Delphinidin is a novel inhibitor of lymphangiogenesis but promotes mammary tumor growth and metastasis formation in syngeneic experimental rats

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We have recently demonstrated that the anthocyanidin delphinidin (DEL), one of the most abundant dietary flavonoids, inhibits activation of ErbB and vascular endothelial growth factor receptor family members. These receptors play crucial roles in the context of tumor progression and the outgrowth of blood and lymphatic vessels. Here, we have developed an improved chemical synthesis for DEL in order to study the effects of the aglycon and its degradation product gallic acid (GA) on endothelial and tumor cells in vitro and in vivo. We found that DEL blocked the proliferation in vitro of primary human blood and lymphatic endothelial cells as well as human HT29 colon and rat MT-450 mammary carcinoma cells in a dose-dependent manner. In contrast, its degradation product GA had little effect. At higher concentrations, DEL induced apoptosis of endothelial and tumor cells. Furthermore, DEL potently blocked the outgrowth of lymphatic capillaries in ex vivo lymphangiogenesis assays. In the MT-450 rat syngeneic breast tumor model, it also significantly reduced angiogenesis and tumor-induced lymphangiogenesis when administered in vivo. These data reveal DEL to be a novel antilymphangiogenesis reagent. Surprisingly, however, the application of DEL unexpectedly promoted tumor growth and metastasis in the MT-450 tumor model, suggesting that the antiproliferative effect of DEL on cultured cells does not necessarily reflect the responses of tumors to this anthocyanidin in vivo. Furthermore, while DEL may have utility as a cancer chemopreventative agent, its ability to promote tumor growth once a neoplasm develops also needs to be taken into consideration.

Introduction

Anthocyanins and their aglycons, the anthocyanidins, are naturally occurring plant pigments that can be found in petals, vegetables and fruits such as berries and grapes (1,2). An anthocyanidin-rich diet or the intake of fruit extracts is thought to have chemopreventative benefits in the context of cancer (reviewed in refs 3,4). The potential benefits have prompted the commercial development of food supplements with a high content of bioactive anthocyanins. The delphinidin (DEL) content, one of the most abundant anthocyanidins in food, is considered to be especially beneficial (5).

The fate of anthocyanins upon dietary intake is complex and not entirely understood (reviewed in refs 6,7). Although intact anthocyanins are absorbed rapidly and can be found in the blood within minutes after consumption, a significant proportion of ingested anthocyanins is thought to be deglycosylated and further metabolized in the intestine (6). Within the stomach, anthocyanins are initially stabilized by the low pH, but the neutral pH milieu of the small intestine and colon may promote their hydrolysis and degradation by intestinal enzymes such as lactase-phlorizin hydrolase (8,9), and the microbiota in the large intestine and colon (7,10,11). The aglycons generated by microbial degradatives of anthocyanins in the intestinal microflora (e.g. 6-O-rhamnosidase and β-d-glucosidase) are less stable than their corresponding glycosylated anthocyanin counterparts and can be degraded into phloroglucinol aldehyde and phenolic acids (7,10,12,13). The identity of the phenolic acid product depends on the initial anthocyanidin. In the case of DEL, the specific phenolic acid is gallic acid (GA) (11,14). Thus, some of the effects attributed to dietary DEL could conceivably be mediated by its metabolic degradation products.

Degradation of DEL may also occur in tissue culture medium in vitro, as DEL rapidly disappears under these conditions (14). However, factors other than degradation such as protein binding may account for this disappearance, as the rate of disappearance of DEL exceeds by far the formation of GA (14). Nevertheless, GA has also been reported to inhibit tumor cell proliferation and induce their apoptosis in vitro (e.g. refs 15–17) and has been shown to reduce tumor growth and progression in vivo (18,19). Thus, the production of GA as a consequence of DEL degradation may account for some of the biological activities attributed to DEL.

Previously, we have shown that DEL strongly inhibits the kinase activity of several receptor tyrosine kinases (RTKs), and that DEL is able to block ligand-induced phosphorylation of epithelial growth factor receptor (EGFR) family members EGFR, ErbB2, ErbB3, as well as vascular endothelial growth factor receptors (VEGFR)-2 and -3 in cellular phosphorylation assays (20). EGFR and VEGFR families play crucial roles in cancer. In many types of cancer enhanced EGFR signaling, for example induced by overexpression of the ligands or mutations in the receptor that lead to constitutive EGFR signaling, promotes tumor proliferation and progression (21). VEGFRs on the other hand are predominantly found on endothelial cells, and VEGFR-2 and VEGFR-3 are key regulators of the outgrowth of new vessels from preexisting ones during angiogenesis and lymphangiogenesis, respectively (22). Angiogenesis supports tumor growth, and tumor-induced lymphangiogenesis promotes metastasis formation (23). Thus, both VEGFRs and EGFRs represent important and attractive targets for cancer therapies (23–25).

