Am J Transl Res 2023;15(3):1831-1841
www.ajtr.org /ISSN:1943-8141/AJTR0148437

Original Article

EGT022, an RGD-containing recombinant disintegrin, inhibits the VEGF-induced angiogenic process by targeting integrin β3 in endothelial cells

Bong-Kyu Kim[1], Sung Jin Lee[2], Tae-Woo Kim[1], Hyojung Kim[1], Hyunjung Lee[1], Eun Young Hong[1], Yang Je Cho[1], Sunjong Kwon[1*], Seok-Hyun Kim[1*]

[1]R&D Center, EyeGene Inc., Goyang-si, Gyeonggi-do 10551, Republic of Korea; [2]Department of Ophthalmology, Soonchunhyang University College of Medicine, Soonchunhyang University Hospital, Seoul 04401, Republic of Korea. [*]Equal contributors.

Received December 12, 2022; Accepted March 1, 2023; Epub March 15, 2023; Published March 30, 2023

Abstract: Objectives: EGT022, an RGD-containing recombinant disintegrin from human ADAM metallopeptidase domain 15 (ADAM15), has been reported to stimulate vascular maturation of retinal blood vessels with promotion of pericyte coverage through binding to integrin αIIbβ3. Previous studies have reported that angiogenesis can be inhibited by several RGD motif-containing disintegrins; however, the effect of EGT022 on Vascular endothelial growth factor (VEGF)-induced angiogenesis has not yet been determined. This study was conducted in order to evaluate the anti-angiogenic function of EGT022 in VEGF-induced endothelial cells. Methods: A proliferation and migration assay was performed using human umbilical vein endothelial cells (HUVEC) cells stimulated with VEGF to determine whether the angiogenic process was suppressed by EGT022. An *in vitro* trans-well assay and Mile's permeability assay were performed to determine the effect of EGT022 on permeability. Western blot was performed in order to further determine whether EGT022 can inhibit phosphorylation of VEGF receptor-2 (VEGFR2) and Phospholipase C gamma1 (PLC-γ1). An integrin binding assay and luciferase assay were performed for identification of the integrin target of EGT022. Results: Angiogenesis including proliferation, migration, tube formation, and permeability was significantly inhibited by EGT022 in HUVEC cells. Our findings also demonstrated that EGT022 binds directly to integrin αvβ3, induces dephosphorylation of integrin β3, and inhibits phosphorylation of VEGFR2. In addition, phosphorylation of PLC-γ1 and activation of Nuclear Factor of Activated T-cell (NFAT), a downstream pathway of VEGF, are inhibited by EGT022 in HUVEC cells. Conclusion: These results clearly demonstrate the anti-angiogenic role played by EGT022 as a potent antagonist of integrin β3 in endothelial cells.

Keywords: EGT022, integrin β3, VEGF signaling, tubulogenesis, endothelial cell, disintegrin, RGD motif

Introduction

Angiogenesis, an essential process, includes endothelial growth and development, wound healing, and inflammation through formation of capillaries from pre-existing blood vessels [1]. Angiogenesis is induced by vascular endothelial growth factor (VEGF), a primary factor involved in angiogenesis in endothelial cells, through binding of VEGF receptor-2 (VEGFR2), a tyrosine kinase receptor [2]. Activation of VEGFR2 leads to stimulation of various intracellular signals such as Phospholipase C gamma1 (PLC-γ1), Serine/threonine kinase (AKT), and the extracellular signal-regulated kinase (ERK) pathway, thereby inducing angiogenesis [3, 4].

Integrins, which are transmembrane receptors, are essential to adhesion of cells to extracellular matrix (ECM) [5]. Integrins are heterodimers composed of α and β subunits. In mammals, integrins are assembled from 18 α and eight β subunits and different combinations of the α and β subunits, resulting in 24 unique integrins [6]. Most integrins contain short peptide sequences such as Arg-Gly-Asp (RGD), Glu-Ile-Leu-Asp-Val (EILDV), or Arg-Glu-Asp-Val (REDV) for recognition of ECM proteins [6]. Upon binding of ligand, various signal transduction path-

ways including angiogenesis are regulated by integrins [7].

Disintegrins are a family of small proteins (45-84 amino acids in length) mainly derived from snake venoms [8]. Several studies have reported on the important role of RGD-containing disintegrins in inhibition of platelet aggregation and angiogenesis [9, 10]. Tumor-induced angiogenesis and platelet aggregation are inhibited by saxatilin, a disintegrin derived from snake venom, and human ADAM metallopeptidase domain 15 (ADAM15) disintegrin-like domain derivatives [11, 12]. High similarity of the sequence of human ADAM15 with that of the snake venom disintegrin family, such as saxatilin, has been reported [11].

Based on these findings, EGT022, an RGD-containing recombinant disintegrin from human ADAM15, was developed as a replacement for a functional RGD-containing disintegrin from snake venom. EGT022, which contains 58 amino acids, stimulated vascular maturation of retinal blood vessels through promotion of pericyte coverage [13]. It was also reported to suppress abnormal neovascular sprout and blood vessel permeability [13]. However, the effect of EGT022 on VEGF-induced angiogenesis has not yet been evaluated.

In the current study, an analysis of the anti-angiogenic mechanism of EGT022 in VEGF-induced endothelial cells was conducted. Our findings demonstrated that VEGF-induced angiogenesis is significantly inhibited by EGT022. In addition, our findings also demonstrated that EGT022 binds directly to integrin β3 and causes impairment of the crosstalk between VEGFR2 and integrin β3, consequently inhibiting phosphorylation of PLC-γ1 and activation of Nuclear Factor of Activated T-cell (NFAT). These findings provide strong evidence indicating that EGT022 is an anti-angiogenic inhibitor as an antagonist of integrin β3 in endothelial cells.

Materials and methods

EGT022 expression and purification

Purification of EGT022 was performed using a previously described method [1]. In brief, full-length EGT022 was cloned from the human fetal liver cDNA library into the expression vector pPIC9, then transformed into the yeast *Pichia pastoris,* and cultured in methanol minimal medium (1.34% YNB with ammonium sulfate, 4×10^{-5} M biotin and 0.5% methanol). To induce expression of EGT022, methanol (0.5%) was added every 12 hr for three days followed by purification using Superdex G-75 gel filtration chromatography.

