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The tetrapeptide Arg-Leu-Tyr-Glu inhibits VEGF-induced angiogenesis

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ABSTRACT

Kringle 5, derived from plasminogen, is highly capable of inhibiting angiogenesis. Here, we have designed and synthesized 10 tetrapeptides, based on the amino acid properties of the core tetrapeptide Lys-Leu-Tyr-Asp (KLYD) originating from anti-angiogenic kringle 5 of human plasminogen. Of these, Arg-Leu-Tyr-Glu (RLYE) effectively inhibited vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation, migration and tube formation, with an IC_{50} of 0.06–0.08 nM, which was about ten-fold lower than that of the control peptide KLYD (0.79 nM), as well as suppressed developmental angiogenesis in a zebrafish model. Furthermore, this peptide effectively inhibited the cellular events that precede angiogenesis, such as ERK and eNOS phosphorylation and nitric oxide production, in endothelial cells stimulated with VEGF. Collectively, these data demonstrate that RLYE is a potent anti-angiogenic peptide that targets the VEGF signaling pathway.

1. Introduction

Angiogenesis, the formation of new blood vessels from established vessels, occurs under a variety of normal and pathological conditions [1]. In physiological states such as embryonic development and wound healing, angiogenesis is activated for a brief period and then completely inhibited by the balance between stimulatory and inhibitory factors [2]. However, pathological conditions that elicit abnormal angiogenesis cause many human diseases including tumor growth and metastasis, rheumatoid arthritis and diabetic retinopathy [2]. In general, angiogenesis is induced by sequential processes, including endothelial cell activation, proliferation and migration and tube formation, which are triggered by various pro-angiogenic growth factors [3].

Among the many angiogenic factors, vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis by activating various signaling cascades that lead to endothelial cell proliferation, migration, and differentiation [4]. Under pathological conditions, VEGF induces abnormal angiogenesis to promote cell growth and vascular leakage in tumors and retinas, resulting in tumor progression and diabetic retinopathy [2,6]. Thus, interference with the biological activity of VEGF and subsequent signal cascades using neutralizing VEGF antibodies and signal inhibitors negatively regulates tumor angiogenesis and retinal neovascularization [7]. Anti-angiogenic therapy, which targets VEGF and its receptor, is a potential strategy for angiogenesis-associated human diseases [5–7].

Although several anti-angiogenic antibodies, proteins and chemicals have been developed and are currently clinically used for treating angiogenesis-related diseases [7–14], including tumors and diabetic retinopathy, they have some therapeutic limitations due to unfavorable side effects, non-specificity, poor bioavailability,

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antigenicity and unfavorable pharmacokinetics [11,15]. In general, small peptides have some advantages such as easy mass production, low antigenicity and high solubility, as well as increased specificity and bioavailability through chemical modification. Thus, small peptides are suggested as a good candidate for drug development.

In this study, we synthesized 10 tetrapeptides, based on biochemical properties of the core peptide Lys-Leu-Tyr-Asp (KLYD) derived from the anti-angiogenic kringle 5 domain of plasminogen [9], and among these, Arg-Leu-Tyr-Glu (RLYE) showed the best inhibitory activity (even higher than KLYD) in VEGF-induced angiogenesis *in vitro*. These results suggest that RLYE can be used as an anti-angiogenic drug in the treatment of angiogenesis-associated diseases.

2. Materials and methods

2.1. Materials

Cell culture media and supplements were purchased from Invitrogen Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT), and VEGF-A and basic fibroblast growth factor (bFGF) were from Upstate Biotechnology (Lake Placid, NY). 4-amino-5methylamino-2,7-difluorofluorescein (DAF-FM) diacetate was purchased from Molecular Probes (Eugene, OR). Antibodies against phospho-ERK (Thr-202/Tyr-204) and phospho-eNOS, ERK, eNOS and actin were purchased from Cell Signaling Technology (Danvers, MA) and BD Biosciences (San Jose, CA). Peptides were purchased from Peptron (Daejeon, South Korea).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were maintained and cultured in M199 as described previously [16], and only passages 2–7 were used in all experiments.

