Diet-derived polyphenols inhibit angiogenesis by modulating the interleukin-6/STAT3 pathway

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Several epidemiological studies have indicated that abundant consumption of foods from plant origin is associated with a reduced risk of developing several types of cancers. This chemopreventive effect is related to the high content of these foods in phytochemicals, such as polyphenols, that interfere with several processes involved in cancer progression including tumor cell growth, survival and angiogenesis. In addition to the low intake of plant-based foods, increased body mass and physical inactivity have recently emerged as other important lifestyle factors influencing cancer risk, leading to the generation of low-grade chronic inflammatory conditions which are a key process involved in tumor progression. The objectives of the current study are to investigate the inhibitory effects of these polyphenols on angiogenesis triggered by an inflammatory cytokine (IL-6) and to determine the mechanisms underlying this action. We found that, among the tested polyphenols, apigenin and luteolin were the most potent angiogenesis inhibitors through their inhibitory effect on the inflammatory cytokine IL-6/STAT3 pathway. These effects resulted in modulation of the activation of extracellular signal-regulated kinase-1/2 signaling triggered by IL-6, as well as in a marked reduction in the proliferation, migration and morphogenic differentiation of endothelial cells. Interestingly, these polyphenols also modulated the expression of IL-6 signal transducing receptor (IL-6Rα) and the secretion of the extracellular matrix degrading enzyme MMP-2 as well as the expression of suppressor of cytokine signaling (SOCS3) protein. Overall, these results may provide important new information on the role of diet in cancer prevention.

Introduction

Various lifestyle factors, such as tobacco use, consumption of alcohol, exposure to environmental pollution and radiation, a high-calorie diet, obesity, physical inactivity, microbial and viral infections, can cause chronic inflammation and lead to increased risk of cancer [1]. The link between inflammation and cancer has recently been proposed to be the seventh hallmark of cancer [2], supported by epidemiological and experimental data [3] and by anti-inflammatory therapies that show efficacy in cancer prevention and treatment [4]. Recent efforts have shed new light on the molecular and cellular events linking inflammation and cancer [5]. Chronic...
inflammation has been linked to a series of steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [6].

The formation of a tumor-associated vasculature, a process called tumor angiogenesis, is a critical key step by which tumor cells stimulate the formation of a new blood vessel network that sustains the development of cancer by providing oxygen and nutrients to tumor cells [7]. This neovascularization occurs through a series of steps, including stimulation of endothelial cells (ECs) by autocrine and/or paracrine growth factors, proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC migration and proliferation, and structural reorganization into a three-dimensionally tubular structure [8]. Angiogenesis is one of the molecular events bridging the gap between chronic inflammation and cancer growth and expansion [9]. This concept was reinforced by evidence that inhibition of inflammation by the use of non-steroidal anti-inflammatory drugs prevented angiogenesis [10]. Recent studies have reported that the inflammatory cells infiltrating the tumor, which seem to be part of a normal response to tissue remodeling, besides being a defense mechanism, can also excite and recruit ECs to stimulate angiogenesis, tumor progression and metastasis, and maintain tumor-promoting inflammation [11].

Interleukin-6 (IL-6) is a multifunctional cytokine that controls many biological processes such as inflammation, differentiation of immune cells, and hepatic regeneration in response to diverse stimuli [12]. IL-6 also plays a crucial role in angiogenesis since it has been demonstrated that in vivo expression of IL-6 accompanies vascularization during wound healing, psoriasis and tumor growth [13]. Its biological activity is mediated through its binding to the membrane-bound glycoprotein IL-6 receptor chain (gp80), which is present on several target cells, including ECs. This leads to dimerization of the ubiquitously expressed gp130 and to the activation of several intracellular signal transduction events, including Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) signaling cascade [14]. Several studies have demonstrated the role of STAT3 as a mediator and biomarker in endothelial activation that regulates many aspects of angiogenesis [15]. For instance, constitutively activated STAT3 has been reported to up-regulate the expression of one of the most important mediators of neovascularization, vascular endothelial growth factor (VEGF), and thereby induces tumor angiogenesis [16]. The identification of molecules that interfere with IL-6 signaling at the EC level may thus be of considerable importance for the development of novel chemopreventive and chemotherapeutic approaches.