Cell culture

Human umbilical vein endothelial cells (HUVEC) (ATCC) were cultured in an Endothelial Cell Growth Medium-2 Bullet Kit (Lonza). HUVEC/TERT2 cells (Evercyte) were cultured in endothelial growth medium (Lonza) supplemented with EGM SingleQuot Kit (Lonza). HEK293 cells (Korean Cell Line Bank) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum.

Cell proliferation assay

For the assessment of proliferation, HUVEC cells were starved for 6 hr, pre-treated with or without EGT022 (1 μM) for 30 min, and treated with either VEGF (80 ng/ml) or PBS, followed by incubation for 24 hr. Measurement of the relative viability of the cells was performed using the CellTiter-Glo Luminescent Assay kit (Promega) according to the manufacturer's instructions.

Wound healing assay

For assessment of migration, HUVEC/TERT2 cells were seeded onto a 24-well plate with 90% confluency for 24 hr, and the middle of the plate was scratched using Scar™ Scratcher (SPL). Mitomycin C (4 μM/mL, Sigma) was added for 1 hr to inhibit proliferation. After washing three times with PBS, cells were pre-treated with or without EGT022 (1 μM) for 30 min, then treated with either VEGF (80 ng/ml) or PBS, followed by incubation at 37°C for 12 hr. The wound closure rate was determined using IMAGEJ software.

Tube formation assay

For assessment of tubulogenesis, μ-Slide Angiogenesis (ibidi) was coated with matrigel (Corning) for 10 min at 37°C. HUVEC cells were pre-treated with or without EGT022 (1 μM) for 30 min, treated with either VEGF (80 ng/ml) or PBS, and then seeded on the matrigel coated

μ-Slide. Following incubation for 18 hr, the formation of tubes was visualized using a CKX53 microscope (Olympus) with a 4 × objective lens (NA = 0.13). The total numbers of nodes, master junctions, master segments, and meshes were quantified using the Angiogenesis Analyzer (ImageJ software).

In vitro transwell assay

An *in vitro* endothelial cell monolayer permeability assay was used for determination of permeability [14]. In brief, HUVEC cells were plated on collagen I (50 ug/ml)-coated trans-well inserts (Corning) and cultured for five days until confluence. Cells located on the inserts were starved for 6 hr, and pre-treated with or without EGT022 (1 μM) for 30 min, followed by treatment with either VEGF (80 ng/ml) or PBS for 6 hr. Streptavidin-horseradish peroxidase (HRP) (R&D systems) was then added, followed by incubation for 5 min, and then reacted with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for the last 5 min. The amount of HRP passing through the cells located on the inserts into the lower chamber was measured at 450 nm (Epoch).

Immunocytochemistry

HUVEC cells were pre-treated with or without EGT022 for 30 min, followed by treatment with either VEGF (80 ng/ml) or PBS for 5 min. The cells were fixed using 4% paraformaldehyde for 10 min at RT, followed by treatment with 0.1% Triton X-100 for 30 min. After blocking with 3% BSA, the cells were incubated with antibodies against VE-cadherin (Cell Signaling; 1:500) overnight at 4°C. After application of Alexa Flour 594 anti-rabbit IgG antibody (Thermo Scientific, 1:500), the cells were incubated for 1 hr followed by mounting with VECTASHIELD PLUS Antifade Mounting Medium with DAPI (Vector Laboratories). Fluorescent signals were obtained using a TCS SP8 confocal microscope (Leica) with a 20 × objective lens (NA = 0.75).

Animals

C57BL/6 mice were bred under constant temperature (22 ± 2°C) and 50 ± 10% humidity, and lighting was adjusted every 12 hr. All animal experiments were approved by the EyeGene Research Center IACUC and performed in accordance with the guidelines for animal experimentation.

Mile's assay

A modified Mile's assay was used for measurement of vascular permeability. In brief, subcutaneous injection of PBS (control) or EGT022 (300 μg/kg) was performed once daily for five days in 8-week-old C57BL/6 (male) mice. Evans blue dye (1%) was injected through the caudal vein after the daily injection, followed by subcutaneous injection of 1 ng/ml VEGF (R&D Systems) or PBS into the back skin of mice. Measurement by Evans blue dye was performed after formamide extraction from the skin of sacrificed mice and absorbance was determined at 620 nm (Epoch).

Western blot

Cell lysates were prepared using RIPA buffer according to a standard method. A Bradford assay was used to estimate the protein concentration. Proteins present in cell lysates (20 μg) were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the following primary antibodies: anti-VEGFR2 antibody (1:1,000, Cell Signaling), anti-phospho-VEGFR2 antibody (1:1,000, Cell Signaling), anti-Integrin β3 (1:1,000, Cell signaling), anti-phospho-Integrin β3 (1:1,000, Cell Signaling), and anti-GAPDH antibody (1:1000, Cell Signaling). HRP-conjugated secondary antibodies and an enhanced chemiluminescence system (Amersham Bioscience) were used for detection of specific protein bands, which were quantified using ImageJ. A human phospho-kinase array kit (R&D Systems) was used according to the manufacturer's protocol for screening phosphoproteins of VEGF-induced signaling pathways affected by EGT022. For the knockdown of integrin β3, HUVEC cells were treated with 50 nm of specific or control siRNAs (Thermo) followed by incubation for 24 hr.

ELISA

HUVEC cells were plated in 6-well plates with 70% confluency. The cells were pre-treated with or without EGT022 (1 μM) for 30 min followed by treatment with either VEGF (80 ng/ml) or PBS for 5 min. Whole-cell lysates were harvested and ELISA was performed using the PathScan® Phospho-VEGFR2 Sandwich ELISA Kit (Cell Signaling) according to the manufacturer's instructions.

Binding assay

Plates were coated with various concentrations of recombinant human integrin (R&D Systems, 0.5-1.5 μg/mL) at 4°C overnight. After blocking with 4% Block Ace (Bio-Rad) for 1 hr at RT, plates were incubated with EGT022 (2 μg/mL) for 2 hr at RT, followed by incubation with mouse anti-EGT022 antibody (2 μg/mL) for 2 hr at RT. Anti-mouse IgG-HRP (0.8 μg/mL) was added, followed by incubation for 2 hr at RT and quantitation of signals at 450 nm was performed using TMB (Bio-Rad).

