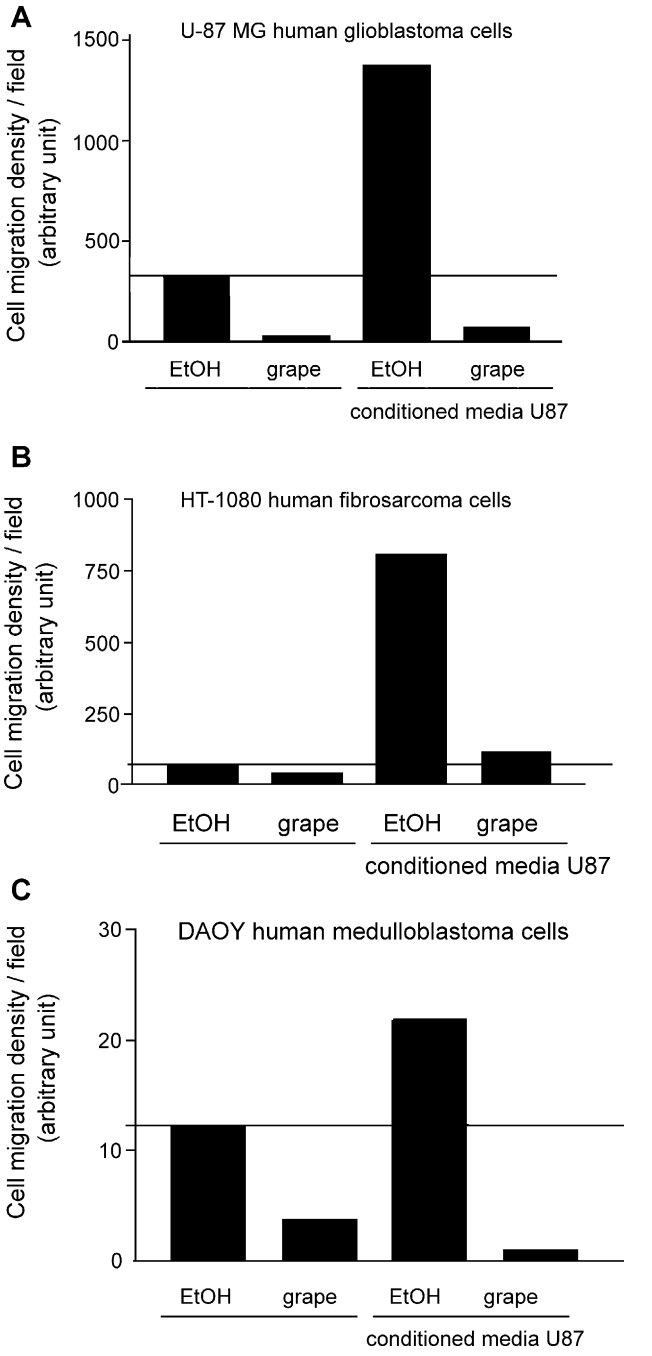

Fig. 4. Exposure to SGE prevents the migration of DAOY (human medulloblastoma), U-87 MG (human glioblastoma), and HT1080 (human fibrosarcoma) cells. Tests were done as previously described for endothelial cells. The only difference was the use of U87-conditioned medium as chemoattractant in place of VEGF or S1P.

