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Didymin by suppressing NF-κB activation prevents VEGF-induced angiogenesis in vitro and in vivo

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ABSTRACT

Although didymin, a dietary flavonoid glycoside from citrus fruits, known to be a potent antioxidant with anti-cancer activities, its role in angiogenesis is not known. In this study, we examined the effect of didymin on VEGF-induced angiogenesis in vitro and in vivo models. Our results suggest that treatment of human umbilical vein endothelial cell (HUVECs) with didymin significantly prevented the VEGF-induced cell proliferation, migration, and invasion. Further, didymin significantly prevented the VEGF-induced endothelial tube formation in culture. Didymin also attenuated the VEGF-induced generation of ROS, activation of NF-κB and the expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin in HUVECs. Further, didymin also prevented the VEGF-induced microvessel sprouting in ex vivo mouse aortic rings. Most importantly, didymin significantly prevented the invasion of endothelial cells and formation of blood capillary-like structures in Matrigel plug model of angiogenesis in mice. Thus, our results suggest a novel antiangiogenic efficacy of didymin in addition to its reported anti-cancer properties, which warrant further development of this agent for cancer therapy.

1. Introduction

Angiogenesis, a process of formation of new blood vessels from the existing vascular network, is a critical process in various physiological and pathological conditions. Angiogenesis is involved in the transition of premalignant lesions to the malignant tumor growth and metastasis, [1,2]. Endothelial cell proliferation, migration, and invasion are the major steps involved in the angiogenic process initiated by growth factors such as VEGF and FGF [3–5]. The hyper-proliferating cancer cells in a solid tumor mass require increased the supply of oxygen and nutrients to cope with their metabolic rate [6]. Cancer cells as well as normal cells express angiogenic stimulators such as VEGF, which play a critical role in the regulation of new blood vessel formation. Elevated expression of VEGF is correlated with the increased angiogenesis and has been observed in various human cancers of lung, breast, renal, colon and glioblastoma [7–10]. Therefore, anti-VEGF treatments have been shown to be effective in treating multiple cancer forms including metastatic cancers [11,12].

Recent studies have demonstrated the therapeutic significance of plant-derived polyphenols and flavonoids as potential anti-angiogenic and chemopreventive agents. Antioxidants such as curcumin, quercetin, and resveratrol have been shown to downregulate various redox-sensitive transcription factors and expression of various inflammatory cytokines, chemokines and growth factors responsible for tumor growth, metastasis, and angiogenesis [13–15].

Didymin, a bioactive flavonoid-glycoside compound, found in the majority of citrus fruits such as oranges, lemons, and mandarins, is a potent antioxidant that regulates a number of pro-inflammatory pathways [16–20]. Several studies suggest that didymin possess anti-cancer properties. It has been shown that didymin prevents cancer cell proliferation and induces apoptosis via mitochondrial dysfunction in HepG2 liver carcinoma cells [21]. Hsu et al. 2016 reported that didymin prevents phthalate ester-associated breast cancer cell proliferation and migration [22]. Didymin prevents neuroblastoma cell proliferation by inhibiting the N-myc and stimulating the Raf kinase inhibitor protein expression [18,23,24]. Didymin has been shown to prevent the growth of NSCLC by regulating Fas/Fas L apoptotic signals [25]. Further, didymin also prevents CCl_4-induced liver injury by attenuating NF-κB, MAP kinase and PI3K and upregulating RKIP pathway [19,26]. Although these studies suggest that didymin is anti-

Abbreviations: DD, Didymin; ECM, Endothelial Cell Medium; HBSS, Hank's balanced salt solution; HUVECs, Human umbilical vein endothelial cells; VEGF, Vascular endothelial growth factor; vWF, Von Willebrand factor; ICAM-1, Intercellular adhesion molecule 1; MAPK, Mitogen-activated protein kinases; VCAM-1, Vascular cell adhesion molecule 1; ROS, Reactive oxygen species.

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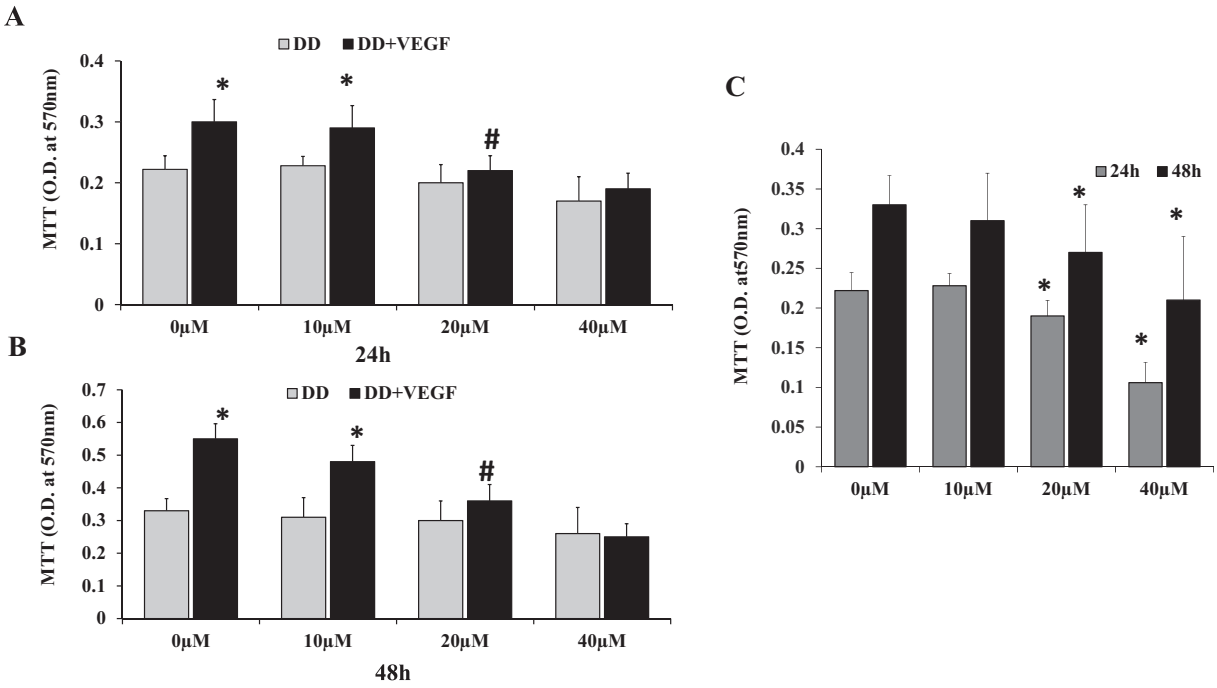