Epidemiological studies suggested that abundant consumption of foods from plant origin could decrease the incidence of many cancers [17], a chemopreventive effect that is related to the high content in these foods of several phytochemicals with potent anticancer properties [18]. Among them, polyphenols found in fruits, vegetables, tea, cocoa and wine have received considerable attention in recent years [19]. Following ingestion, these molecules are bioavailable to a variety of tissues [20,21], and may thus exert their biological effects [18]. The beneficial health effects of these compounds have been attributed, in part, to their antioxidant properties, but there is now convincing evidence that these molecules also inhibit key processes associated with inflammation, tumor growth and angiogenesis [22]. Here, we attempted to determine whether angiogenesis induced by IL-6 is affected by polyphenols in human ECs, and to characterize the mechanisms involved. Five polyphenols were evaluated: delphinidin (an anthocyanidin from berries), epigallocatechin gallate (a catechin from green tea), ellagic acid (a phenolic acid from raspberries), apigenin and luteolin (flavones from parsley and celery). The rationale underlying the selection of these polyphenols was dictated by their known anti-angiogenic activities [22]. Here, we report for the first time that some of these polyphenols inhibit angiogenesis by modulating the IL-6 signaling pathways in ECs.

Materials and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, ON) and serum was purchased from HyClone Laboratories (Logan, UT). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Apigenin, delphinidin, ellagic acid and luteolin were purchased from Extrasynthese (Lyon, France). Human recombinant IL-6 was obtained from R&D Systems (Minneapolis, MN). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The MEK kinase inhibitor PD98059 and the JAK family tyrosine kinase inhibitor AG490 were from Calbiochem (La Jolla, ON). The anti-phospho-gp130 (Ser 782)-R and anti-ERK-1/2 (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies for JAK1 (6G4), JAK2 (D2E12), STAT3 (79D7), SOCS3 (L210) and anti-phospho-p44/42 mitogen-activated protein kinase (MAPK), anti-phospho-JAK1 (Tyr 1002/1023), anti-phospho-JAK2 (Tyr 1007/1008) and anti-phospho-STAT3 (Tyr 705) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Advanced Immunochemical (Long Beach, CA). Anti-mouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Micro bichloronic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich (Oakville, ON).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs-d-Ad) were purchased from Clonetics (San Diego, CA). Cells were maintained in EC basal medium-2 (EBM-2; Lonza, Walkersville, MD) supplemented with 2% fetal bovine serum (FBS) for HUVECs and 5% FBS for HMVECs and with EGM-2 growth factor mixture (Lonza). Cells used in this study were restricted to use between passage 3 and 6. They were cultured at 37 °C under a humidified 95%–5% (v/v) mixture of air and CO2. Cells were treated with vehicle or with polyphenols and stimulated with IL-6.

Migration assays

Transwells (8-μm pore size; Costar) were precoated with 0.15% gelatin in phosphate buffered saline (PBS) by adding 600/100 μL in the lower/upper chambers followed by overnight incubation 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 (10^6 cells/mL) and HUVECs were allowed to adhere for 30 min. The monolayers were then treated for 2 h by adding 50 μL of 2-fold concentrated polyphenol solution prepared in serum-free medium into the upper chamber and 600 μL of the compound solution (1 x) into the lower chamber. After 2 h, cell migration was initiated by adding 50 ng/mL IL-6 to the lower chamber. The plate was placed at 37 °C in 5% CO2/95% air for another 18 h. Cells that had migrated to the lower surface of the incubation, [3H]-thymidine (PerkinElmer, Boston, MA) was added at 0.5 μCi/well at 37 °C in 5% CO2/95% air. Afterwards, cells were washed two times with PBS (37 °C), fixed in EtOH/acetic acid (3:1) for 5–10 min and air dried. Incorporated [3H]-thymidine was extracted in Microscint™-O cocktail and measured in a TopCount microplate scintillation counter.

Cell proliferation assay

Cell proliferation was determined by [3H]-thymidine incorporation assay. HUVECs were plated in 96-well plates at 10,000 cells/well in 200 μL complete medium and incubated at 37 °C under a humidified atmosphere containing 5% CO2 for 24 h. Cells were rendered quiescent by incubation for 18 h in EBM-2 containing 2% FBS. Then cells were incubated with serum-free medium containing (or lacking) IL-6 (50 ng/mL) or polyphenols for 48 h. During the last 4 h of the incubation, [3H]-thymidine (PerkinElmer, Boston, MA) was added at 0.5 μCi/well at 37 °C in 5% CO2/95% air. Afterwards, cells were washed twice with PBS (37 °C), fixed in EtOH/acidic acid (3:1) for 5–10 min and air dried. Incorporated [3H]-thymidine was extracted in Microscint™-O cocktail and measured in a TopCount microplate scintillation counter.