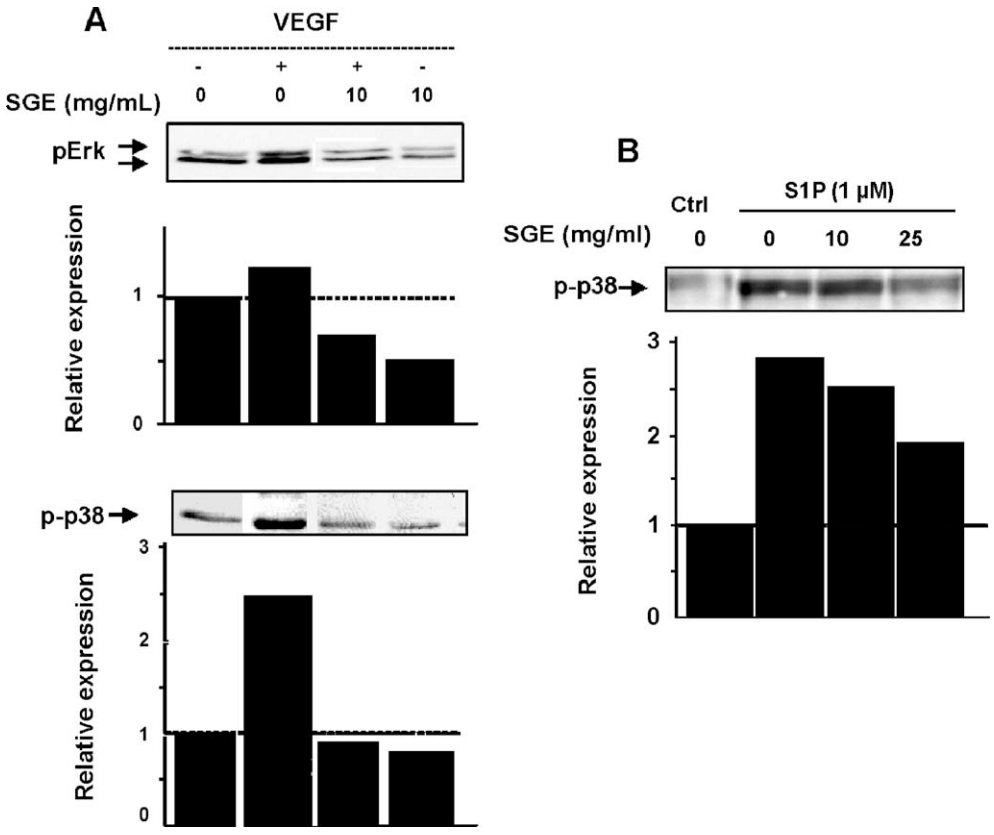

Fig. 5. SGE prevents p42/44 MAPK and p38 MAPK phosphorylation. Confluent BAECs were treated with vehicle or 10 μg/ml SGE for 2 h and then stimulated with 50 ng/ml VEGF (A) or 1 μM S1P (B) for 10 min as described under Materials and methods. Cell lysates [20 μg (A) or 30 μg (B)] were loaded onto 10% SDS-PAGE followed by Western blotting and immunodetection with anti-phospho-ERK1/2 or anti-phospho-p38 antibody, revealed with HRP-conjugated anti-rabbit antibodies. The signals were visualized with an ECL detection system.

stimulatory effect by 89%, and maintained PAF level around the control value.

Discussion

Neoangiogenesis, the growth of novel capillaries in response to proangiogenic cytokines synthesized by tumor cells deprived of oxygen or nutrients, plays a crucial role in tumor growth and metastasis [11]. Inhibiting angiogenesis has thereby become a target for anticancer strategies. In the present study, we have demonstrated that SGE inhibits the formation of capillary-like structures in the Matrigel model. The observation that the tube network is dose dependently inhibited when HUVECs are exposed to SGE after or before cell adhesion to Matrigel supports the hypothesis that SGE both down-regulates and prevents angiogenesis and, consequently, may contribute to prevent and treat cancer. The effect of SGE is decreased by serum. However, as a preexposure to 15 μg/ml SGE is sufficient to completely abolish tubulogenesis in the presence of serum, it can be hypothesized that SGE would exhibit in vivo activity.

Having the knowledge (i) that VEGF increases vascular permeability and induces EC proliferation and migration through high affinity binding to its receptor VEGFR-2 [Flk-1/KDR] at the endothelial cell surface [29], and (ii) that the increased synthesis of PAF by VEGF and S1P contributes to EC migration and angiogenesis [4,16], we have then investigated the effect of SGE on the migration of BAEC mediated by S1P and VEGF. Under our experimental conditions, a 2-h preincubation with 25 μg/ml SGE prevented the chemotactic effect of S1P and VEGF and decreased by up 50% the basal motility of BAEC. The EC migration induced by 10 ng/ml

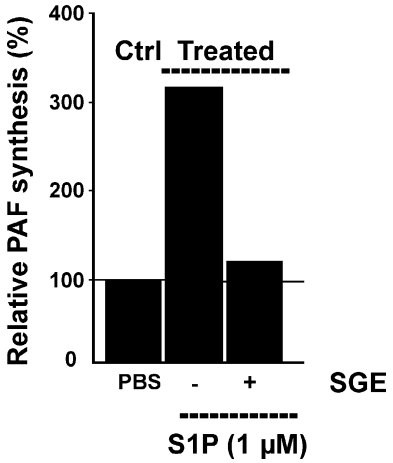

Fig. 6. SGE prevents S1P-induced PAF synthesis. Confluent BAECs were treated with 0 or 25 μg/ml SGE before stimulation with either S1P (1 μM) or the vehicle (PBS alone). Results are expressed in relative PAF synthesis (%) measured as described under Materials and methods. The values are means of three experiments in duplicate.