Fig. 1. Didymin prevents VEGF-induced HUVECs proliferation. (A & B) Serum-starved and growth-arrested HUVECs were pre-treated with different concentrations of didymin (10 μM, 20 μM, and 40 μM) followed by incubation without or with VEGF for 24 h and 48 h respectively and cell viability were determined by MTT assay. (C). Bars showing effect of Didymin on HUVEC proliferation after treatment with different concentration of Didymin for 24 h and 48 h. Data represent mean ± SD ($n = 6$ for each group). *$p < .01$ vs control, #p < .01 vs VEGF-treated.

carcinogenic due to its anti-oxidative and anti-inflammatory actions, the anti-angiogenic role of didymin is not known. Therefore, in this study, we examined the effect of didymin on VEGF-induced angiogenesis in vitro and in vivo. Our results reveal that treatment of HUVECs with didymin significantly prevents VEGF-induced cell proliferation, migration, tube formation in HUVECs, microvascular sprouting in mice aortic rings and prevents the expression of angiogenic markers in mouse matrigel-plug model. Thus, our data suggest that didymin could be developed as a potential therapeutic agent for the prevention of angiogenesis.

2. Material and methods

2.1. Materials

Didymin (#38964) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (#M5655) was purchased from Sigma-Aldrich. The structure of didymin is reported elsewhere [27,28]. Endothelial Cell Medium (ECM) and endothelial cell growth supplement were purchased from Sciencell Research laboratories (#1001). Matrigel growth factor reduced (GFR) basement membrane matrix, 12 well 8.0 μm transwell permeable membrane insert were obtained from Corning Inc., USA. PBS, fetal bovine serum (FBS), penicillin/streptomycin solution, trypsin/EDTA, Annexin V Alexa Fluor-488 conjugate (#A13201), Calcein-AM (#C3100MP), CM-H2DCFDA (#C6827) were obtained from Invitrogen. RIPA buffer (#SC24948), anti-VCAM-1 (#SC8304), anti-ICAM-1 (#SC107), anti-E-selectin (#SC137054), antibodies were obtained from Santa Cruz Biotechnology. Vascular endothelial growth factor (VEGF-165), anti-p-NF-kB-p65 (#3033), anti-Von Willebrand Factor (vWF) (#65707), anti-histone H3(#4499), anti Rabbit IgG isotype control (#3900S) and anti-GAPDH (#14C10) were obtained from Cell Signaling technology. The anti-CD31 antibody (#ab28364) was purchased from Abcam.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) cells were purchased from the ScienCell Research Laboratories (#8000). Cells were grown in ECM media (ScienCell #1001) containing 1× Endothelial Cell Growth Supplement (ECGS), 5% FBS and 1% penicillin/streptomycin and cultured in a humid incubator at 37 °C and 5% CO_2. HUVECs in the 6–8 passages were used in this study.

2.3. Cytotoxicity assays

HUVECs (3000 cells/well) were seeded in 96-well plates. Next day, cells were growth arrested in 0.1% FBS ± three different concentration of didymin (10 μM, 20 μM, and 40 μM, $n = 6$–8/group) for overnight. Subsequently, the cells were incubated with VEGF (10 ng/mL) for another 24 h and 48 h. Cell viability was determined by MTT assay [29]. To examine the cell death, the treated HUVECs were washed and re-suspended in HBSS containing 0.5 μg/mL Annexin-V and 5 μg/mL PI for 15 min in dark and subjected to flow cytometry analysis (BD LSRII Fortessa) as described earlier [16].

2.4. Immunofluorescence assays of ROS in HUVECs

Growth-arrested HUVECs were treated VEGF (10 ng/mL) ± didymin (20 μM) for 3 h. After the incubation, the cells were stained with ROS dye, CM-H2DCFDA, for 15 min and analyzed immediately by flow cytometry (BD LSRII Fortessa). Fold change of mean fluorescence intensity (MFI) were quantified by using Flow Jo software (Treestar, Ashland, OR, USA).