Luciferase reporter assay

HUVEC cells were seeded in 12-well plates with 70% confluency. After 24 hr, cells were co-transfected with 100 ng of pGL4.30 [luc2P/NFAT-RE/Hygro] vector (Promega) and 10 ng of pRL-TK renilla vector (Promega) using X-tremeGENE HP (Roche) according to the manufacturer's instructions. After 24 hr, cells were starved for 4 hr, pre-treated for 30 min with or without EGT022 (1 μM), followed by treatment with either VEGF (80 ng/ml) or PBS for 4 hr.

For VEGFR2 and Integrin (αv, α5, β1, β3) overexpression, transfection of 300 ng of VEGFR2 natural ORF (Sino Biological) constructs with or without 100 ng Integrin αv/α5/β1/β3 ORF (Sino biological) constructs was performed in HEK293 cells using 100 ng of pGL4.30 [luc2P/NFAT-RE/Hygro] vector. The dual luciferase assay system (Promega) was used according to the manufacturer's protocol for determination of luciferase activity. The relative firefly activity was normalized against transfection efficiency determined according to TK renilla activity.

Statistical analysis

Evaluation of statistical differences was performed using the Student's t-tests included in Excel software. Statistical significance was considered as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

EGT022 inhibits VEGF-induced proliferation, migration, and tube formation

Expression and purification of EGT022 were performed as previously described in order to examine the biologic function of EGT022 [12]. Purified EGT022 with a molecular weight of 6.1 kDa was identified (**Figure 1A**).

Previous studies reported that VEGF-induced tubulogenesis is inhibited by RGD-disintegrins in endothelial cells [15-17]. Therefore, we attempted to determine whether EGT022 can also suppress angiogenic processes such as proliferation and migration using HUVEC cells stimulated with VEGF. Compared to non-treated control cells, treatment with VEGF resulted in increasing proliferation (1.84-fold) and migration (2.59-fold) of the cells, respectively. However, treatment with EGT022 resulted in suppressive proliferation (0.76-fold) and migration (0.73-fold) of VEGF-induced cells (**Figure 1B-D**). A tube formation assay was then performed for analysis of the role of EGT022 in endothelial tubulogenesis induced by VEGF. As shown in **Figure 1E**, treatment with EGT022 resulted in inhibition of the number of nodes (0.82-fold), master junctions (0.84-fold), and master segments (0.77-fold) as compared with VEGF-treated control cells. These results indicate that the VEGF-induced angiogenic process is inhibited by EGT022.

EGT022 inhibits VEGF-induced endothelial permeability

VEGF is a critical factor of increasing vascular permeability [18] and we attempted to determine whether EGT022 can affect permeability using an *in vitro* trans-well assay. The results of pretreatment of HUVEC cells with EGT022 showed a 0.61-fold VEGF-stimulated permeability (**Figure 2A**).

VE-cadherin plays an essential role in maintenance of vascular integrity [19]. Treatment with VEGF results in reduced expression of VE-cadherin with diffusion of F-actin for bundling into stress fibers, thereby increasing endothelial permeability [19, 20]. Immunocytochemistry was performed to determine whether expression of VE-cadherin and F-actin is altered by EGT022. Of particular interest, our findings showed that pretreatment with EGT022 resulted in enhanced expression of VE-cadherin, which was reduced by VEGF alone (**Figure 2B**). These results clearly demonstrate that dynamic movement of HUVEC cells is stimulated by treatment with VEGF, which can be inhibited by pre-treatment with EGT022. Mile's permeability assay was also performed in order to