2.3. Cell proliferation assay

Endothelial cell proliferation was measured by [^{3}H]-thymidine incorporation assays as described previously [16]. Briefly, HUVECs were seeded at a density of 2×10^4 cells/well in gelatin-coated 24-well plates. After overnight culture, cells were cultured for another 6 h in M199 containing 1% FBS. Cells were preincubated for 30 min with various concentrations of peptides and stimulated with 10 ng/ml VEGF for 24 h, followed by the addition of 0.5 μCi/ml [^{3}H]-thymidine (Amersham, Aylesbury, UK) and incubation for another 6 h [^{3}H]-Thymidine incorporated into high molecular weight DNAs was determined using a liquid scintillation counter.

2.4. Cell migration assay

Chemotactic motility of HUVECs was assayed using Transwell plates (Corning Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (8 μm pore size) as described previously [16]. Briefly, the lower surface of the filter was coated with 10 μg gelatin. Fresh M199 media (1% FBS) containing VEGF was placed in the lower wells. The cell suspension (1×10^6 cells/ml) was incubated with various concentrations of peptides for 30 min at room temperature. One hundred μl of the cell suspension was loaded into the upper wells. The chamber was incubated at 37 °C for 4 h, and the migrated cells were quantified by counting the cells using an optical microscope.

2.5. Tube formation assay

Tube formation of HUVECs was assayed on growth factor-reduced Matrigel as described previously [16]. Briefly, growth factor-reduced Matrigel (250 μl) was pipetted into 24-well culture plates and polymerized at 37 °C for 30 min. HUVECs cultured in M199 containing 1% FBS for 6 h were plated onto the layer of Matrigel at a density of 2.0×10^5 cells/well, followed by incubation with VEGF (10 ng/ml) alone or in combination with peptide (0.15 nM) at 37 °C for 20 h. Tube formation was observed using an inverted phase contrast microscope, and images were captured with a video graphic system. The degree of tube formation was quantified by measuring the length of the tubes in 5 randomly chosen low-power fields (x100) from each well using the Image-Pro Plus v4.5 (Media Cybernetics, San Diego, CA).

2.6. Western blot analysis

Cells were lysed in RIPA buffer, and the lysates (50 μg protein) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies against target proteins for 2 h. After washing twice, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody, and protein levels were detected by an enhanced chemiluminescence system as described previously [16].

2.7. Nitric oxide (NO) measurement

Intracellular NO levels were measured *in situ* using DAF-FM diacetate according to the manufacturer's instructions. HUVECs were pretreated with 0.15 nM peptide and stimulated with 10 ng/ml VEGF for 4 h. Cells were incubated with 5 μM (final concentration) DAF-FM diacetate for 30 min in a CO_2 incubator. Intracellular NO levels were determined using a confocal laser microscope as described previously [16].

2.8. Zebrafish angiogenesis assay

Adult zebrafish and embryos were raised and maintained under standard laboratory conditions [17]. Zebrafish work was approved by Chungnam University animal research ethics committee. To analyze vascular formation, embryos were anaesthetized by adding tricaine (MS-222, 0.02%) to the embryo buffer at 48 h post fertilization (hpf). The embryos were injected with each peptide and subsequently with FITC-dextran (2000 kDa, Sigma). Vascular images were taken using a Leica DM5000B system at 72 hfp. For whole-mount *in situ* hybridization, digoxigenin (DIG)-labeled RNA probes were prepared with *cad5* using a commercial kit (Fermentas, Glen Burnie, MD). Embryos at 18 hpf were injected with peptides and harvested at 25 hpf. Embryos fixed with 4% paraformaldehyde were washed in phosphate-buffered saline (PBS) with 0.1% Tween 20 and hybridized with the DIG-labeled riboprobes in hybridization buffer, followed by incubation with an alkaline phosphatase (AP)-conjugated anti-DIG antibody. After incubation in the AP staining solution, images were visualized under the dissecting microscope (Leica, MZ-16) as reported previously [17].

2.9. Statistical analysis

Quantitative data are expressed as mean ± standard deviation (SD) of at least three separate experiments. Statistical significance was determined using the unpaired Student's *t* test, depending on

the number of experimental groups analyzed. Significance was established at a p value <0.05.