2.5. Transwell migration assay

Transwell migration assay was performed in 12 well plate containing translucent polycarbonate membrane insert of 8 μm membrane pore size. The HUVECs cells (40,000 cells/mL 0.1% ECM) were added on top of the insert. Subsequently, in the bottom well VEGF (10 ng/

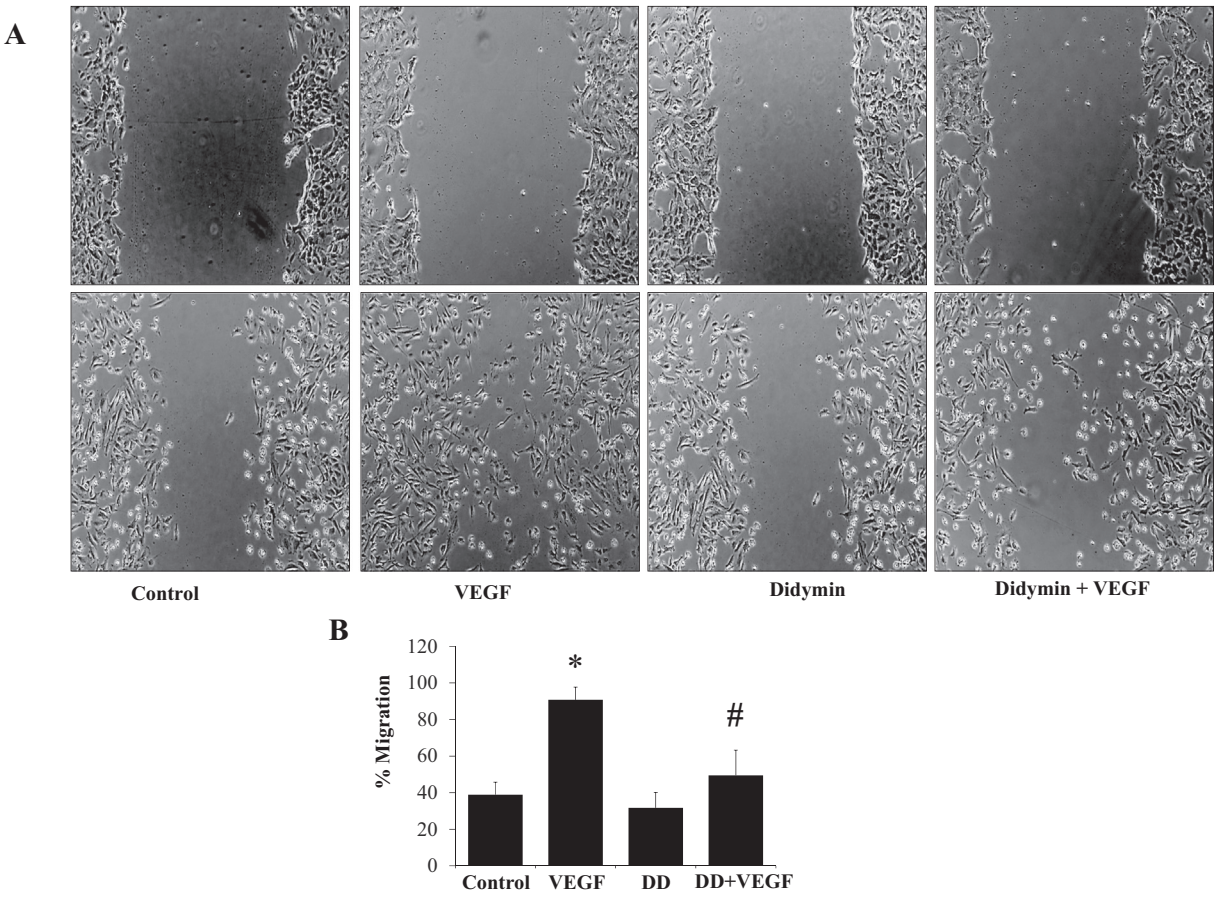

Fig. 2. Didymin inhibits VEGF-induced HUVECs migration. Effect of didymin on VEGF-induced HUVECs migration was determined by in vitro wound healing assay. (A) HUVECs were grown in 6 well plates and allowed to form a monolayer. A longitudinal uniform scratch was made and further incubated with VEGF (10 ng/mL) without and with didymin (20 μM) for 18 h. A representative image was shown in each group. (B) Percentage change in cell migration was calculated by ($Width_{0h}$ – $Width_{18h}$)/$Width_{0h}$ × 100. Data represent mean ± SD (n = 3 for each condition). Representative data from 3 independent analysis is shown *p < .001 vs control, #p < .01 vs VEGF-treated.