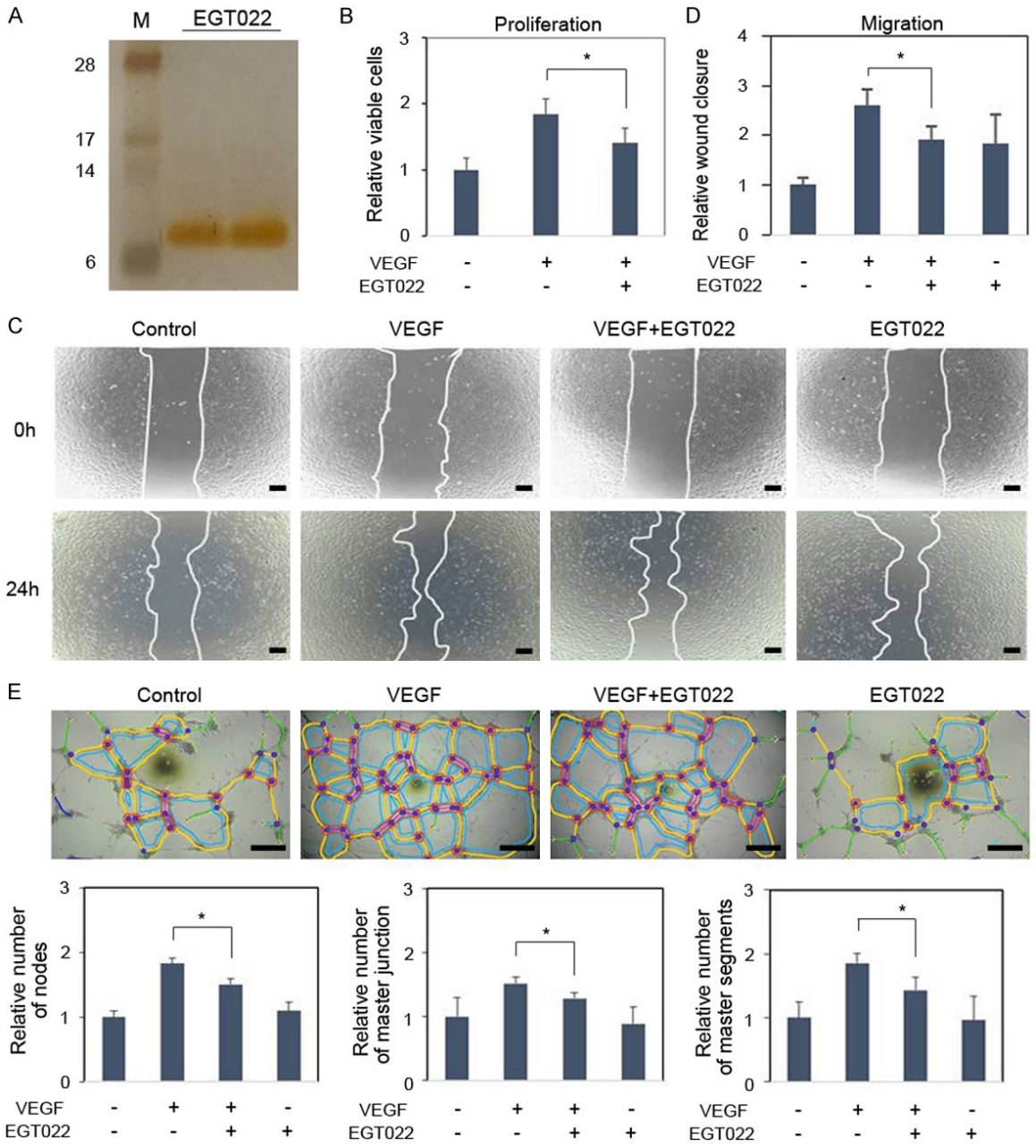

Figure 1. EGT022 inhibits VEGF-induced proliferation, migration, and tubulogenesis. A. Purified EGT022 was assessed by silver staining as a 6.1 kDa. M: size marker. B. Viable VEGF-induced HUVEC/TERT2 cells were decreased by treatment with EGT022 compared with untreated cells. C, D. Results of a wound healing assay showed that treatment with EGT022 inhibits migration compared to the control. Relative wound closure was analyzed using ImageJ software. Migrated cells observed under a microscope (40 ×). Scale bar = 50 μM. E. Results of a tube formation assay of HUVEC cells showed that the number of nodes, master junctions, and master segments was decreased following pretreatment with EGT022. ImageJ Angiogenesis Analyzer was utilized for quantitative analysis. Results are presented as the average of three independent experiments. $^{*}P < 0.05$. Cells observed under a microscope (20 ×). Scale bar = 200 μM. Vascular endothelial growth factor (VEGF), Human umbilical vein endothelial cells (HUVEC).

further evaluate the reduced permeability caused by EGT022 *in vivo*. We found that the absorbance of Evans blue leaked from the mice was decreased by 0.56-fold by pre-treatment with EGT022 as compared to mice treated with VEGF alone (**Figure 2C**). These experimental results suggest that EGT022 can prevent endothelial permeability *in vivo*.

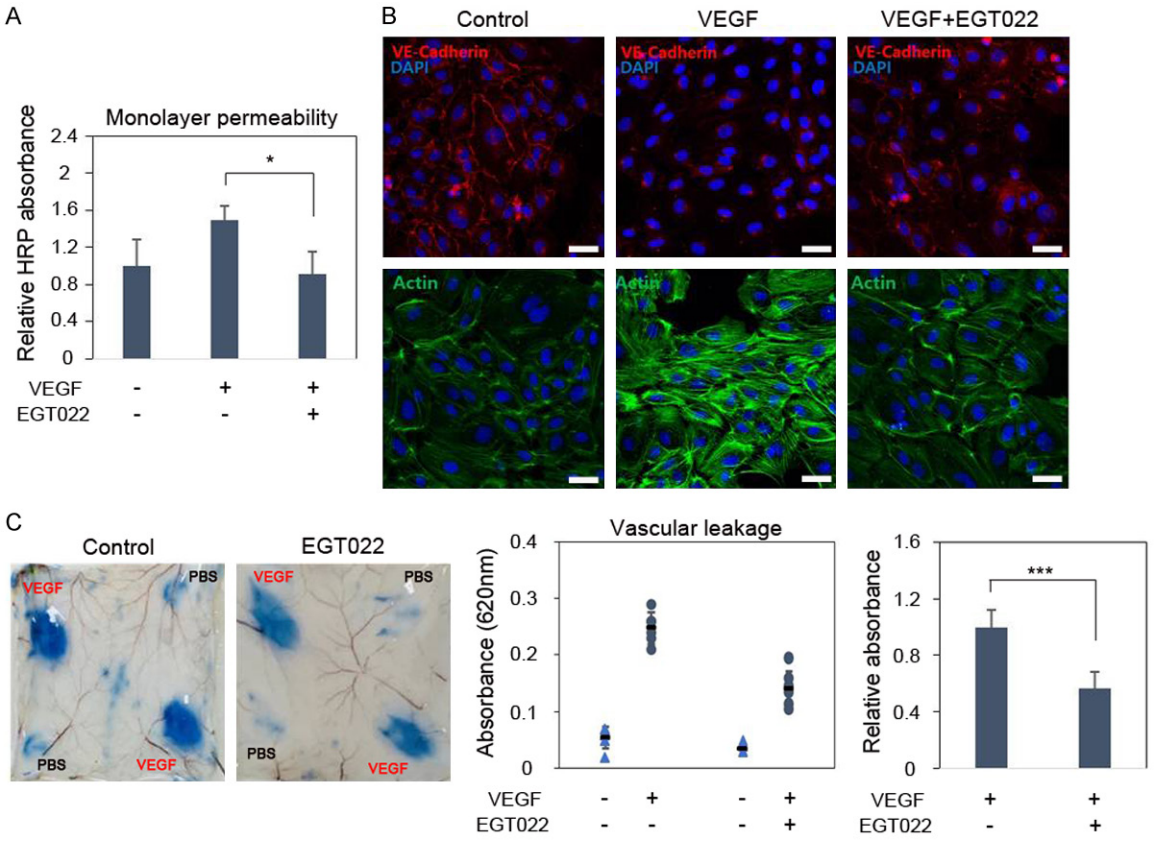

Figure 2. EGT022 inhibits the VEGF-induced endothelial permeability. A. Results of a monolayer permeability assay showed that Streptavidin-HRP diffusion to the lower chamber was decreased by EGT022 in VEGF-induced HUVEC/TERT2 cells. Results are presented as the average of three independent experiments. B. Confocal microscopy showed increased expression of intracellular VE-cadherin by EGT022 in VEGF-induced HUVEC cells. In contrast, F-actin expression was reduced. Blue: DAPI, Green: Actin, Red: VE-Cadherin. Cells were photographed at a magnification of 200 ×. Scale bar = 10 μM. C. In the Mile's Assay treatment with EGT022 reduced the leakage of Evans Blue from the skin (N = 6). Relative absorbance was determined by normalizing according to the absorbance value of the PBS treated skin. $^{*}P < 0.05$; $^{***}P < 0.001$. Vascular endothelial growth factor (VEGF), Human umbilical vein endothelial cells (HUVEC).