Based on our previous finding that DEL treatment inhibits RTK activity (20), here we tested and compared the effects of DEL and GA on endothelial and tumor cells that express DEL-sensitive RTKs. We also established an improved chemical synthesis for DEL to allow sufficient quantities of DEL to be produced for the testing of its effects on tumor growth and metastasis in experimental animals in vivo. Our results show that DEL but not its degradation product GA is a potent inhibitor of endothelial and tumor cell proliferation in vitro, and at higher concentrations induces their apoptosis. In accordance with our previous findings that DEL inhibits VEGFR-2 and VEGFR-3 activation (20), we also show that it blocks the outgrowth of lymphatic capillaries in ex vivo lymphangiogenesis assays and can reduce lymphangiogenesis and angiogenesis in the MT-450 syngeneic rat mammary tumor model in vivo. Counterintuitively, however, we found that...
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DEL promoted tumor growth and metastasis in this model. These data demonstrate that the inhibitory effects of DEL observed in cultured cells do not necessarily reflect the activity of DEL in vivo and suggest that the use of DEL as a dietary supplement or potential therapeutic needs to take into account possible protumorigenic effects of the substance.

Materials and methods

Chemistry

Full details of the synthesis route and methodology for the synthesis of DEL chloride as outlined in Figure 1 can be found in the Supplementary Materials and methods, available at Carcinogenesis Online. Purified GA was purchased from Sigma–Aldrich (Taufkirchen, Germany).

Cell culture

Primary human dermal lymphatic endothelial cells (LECs) and human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex or Promocell (Heidelberg, Germany) and were maintained in endothelial cell growth medium (Cambrex) supplemented with 5% fetal calf serum (26). The rat mammary tumor cells MT-450 and HT29 human colon carcinoma cells were maintained as described previously (27,28).

In vitro kinase assays

In vitro kinase assays were performed as described previously (20). Briefly, the tyrosine kinase substrate (poly-Glu,Tyr) 4:1 (molecular weight: 20 000–50 000) was used to coat 96-well microtiter plates. A reaction mix was prepared by combining either DEL or GA with recombinant glutathione-S-transferase kinase and adenosine triphosphate. Phosphorylation was allowed to proceed at 30°C for 90 min, then detected using peroxidase-conjugated antiphospho-tyrosine antibodies (Becton Dickinson, Heidelberg, Germany) followed by ABTS substrate. Absorbance was measured photometrically at 405 nm. All data points were performed in triplicate.

Ligand-induced EGFR and ErbB3 phosphorylation assays

Aliquots of 2 × 10^6 HT29 cells were seeded into Petri dishes and allowed to grow for 48 h. Thereafter, cells were further cultivated under serum-reduced (1% fetal calf serum) conditions for 24 h and incubated with either DEL or GA for 45 min in serum-free medium in the presence of catalase (100 U/ml). Receptors were stimulated with their respective ligand [EGF (100 ng/ml) for EGFR and ErbB2, heregulin (20 ng/ml) for ErbB3] for the final 15 min of incubation. Cells were lysed at 4°C in 0.2 ml radioimmunoprecipitation assay buffer [50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM NaF, 1% (vol/vol) Igepal, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 2% (vol/vol) protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. Thereafter, the lysate was homogenized thoroughly and subsequently centrifuged for 10 min (20 000g, 4°C). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by western blotting. The membranes were probed with antibodies against human EGFR, ErbB2, ErbB3, phospho-EGFR (Tyr 1173) (Cell Signaling Technology, Beverly, MA), phospho-ErbB2 (Tyr 1248) or phospho-ErbB3 (Tyr 1228) (Santa Cruz, Heidelberg, Germany). Alpha-tubulin served as a loading control. Anti-mouse or anti-rabbit immunoglobulin (Ig) G peroxidase conjugates (Santa Cruz) were used as appropriate as secondary antibodies.

Ligand-induced VEGFR phosphorylation assays

Ligand-induced VEGFR phosphorylation assays were performed as described previously (29). Briefly, porcine aortic endothelial cells transfected with either human VEGFR-2 or murine VEGFR-3 were preincubated with either DEL or GA at the appropriate concentration, the cells were stimulated with either ΔNΔC/VEGF-C/Cys152->Ser (VEGF-C-Cys), a mutant form of VEGF-C protein that specifically activates VEGFR-3 but not VEGFR-2, or with human recombinant VEGF_165 (Reliatech, Braunschweig, Germany) as appropriate. Lysates of the cells were then immunoprecipitated with anti-VEGFR-2 (2479L; Cell Signaling Technology, Frankfurt, Germany) or anti-VEGFR-3 antibody (AF743; R&D Systems) and protein G–Sepharose (Amersham). Half the volume of each sample was loaded onto two sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and western blotted. The blots were probed with monoclonal antibodies against human VEGFR-2 (Cell Signaling Technology, Beverly, MA) and α-tubulin (Cell Signaling Technology, Beverly, MA).