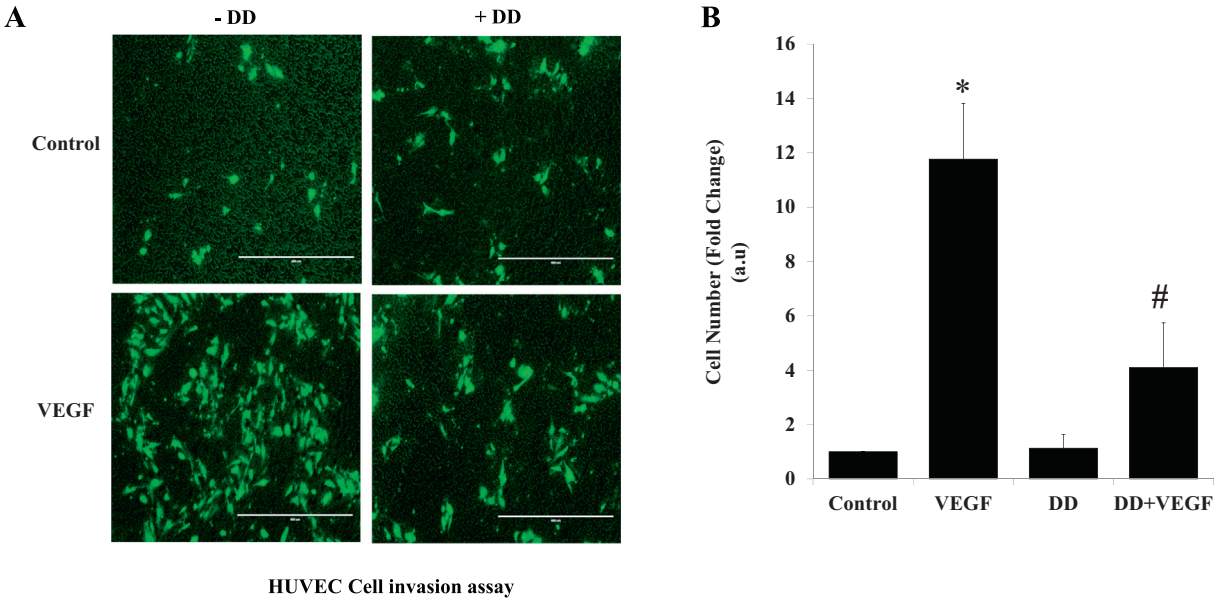

Fig. 3. Didymin abrogates VEGF-induced HUVECs transmigration. Transwell migration assay was performed to examine the HUVEC invasion. (A) HUVECs were seeded onto 8 μM transwell membrane inserts and stimulated with VEGF (10 ng/mL) without or with didymin (20 μM) for 18 h; The membranes were stained with calcein-AM to visualize the migrated cells and images were taken using a Fluorescence microscope. (B) The number of invaded cells was counted using a hemocytometer and data presented as fold change compared to control. Data represent mean ± SD (n = 3 for each condition). *p < .001 vs control, #p < .001 vs VEGF-treated.

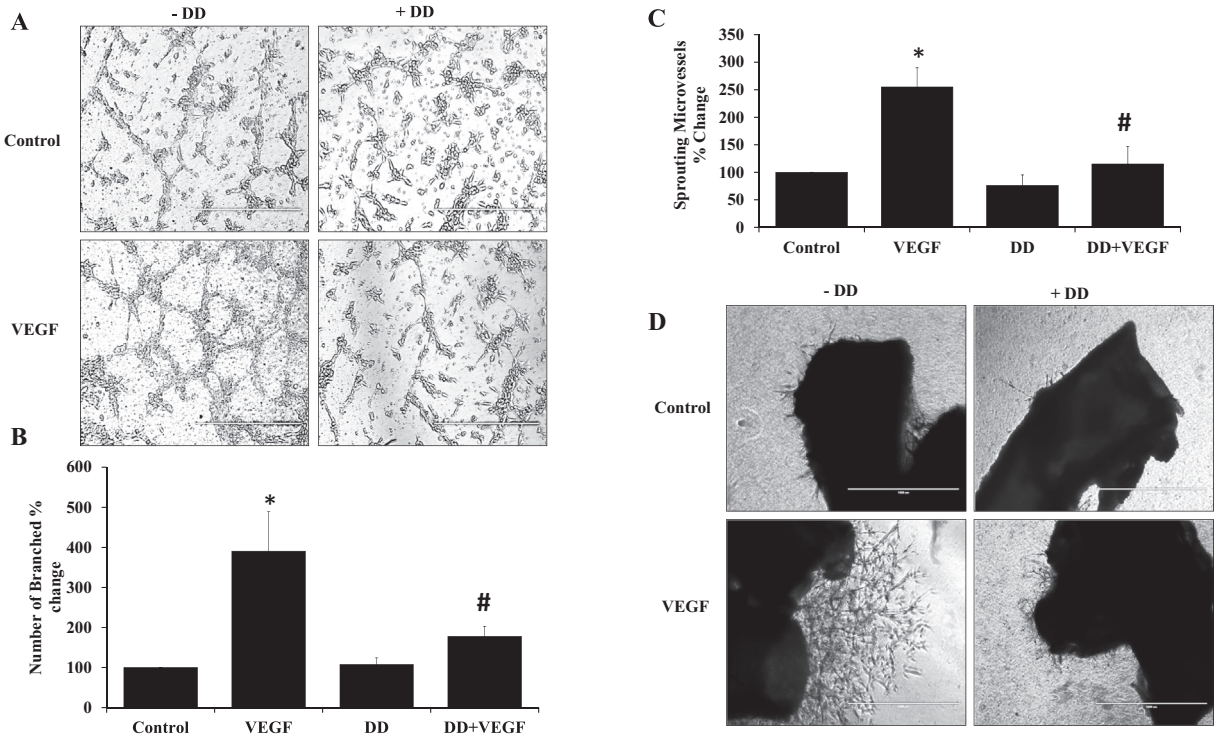

Fig. 4. Didymin prevents VEGF-induced capillary like-tube formation in vitro and microvessel sprouting in mice aortic rings ex vivo. (A) HUVECs cells seeded on growth factor-reduced Matrigel-coated 96 well plates and incubated with VEGF without or with didymin and incubated for 6 h and analyzed under a bright field microscope. A representative image is shown for each group. (B) The angiogenic effect was determined by counting the relative branched nodes and bar graphs show the percentage change in branched nodes with various treatments. Random fields were counted in each group and tube length was plotted. Representative data from 3 independent experiments is shown (C and D) Mouse aortic rings were embedded on growth factor-reduced Matrigel followed by incubation with VEGF (10 ng/mL) in absence and presence of didymin (20 μM) for 5 days, as described in the methods. The number of sprouting micro vesselswere counted arbitrarily and data presented as a percentage change. Data represent mean ± SD (n = 3). *p < .001 vs control, #p < .001 vs VEGF-treated.

mL) ± didymin (20 μM) was added and incubated for 18 h. Next, the unmigrated HUVECs on the top chamber of the transwell insert were wiped using a cotton swab, and the cells on the bottom layer were left undisturbed. Calcein-AM florescent dye was used to determine the transmigration of cells towards the bottom side of the membrane and photographs were taken using an EVOS fluorescence microscope. In another set of experiment, transwell membranes were stained with Giemsa stain and pictures were taken. Furthermore, cells on the membranes were harvested and counted by a hemocytometer.