3. Results

3.1. Identification of the potent anti-angiogenic peptide RLYE

Since the tetrapeptide KLYD, derived from the plasminogen kringle domain 5, has been shown to possess anti-angiogenic activity [18], we synthesized 10 new tetrapeptides based on the chemical properties of its amino acid composition (Fig. 1A). We first examined the inhibitory effects of the synthetic peptides on proliferation, as a typical angiogenic property *in vitro*, of cultured HUVECs in response to VEGF. As expected, KLYD as a positive control peptide significantly inhibited VEGF-induced HUVEC proliferation. Among the other 9 peptides, KLYE, RLYE, RLYD and RVYE showed a higher inhibitory effect on VEGF-induced endothelial cell proliferation than KLYD, while KLFD and KIYD revealed an inhibitory effect, but were comparable to that of KLYD (Fig. 1B). However, KLWD, RLME and EYLR (scrambled peptide against RLYE) had no significant effect on VEGF-induced endothelia cell proliferation. We next examined the effect of the peptides on another angiogenic property, such as endothelial cell migration. We found that the inhibitory effects of all the peptides on VEGF-induced chemotactic endothelial cell migration (Fig. 1C) were highly correlated with their anti-proliferation activity (Fig. 1B). These results indicate that four tetrapeptides KLYE, RLYE, RLYD and RVYE have a more potent anti-angiogenic activity compared with the control peptide KLYD.

3.2. RLYE is the most potent anti-angiogenic peptide

We further examined the inhibitory effects of the four tetrapeptides KLYE, RLYE, RLYD and RVYE on ERK phosphorylation, as a key angiogenic signal event, in endothelial cells stimulated with VEGF. Treatment with VEGF significantly increased phosphorylation-dependent activation of ERK in cultured HUVECs, and this activation was effectively inhibited by treatment with KLYE, RLYE, RLYD or RVYE, but not KLWD and EYLK, as compared with the control KLYD (Fig. 2A). However, the inhibitory activity of RLYE was more potent than of the other peptides. To further compare their anti-angiogenic activities, we determined their IC_{50} values against VEGF-induced endothelial cell proliferation. The IC_{50} values of KLYE, RLYE, RLYD and RVYE were 0.30, 0.08, 0.14, and 0.39 nM, respectively (Fig. 2B), and the IC_{50} of RLYE was about ten-fold lower than that (0.79 nM) of KLYD. We also examined the dosage effect of RLYE on VEGF-induced endothelial cell migration. Treatment with RLYE inhibited VEGF-induced chemotactic migration of HUVECs in a concentration-dependent fashion, with an estimated IC_{50} of 0.06 nM (Fig. 2C and D). Since endothelial nitric oxide synthase (eNOS)-derived NO plays an important role in vascular function and angiogenesis [19], we examined the effects of RLYE on eNOS activation and endothelial NO production. RLYE effectively suppressed Akt phosphorylation and NO synthesis in HUVECs stimulated with VEGF, whose effect was significantly higher than KLYD (Fig. 2E and F). These results indicate that RLYE is the most potent anti-angiogenic peptide when compared with the other 9 peptides.

3.3. RLYE inhibits VEGF-induced angiogenic signaling and tube formation

Although VEGF is a strong stimulator of endothelial cell proliferation and migration, this growth factor also potently induces morphological differentiation associated with tube-like structure formation of endothelial cells [5]. Thus, we examined the effect of RLYE on tube-like structure formation of endothelial cells using a two-dimensional Matrigel array. HUVECs stimulated with VEGF on growth factor-reduced Matrigel promoted formation of elongated and robust tube-like structures, which were organized by a much larger number of cells compared with the control cells (Fig. 3A and B). However, treatment with RLYE effectively inhibited the width and length of endothelial tubes induced by VEGF, and this effect was stronger than that of KLYD (Fig. 3A and B). These results indicate that RLYE is a potent inhibitor of VEGF-induced angiogenesis *in vitro*.

3.4. RLYE inhibits angiogenesis in a zebrafish model

Since the angiogenic process and anatomical vascular pattern are highly conserved between zebrafish and other vertebrates including mammals, the zebrafish angiogenesis model has been used to develop regulatory molecules and drugs of angiogenesis, as well as to study the regulation of vascular development in vertebrates [20]. Thus, we employed a zebrafish angiogenesis model to evaluate the anti-angiogenic activity of RLYE *in vivo*. Treatment