Fig. 1. Synthesis of DEL. 1: GA; 2: 3,4,5-Triacetoxybenzoic acid; 3: 5-(Chlorocarbonyl)benzene-1,2,3-triyl triacetate; 4: 5-Acetylbenzene-1,2,3-triyl triacetate; 5: 5-(2-Bromoacetyl)benzene-1,2,3-triyl triacetate; 6: 5-(2-Acetoxyacetyl)benzene-1,2,3-triyl triacetate; 7: 2,4,6-Trihydroxybezaldehyde; 8: 2-Formyl-3,5-dihydroxyphenyl benzoate; 9: DEL.
with antiphosphotyrosine Ig (PY20-hrho; Becton Dickinson) or with specific antireceptor Ig (VEGFR-3: AFL4; Bioscience, VEGFR-2: C-1158; Santa Cruz), respectively, to control the loading of the samples.

**Proliferation assay**

Cells were seeded into 96-well plates at a density of 1 × 10⁴ cells and incubated for 24 h in the presence or absence of DEL, GA or solvent control. Each sample was performed in triplicate. The cells were then labeled for 16 h with 1 μCi [3H]-thymidine, trypsinized for 30 min and then harvested onto a glass fibre filter (Wallac, Turku, Finland) using a Harvester 96 cell harvester (Tomtec, Hamden, CT). The filter-immobilized radioactivity was quantified using MicroBeta TriLux Liquid Scintillation together with a Luminescence counter (Wallac).

**Cell cycle analysis**

Cells were harvested, mixed with ice-cold 70% ethanol and fixed overnight at −4°C. Cells were then pelleted, washed once with phosphate-buffered saline (PBS) and then stained with Draq5 (Biostatus Ltd, Shpeshed, UK) at a final concentration of 10 μM for 15 min in the dark. Cell cycle analysis was then performed flow cytometrically using a FACScan instrument (Becton Dickinson) to measure the DNA content of the cells.

**Three-dimensional lymphatic ring cultures**

Lymphatic ring cultures were performed as described previously (26,30). Briefly, thoracic ducts were dissected from 2 to 3 months old C57Bl/6 mice and cut into 1 mm long pieces. The explants were embedded in rat-tail interstitial collagen gel (Serva Electrophoresis) and then polymerized in cylindrical agarose wells. They were then cultivated in the presence of appropriate concentrations of DEL, GA or dimethyl sulfoxide (DMSO) for 10 days. To quantify vessel outgrowth, binary images of photographs taken under identical conditions of light, contrast and magnification were analyzed using NIH ImageJ software.

**Animal experiments**

All animal experiments were approved by the local regulatory authorities and performed according to the German legal requirements. Wistar-Furth rats were injected subcutaneously with 5 × 10⁴ MT-450 cells in PBS. Treatment was initiated 4 days after tumor injection. The animals (eight per group) were injected with 1.18 × 10⁻⁵ mol of DEL or GA dissolved in DMSO, or DMSO as a solvent control. Daily peritumoral injections were administered subcutaneously for 21 days. For the oral application of DEL and GA in vivo, Wistar-Furth rats were first injected subcutaneously with 5 × 10⁵ MT-450 cells in PBS. Treatment was initiated in parallel with tumor cell injection. The animals (eight per group) were treated orally (16 G feeding needle) with 1.18 × 10⁻⁵ mol of DEL or GA dissolved in sunflower oil, or with sunflower oil or PBS as controls. The oral application of the substances was performed daily for 28 days. Tumor size was measured with a micrometer caliper. The animals were then killed and an autopsy was performed. The mass of the draining ipsilateral inguinal and axillary lymph nodes was assessed as a measure for lymph node metastases; contralateral lymph nodes served as a control. In addition, longitudinal transverse paraffin-embedded sections were taken from the central portion of the lymph nodes to ensure that corresponding regions of the lymph nodes were compared. Sections were hematoxylin and eosin (H&E) stained, photographed and analyzed for the presence of tumor cells with a light microscope (Olympus, Münster, Germany). The tumor-infiltrated area was quantified using Olympus Cell^B^ software. The lung was examined for the occurrence of metastases and nodules were counted. The tumors and surrounding tissue were isolated and snap frozen in liquid nitrogen for histochemical studies.