VEGF was completely abolished while the migration induced by 1 μM S1P was decreased by 40%. Agarwall's team has reported that grape seed anthocyanins decrease VEGF activation and tubulogenesis [21,22]. Our data show that SGE has a similar effect, and presents the complementary interest to inhibit the migration of EC induced by S1P, which amplify angiogenesis. To our knowledge, this is the first time that grape polyphenols are reported to exhibit this latter property.

Cell migration is dependent on local proteolysis of extracellular matrix (ECM) by metalloproteases (MMPs) [5,30]. MMPs not only facilitate the migration of the endothelial through the extracellular matrix but also that of tumor cells [28–33]. Inhibiting these proteases thus contribute to decrease tumor proliferation and metastasis. It is established that MMP expression and activity is in large part dependent on VEGF signaling [5]. By increasing ERK, p38/ MAPK, and phosphatidylinositol 3-kinase (PI3K) phosphorylation, S1P amplify both MMP expression and activity. S1P-induced biological effects are mediated through high affinity binding to G-protein-coupled receptors of the endothelial differentiation gene (EDG) family localized on the plasma membrane of EC and of a wide range of cell lines, including cancer cells [34]. Consistent with the role of S1P in the promotion of angiogenesis, a recent study has evidenced a key role of the S1P/EDG-1 cascade in ECM proteolysis, and in endothelial and nonendothelial cell migration [27]. The inhibition of S1P and VEGF-induced endothelial and cancer cell migration by SGE indicates that SGE not only inhibits VEGF signaling but also down-regulates the activation of the S1P/EDG-1 cascade. This supports the hypothesis that SGE not only decreases MMP expression and activity, but may also positively affect various other S1P-dependent biological effects. This is of interest because S1P is not only secreted by EC but also by platelets, macrophages, and different types of tumor cells and mediates multiple biological effects. It contributes to cell proliferation mediated by platelet-derived growth factor (PDGF) and FCS mitogens [16,27,33], acts as a chemoattractant for hematopoietic precursor cells and immature dendritic cells [34], stimulates MT1-MMP-dependent EC migration and morphogenic differentiation [16,27,31,33,34], controls the recruitment of inflammatory cells (including activated platelets) to sites of inflammation [35], and contributes to the metastatic cascade [35]. In addition, by regulating bone marrow stromal cell migration/recruitment, S1P contributes to diseases in which platelet-endothelial interactions are critical such as thrombosis, angiogenesis, and atherosclerosis [35,36]. S1P is therefore a proinflammatory and proangiogenic agent that contributes to enhance cancer proliferation and metastasis. By down-regulating VEGF and S1P/EDG signaling, SGE may prevent or down-regulate invasive cancer growth and metastasis. Accordingly, tests on BAECs, HT-1080 human fibrosarcoma, U-87 human gliobastoma, and DAOY human medulloblastoma cells have demonstrated that a 2-h preincubation with 25 μg/ml SGE reduces not only the migration of endothelial but also that of cancer cells under basal conditions. Under stimulated conditions, the cell motility of DAOY and U-87 cells is almost completely suppressed while the migration of HT-1080 cells is decreased to the control level. The migration of endothelial and cancer cell is a hallmark of cancer invasion [5,28]. Despite significant advances in neuroimaging, neurosurgery, and radiotherapy, the median survival for patients with malignant glioma is still less than 1 year [37]. It has been reported that a combination of citrus flavonoids, chokeberry extract, lycopene, selenium, red clover extract, and red grape seed extract may reduce the progression of highly invasive malignant glioma [37]. The capacity of SGE to inhibit both the S1P- and the VEGF-induced EC migration, and the migration of U-87 glioblastoma cell induced by conditioned medium, suggests that SGE may also be helpful against this type of cancer, characterized by an intensive vascularization and a rapid tissue invasion [37]. Furthermore, by inhibiting VEGF- and S1P-induced signaling, it is likely that SGE may also be useful against inflammatory diseases associated with platelet–endothelial cell interaction, ischemia and reperfusion injury, and pathological angiogenesis. Indeed, compelling evidence has shown that high VEGF levels enhance the intensity of atherosclerotic lesions [38–40] and favor ischemia and reperfusion injury as well as excessive development of new blood vessels and tumor aggressivity [1,7–9] and that S1P contributes to increase VEGF signaling, amplifying inflammation and angiogenesis [16,33].