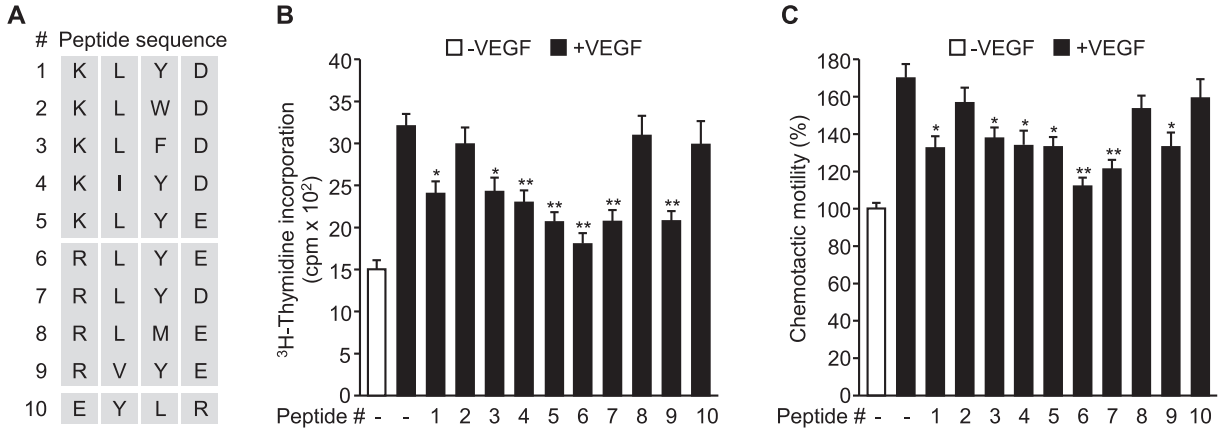

Fig. 1. Tetrapeptides that inhibit endothelial cell proliferation and migration. (**A**) Amino acid sequences of 10 synthetic tetrapeptides. (**B**) HUVECs were pretreated with each peptide (0.15 nM) for 30 min and stimulated with VEGF (10 ng/ml) for 24 h, followed by the addition of 0.5 μCi/ml of [^{3}H]-thymidine for 6 h. Levels of ^{3}H-Labeled high molecular DNAs were determined using a liquid scintillation counter. (**C**) HUVECs were treated with VEGF for 4 h following pretreatment with each peptide (0.15 nM) for 30 min in Transwell plates. Cells that migrated to the lower side of the filter were stained with H&E and counted. Data shown in the graphs are the mean ± SD (n ≥ 3). *P < 0.05 and **P < 0.01 versus VEGF alone.