**Immunostainings**

Pieces of tumor were either formalin-fixed and embedded in paraffin wax or frozen in cryosectioning medium using standard methods. Paraffin wax-embedded sections were stained with either anti-mouse proliferating cell nuclear antigen antibodies (DAKO, Hamburg, Germany) or with the ApopTag staining kit (Millipore, Schwalbach, Germany) as described by the manufacturer. MT-450 primary tumor cryosections (7–10 μm thick) were fixed in 100% ice-cold acetone for 10 min, blocked with 10% goat serum in PBS and incubated with primary antibodies against LYVE-1 (Reliatech, Wolfenbüttel, Germany) or CD31 (Becton Dickinson) overnight at 4°C. Binding of the primary antibody was visualized using Alexa-coupled fluorescent secondary antibodies (Molecular Probes, Eugene, OR). Cell nuclei were counterstained with Hoechst bisbenzimide (Sigma–Aldrich). Specimens were examined using a light microscope (LEICA, Wetzlar, Germany), and images were captured with a digital camera. The number of positively stained lymphatic microvessels in the peritumoral region was evaluated in five independent fields (1 mm² each) for each of the eight tumors per group using NIS-ELEMENTS software (Nikon, Düsseldorf, Germany).

**Results**

**Chemical synthesis of DEL**

To further study the molecular and cellular effects of DEL on endothelial and tumor cells *in vitro* and *in vivo*, we set out to chemically synthesize pure DEL in the requisite amounts. Given the purity and quantity of DEL required, the existing protocols for the synthesis of DEL and its derivatives (31) were inadequate in terms of yield, practicability and safety. We therefore developed an improved and optimized scale-up synthesis (Figure 1). Several important modifications were introduced. The elongation of compound 3 used for the first time a Grignard reagent as the source for the additional carbon chain. The synthesis of 5-(2-acetoxyacetyl)benzene-1,2,3-triylic tricarboxylic acid (compound 6) was also successfully achieved without the use of highly toxic diazomethane. Furthermore, this synthesis route has additional advantages as it avoids the use of diethyl malonate, which needs harsher reaction conditions and involves more synthetic steps. The optimized synthesis route presented here enabled us to produce large quantities of pure and defined DEL aglycon for use in our study.

**GA only weakly impairs phosphorylation of a range of RTKs in *in vitro* kinase assays but inhibits ligand-induced autophosphorylation of VEGFR-2 in cellular assays**

We have previously demonstrated that DEL inhibits ligand-induced activation of several RTKs *in vitro* (20). However, DEL may be degraded to GA in tissue culture media (14). GA has also been reported to possess chemopreventative and antimetastatic properties (15–19,32). We therefore reasoned that GA rather than DEL might be responsible for the anti-RTK activity attributed to DEL. To determine whether this is the case, we assessed the ability of GA to inhibit the phosphorylation of ErbB2, EGFR, VEGFR-2 and VEGFR-3 in cell-free *in vitro* kinase assays. GA only slightly inhibited the kinase activity of all RTKs tested at 300 μM (Figure 2A), the highest concentration used. In contrast, DEL inhibits these RTKs in similar assays at low micromolar concentrations (20).

To test if GA can block RTK activity in the cellular context, we performed ligand-induced autophosphorylation assays using HT29 colon carcinoma cells that endogenously express EGFR, ErbB2 and ErbB3, and porcine aortic endothelial cells that ectopically express either VEGFR-2 or VEGFR-3 (29). No effect on ligand-induced autophosphorylation was observed for EGFR, ErbB2 and VEGFR-3 (Figure 2B, C and F), and phosphorylation of ErbB3 was only slightly reduced by GA (Figure 2D). However, GA markedly impaired VEGF-induced autophosphorylation of VEGFR-2 in a concentration-dependent manner (Figure 2E). Our previous work shows that DEL inhibits ligand-induced autophosphorylation of ErbB and VEGFR family members with an IC₅₀ between 20 and 50 μM (20). Together, these data suggest that DEL and GA inhibit a different spectrum of RTKs, and that DEL is a more potent inhibitor than GA, at least for the RTKs tested here. The data also show that the RTK inhibitory activity of DEL we have previously reported cannot be attributed to its degradation to GA.

**DEL and GA inhibit the proliferation of tumor and endothelial cells and induce apoptosis at higher concentrations**

To investigate and compare potential cellular effects of DEL and GA, we analyzed the ability of both substances to inhibit the proliferation of endothelial and tumor cells, as the RTKs inhibited by these substances regulate the proliferation of these cells. To this end, we chose primary HUVECs, primary human dermal LECs, human HT29 colon carcinoma cells and MT-450 rat mammary carcinoma cells and assessed the influence of DEL and GA on their proliferation as measured by [³H]-thymidine incorporation. Both DEL and GA showed an antiproliferative effect on all four cell types tested. However, GA was again much less potent than DEL (Figure 3A and B). Furthermore, we found that blood and LECs were more sensitive to both substances in comparison with the carcinoma cells tested.
Cell cycle analysis of DEL-treated cells confirmed the observations made in the proliferation assays and showed that higher concentrations of DEL between 50 and 200 μM induced apoptosis, as indicated by an increase in cells with a sub-G1 DNA content (Figure 3C). Together, these results show that while both DEL and GA can inhibit the proliferation of tumor cells, GA is much less potent than DEL, and demonstrate that DEL has substantial antiproliferative and proapoptotic effects on endothelial cells as well as tumor cells in vitro.