Cytotoxicity assays measuring LDH release

To assess the effect of polyphenols on cell viability, the presence of lactate dehydrogenase (LDH) (released by damage to the plasma membrane) was analyzed in the culture medium of HUVECs. Triplicate samples from the cell medium were taken after culture of the cells maintained in various concentrations of polyphenols in the presence or absence of IL-6 (50 ng/mL) for 48 h. LDH activity was measured at 30 °C by a continuous optical test based on the extinction change of pyridine nucleotide at 340 nm as described by the manufacturer's instructions (Promega).

Western blot analysis

After treatment with polyphenols or synthetic inhibitors (PD98059 or AG490) for 18 h in 1% FBS, HUVECs or HMVECs were starved 30 min in EBM-2 before their stimulation with IL-6 (100 ng/mL, 10 min). Following this, cells were washed once with ice-cold PBS containing 1 mM each of NaF and Na3VO4 and were incubated in the same medium for 30 min 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 ethylene glycol-O, -bis(2-aminoethyl)-N, N', N'-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100] and the resulting lysates (25 μg protein) were solubilized in Laemmli sample buffer [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 10 β-mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were then blocked overnight at 4 °C with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147 mM NaCl, 20 mM Tris–HCl, pH 7.5, and 0.1% Tween 20). Membranes were further washed in TBS-T and incubated with the primary antibody in TBS-T containing 3% bovine serum albumin (BSA), followed by a 1 h incubation with HRP-conjugated anti-mouse antibodies in TBS-T containing 5% nonfat dry milk. Immunoreactive material was visualized with an ECL detection system.

Gelatin zymography

To assess the level of the extracellular matrix degrading enzyme MMP-2 (proMMP-2) activity, quiescent HUVECs were serum-starved for 18 h in the presence or absence of IL-6 (100 ng/mL) or polyphenols (20 μM). The conditioned media were then collected and clarified by centrifugation. An aliquot (30 μL) of the culture medium was subjected to SDS-PAGE in a gel containing 1 mg/mL gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H2O. The gels were further incubated at 37 °C for 20 h in 200 mM NaCl/5 mM CaCl2/0.02% Brij-35/50 mM Tris–HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% acetic acid/30% methanol. Gelatinolytic activity was detected as unstained bands on a blue background.

Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

HUVECs were plated onto 55-cm2 Petri dishes and exposed to EBM-2 medium with or without IL-6 (100 ng/mL) or polyphenols (20 μM). After 18-h treatment, total RNA was extracted from HUVECs using TRIzol reagent from Invitrogen (Burlington, ON) using standard procedures. RT-PCR reactions were performed using SuperScript™ One-Step RT-PCR from Invitrogen. The upstream and downstream primers used were as follows: for IL-6R: 5'-GCT CCT CAT TGC CAT TG-3' and 5'-GCA TCT GGT CTT TGG CTG CT-3' (expected product 216 bp); for gp130: 5'-CTG TAT CAC AGA CTG GCA ACA AG-3' and 5'-GCA TTT GCT CTC TGG TAA GTC CC-3' (expected product 79 bp); and for GAPDH: 5'-CATCACTCATCTCTAGGAC-3' and 5'-CTGCTCTACCCACTCTGTTG-3' (expected product 540 bp). RT-PCR conditions were optimized so that the gene products were obtained during the exponential phase of the amplification. Gene product amplification was performed for 35 cycles of PCR for GAPDH or 40 cycles of PCR for IL-6R or gp130 (94 °C for 15 s, 55 °C for 30 s, 70 °C for 45 s) and amplification products were fractionated on 2% (w/v) agarose gels and visualized by ethidium bromide.