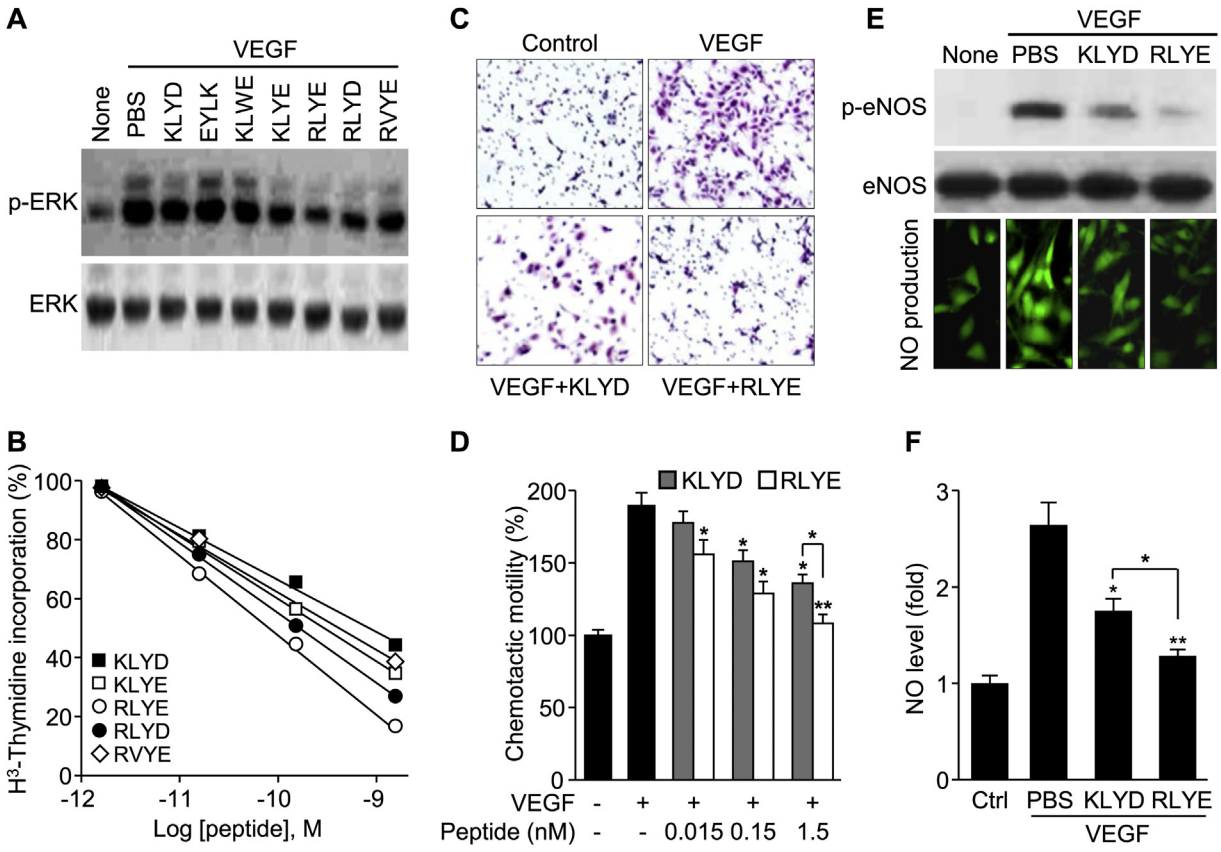

Fig. 2. RLYE effectively inhibits ERK phosphorylation, endothelial cell proliferation and migration and NO production. (**A**) HUVECs were pretreated with each peptide (0.15 nM) for 30 min and stimulated with VEGF (10 ng/ml) for 30 min. ERK phosphorylation was determined in cell lysates by Western blotting. (**B**) Cells were pretreated with the indicated concentrations of peptides and VEGF (10 ng/ml). Cell proliferation was determined by a [^{3}H]-thymidine incorporation assay. Data are average values from two independent experiments performed in triplicate. (**C** and **D**) HUVECs were pretreated with each peptide (0.15 nM in **C** and the indicated concentrations in **D**) for 30 min and stimulated with VEGF in Transwell plates for 4 h. Migrated cells were counted after staining with H&E. (**E** and **F**) Cells were stimulated with peptides (0.15 nM) and VEGF. Levels of eNOS phosphorylation and intracellular NO production were determined by Western blotting and confocal microscopy. Data shown in D and F are the mean ± SD (n ≥ 3). $^*P < 0.05$ and $^{**}P < 0.01$ versus VEGF alone.

with RLYE significantly inhibited subintestinal vessel (SIV) formation, compared with PBS-injected embryos, while the scrambled peptide had no inhibitory effect on developmental SIV formation (Fig. 4A). Moreover, injection with RLYE also effectively inhibited intersegmental vessel (ISV) growth, compared with PBS-injected control embryos, while the scrambled peptide did not affect ISV formation (Fig. 4B). Taken together, these data demonstrate that RLYE inhibits angiogenesis *in vivo*.

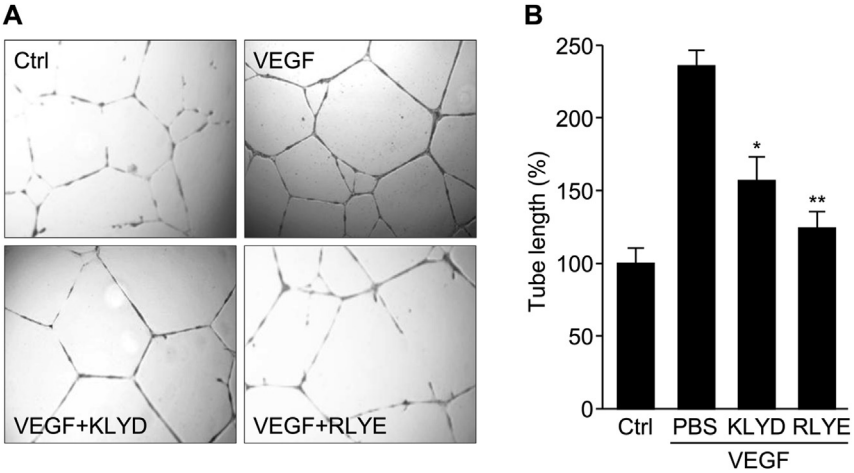

Fig. 3. RLYE inhibits tube-like structure formation of endothelial cells. HUVECs plated on Matrigel-coated plates were treated with VEGF in the presence or absence of peptides (0.15 nM) for 30 min and treated with VEGF (10 ng/ml) for 20 h. Images of tube-like structure were photographed using an inverted phase contrast microscope, and the tube length was quantitated using Image-Pro Plus software. (A) Representative image of the tube-like structure. (B) Quantitation of the tube length. Data shown are the mean ± SD (n ≥ 3). $^*P < 0.05$ and $^{**}P < 0.01$ versus VEGF alone.