DEL and GA inhibit the outgrowth of LECs in ex vivo lymphatic ring assays

The finding that DEL blocks VEGF-C-induced autophosphorylation of VEGFR-3 (20), and has antiproliferative and proapoptotic effects on LECs (Figure 3), suggested to us that DEL could potentially possess antilymphangiogenic properties. To test this hypothesis, we performed ex vivo lymphangiogenesis assays and analyzed the influence of DEL and GA on the outgrowth of lymphatic capillaries from murine thoracic ducts. As expected, DEL robustly reduced lymphatic capillary outgrowth in a concentration-dependent manner (Figure 4A). At 1 μM, DEL significantly impaired lymphatic capillary outgrowth and almost completely inhibited outgrowth at 10 μM (P < 0.001). DEL was a much more potent inhibitor of lymphangiogenesis than GA (Figure 4B). These data demonstrate that DEL can inhibit lymphangiogenesis.

DEL inhibits tumor-induced angiogenesis and lymphangiogenesis in vivo but promotes the growth and metastasis of MT-450 tumors

In addition to inhibiting tumor cell and blood endothelial cell proliferation, the data above suggest that DEL can inhibit lymphangiogenesis. Tumor-induced lymphangiogenesis has been correlated with metastasis and poor prognosis (23). In order to investigate the ability of DEL to inhibit tumor growth, angiogenesis and lymphangiogenesis in vivo, MT-450 mammary carcinoma cells were implanted into syngeneic experimental rats. Tumors derived from MT-450 cells robustly induce lymphangiogenesis and metastasize to the draining lymph nodes and lung in vivo, and are therefore a suitable tool to investigate the properties of potentially antilymphangiogenic substances (26,27,33). Based on our previous experience with RTK inhibitors in this model (29,33), the animals were treated daily with 1.18 × 10⁻⁵ mol of DEL or GA, or with DMSO as a solvent control for 21 days by peritumoral injection, starting 4 days after implantation of the tumor cells (Figure 5). The growth of the tumors was monitored regularly (Figure 5A and B). The rats were killed 26 days after the injection of the tumor cells, and an autopsy was performed. Immunofluorescence analysis of histological sections of the primary tumors showed that peroxidase-conjugated antiphosphotyrosine antibodies and ABTS substrate. Absorbance was measured photometrically at 405 nm. Arbitrary light units are plotted as test over control (%), n = 3 independent experiments. Error bars represent standard error. Statistics were performed using Student’s t-test, assuming equal variation. (B–D) GA has no effect on activation of EGFR (B) or ErbB2 (C) in cellular phosphorylation assays but slightly impairs ligand-induced phosphorylation of ErbB3 (D). Starved HT29 cells were preincubated with either DEL or GA at different concentrations and then stimulated with 100 ng/ml EGF (EGFR, ErbB2) or 20 ng/ml heregulin (ErbB3). Cells were lysed in radioimmunoprecipitation assay buffer and the resulting lysates analyzed in western blots using EGFR, ErbB2 or ErbB3 and phospho-EGFR-, phospho-ErbB2- or phospho-ErbB3-specific antibodies, respectively. Alpha-tubulin served as an additional loading control. (E and F) GA has no effect on ligand-induced autophosphorylation of VEGFR-3 but inhibits VEGFR-2 activation. Porcine aortic endothelial cells transfected with either human VEGFR-2 or murine VEGFR-3 were cultured in serum-free medium. After preincubation with either DEL or GA at the appropriate concentration, the cells were stimulated with 30 ng/ml VEGFα or 400 ng/ml VEGF-C-Cys. After growth factor stimulation, the cells were lysed. The resulting lysates were immunoprecipitated with anti-VEGFR-2 or anti-VEGFR-3 antibodies. Precipitates were analyzed in western blots that were probed with antiphosphotyrosine Ig or with specific antireceptor Ig to control the loading of the samples.
both peritumoral lymphatic and intratumoral blood vessel density was significantly reduced ($P < 0.05$) in DEL-treated animals, whereas treatment with GA only significantly reduced intratumoral blood vessel density (Figure 5F and G), consistent with the finding that GA can block VEGFR-2 signaling. These data also confirm the in vitro findings made with endothelial cells. In additional experiments, we examined the ability of DEL to inhibit tumor-induced lymph node lymphangiogenesis but observed no effect (Supplementary Figure 1, Fig. 3. DEL and GA both inhibit the proliferation of endothelial and tumor cells, and DEL induces apoptosis of endothelial and tumor cells in vitro. (A) Primary human LECs, HUVECs, HT29 colon carcinoma or MT-450 mammary carcinoma cells were incubated for 24 h in the presence or absence of DEL, GA or solvent control. The cells were then labeled for 16 h with $^3$H-thymidine and analyzed for the amount of incorporated radioactivity. Each sample was performed in triplicate. Error bars represent standard error. (B) Inhibitory effect of DEL and GA on endothelial and tumor cell proliferation. The IC$_{50}$ values for primary human LECs, HUVECs, HT29 colon carcinoma and MT-450 mammary carcinoma cells based on (A) are shown. (C) Primary human LECs, HUVECs, HT29 colon carcinoma or MT-450 mammary carcinoma cells were incubated for 30 h in the presence or absence of DEL or solvent control. The cells were then harvested, fixed and their DNA stained with Draq5. Cell cycle analysis was then performed using flow cytometry, $n = 3$ independent experiments. Error bars represent standard error.
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**Fig. 4.** DEL significantly inhibits the outgrowth of three-dimensional *ex vivo* lymphatic ring cultures. Thoracic ducts were isolated from mice and cut into 1 mm long pieces. The explants were embedded in collagen gels and maintained in the presence or absence of appropriate concentrations of DEL (A), GA (B) or DMSO, as a solvent control. After 10 days, pictures were taken and converted into binary images. The total area of outgrown vessels was then calculated and plotted: *n* = 4 (DEL) or *n* = 3 (GA) independent experiments, respectively. Error bars represent standard error. Statistics were performed using Student’s *t*-test, assuming equal variation. *P* < 0.05, ***P* < 0.001; relative to DMSO controls.