2.6. Cell migration by wound healing assay

Migration of HUVECs was performed by scratch wound healing assay [29]. Briefly, HUVECs cell was seeded in 6 well plates and allowed to grow to form a monolayer. The cells were growth arrested overnight in the absence and presence of didymin (20 μM). Next day, a longitudinal uniform scratch was made with a fine needle at the center of the monolayer. Cells were washed to remove detached cells and incubated further with VEGF (10 ng/mL) ± didymin (20 μM) for 18 h. To determine the migration, the photographs of each well were taken at 0 and 18 h using an EVOS bright field microscope. Percentage change in the cell migration was calculated by $(Width_{0h} - Width_{18h})/Width_{0h} \times 100$.

2.7. Tube formation assay

Growth factor reduced Matrigel was thawed on ice, and 50 μL Matrigel was added into each well of pre-chilled 96 well plate and allowed to polymerize in a humid incubator for 30 min. HUVEC cells were serum starved overnight without or with didymin (20 μM). Further ~5000 cells were suspended in culture medium containing VEGF ± didymin and added into each well carefully and incubated at 37 °C 5% CO_2 for 6 h. Photograph of tube formation was taken using an EVOS microscope.

2.8. Ex vivo mice aortic rings angiogenesis assay

Ex-vivo angiogenesis was determined in mice aortic rings. Briefly, the aortic rings ~1.0 mm long obtained from the normal C57BL/6 mice were incubated with the growth factor reduced Matrigel. Subsequently, 300 μL of DMEM media containing VEGF (10 ng/mL) ± didymin (20 μM) was added to each well and incubated at 37 °C in a CO_2 incubator for 5 days. At the end of the experiment, the vascular sprout formations were observed under an EVOS bright field microscope.

2.9. In vivo Matrigel plug angiogenesis assay

All animal experiments were conducted under aseptic conditions following the guidelines of the institutional animal ethical committee. About 5 weeks old male C57BL/6 mice were obtained from Jackson Laboratory, USA and housed for another two weeks to acclimatize. Animals were randomly assigned to each treatment group. Matrigel plug assay was used to determine in vivo angiogenesis [30]. Briefly, Matrigel was mixed with VEGF (10 ng/mL) ± didymin (20 μM) and injected subcutaneously in mice to form a solid gel plug (3mice/group). Ten days after Matrigel injection, the mice were euthanized, Matrigel plugs were dissected out and photographed. The formalin-fixed paraffin-embedded Matrigels were cut into 5 μm sections and stained with hematoxylin and eosin to visualize the cells and blood vessels. Further, immunohistochemistry analysis was performed by using monoclonal antibodies against CD31, vWF and Anti-Rabbit IgG using DakoCytomation LSAB + system-HRP kit or using a ABC reagent from Vector labs.