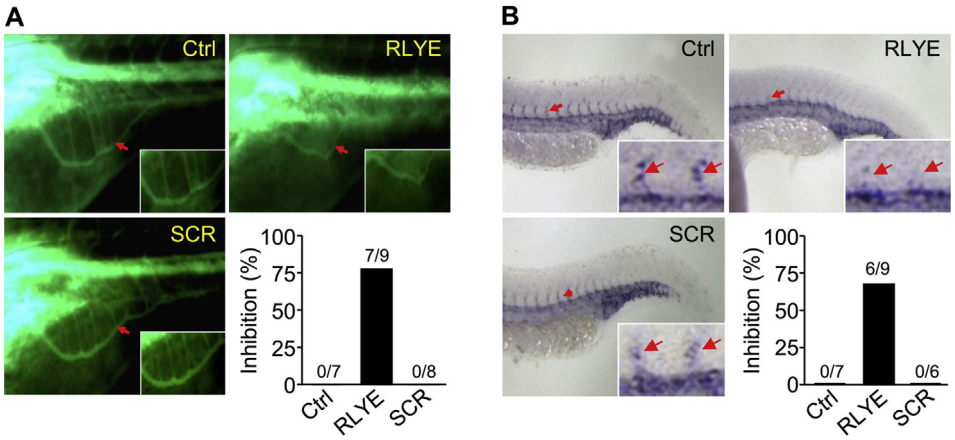

Fig. 4. RLYE inhibits angiogenesis in a zebrafish model. (A) Zebrafish embryos were injected with 9 nl of RLYE (150 mM), scrambled tetrapeptide (SCR) (150 mM) or PBS. Microangiography was performed in order to visualize the formation of subintestinal vessels (indicated by arrows) using a dextran-FITC. Angiogenic inhibition is expressed as a ratio of SIV-defective embryos/total embryos. (B) Embryos injected with RLYE, SCR or PBS were *in situ* hybridized with a DIG-labeled riboprobe for *cad5* and incubated with an AP-conjugated anti-DIG antibody. After incubation in AP staining solution, images of intersegmental vessels (indicated by arrows) were visualized under the dissecting microscope, and angiogenic inhibition is expressed as a ratio of ISV-defective embryos/total embryos.

4. Discussion

Angiogenesis is crucial for the pathogenesis or progression of human diseases, particularly solid tumors. Solid tumors produce VEGF that promotes tumor angiogenesis, leading to growth, invasion and metastasis of tumors [6,21]. Therefore, therapies using neutralizing antibodies against VEGF and blockers of VEGF-induced signal transduction inhibit tumor growth and metastasis, leading to the improvement of cancer patient survival by the blockade of tumor angiogenesis [21]. Due to the fact that some of these therapeutic drugs cause problems with bleeding, blood clotting, heart function, the immune system, and the reproductive system [15,22,23], new anti-angiogenic drugs that overcome these unfavorable profiles should be developed for the treatment of patients suffering from angiogenesis-associated human diseases, including solid tumors. Here, we have synthesized the new tetrapeptide RLYE as a potent inhibitor of *in vitro* angiogenesis in HUVECs stimulated with VEGF and developmental angiogenesis in a zebrafish model.

An initial study conducted by O'Reilly et al. reported that angiostatin consisting of kringles 1-4, a proteolytic fragment of plasminogen, profoundly inhibits angiogenesis *in vitro* and tumor growth *in vivo* [24]. Thereafter, kringle 5 of plasminogen revealed a higher anti-endothelial proliferative activity than angiostatin [18]. From the sequence homology of kringle 1 through kringle 5, KL(R)YDY has been confined as an important peptide possessing anti-angiogenic activity [9]. Moreover, the tetrapeptide KLYD was core sequence in negatively regulating endothelial cell angiogenesis [25]. To improve anti-angiogenic activity of this peptide, we synthesized 9 new peptides, based on the acidic-hydrophobic-aromatic-basic sequence of KLYD. Our data shows that RLYE has the strongest anti-angiogenic activity, as assessed by cell proliferation and migration in HUVECs stimulated VEGF. Its anti-proliferative effect was much higher than that of KLYD and recombinant plasminogen kringle 5 [25].