Endothelial cell tube formation assays

HUVECs were embedded within fibrin gels at a concentration of 10^5 cells/mL using the fibrin gel in vitro angiogenesis assay kit (Millipore, Temecula, CA). In brief, 30 μL of fibrinogen solution was dispensed into each well of a 96-well plate and 20 μL of thrombin solution was added to the fibrinogen. This was allowed to solidify for 60 min at 37 °C. Once solid, the wells were incubated for 30 min with 100 μL of cells (10,000 cells/well) in EBM-2 special medium (serum-free basal medium containing bovine serum albumin (BSA, 0.5%) and EGM-2 (0.5%)). After adhesion of the cells, HUVECs were then pretreated with IL-6 (250 ng/mL) in the presence or absence of polyphenols (20 μM) for 18 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 following which 100 μL of fresh EBM-2 special medium, supplemented with IL-6 and containing (or lacking) polyphenols,
were added to each well. After 24 h, the cultures were photographed (100×) using a Retiga 1300 camera and a Zeiss Axiovert S100 microscope.

**Statistical analysis**

Statistical analyses were performed using 1-way ANOVA with a post hoc Dunnett’s test. Differences with $P<0.05$ were considered significant.

**Results**

**Polyphenols inhibit IL-6-induced EC migration**

Migration of ECs represents a critical step in angiogenesis, allowing cells to disseminate from pre-existing vessels and to form new vessels. To better understand the inhibitory action of polyphenols on the induction of in vitro angiogenesis, we first examined their effect on the chemotactic motility of HUVECs in the presence of the most common inflammatory cytokine, IL-6. Cells were allowed to adhere to gelatin-coated Transwells, were incubated with various concentrations of polyphenols and, after 2 h, IL-6 was added to the lower chamber as a chemoattractant. We studied the effect of polyphenols on cell migration only after IL-6 stimulation since, under control conditions, HUVEC apoptosis is induced by deprivation of growth factors [24,25] whereas IL-6 has cytoprotective effects on HUVECs [26]. As shown in Fig. 1A, IL-6 stimulated cell migration; maximum activation was reached with ~50 ng/mL IL-6. The increase in cell migration was completely inhibited by four polyphenols: EGCG, Dp, Api and Lut (Fig. 1B). Each potent inhibitory polyphenol caused a dose-dependent inhibition of cell migration in the presence of IL-6 with a half-maximal inhibition ($IC_{50}$) of 16.8 μM for EGCG (Fig. 1C), 14.4 μM for Dp (Fig. 1D), 8.2 μM for Api (Fig. 1E) and 3.6 μM for Lut (Fig. 1F).

**Polyphenols inhibit IL-6-induced EC proliferation**

The proliferation of vascular ECs is another important event during angiogenesis [7]. In order to investigate whether polyphenols were able to inhibit EC proliferation in the presence of IL-6, [3H]-thymidine incorporation was measured into HUVECs. As shown in Fig. 2A, 50 ng/mL IL-6 is sufficient to stimulate the synthesis of DNA in HUVECs. Our results revealed that all the polyphenols potently inhibited [3H]-thymidine incorporation in the presence of IL-6 (Fig. 2B) with the notable exception of EA, which was much less potent. In the presence of Lut, EGCG, Api and Dp, the EC IL-6 dependent proliferation was significantly reduced, in a concentration-dependent manner with $IC_{50}$ values of 9.9 μM (Fig. 2C), 7.5 μM (Fig. 2D), 7.2 μM (Fig. 2E), and 3.0 μM (Fig. 2F), respectively. To clarify whether the observed reduction in proliferation of HUVECs resulted from increased cell death at higher concentrations, we studied the effects of polyphenols on cell viability. The release of LDH in the culture media of HUVECs was analyzed in the presence of 50 ng/mL IL-6. Fig. 2G shows that HUVECs were significantly more sensitive to the cytotoxic effects of EGCG (10 μM, 81% cell survival; 20 μM, 55% cell survival), EA (10 μM, 86% cell survival; 20 μM, 65% cell survival) and Dp (20 μM, 74% cell survival) compared to the control cells after 48 h of treatment. Therefore, these results could partially explain the inhibitory effects of EGCG, EA and Dp on cell proliferation at 20–25 μM. Nevertheless, Api and Lut did not induce significant cell death, confirming the specific antiangiogenic effects of these compounds on cell proliferation. Overall, the IL-6 induced growth of HUVECs was strongly inhibited by polyphenols.