available at *Carcinogenesis* Online). Surprisingly, however, DEL strongly promoted tumor growth (Figure 5A) and increased metastasis to the lymph nodes draining the primary tumor and to the lung in comparison with both the GA-treated animals and the DMSO control group (Figure 5C–E). This was accompanied by a reduced infiltration of CD4-positive T cells into the tumors from DEL-treated animals (Supplementary Figure 2, available at *Carcinogenesis* Online).

DEL is normally ingested orally as a dietary constituent. To determine whether this route of application also promotes tumor growth and metastasis, MT-450 tumor-bearing rats were treated daily orally with 1.18 × 10⁻² mol of DEL or GA dissolved in sunflower oil. Groups of MT-450 tumor-bearing rats were also treated similarly with sunflower oil alone or with PBS as controls. As before, DEL but not GA significantly promoted tumor growth in comparison with the control groups during early tumor growth (Figure 6A and B), and the tumors remained tendentially bigger in size throughout the experiment. Furthermore, treatment with DEL and GA promoted metasta-sis to the tumor-draining lymph nodes (Figure 6C and D), although GA did so less potently than DEL. Although no differences in the number of lung metastases were observed (Figure 6E), sunflower oil alone inhibited metastasis formation in both lymph nodes and lungs (Figure 6F–I), suggesting that the solvent obscured at least in part prometastatic effects of DEL and GA.

Together, these data demonstrate in a syngeneic rat model of breast cancer that DEL can promote tumor growth and metastasis when administered peritumorally or orally.

**Discussion**

DEL inhibits a variety of RTKs that have been implicated in the growth of tumor and endothelial cells (20). With the possible exception of VEGFR-2 inhibition, the data presented here demonstrate that this inhibitory activity cannot be ascribed to GA, a breakdown product of DEL. Consistently, we found that DEL potently inhibited the proliferation of endothelial and tumor cells *in vitro*, and at higher concentrations induced their apoptosis. GA only exhibited weak inhibitory effects in these assays. DEL also blocked the outgrowth of lymphatic capillaries in *ex vivo* lymphangiogenesis assays and significantly reduced angiogenesis and lymphangiogenesis *in vivo* in the MT-450 syngeneic mammary tumor model. However, DEL also promoted primary tumor growth and metastasis in this model. These findings indicate that the proposed use of DEL for cancer chemoprevention and therapy needs to take into account possible protumorigenic and metastasis-promoting activities of DEL.