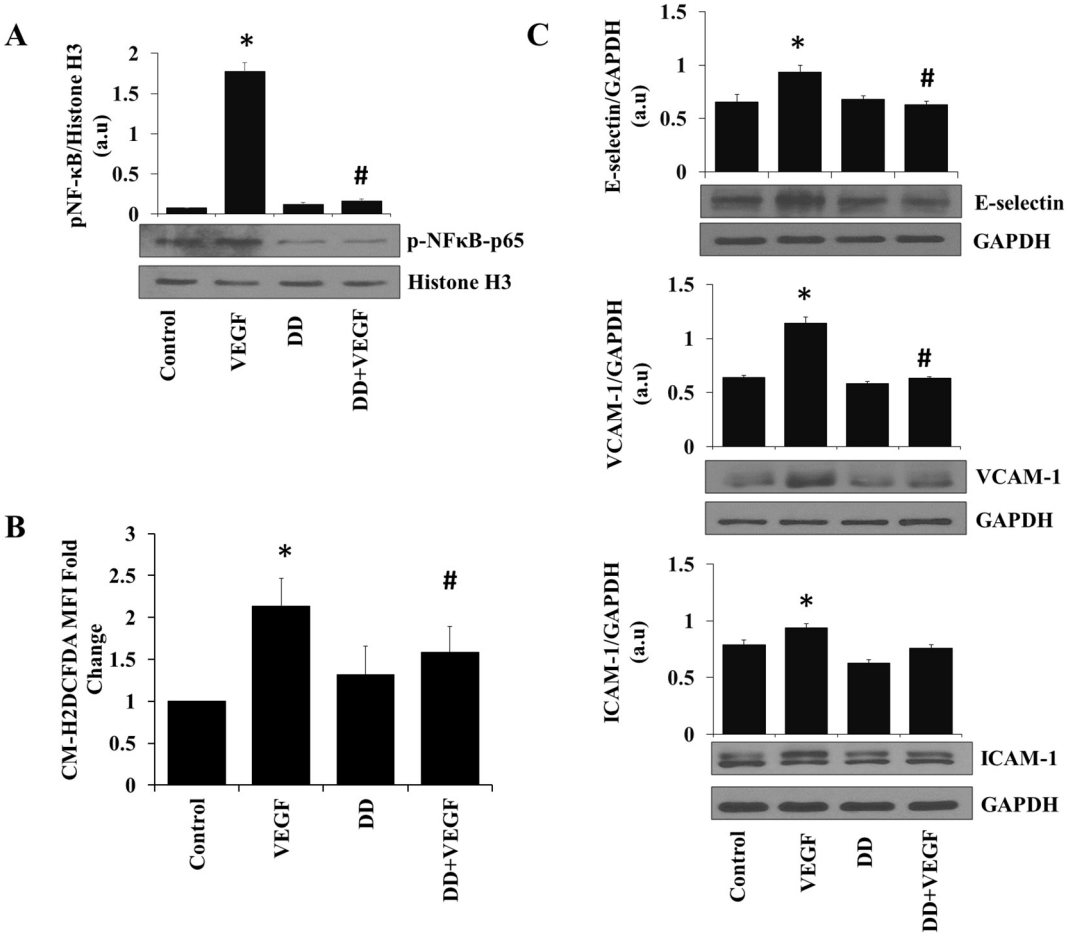

Fig. 5. Didymin prevents VEGF-induced NF-κB activation and expression of adhesion molecules in HUVECs. Growth-arrested HUVECs were pretreated without or with didymin (20 μM) overnight followed by stimulation with VEGF (10 ng/mL) for 2 h. (A) Equal amount of nuclear protein was subjected to Western Blot using phospho-NF-κB antibodies. (B) After the treatments, ROS levels were determined by incubating the cells with CM-H2DCFDA for 15 min and subjected to flow cytometry analysis. (C) HUVECs were pretreated without or with didymin (20 μM) overnight followed by stimulation with VEGF (10 ng/mL) for 24 h. Equal amount protein from the cell lysates was subjected to Western Blot analysis using specific VCAM-1, ICAM-1 and *E*-selectin antibodies. A representative blot from 3 independent experiment is shown. Blots were quantified using Image J. Data represent mean ± SD (n = 3). *p < .01 vs control, #p < .01 vs VEGF-treated.

The photographs were taken using EVOS fluorescence microscope.

2.10. Western blot analysis

Nuclear protein from the treated HUVECs were isolated by using nuclear extraction kit from Cayman Chemicals, and total protein was extracted by using RIPA buffer. Protein concentration was measured by Bradford protein assay (Bio-Rad, USA). An equal amount of protein was subjected to Western blot analysis using antibodies against VCAM-1, ICAM-1, *E*-selectin and phosho-NF-κB. Immunolabelled antibodies were detected by using SuperSignal West Pico Chemiluminescent substrate (ECL) from Thermo Scientific. Densitometric quantification of blots was performed using Image J software.

2.11. Ethics statement

All the experiments using mice were performed following the relevant guidelines and protocols approved by Institutional Animal Care and Use Committee (IACUC), UTMB, Galveston. After 10 days of in vivo matrigel injection, the animals were euthanized by CO_2 asphyxiation followed by cervical dislocation, and the Matrigel plugs were harvested for further analysis.

2.12. Statistical analysis

Data were presented as mean ± SD ($n = 6$), and p-values were determined by using Student's t-test analyzed using the GraphPad software. Multiple comparisons were analyzed by ANOVA. The $p < .01$ was considered as statically significant.

3. Results

3.1. Didymin prevents VEGF-induced HUVECs growth

We first examined the effect of didymin on VEGF (10 ng/mL)-induced cell viability. HUVECs were stimulated with VEGF ± didymin (10 μM, 20 μM, and 40 μM) for 24 h and 48 h and cell viability was determined by MTT assay. Treatment of HUVECs with VEGF increased the cell growth after 24 h, and 48 h (Fig. 1A and B), and pre-treatment of HUVECs with didymin significantly prevented the VEGF-induced increase in cell growth in a concentration-dependent manner. Didymin alone did not affect the growth of HUVECs up to 20 μM. Fig. 1C showing time course effect on HUVEC proliferation after treatment with different concentration of didymin alone for 24 h and 48 h. However, nonsignificant cell death was observed at 40 μM didymin alone -treated cells. We have selected 20 μM of didymin for all other experiments as this concentration didymin showed maximal efficacy and seemed optimal.