EGT022 inhibits phosphorylation of VEGFR2 and PLC-γ1 after stimulation of cells with VEGF

In order to further determine the anti-angiogenic mechanism by EGT022, western blot was performed to determine its effect on phosphorylation of VEGFR2. As shown in **Figure 3A** and **3B**, phosphorylation of VEGFR2 by pre-treatment with EGT022 was significantly decreased by 0.49-fold compared to VEGFR2 phosphorylation of VEGF-induced HUVEC cells. Similarly, the results of ELISA showed a pattern of suppressed phosphorylation (0.65-fold) of VEGFR2 (**Figure 3C**). A human phosphokinase array was used for screening the phosphorylation of various proteins in order to examine the target downstream pathways affected by EGT022 in endothelial cells. Our findings demonstrated that the phosphorylation profile of several proteins associated with angiogenesis, including ERK1/2, CREB, c-Jun, eNOS, and phospholipase C gamma1 (PLC-γ1), is altered by pre-treatment with EGT022 compared to VEGF-induced HUVEC cells (**Figure 3D**). Western blot was performed in order to confirm the results of the phospho-kinase array (**Figure 3E**). As shown in **Figure 3F**, phosphorylated PLC-γ1, activated by VEGF, was decreased to 0.75-fold by EGT022. However, other proteins did not show significant alteration of phosphorylated status (data not shown). VEGF-induced activation of PLC-γ1 has been reported to mediate nuclear translocation of NFAT, resulting in angiogenesis [21]. We attempted to determine whether decreased phosphorylation of VEGFR2 and PLC-γ1 by EGT022 would affect nuclear