GA has been shown to inhibit tumor cell proliferation and induce their apoptosis *in vitro* (15–17). Consistent with these studies, we found that GA indeed exerts antiproliferative and proapoptotic effects on tumor cells *in vitro*, albeit at very high concentrations in comparison with DEL (Figure 3). Similarly, with the exception of VEGFR-2 that was potently inhibited by GA (Figure 2E), GA only slightly inhibited the kinase activity of the RTKs tested here at 200 μM (Figure 2), whereas DEL inhibits these RTKs at low micromolar concentrations (20). Nevertheless, GA has a pronounced effect on the proliferation of tumor cells at a concentration of 200 μM (e.g. refs 17,34), suggesting that GA may inhibit the activity of other RTKs not tested in this present study or may inhibit tumor cell proliferation by an alternative mechanism. Either way, in all experiments presented here, DEL had much more potent effects than GA, be that on inhibition of RTK activity, cell proliferation, lymphangiogenesis or tumor growth and metastasis. Thus, we conclude that biological effects observed with DEL in this and other studies cannot be ascribed to its phenolic acid degradation product GA.

Oral application of GA has been reported to suppress tumor growth and progression in TRAMP (transgenic adenocarcinoma of the mouse prostate) mice that develop prostate tumors (19), and in prostate (35) and lung (17) carcinoma xenografts in nude mice. These studies used GA doses between 20 (17) and 120 mg/kg (19). In our studies, we did not observe any effect of GA on tumor growth but used much lower GA doses (7 mg/kg) to allow us to compare the effects of GA and DEL. Thus, we cannot exclude that we would have seen an effect on tumor growth had we used higher doses of GA. Nevertheless, GA significantly reduced the number of intratumoral CD31-positive blood vessels (Figure 5E), consistent with its ability to impair activation of VEGFR-2 (Figure 2E), a key mediator of angiogenesis. Oral application of GA also significantly increased tumor-draining lymph node weight, a measure for lymph node metastasis (Figure 6C). Reduced liver metastasis at much higher GA doses than we used (50 mg/kg) has also been reported in a mouse mastocytoma tumor model (18).

A novel activity of DEL we report here is its ability to inhibit lymphangiogenesis. This activity was demonstrated by the ability of DEL to suppress LEC proliferation, to inhibit the outgrowth of lymphatic capillaries in *ex vivo* lymphangiogenesis assays and to reduce tumor-induced lymphangiogenesis *in vivo*. The ability of DEL to inhibit key regulatory RTKs such as VEGFR-3 is likely to mediate this antilymphangiogenesis effect. Other natural products such as hyperforin from St John’s Wort have also been reported to inhibit lymphangiogenesis (26). The inhibition of lymphangiogenesis has attracted attention as a possible way of limiting tumor metastasis (25), but the data we present here suggest that DEL is unlikely to find application in this context due to its ability to promote tumor growth and metastasis. GA also inhibited outgrowth of lymphatic capillaries in the *ex vivo* assays (Figure 4B) although at much higher concentrations than DEL. This activity may be due to the ability of GA to inhibit VEGFR-2, an RTK that is also expressed on LECs and which can contribute to LEC proliferation (23).

An important and unexpected finding was that DEL promotes tumor growth and metastasis in the MT-450 rat syngeneic breast cancer model, despite inhibiting tumor cell and endothelial cell proliferation *in vitro*, and inhibiting angiogenesis and lymphangiogenesis *in vivo*, although premetastatic intranodal lymphangiogenesis
in tumor-draining lymph nodes was not significantly affected by DEL (Supplementary Figure 1, available at Carcinogenesis Online). Besides RTK inhibition, various other biological effects including antioxidative and anti-inflammatory properties have been ascribed to DEL (36,37), which may contribute to its activity in vivo. A variety of studies based on in vitro experiments with different cell lines have also reported that DEL inhibits tumor cell proliferation and form the basis for the conclusion that DEL has a chemopreventative effect (38–46).

The results we present here suggest that caution should be taken when drawing conclusions from studies that only use cultured cells, as the in vivo effects of DEL may not reflect its inhibitory activity in vitro.

In contrast to our findings, Hafeez et al. (5) showed that DEL significantly reduced the growth of human PC3 prostate carcinoma xenografts in vivo. A number of differences in the experimental design of the two studies may account for the divergent outcomes.