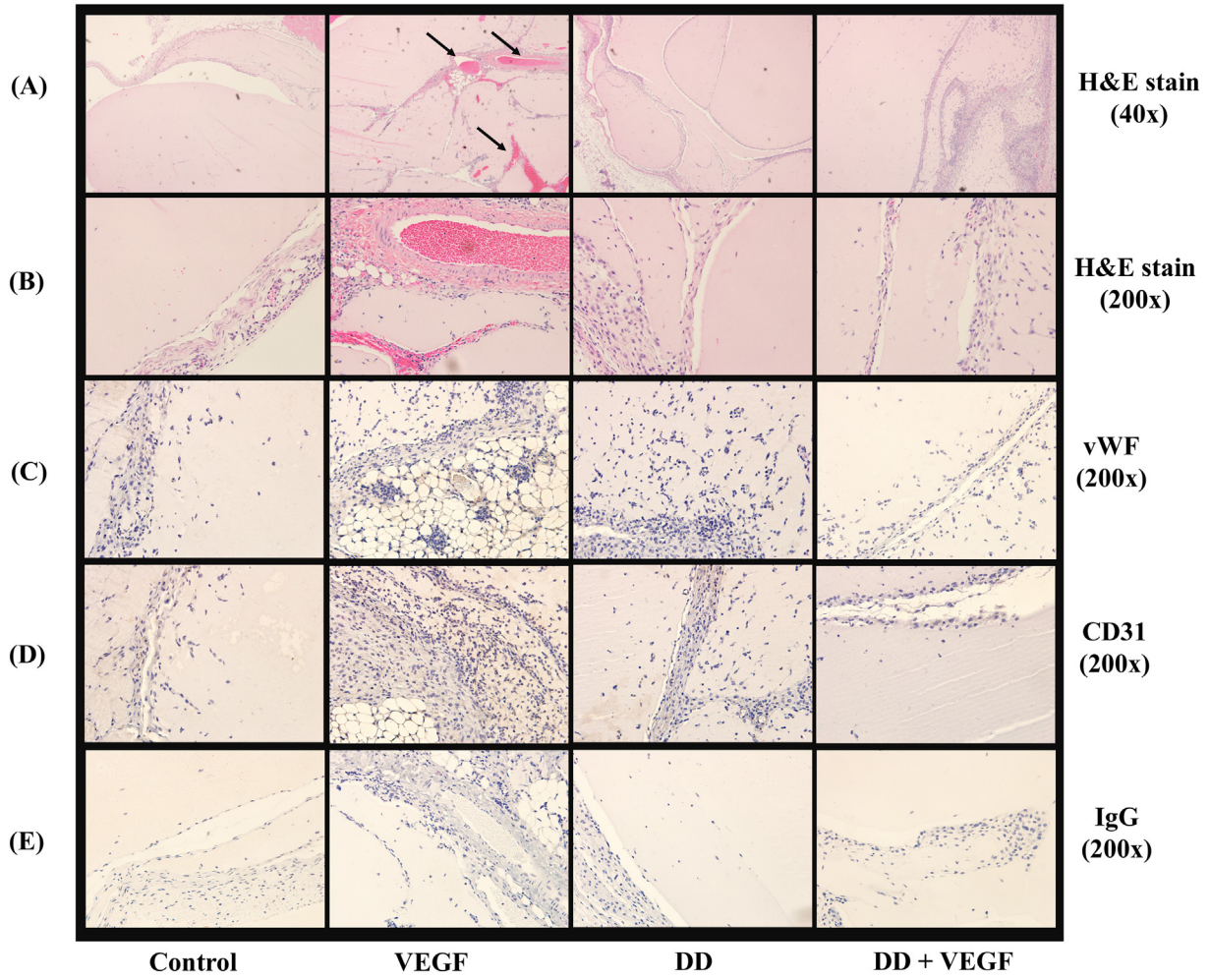

Fig. 6. Treatment with didymin prevents VEGF-induced angiogenesis in mice matrigel plug assay. Growth factor-reduced Matrigel without or with VEGF (10 ng/mL) and didymin (20 μM) was injected subcutaneously in C57Bl/6 mice. After one week, Matrigel plugs were excised and photographed. Matrigel sections were stained with (A) hematoxylin/eosin 40× magnification (B) matrigel hematoxylin and eosin stained sections 200×. Immunohistochemical staining of matrigel sections with antibodies against (C) anti-Von Willebrand factor (vWF), (D) anti-CD31/PECAM-1 and (E) rabbit anti-IgG control (Nuclei are counterstained blue) and photographs were taken under a light microscope. A representative image is shown (n = 3). Magnification 200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Didymin prevents VEGF-induced HUVECs migration

Since endothelial cell migration is an essential step of neovascularization and wound healing, we next examined the effect of didymin on VEGF-induced HUVECs motility using scratch wound-healing migration assay. Incubation of HUVECs with VEGF showed a significant increase in the migration of cells as compared to the untreated and didymin alone -treated cells (Fig. 2A and B). Further, HUVECs pretreated with didymin significantly prevented VEGF-induced HUVEC migration.

3.3. Didymin prevents VEGF-induced HUVECs Transwell migration

Transwell chemotaxis migration assay was used to examine the effect of didymin on VEGF-induced endothelial transmigration. Treatment of didymin prevents VEGF-induced transwell migration of HUVECs across the porous transwell membranes. A clear inhibition of HUVEC migration was observed after 18 h in VEGF-treated HUVEC in combination with didymin. At the end of the incubation period, the membranes were stained with Calcein AM and examined for live cells by using fluorescence microscopy (Fig. 3A). Significantly increased Calcein AM -stained cells were observed in VEGF-treated wells but not in the didymin plus VEGF- treated cells. Similar results were observed when we harvested the cells from the membranes and counted the cell number by using a hemocytometer (Fig. 3B). Thus, these results suggest that didymin significantly prevents the VEGF-induced HUVECs transwell migration.

3.4. Didymin prevents VEGF-induced capillary-like tube formation of HUVECs

Invasion and migration are the critical steps required for angiogenesis, and our results suggest that didymin prevents VEGF-induced migration of HUVECs. Therefore, we next examined the effect of didymin on VEGF-induced tube formation, an in vitro angiogenesis assay. HUVECs were seeded on 96 well plates containing growth factor-reduced Matrigel and incubated with VEGF (10 ng/mL) without or with didymin (20 μM) for 24 h. As shown in Fig. 4A and B, HUVECs incubated with VEGF showed significantly increased tube formation as compared to untreated control and didymin alone treated cells. However, pretreatment with didymin significantly attenuated VEGF-induced tube formation of HUVECs indicating that didymin prevents VEGF-induced neovascularization.

3.5. Didymin prevents VEGF- induced microvessel sprouting in ex vivo mice aortic rings

We further examined the effect of didymin on VEGF-induced angiogenesis using an established ex vivo model of microvessel sprouting of mice aortic rings. Our results shown in the Fig. 4C and D indicate a significantly increased microvessel sprouting in ex vivo mice aortic rings treated with VEGF alone as compared to control or didymin alone treated aortic rings. However, pretreatment of didymin significantly prevented the VEGF-induced microvessel sprouting in the aortic rings.