Further investigations have been conducted to understand how SGE decreases S1P and VEGF chemotactic activity. It has previously been reported that (i) S1P and VEGF stimulates the synthesis of PAF, a potent inflammatory mediator contributing to their chemotactic activity [4,16]. Notably, S1P-induced EC migration is PAF dependent [16]. To test the hypothesis that SGE may inhibit PAF synthesis mediated by S1P and VEGF, we have first investigated the effect of a preexposure of BAEC to SGE on the activation of ERK and p38/MAPK previously identified as the essential regulators of PAF synthesis in this cell line [4,16]. This approach presented an interest in testing SGE on native nontransfected EC which possess the intracellular pathways found in normal endothelium. The down-regulation of VEGF signaling by SGE, and the capacity of SGE to down-regulate the S1P/EDG-1 cascade, is evidenced by the reduced phosphorylation of p38/MAPK activated by VEGF and S1P and by the decreased VEGF-induced phosphorylation of ERK observed in cells preexposed to SGE. We have unambiguously demonstrated that SGE antagonizes the synthesis of PAF in response to S1P. A direct relation between the decreased synthesis of PAF, the inhibition of p38/MAPK and p42/44/MAPK signaling, the inhibition of S1P-induced EC chemotaxis by SGE is in accord with reports of other teams showing that PAF synthesis by VEGF and S1P enhances p42/44 and p38/MAPK phosphorylation in EC [4,16] and that S1P-induced PAF synthesis is specifically antagonized by the p38/MAPK inhibitor SB203580 [16]. By down-regulating PAF synthesis, it is obvious that SGE decreases VEGF- and S1P-driven inflammation. Furthermore, because SGE down-regulates both ERK and p38/MAPK, it is likely that inhibition of PAF synthesis by SGE results from the inhibition of enzymes involved in the phospholipid remodeling pathway activated by S1P and VEGF and leading to the synthesis of PAF. This

remains to be demonstrated, but is supported by various lines of evidences: (i) the induction of PAF synthesis by VEGF and S1P requires the activation of the phospholipase A_2 isoforms: $sPLA_2$ and $cPLA_2$ and lyso-PAF acetyltransferase [16,41]; (ii) p38/MAPK is capable of directly phosphorylating and activating various PLA2 isoforms and the lyso-PAF acetyltransferase involved in PAF synthesis [42]. (iii) As do inhibitors of cPLA2 or sPLA2 isoforms, inhibitors of p38/MAPK inhibit S1P-induced PAF synthesis [16].

Although these findings are not expected to be directly representative of physiological effects because neither the bioavailability nor the systemic metabolism of SGE are taken into account, they suggest that SGE may contribute to prevent (or to delay) the progression of diseases associated with pathological angiogenesis, including cancer. Furthermore, they demonstrate for the first time that polyphenols from *Vitis vinifera* skin antagonizes the chemotactic effects of VEGF and S1P in part through a decreased synthesis of PAF. This indicates that they are capable of preventing VEGF signaling, the activation of the S1P/EDG-1 cascade, and the synthesis of PAF which amplify inflammation and angiogenesis. These results provides a new explanation for the lower prevalence of coronary heart disease in the French population consuming moderate and regular amounts of wine, a phenomenon called “French paradox” [22] and for the prevention of inflammatory diseases, pathologic angiogenesis, and cancer by wine polyphenols.

References

[1] Ferrara, N. Vascular endothelial growth factor: molecular and biological aspects. *Curr. Top. Microbiol. Immunol.* **237:**1–30; 1999.

[2] Brauchle, M.; Funk, J. O.; Kind, P.; Werner, S. Ultraviolet B and H_2O_2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J. Biol. Chem.* **271:**21793–21797; 1996.

[3] Kuroki, M.; Voest, E. E.; Amano, S.; Beerepoot, L. V.; Takashima, S.; Tolentino, M.; Kim, R. Y.; Rohan, R. M.; Colby, K. A.; Yeo, K. T.; Adamis, A. P. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo. *J. Clin. Invest.* **98:**1667–1675; 1996.

[4] Bernatchez, P. N.; Allen, B. G.; Gelinas, D. S.; Guillemette, G.; Sirois, M. G. Regulation of VEGF-induced endothelial cell PAF synthesis: role of p42.44 MAPK, p38/MAPK and pI3K. *Br. J. Pharmacol.* **134:** 1252–1262; 2001.