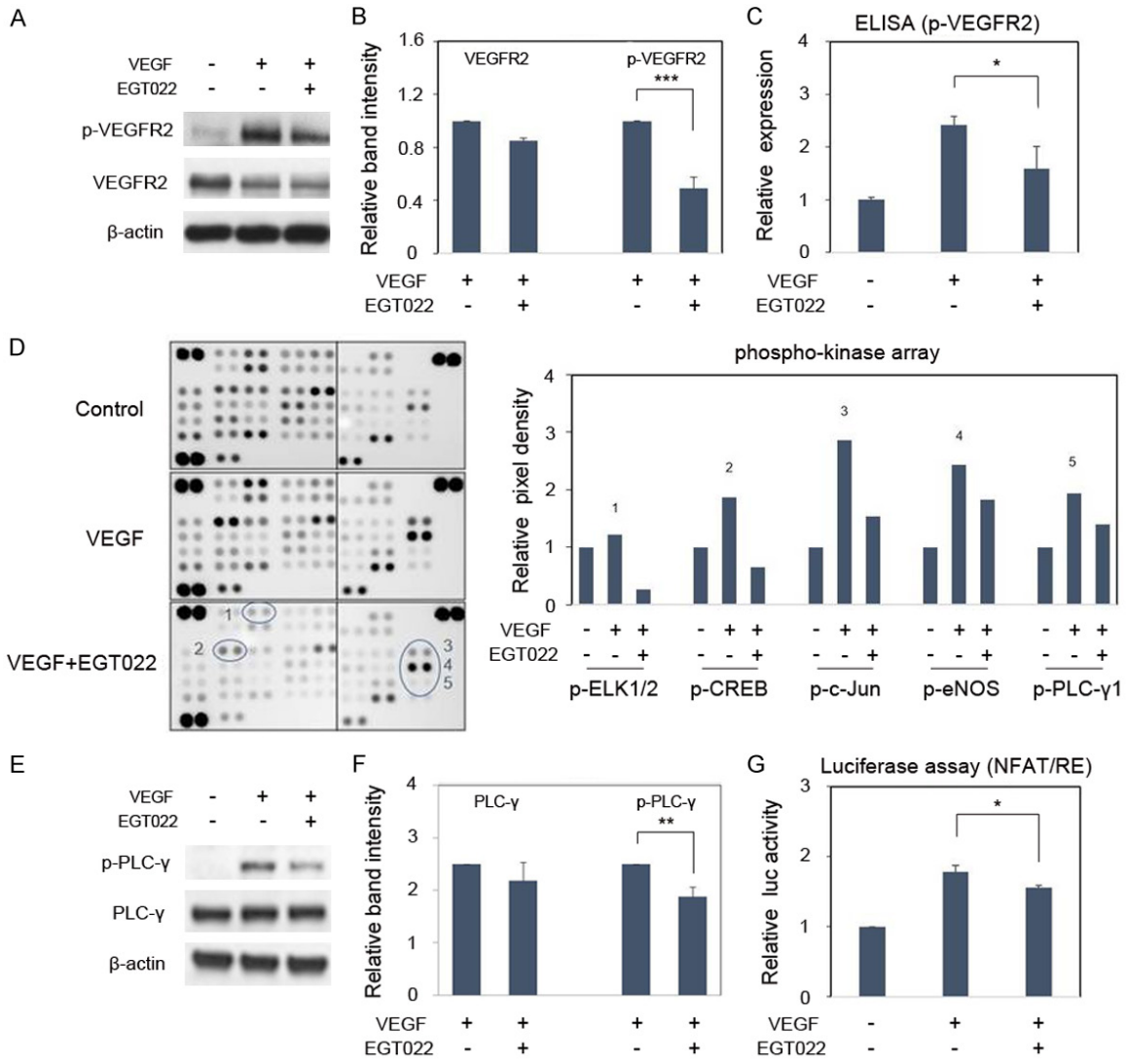

Figure 3. EGT022 inhibits both VEGFR2 and PLC-γ1 phosphorylation mediated by VEGF. (A, B) Results of western blot analysis showed that pretreatment with EGT022 significantly inhibited phosphorylation of VEGFR2 in VEGF-induced HUVEC cells. β-actin was used as a loading control. (C) Inactivation of phosphorylation of VEGFR2 by EGT022 in VEGF-induced HUVEC cells was also detected by Sandwich ELISA. (D) Phospho-kinase array was performed to screen the change in phosphorylation levels of different kinases by EGT022 in VEGF-induced HUVEC cell lysates. Phosphorylation of several protein kinases related to angiogenesis was altered by EGT022 in VEGF-induced HUVEC cells. (E, F) Phosphorylated PLC-γ1 was decreased by EGT022 in VEGF-induced HUVEC cells. (G) EGT022 inhibited luciferase activity in VEGF-induced HUVEC cells containing NFAT/RE promoter. Relative expression of each protein was quantified using ImageJ software (B, D, F). Results are presented as the average of three independent experiments (B, C, F, G). $^{*}P < 0.05$; $^{**}P < 0.01$. NS, not significant. Vascular endothelial growth factor (VEGF), Human umbilical vein endothelial cells (HUVEC).

translocation of NFAT. Results of a reporter assay with NFAT response elements showed that luciferase activity was reduced by 0.85-fold after treatment with EGT022 compared to that of VEGF-induced HUVEC cells (**Figure 3G**). These findings suggest that phosphorylation of PLC-γ1 is specifically decreased by EGT022 with subsequent inhibition of NFAT-mediated transcription activated by VEGF in endothelial cells.

EGT022 binds integrin β3 and suppresses VEGF-stimulated phosphorylation

Crosstalk between VEGF and integrin cell adhesion receptors is critical in the regulation of

angiogenesis [6]. Using an integrin binding assay, we attempted to determine whether EGT022 could bind directly to a specific integrin. We observed strong binding of EGT022 with integrin αIIbβ3 (Kd^{app} = 292 nm) and αvβ3 (Kd^{app} = 29 nm) (**Figure 4A**). Next, transient transfection of a single integrin subunit was performed in HEK293 cells with a low level of integrin expression in order to screen for integrins that could be directly affected by pretreatment with EGT022. As observed in the integrin binding assay, transfection of integrin β3 resulted in reduced VEGF-meditated activation of NFAT/RE promoter after pre-treatment with EGT022 (**Figure 4B**). Western blot was performed to determine whether EGT022 can affect phosphorylation of integrin β3. The experimental results showed that phosphorylation of integrin β3 is induced by VEGF in HUVEC cells. Of particular interest, however, pre-treatment of the cells with EGT022 resulted in a 0.52-fold decrease of VEGF-stimulated phosphorylation of β3 (**Figure 4C**, **4D**). We also examined the role of EGT022 in activation of NFAT/RE in the knock-down of integrin β3 by treatment of HUVEC cells with specific β3 siRNAs. The results of western blot analysis showed suppressed expression of integrin β3 in specific siRNA-treated cells (**Figure 4E**). The results of the luciferase assay showed that pretreatment with EGT022 had no effect on VEGF-induced NFAT/RE in cells with knock-down of integrin β3 (**Figure 4F**). Collectively, these results suggest that crosstalk between integrin αvβ3 and VEGFR2 is impaired by the direct interaction between EGT022 and integrin β3 and their phosphorylation is inhibited, thereby preventing VEGF-induced angiogenic effects in endothelial cells.

Discussion

Crosstalk between VEGFR2 and integrin αvβ3 in endothelial cells is fundamental to the process of angiogenesis, and impairment of this interaction could lead to development of a potent anti-angiogenic therapy. The strategy using an antagonist of RGD integrin has been proposed and many studies have demonstrated that angiogenesis is inhibited by RGD-disintegrins in endothelial cells [6, 15-17]. For example, VEGF-mediated angiogenesis including migration, invasion, and tubulogenesis was inhibited by Dis*Ba*-01, an inhibitor of RGD-disintegrin and αvβ3 [15]. Network formation induced by pro-angiogenic growth factors was decreased by *Cyclo* [DKP-RGD] 1, a cyclic RGD peptidomimetic [16]. Adhesion, migration, and tube formation were inhibited by a fragment of BIGH3 containing an RGDRGD motif, with induction of cell apoptosis in HUVEC cells [17].

EGT022, an RGD-containing disintegrin originating from human ADAM15, has been developed as a treatment for several vascular related diseases including diabetic retinopathy, pressure ulcer, and myocardial ischemia. Phase 2 clinical trials have been conducted to evaluate the effectiveness and safety of an EGT022 for treatment of those diseases. In clinical trials of diabetic retinopathy and myocardial ischemia, EGT022 is administered subcutaneously, not into the eye or directly into the heart. Findings of our previously reported study demonstrated that EGT022 binds directly to integrin αIIbβ3 on the surface of platelets and promotes the recruitment of pericytes, thereby restoring retinal ischemic capillaries and reducing the ischemic area [13]. By contrast, in the case of pressure ulcer, EGT022 is administered in the form of an ointment and applied directly to the ischemic area. However, the mechanism of the direct action of EGT022 to induce vascular normalization in the injured area has not yet been determined.

In this study, we examined the question of whether EGT022 can also effectively suppress VEGF-induced angiogenesis in HUVEC cells. In agreement with findings on other RGD-disintegrins, we observed that proliferation, migration, tube formation, and vascular permeability were prevented by EGT022 in HUVEC cells. These results suggested that EGT022 is an effective inhibitor of angiogenesis.