**Polyphenols inhibit IL-6-induced JAK/STAT3 and MAPK signaling pathways**

To further investigate the cellular mechanisms involved in the inhibition of migration and growth by polyphenols, we next explored the potential involvement of two major signaling pathways: the JAK/STAT3 pathway and the MAPK pathway, both being involved in IL-6 mediated functions in several cell types [12]. Quiescent HUVECs were incubated for 18 h in EBM-2 medium containing 1% FBS in the presence or absence of polyphenols (20 μM). The medium was then replaced with fresh, serum-free medium without polyphenols for 30 min and the cells were stimulated with IL-6 for 10 min. Protein expression and phosphorylation of the downstream signaling pathways targeted by polyphenol treatment were assessed by immunoblotting using specific antibodies. As shown in Fig. 3A, IL-6 caused a marked increase in the tyrosine phosphorylation state of STAT3 in untreated HUVECs, maximal activation being reached at 100 ng/mL. Moreover, this stimulation was rapid, being observed after as little as 5 min (Fig. 3B). Under these experimental conditions, Fig. 3C shows that polyphenols affected the relative levels of phosphorylation of JAK/STAT3 and MAP kinase, as determined by the ratio of unphosphorylated and phosphorylated proteins. We observed that Api and Lut were the most potent inhibitors; the phosphorylation of JAK1, as well as of JAK2 and STAT3, was significantly decreased. The phosphorylation of STAT3 was also inhibited by AG490, a specific JAK2 inhibitor, confirming that these effects are mediated through JAK/STAT pathway (Fig. 3D). Interestingly, these polyphenols activated the phosphorylation of ERK in the presence of IL-6 (2.1 ×-fold for Api and 1.4 ×-fold for Lut), whereas Dp, EGCG and EA diminished this event by ~51%, ~32% and ~44%, respectively. Pretreatment of ECs with an inhibitor of MEK (the direct upstream regulator for ERK-MAPK, PD98059), showed similar result to that obtained with Api and Lut on the phosphorylation of STAT3 (Fig. 3D), indicating that ERK pathway was also involved. In order to strengthen these results, we next used another EC model, HMVECs. As shown in Fig. 3E, Api and Lut are also capable of inhibiting completely STAT3 phosphorylation and stimulated ERK phosphorylation (1.7 ×-fold for Api and 1.5 ×-fold for Lut). Overall, these results suggest that the effects of these polyphenols toward the proliferation and the migration of ECs are indeed regulated through IL-6/STAT3/ERK signaling pathways.

**Apigenin and luteolin affect STAT3 gene targets**

STAT3, a member of the JAK-STAT signaling pathway, is a signal transducer and activator of transcription. Upon activation, STAT3 is phosphorylated at tyrosine residues by activated JAK kinases in receptor complexes, leading to its dimerization, nuclear translocation, and activation of the transcription of genes with STAT3 recognition sites in their promoters [27]. Since STAT3 modulates the transcription of a variety of genes involved in the regulation of angiogenesis, tumor invasion and metastasis such as MMPs [27,28], we used gelatin zymography of the conditioned media of serum-starved HUVECs to assess whether the most potent inhibitors, Api and Lut, could affect pro-MMP-2 secretion. Fig. 4A shows that these
two polyphenols effectively downregulated IL-6-induced proMMP-2 levels by ~60% and ~93%, respectively. We next investigated the effects of these polyphenols on another STAT-3 transcriptional target, the SOCS3 protein, a key negative regulator of IL-6 signaling pathways [29]. SOCS3 proteins are present in cells at very low levels but are rapidly transcribed after exposure of cells to cytokines. They act in a negative feedback loop to inhibit JAK activation. Since JAK/STAT3 signaling pathways are also inhibited by Api and Lut, we explored the hypothesis that SOCS3 mediates the inhibitory effects of these polyphenols on EC signaling. As shown in Fig. 4B, minimal SOCS3 induction by IL-6 (1.3 ×-fold) was observed compared to untreated cells. However, Lut potently induced SOCS3 expression (3.9 ×-fold) though this increase was not be observed following Api treatment.