[5] Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1:**27–31; 1995.

[6] Folkman, J. Fundamental concepts of the angiogenic process. *Curr. Mol. Med.* **3:**643–651; 2003.

[7] Takahashi, Y.; Tucker, S. L.; Kitadai, Y.; Koura, A. N.; Bucana, C. D.; Cleary, K. R.; Ellis, L. M. Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Arch. Surg.* **132:**541–546; 1997.

[8] Gasparini, G. Prognostic value of vascular endothelial growth factor in breast cancer. *Oncologist* **5:**37–44; 2000.

[9] Solorzano, C. C.; Jung, Y. D.; Bucana, C. D.; McConkey, D. J.; Gallick, G. E.; McMahon, G.; Ellis, L. M. In vivo intracellular signaling as a marker of antiangiogenic activity. *Cancer Res.* **61:**7048–7051; 2001.

[10] Bergers, G.; Song, S.; Meyer-Morse, N.; Bergsland, E.; Hanahan, D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J. Clin. Invest.* **111:**1287–1295; 2003.

[11] Harvey, K.; Siddiqui, R. A.; Sliva, D.; Garcia, J. G.; English, D. Serum factors involved in human microvascular endothelial cell morphogenesis. *J. Lab. Clin. Med.* **140:**188–198; 2002.

[12] English, D.; Welch, Z.; Kovala, A. T.; Harvey, K.; Volpert, O. V.; Brindley, D. N.; Garcia, J. G. Sphingosine 1–phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *FASEB J.* **14:**2255–2265; 2000.

[13] Pyne, S.; Chapman, J.; Steele; Pyne, N. J. Sphingomyelin-derived lipids regulate the extracellular signal-regulated kinase-2 (ERK-2) and c-Jun N-terminal kinase (JNK) signal cascades in airway smooth muscle. *Eur. J. Biochem.* **237:**819–826; 1996.

[14] Idzko, M.; Panther, E.; Corinti, S.; Morelli, A.; Ferrari, D.; Herouy, Y.; Dichmann, S.; Mockenhaupt, M.; Gebicke-Haerter, P.; Di Virgilio, F.; Girolomoni, G.; Norgauer, J. Sphingosine 1–phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *FASEB J.* **16:**625–627; 2002.

[15] Nash, G. F.; Turner, L. F.; Scully, M. F.; Kakkar, A. K. Platelets and cancer. *Lancet Oncol.* **3:**425–430; 2002.

[16] Bernatchez, P. N.; Tremblay, F.; Rollin, S.; Neagoe, P. E.; Sirois, M. G. Sphingosine 1-phosphate. Effect on endothelial cell PAF synthesis: role in cellular migration. *J. Cell. Biochem.* **90:**719–731; 2003.

[17] Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339:**1523–1526; 1992.

[18] Bagghi, D.; Bagghi, M.; Stohs, J.; Ray, S. D.; Sen, C. K.; Preuss, H. G. Cellular protection with proanthocyanidins from grape seeds. *Ann N.Y. Acad. Sci.* **957:**260–270; 2002.

[19] Oak, M.-H.; El Bedoui J.; Schini-Kerth, B.V. Antiangiogenic properties of natural polyphenols from red wine and green tea. *J. Nutr. Biochem.* **16:**1–8.

[20] Sartor, L.; Pezzato, E.; Dell’Aica, I.; Caniato, R.; Biggin, S.; Garbisa, S. Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design. *Biochem. Pharmacol.* **64:**229–237; 2002.

[21] Agarwal, C.; Singh, R. P.; Dhanalakshmi, S.; Agarwal, R. Anti-angiogenic efficacy of grape seed extract in endothelial cells. *Carcinogenesis* **26:**1–10; 2005.

[22] Singh, R. P.; Tyagi, A. K.; Dhanalakshmi, S.; Agarwal, R.; Agarwal, C. Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3. *Int. J. Cancer* **108:**733–740; 2004.

[23] Khanna, S.; Roy, S.; Bagchi, D.; Bagchi, M.; Sen, C. K. Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. *Free Radic. Biol. Med.* **31:**38–42; 2001.

[24] Khanna, S.; Venojarvi, M.; Roy, S.; Sharma, N.; Trikha, P.; Bagchi, D.; Bagchi, M.; Sen, C. K. Dermal wound healing properties of redox-active grape seed proanthocyanidins. *Free Radic. Biol. Med.* **33:**1089–1096; 2002.