First, we employed a mammary tumor model, whereas Hafeez et al. used a prostate cancer model. DEL may exhibit cancer type-specific effects on tumor growth in vivo. Second, Hafeez et al. used intraperitoneal injections to administer DEL, whereas we saw the most pronounced effects on tumor growth with peritumoral injections. We also tested intraperitoneal administration to rule out that the route of DEL delivery affects the outcome of the experiment but found that intraperitoneal administration of DEL was not tolerated by the animals. Third, DEL can inhibit the activity of several RTKs (20). The spectrum of RTKs sensitive to DEL might well be even broader than those tested in this study. It is therefore conceivable that PC3 cells might express DEL-sensitive RTKs that are not present on MT-450 cells, but which are critical for the growth of PC3 tumors. Finally, and possibly most importantly, Hafeez et al. used athymic nude mice and xenograft in their experiments, whereas we used a...
Fig. 6. Oral application of DEL and GA has no significant effects on tumor growth but promotes lymph node metastasis in vivo. MT-450 cells were subcutaneously injected into experimental rats (eight per group). Oral application of DEL or GA or sunflower oil as a solvent control was initiated in parallel to tumor cell injection and then performed daily for 28 days. Tumor size was monitored regularly. Tumor volumes up to day 20 are depicted (A and B). At day 29, the animals were killed and an autopsy was performed. The mass (C) of the draining ipsilateral axillary (LN ax. ips.) lymph nodes and quantification of tumor-infiltrated nodal area (D) served as a measure for lymph node metastases (representative pictures of H&E-stained sections from lymph node metastases are shown). Lung nodules were counted (E). Error bars represent standard error. Statistics were performed using the Student’s t-test, assuming equal variation. *P < 0.05, **P < 0.01; relative to the sunflower oil control. To test the influence of sunflower oil on tumor growth and metastasis, the experiment described in (A) was repeated, but instead sunflower oil or PBS was applied orally. Tumor size was monitored regularly. Tumor volumes up to day 22 are depicted (F). At day 29, the animals were killed and an autopsy was performed. The mass (G) of the draining ipsilateral axillary (LN ax. ips.) lymph nodes and quantification of tumor-infiltrated nodal area (H) served as a measure for lymph node metastases (representative pictures of H&E-stained sections from lymph node metastases are shown). Lung nodules were also counted (I). Error bars represent standard error. Statistics were performed using the Student’s t-test, assuming equal variation. *P < 0.05, relative to PBS control.
syngeneic immunocompetent model. The impact of the immune system on tumor growth has been increasingly appreciated during the last 10 years (47). Certain immune cells are able to suppress and eliminate tumor cells, while others promote tumor growth and metastatic progression (47–50). In this regard, it is interesting to note that DEL itself has been suggested to possess anti-inflammatory properties (37). Consistently, we found that DEL but not GA significantly reduced the number of peritumoral CD4+ cells in primary tumors (Supplementary Figure 2, available at Carcinogenesis Online). Several CD4+ T-cell subsets have been reported to be involved in the antitumoral immune response (51–53). It therefore seems plausible that the reduction of CD4+ cells by DEL might be responsible for the increased tumor growth and metastasis formation we observed upon DEL treatment. This would not only help to explain the divergent outcome of our study and the study of Hafeez et al. (5) who used nude mice lacking CD4+ T cells, but also suggest a mechanism through which DEL might promote metastasis despite its antiangiogenic and antilymphangiogenic properties.

Another surprising finding in this study was that sunflower oil reduced metastasis significantly (Figure 61). Linoleic acid and vitamin E are major constituents of sunflower oil that have been reported to exert opposing effects on metastasis. The term vitamin E describes a group of several lipophilic tocopherol and tocotrienol compounds. Studies examining the effects of these substances on tumor growth and progression in experimental animals demonstrate that they can inhibit both tumor growth and metastasis (54). Conversely, it has been demonstrated that dietary linoleic acid can promote tumor progression in xenografted human mammary tumors, possibly by induction of matrix metalloproteinase-9 expression (55). In syngeneic mouse mammary carcinoma models, however, linoleic acid had no significant effects on primary tumor growth or metastasis (56). It therefore remains unclear how sunflower oil might suppress metastasis formation.

DEL is one of the most abundant anthocyanidins in food, and accounts for ~12% of the total anthocyanidin content in the diet (3). Estimates of daily mean consumption of anthocyanins vary greatly from source to source, ranging from 10 mg up to 1 g (57,58). Given an anthocyanin content of 700–800 mg/l in red wine, and a bioavailability of >5%, it has been calculated that even moderate wine consumption (0.4 l) would result in biologically relevant anthocyanidin plasma levels of ~10 μM (59). Nevertheless, the amounts of DEL ingested as part of a normal diet are considerably below the doses administered in animal experiments. However, reports about possible beneficial health effects of anthocyanins have prompted the commercial production of plant extracts with a high anthocyanin content as nutritional supplements, whose consumption could substantially increase the amounts of DEL in the body over and above that acquired through a normal diet. Furthermore, the use of anthocyanins and anthocyanidins as potential drugs has been discussed (4,20), which would entail the ingestion of artificially high doses. Chemopreventive properties of these substances in limited amounts have been inferred from a variety of preclinical and clinical studies (reviewed in ref. 4), but an unrestricted beneficial value of pure DEL aglycon has to be reconsidered in the light of our findings, as we clearly show that in certain contexts DEL can have undesirable positive effects on tumor growth and metastasis. Future work will focus on understanding the molecular mechanisms through which DEL exerts its tumor- and metastasis-promoting functions.

**Supplementary material**

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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**References**