**Apigenin and luteolin reduce IL-6 receptor gene expression**

To further investigate the mechanisms involved in the inhibitory actions of Api and Lut on the IL-6 signaling pathway, we next examined whether these polyphenols affected IL-6 receptor gene expression. RT-PCR analysis revealed that the IL-6-mediated increase of IL-6Rα mRNA was reduced by Api and Lut (Fig. 5). Moreover, these polyphenols also attenuated the basal levels of the receptor. However, gene expression of the gp130 receptor was not affected.
Fig. 2 – Effects of polyphenols on IL-6-induced proliferation of HUVECs. (A) HUVECs were seeded into wells of a 96-well plate. After 24 h, cells were rendered quiescent by incubation for 18 h in low serum and then incubated with serum-free medium containing various concentrations (0, 10, 25, 50, 100 or 250 ng/mL) of IL-6 or indicated polyphenols (25 μM) (B) or different concentrations of Lut (C), EGCG (D), Api (E) or Dp (F) in the presence of IL-6 (50 ng/mL) for 48 h. During the last 4 h of the incubation, 0.5 μCi/well [3H]-thymidine was added to cells. The incorporated [3H]-thymidine was extracted in Microscint™-O cocktail and measured in a TopCount microplate scintillation counter. (G) Cellular toxicity was estimated by analyzing the content of LDH in the culture medium of each well as described in Materials and methods section. Values are means of three independent experiments (*p < 0.05; **p < 0.01; ***p < 0.001 versus IL-6 alone); bars, ± SEM.
Apigenin and luteolin inhibit IL-6-induced tube formation in 3D fibrin gels

In order to verify that Api and Lut could inhibit angiogenesis in vitro, we further investigated the antiangiogenic effects of these polyphenols on vessel formation in a 3D EC model, as previously described [23]. When IL-6 was added to HUVECs embedded in 3D fibrin gels, IL-6 led to the formation of elongated tube-like structures with lumina and invasive sprouts, compared to the control cells (Fig. 6, top panel). Using this model, we observed that Api and Lut potently inhibited IL-6-induced capillary-like structure formation by HUVECs (Fig. 6, bottom panel), confirming the specific antiangiogenic effects of these polyphenols on IL-6-induced angiogenesis.

Discussion

A close association between inflammation and angiogenesis has recently been established and these processes are now recognized to play interdependent roles in orchestrating tumor development and growth [9,10]. Although great advances have been made in understanding the functions of VEGF in promoting tumor angiogenesis, the issue as to whether growth factors and cytokines play a significant role in the VEGF regulation of angiogenic processes remains incompletely understood. Accumulating evidence suggests that IL-1β, IL-6, transforming growth factor-β, and platelet-derived growth factor (PDGF)-B can participate in VEGF-mediated angiogenesis via diverse signaling pathways in human cancers [30,31]. The role of IL-6 in angiogenesis is supported by a recent report showing that a peptide specifically binding to the IL-6R can inhibit vessel formation and tumor growth in severe combined immunodeficient mice [32]. In addition, multiple lines of evidence indicate that IL-6 is an important prognostic factor in angiogenic pathological disorders and cancer [33].

Several reports, published during recent years, have highlighted the beneficial effects of phenolic compounds from various dietary sources for the prevention of chronic disorders such as cancer [18,34]. Polyphenols have a wide range of biological activities and can influence many signaling pathways vital to cellular functions, including anti-inflammatory, antiangiogenic and antitumor
mechanisms [6,19,22]. Here, our study highlights the antiangiogenic effects of polyphenols against angiogenesis induced by the pro-inflammatory cytokine IL-6. Among the polyphenols tested, Api and Lut seem to be the most potent inhibitors of IL-6 actions. We showed that Api and Lut reduced IL-6Rα gene expression and that this effect was associated with an impairment of the downstream signaling events triggered by this receptor, such as phosphorylation of the JAK/STAT3 and MAPK signaling pathways. Importantly, the inhibition of these IL-6-dependent events by both flavones was correlated with a marked reduction in the migratory and proliferation properties of HUVECs, two crucial events involved in the recruitment of these cells to neovessels. Indeed, the inhibitory effects of these flavones also antagonized the activation of STAT3 by IL-6 in ECs. However, Dp and EGCG had no effect on IL-6-induced phosphorylation of STAT3, unlike the results of other studies which found that Dp and EGCG downregulated the activation of STAT3 induced by the HGF [43] and EGFR signaling pathways [44], respectively.