[25] Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemisry* **43:**509–512; 1996.

[26] Vukicevic, S.; Kleinman, H. K.; Luyten, F. P.; Roberts, A. B.; Roche, N. S.; Reddi, A. H. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp. Cell Res.* **202:**1–8; 1992.

[27] Annabi, B.; Thibeault, S.; Lee, Y. T.; Bousquet-Gagnon, N.; Eliopoulos, N.; Barrette, S.; Galipeau, J.; Beliveau, R. Matrix metalloproteinase regulation of sphingosine-1-phosphate-induced angiogenic properties of bone marrow stromal cells. *Exp. Hematol.* **31:**640–649; 2003.

[28] Bergers, G.; Benjamin, L. E. Tumorigenesis and the angiogenic switch. *Nat. Rev.* **2:**401–410; 2002.

[29] Bernatchez, P.; Soker, S.; Sirois, M. G. VEGF effects on endothelial cell proliferation, migration and PAF synthesis is mediated through the activation of Flk-1 receptor. *J. Biol. Chem.* **274:**31047–31054; 1999.

[30] Sternlicht, M. D.; Werb, Z. Neutrophil elastase and cathepsin G. In: Kreis, T., Vale, R. (Eds.), *Extracellular Matrix, Anchor, and Adhesion Proteins*. Oxford Univ. Press, Oxford, pp. 543–545; 1999.

[31] Coussens, L. M.; Fingleton, B.; Matrisian, L. M. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* **295:**2387–2392; 2002.

[32] Palumbo, R.; Gaetano, C.; Melillo, G.; Remuzzi, A.; Capogrossi, M. C. Shear stress downregulation of platelet-derived growth factor receptor-beta and matrix metalloprotease-2 is associated with inhibition of smooth muscle cell invasion and migration. *Circulation* **102:**225–230; 2000.

[33] Langlois, S.; Gingras, D.; Beliveau, R. Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation. *Blood* **103:**3020–3028; 2004.

[34] Hla, T.; Lee, M. J.; Ancellin, N.; Thangada, S.; Liu, C. H.; Kluck, M.; Chae, S. S.; Wu, M. T. Sphingosine-1-phosphate signaling via the EDG-1 family of G-protein-coupled receptors. *Ann. N. Y. Acad. Sci.* **905:**16–24; 2000.

[35] Nash, G. F.; Turner, L. F.; Scully, M. F.; Kakkar, A. K. Platelets and cancer. *Lancet Oncol.* **3:**425–430; 2002.

[36] Ribatti, D. A.; Vacca, B.; Nico, R. Ria; Dammacco, F. Cross-talk between hematopoiesis and angiogenesis signaling pathways. *Curr. Mol. Med.* **2:**537–543; 2002.

[37] Rooprai, H. K.; Christidou, M.; Pilkington, G. J. The potential for strategies using micronutrients and heterocyclic drugs to treat invasive gliomas. *Acta Neurochir. (Wien)* **145:**683–690; 2003.

[38] Ruef, J.; Hu, Z. Y.; Yin, L. Y.; Wu, Y.; Hanson, S. R.; Kelly, A. B.; Harker, L. A.; Rao, G. N.; Runge, M. S.; Patterson, C. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ. Res.* **81:**24–33; 1997.

[39] Inoue, M.; Itoh, H.; Ueda, M.; Naruko, T.; Kojima, A.; Komatsu, R.; Doi, K.; Ogawa, Y.; Tamura, N.; Takaya, K.; Igaki, T.; Yamashita, J.; Chun, T. H.; Masatsugu, K.; Becker, A. E.; Nakao, K. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation* **98:**2108–2116; 1998.

[40] Ruef, J.; Hu, Z. Y.; Yin, L. Y.; Wu, Y.; Hanson, S. R.; Kelly, A. B.; Harker, L. A.; Rao, G. N.; Runge, M. S.; Patterson, C. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ. Res.* **81:**24–33; 1997.

[41] Bernatchez, P. N.; Winstead, M. V.; Dennis, E. A.; Sirois, M. G. VEGF stimulation of endothelial cell PAF synthesis is mediated by group V14 kDa secretory phospholipase A2. *Br. J. Pharmacol.* **134:**197–205; 2001.

[42] Martin-Blanco, E. p38 MAPK signaling cascades: ancient roles and new functions. *Bioassays* **22:**637–645; 2000.