In addition, our previous data demonstrated that interaction of EGT022 with the integrin αIIbβ3 in human platelets results in inhibited aggregation of platelets [13]. Our current and previous results demonstrated a high affinity of EGT022 for both integrin αIIbβ3 and αvβ3 [13]. Abundant expression of Integrin αvβ3 occurs on the surface of HUVECs [22]; the possibility of direct binding of EGT022 to HUVECs has been suggested. In this study, we demonstrated that EGT022 binds specifically to integrin β3, inhibiting phosphorylation of both integrin β3 and VEGFR2. VEGF signaling in HUVEC cells

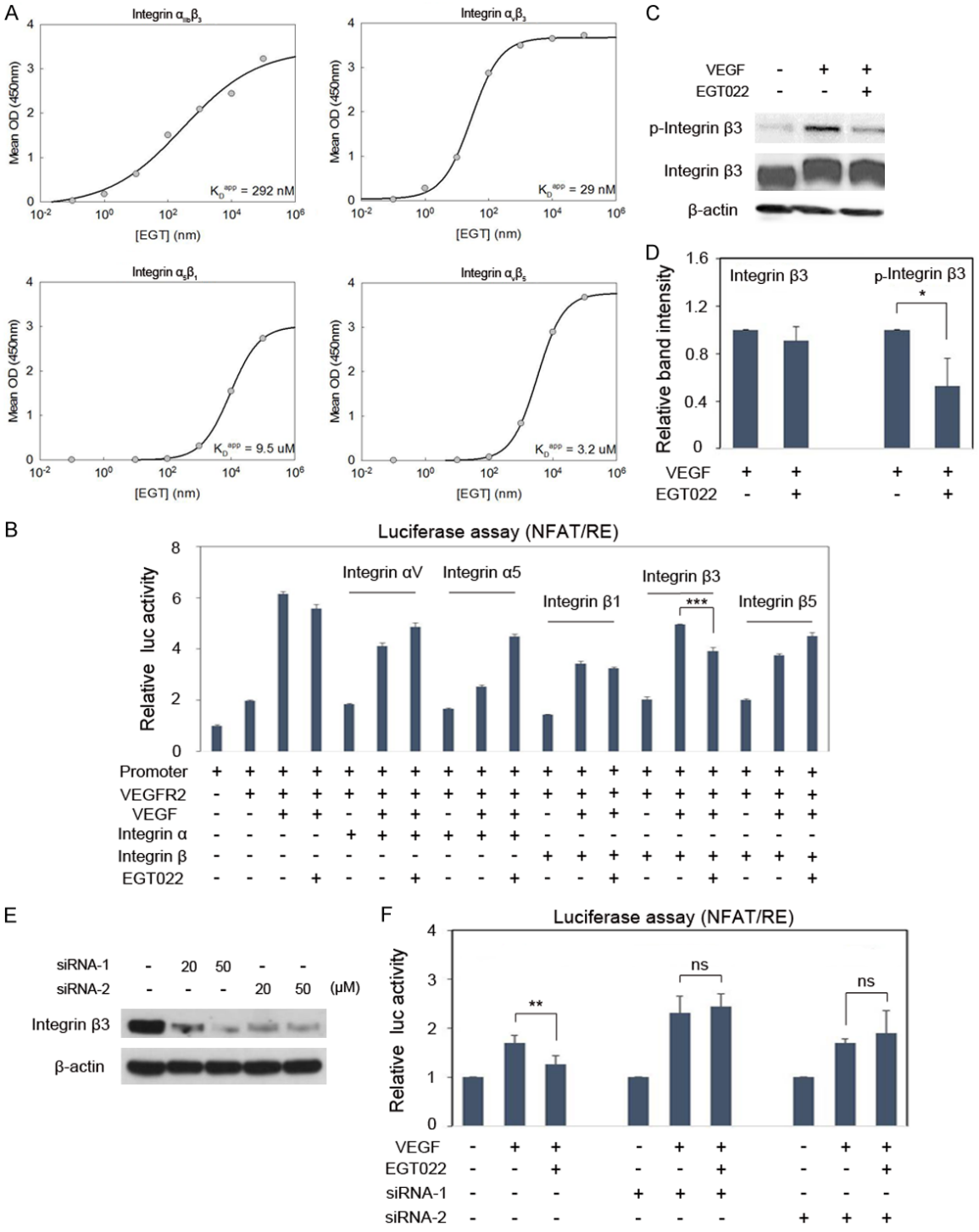

Figure 4. EGT022 binds integrin β3 and inhibits phosphorylation after VEGF stimulation. (A) Results of the integrin binding assay showed strong binding of EGT022 with integrin αIIbβ3 and αvβ3. (B) Transient transfection for each integrin subunit in HEK293 cells. EGT022 inhibited luciferase activity in VEGF-induced HEK293 cells containing the NFAT/RE promoter only in the presence of integrin β3. (C, D) EGT022 inhibits phosphorylation of integrin β3 in VEGF-induced HUVEC cells. (E) Expression of integrin β3 had a marked decrease in HUVEC cells treated with two specific integrin β3 siRNAs (50 nm). (F) Knockdown of integrin β3 by siRNAs did not inhibit the activity of NFAT/RE in VEGF-induced HUVEC cells. Results are presented as the average of three independent experiments (B-E). $^{*}P < 0.05$; $^{**}P < 0.01$. NS, not significant. Vascular endothelial growth factor (VEGF), Human umbilical vein endothelial cells (HUVEC).

pretreated with EGT022 was not affected by knock-down of integrin β3 (**Figure 4E**). These experimental data indicated that binding of EGT022 to integrin β3 is a critical inhibitory step in the process of angiogenesis. The results clearly demonstrate that the direct binding of EGT002 and integrin β3 is the essential element for molecular function of EGT002 in both endothelial cells and platelets.

We have further demonstrated that phosphorylation of PLC-γ1 and activation of NFAT are specifically inhibited by EGT022 in HUVEC cells. PLC-γ1, a member of the PLC family, has various functions as a signal transducer of VEGFR2 [23]. Phosphorylation of PLC-γ1 results in subsequent dephosphorylation of NFAT, a transcription factor involved in regulating the expression of target genes [21]. This VEGF/PLC-γ1-mediated NFAT signaling has been implicated in angiogenesis, including proliferation, migration, and permeability in endothelial cells [21]. Mufti et al. previously reported that downstream PLC-γ1 signaling is impaired by blocking αvβ3 integrin [24]. Considering this role of PLC-γ1 signaling, it seems probable that anti-angiogenetic effects of EGT022 are mediated by subsequent suppression of PLC-γ1 signaling in endothelial cells.

It is also interesting to note that treatment with EGT022 had no effect on phosphorylation of AKT and ERK, which are also involved in VEGF signaling pathways and associated with angiogenesis, while it has been reported that these signals were inhibited by several RGD-disintegrins [15-17]. Further studies will be required in order to determine which structural differences in EGT022 are associated with different regulatory mechanisms among RGD-integrins.