3.6. Didymin prevents VEGF-induced NF-κB activation and expression of adhesion molecules in HUVECs

Since activation of NF-κB and expression of adhesion molecules have been shown to be involved in the VEGF-induced HUVECs invasion, migration and neovascularization, we next examined the effect of didymin on the VEGF-induced activation of NF-κB in HUVECs. As shown in the Fig. 5A treatment of HUVECs with VEGF stimulated NF-κB activity and pretreatment of didymin (20 μM) significantly prevented it. Since NF-κB, is a redox-sensitive transcription factor, we next examined the effect of didymin on VEGF-induced ROS generation in HUVECS. Incubation of HUVECs with VEGF significantly (~2-fold) increased the ROS production when compared to control. However, pretreatment of HUVECs with didymin significantly prevented the VEGF-induced ROS production (Fig. 5B). Since NF-κB is known to be involved in the expression of adhesion molecules; we next determined the effect of didymin on the VEGF-induced expression of VCAM-1, ICAM-1 and *E*-selectin in HUVECs. Treatment of HUVECs with VEGF significantly increased the expression of VCAM-1, ICAM-1, and E-selectin. However, pretreatment HUVECs with didymin significantly prevented the VEGF-induced alterations in the expression of adhesion molecules (Fig. 5C). Thus, these results suggest that didymin prevents VEGF-induced activation of NF-κB and expression of adhesion molecules in HUVECs.

3.7. Didymin prevents VEGF-induced angiogenesis in mice matrigel plug model

In vivo anti-angiogenic effect of didymin was determined in a mouse Matrigel plug model. After one week of injection into mice, the Matrigel plugs were dissected out. The plugs obtained containing VEGF alone showed a high RBC content, which was not observed in plugs containing didymin + VEGF. The plugs were fixed in 10% formalin and sections were made. H&E staining of the matrigel sections, clearly showed capillary-like structures and infiltration of cells. Maximal localization of endothelial cells was significantly observed in VEGF-treated groups but not in the didymin + VEGF treated groups (Fig. 6A and B). Didymin alone -treated groups did not show any effect and was comparable to untreated controls. VEGF-induced endothelial cells infiltration, a marker for angiogenesis, was also observed by staining the sections with anti-vWF and anti-CD31 antibodies (Fig. 6C and D). CD31 and vWF staining of the Matrigel plug sections obtained from didymin + VEGF showed significantly less number of infiltrated endothelial cells, suggesting that didymin prevents angiogenesis in vivo. Anti-rabbit IgG Isotype control stained sections are shown in Fig. 6E.

4. Discussion

Solid tumors depend on new blood vessel network to acquire nutritional supply to support the tumor cell survival, propagation, and invasion [6]. Angiogenesis besides playing a crucial physiological role in wound healing, and organ development, also plays an essential pathological role in tumor growth and metastasis [9,31]. Several anticancer agents have been shown to prevent the angiogenic process [32,33]. Although didymin, a dietary flavonoid glycoside from citrus fruits, has been shown to be an excellent antioxidant with chemopreventive activities, its efficacy in preventing angiogenesis is not known [18,21–26,32]. The present study, to the best of our knowledge, demonstrates the anti-angiogenic effects of didymin and suggests that by preventing angiogenesis didymin could inhibit tumor growth. We have shown that didymin through its potential antioxidant activities prevents VEGF-induced endothelial cell proliferation, migration, invasion, sprouting and capillary-like tube formation.

Increased oxidative stress and formation of ROS have been shown to involved in the growth factor-induced angiogenic process [34,35]. Further, oxidative stress triggers the expression of various pro-angiogenic factors such as growth factors, cytokines, chemokines and adhesion molecules by activating transcription factors NF-κB and AP1, which facilitates increased endothelial cells differentiation and proliferation [34–37]. Growth factors like VEGF and FGF are well characterized for their role in initiating neovascularization and angiogenesis, and antagonists of VEGF are now in the clinical practice to control the tumor growth and spread [11,38]. In addition, several antioxidants, and free radical scavengers which prevent VEGF-induced endothelial cell growth, migration, and tube formation have been tested in the cancer clinical trials [13–15]. Antioxidants such as quercetin, reservatral, curcumin, etc. have been shown to be potent anti-angiogenic agents with chemopreventive activities [13,15,39,40]. These plant-derived antioxidants have been shown to prevent VEGF-induced endothelial cell growth and migration and tube formation. In this study, we have demonstrated that didymin substantially attenuated the VEGF-induced HUVEC cell proliferation, migration, and invasion: the initial steps required for neovascularization. These results are consistent with the findings from other reports that indicate medicinal plant products such as curcumin, polyphenols, and flavonoids prevent the proliferation of endothelial cells [39,40].

Sprouting of microvessels and tube formation have been associated with the neovascularization and VEGF has been shown to be mediate these process in endothelial cells [2]. Our studies showing that didymin prevented VEGF-induced tube formation in HUVECs in vitro and microvessel sprouting in mice aortic rings ex vivo suggest that didymin could antagonize the VEGF effects. Activation of adhesion molecules such as ICAM-1, VCAM-1, *E*-selectin is required for attachment of endothelial cells with extracellular matrix and monocytes and to form new capillaries and invasion to other organs [4,41]. VEGF increases endothelial cell permeability by inducing the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin via NF-κB activation that allows binding of monocytes to endothelial cells. [42–44]. Our data showed that didymin significantly attenuated VEGF-induced NF-κB activation and expression of adhesion molecules. Didymin by preventing the VEGF-induced expression of adhesion molecules could prevent the endothelial invasion and tumor metastasis. Most importantly, didymin also attenuated the formation of new capillaries and infiltration of endothelial cells in Matrigel plug model of angiogenesis in a mouse model.

In summary, our study demonstrates that didymin potentially attenuates VEGF-induced cell angiogenesis by inhibiting endothelial cell proliferation and migration. Our studies suggest that by preventing the VEGF-induced, redox-sensitive NF-κB activation and expression of adhesion molecules, didymin might exert its antiangiogenic effects. Thus, our studies provide a primary mechanism through which didymin prevents angiogenesis in vitro and in vivo and suggest potential anticancer activities of didymin could be attributed to its anti-angiogenic effects.

Conflict of interest

Author's declare no conflict of interest.

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