The phosphorylation of STAT3 modulates the transcription of a variety of genes involved in the regulation of critical cell functions, including differentiation, proliferation, apoptosis, inflammation, invasion, angiogenesis and metastasis [27]. These include MMPs, which play key roles in the processes of matrix remodeling, migration and EC sprouting during angiogenesis [45]. IL-6 can induce the expression and secretion of MMPs such as MMP-2 and MMP-9 in normal and tumor cells [46]. Consistent with these results, we detected an upregulation of pro-MMP-2 in ECs promoted by IL-6. Api and Lut inhibited this secretion, probably via inhibition of the phosphorylation state of STAT3 thus preventing its interaction with the MMP-2 promoter [28]. SOCS3, another target gene regulated by STAT3, is a key negative regulator of IL-6 signal transduction, particularly of the JAK/STAT signaling pathway [29]. Mechanisms by which SOCS3 regulates IL-6 signaling have been proposed; SOCS3 by their effect on IL-6/MAPK pathway and their potential antiangiogenic activities against VEGF and PDGF receptor phosphorylation, leading to suppression of angiogenesis [23,35,36].

IL-6 transcription factor STAT3 is known as an oncogene that is constitutively phosphorylated in many types of human cancer cell lines or primary tumors [16], implicating aberrant STAT3 signaling as an important process in malignant progression. STAT3 regulates cell proliferation and migration, and mediates vascular function [15]. Several natural agents known to be chemopreventive are quite effective in suppressing STAT3 activation [27]. Curcumin has been shown to inhibit the activity of proteins implicated in STAT3 phosphorylation such as JAK2 [37] and IL-6 [38]. Resveratrol was found to inhibit IL-6-induced activation of STAT3 in human multiple myeloma cells [39] and ECs [40]. Lut has been shown to promote the degradation of STAT-3 in human hepatoma cells [41]. Moreover, Api and Lut inhibited hypoxia-induced STAT3 tyrosine phosphorylation in lung cancer squamous cell carcinoma [42]. Our data revealed that these flavones also antagonized the activation of STAT3 by IL-6 in ECs. However, Dp and EGCG had no effect on IL-6-induced phosphorylation of STAT3, unlike the results of other studies which found that Dp and EGCG downregulated the activation of STAT3 induced by the HGF [43] and EGFR signaling pathways [44], respectively.

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binds to the cytokine receptor through its SH2 domain and, to a lesser extent, to JAK kinases. This association allows it to interact with and inhibit the receptor-bound JAKs [29]. Reduced expression of SOCS3 has been observed in several human cancers and is associated with constitutive STAT3 activation, which contributes to tumor angiogenesis [15,16]. Our results showed an overexpression of SOCS3 induced by Lut, compared to cells treated with Api. These results are consistent with a previous report indicating that EGCG inhibited SOCS1-regulated cell signaling [47]. It is noteworthy that Api and Lut differ at a hydroxyl group in the 3′-position of the B ring [48], which might account for their different actions shown here.

Further exploration of the mechanisms of action of Api and Lut indicated a downregulation of pJAKs and pSTAT3 levels as well as an upregulation of pERK levels, suggesting possible cross talk between signaling pathways. Interestingly, Api was also found to sustain the activation of ERK induced by epidermal growth factor stimulation in PC12 cells [49]. Several lines of evidence support a role for ERKs in both positive and negative effects on JAK/STAT signaling in several systems [50–52], where an inhibition of STAT3 activation by agents correlated with the phosphorylation of ERK. However, the molecular basis for the interaction of ERK and the JAK/STAT pathways still remains largely unknown. Some studies report that the mechanisms underlying inhibition of STAT3 activities include not only tyrosine but also serine phosphorylation of STAT3 [53,54]. It is possible that the overexpression of ERK activation affected the homodimer formation of STAT3 and its DNA binding [54] through serine phosphorylation at the C-terminal site (serine-727) of STAT3, which is a good consensus for MAPK [55]. Further studies would be necessary to identify and characterize the serine/threonine kinases activated by IL-6 that are responsible for the serine phosphorylation of STAT3 in ECs.

In conclusion, our study showed that Api and Lut, upon stimulation by the pro-inflammatory cytokine IL-6, efficiently inhibited migration, proliferation and tubulogenesis of HUVECs, an inhibitory effect that was mediated through the JAK/STAT3 and MAPK signaling pathways. The results reported here provide new information regarding the inhibitory effects of polyphenols against angiogenesis and highlight the potency of dietary-derived polyphenols as practical chemopreventive agents.

**Conflict of interest statement**

The authors have no conflict of interest.

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