In conclusion, the experimental results obtained in this study suggest the potency of EGT022 as an antagonist of integrin β3, capable of inhibiting angiogenesis including proliferation, migration, tube formation, and vascular permeability in endothelial cells. Our previous report demonstrated that vascular maturation is stimulated and both abnormal neovascular sprouting and vascular permeability are inhibited by EGT022 [13]. Thus, EGT022 is a strong candidate for use in treatment of diseases with a direct or indirect association with VEGF-induced neovascularization.

Although further studies will be required in order to clarify the detailed mechanism of EGT022 in the crosstalk between VEGFR2 and αvβ3 and to determine the optimal effective concentration of EGT022, the current work provides evidence for determining the novel function of EGT022 and will be useful in development of target-based therapies for VEGF-induced neovascularization.

Acknowledgements

This research was financially supported by the grant from the Industrial Source Technology Development program (Grant No.: 10037336) of the Ministry of Trade, Industry and Energy, Korea. We thank Dr. Doo-Sik Kim for his assistance in proofreading manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Seok-Hyun Kim and Sunjong Kwon, R&D Center, EyeGene Inc., Goyang-si, Gyeonggi-do 10551, Republic of Korea. Tel: +82-2-322-1687; Fax: +82-2-324-8059; E-mail: burgundy@eyegene.co.kr (SHK); charcoal@eyegene.co.kr (SK)

References

[1] Folkman J and D'Amore PA. Blood vessel formation: what is its molecular basis? Cell 1996; 87: 1153-1155.
[2] Ferrara N, Gerber HP and LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9: 669-676.
[3] Nieves BJ, D'Amore PA and Bryan BA. The function of vascular endothelial growth factor. Biofactors 2009; 35: 332-337.
[4] Luengas-Martinez A, Hardman-Smart J, Paus R and Young HS. Vascular endothelial growth factor-A as a promising therapeutic target for the management of psoriasis. Exp Dermatol 2020; 29: 687-698.
[5] Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110: 673-687.
[6] Danhier F, Le Breton A and Preat V. RGD-based strategies to target alpha(v) beta(3) integrin in cancer therapy and diagnosis. Mol Pharm 2012; 9: 2961-2973.
[7] Giancotti FG and Ruoslahti E. Integrin signaling. Science 1999; 285: 1028-1032.
[8] McLane MA, Sanchez EE, Wong A, Paquette-Straub C and Perez JC. Disintegrins. Curr Drug

Targets Cardiovasc Haematol Disord 2004; 4: 327-355.
[9] Perutelli P. Disintegrins: potent inhibitors of platelet aggregation. Recenti Prog Med 1995; 86: 168-174.
[10] Swenson S, Ramu S and Markland FS. Anti-angiogenesis and RGD-containing snake venom disintegrins. Curr Pharm Des 2007; 13: 2860-2871.
[11] Jeon OH, Kim D, Choi YJ, Kim SH, Choi WS and Kim DS. Novel function of human ADAM15 disintegrin-like domain and its derivatives in platelet aggregation. Thromb Res 2007; 119: 609-619.
[12] Hong SY, Koh YS, Chung KH and Kim DS. Snake venom disintegrin, saxatilin, inhibits platelet aggregation, human umbilical vein endothelial cell proliferation, and smooth muscle cell migration. Thromb Res 2002; 105: 79-86.
[13] Jang JW, Cho YJ, Kim HJ, Kim JM, Lee SJ, Kwon OW and Kim DS. Blood vessel maturation by disintegrin in oxygen-induced retinopathy. Curr Eye Res 2016; 41: 689-699.
[14] Chen HR, Chuang YC, Lin YS, Liu HS, Liu CC, Perng GC and Yeh TM. Dengue virus nonstructural protein 1 induces vascular leakage through macrophage migration inhibitory factor and autophagy. PLoS Negl Trop Dis 2016; 10: e0004828.
[15] Danilucci TM, Santos PK, Pachane BC, Pisani GFD, Lino RLB, Casali BC, Altei WF and Selistre-de-Araujo HS. Recombinant RGD-disintegrin DisBa-01 blocks integrin alpha(v)beta(3) and impairs VEGF signaling in endothelial cells. Cell Commun Signal 2019; 17: 27.
[16] Fanelli R, Schembri L, Piarulli U, Pinoli M, Rasini E, Paolillo M, Galiazzo MC, Cosentino M and Marino F. Effects of a novel cyclic RGD peptidomimetic on cell proliferation, migration and angiogenic activity in human endothelial cells. Vasc Cell 2014; 6: 11.
[17] Ge H, Tian P, Guan L, Yin X, Liu H, Xiao N, Xiong Y, Luo X, Sun Y, Qi D, Ni S and Liu P. A C-terminal fragment BIGH3 protein with an RGDRGD motif inhibits corneal neovascularization in vitro and in vivo. Exp Eye Res 2013; 112: 10-20.
[18] Lal BK, Varma S, Pappas PJ, Hobson RW 2nd and Duran WN. VEGF increases permeability of the endothelial cell monolayer by activation of PKB/akt, endothelial nitric-oxide synthase, and MAP kinase pathways. Microvasc Res 2001; 62: 252-262.
[19] Gavard J. Endothelial permeability and VE-cadherin: a wacky comradeship. Cell Adh Migr 2014; 8: 158-164.
[20] Prasain N and Stevens T. The actin cytoskeleton in endothelial cell phenotypes. Microvasc Res 2009; 77: 53-63.
[21] Moccia F, Negri S, Shekha M, Faris P and Guerra G. Endothelial Ca(2+) signaling, angiogenesis and vasculogenesis: just what it takes to make a blood vessel. Int J Mol Sci 2019; 20: 3962.
[22] Baranska P, Jerczynska H, Pawlowska Z, Koziolkiewicz W and Cierniewski CS. Expression of integrins and adhesive properties of human endothelial cell line EA.hy 926. Cancer Genomics Proteomics 2005; 2: 265-269.
[23] Chen D and Simons M. Emerging roles of PLCgamma1 in endothelial biology. Sci Signal 2021; 14: eabc6612.
[24] Mufti RE, Zechariah A, Sancho M, Mazumdar N, Brett SE and Welsh DG. Implications of alphavbeta3 integrin signaling in the regulation of Ca2+ waves and myogenic tone in cerebral arteries. Arterioscler Thromb Vasc Biol 2015; 35: 2571-2578.