Since the blockade of VEGF leads to regression of angiogenesis and vascular networks, several anti-VEGF antibodies have been developed and used as therapeutic drugs for tumor growth and metastasis. A humanized anti-VEGF monoclonal antibody (bevacizumab) has been clinically used for treating a variety of tumors, including colorectal cancer and breast cancer. An initial study has shown that bevacizumab displayed anti-endothelial cell proliferation with an estimated IC_{50} of 22 ng/ml (0.15 nM) in HUVECs treated with a mixture of various concentrations of the antibody and 50 ng/ml of VEGF [26]. Our results demonstrate that RLYE effectively inhibits endothelial cell proliferation with an IC_{50} of 0.06 nM in HUVECs stimulated with 10 ng/ml of VEGF. Although our experimental conditions are slightly different from the previous study, with respect to the VEGF concentration [26], our data indicate that the anti-angiogenic activity of RLYE against VEGF is more potent than those of bevacizumab and recombinant plasminogen kringle 5 [25,26], indicating that this peptide is a potential inhibitor of VEGF-driven angiogenesis. Thus, RLYE is useful for the development of new anti-angiogenic drugs for the treatment of angiogenesis-related human diseases, including solid tumors and diabetic retinopathy.

Bevacizumab inhibits the biological activity of both VEGF receptor 1 (VEGFR-1) and VEGFR-2 (a major angiogenic receptor) by specifically binding to VEGF-A. A previous study showed that bevacizumab completely blocked VEGF-A-induced endothelial cell growth at a molar ratio of 2.6:1 of the antibody to homodimeric VEGF-A [26]. This indicates that a molar binding ratio between bevacizumab and monomeric VEGF-A is approximately 1:1. Here, we found that treatment with 1.5 nM of RLYE completely blocked endothelial cell proliferation in HUVECs treated with 10 ng/ml of VEGF, indicating that a molar ratio of 11:1 of RLYE to monomeric VEGF is necessary to reach maximum inhibition. This ratio suggests that RLYE may not bind directly to VEGF. Thus, we propose that the anti-angiogenic mode of RLYE is not associated with direct inhibition of VEGF activity. We are currently examining the mechanism by which RLYE regulates VEGF-mediated angiogenesis.

Many tyrosine receptor kinase inhibitors (TKIs) have been used to inhibit angiogenesis by blocking the catalytic activity of tyrosine kinase associated with receptors of various angiogenic growth factors, such as VEGF, platelet-derived growth factor (PDGF) and bFGF [10–14]. Some TKIs, such as sunitinib, axitinib, cabozantinib and vandetanib, are progressing to clinical trial investigation or are already approved by the FDA for the treatment of solid tumors, including stomach cancer, renal cancer and colon cancer. However, these inhibitors have some limitations, including low selectivity and cross-inhibition among various tyrosine receptor kinases and the development of hypertension and proteinurea [22,23]. Of them, the FDA-approved TKI sunitinib has low selectivity for some tyrosine receptor kinases with an IC_{50} of 80 nM, 2 nM and 2.9 μM for VEGFR-2, PDGF receptor and bFGF receptor, respectively [14]. Other

TKIs, such as the selective VEGFR inhibitor (vandetanib, IC_{50} of 40 nM) and the potent VEGFR-2 inhibitor (cabozantinib, IC_{50} of 0.035 nM), also nonspecifically inhibit other tyrosine receptor kinases [10,11]. Although not shown here, we found that RLYE did not inhibit HUVEC migration in response to EGF or bFGF, suggesting that this peptide selectively inhibits VEGF-induced angiogenesis. Although the anti-angiogenic activity of RLYE with an IC_{50} of 0.06–0.08 nM is relatively lower than that of the most potent VEGFR-2 inhibitor cabozantinib with an IC_{50} of 0.035 nM [11], this peptide is a strong angiogenic inhibitor compared with other TKIs [10,12–14]. Therefore, our findings suggest that RLYE is a selective and potent angiogenic inhibitor compared with other drugs that are currently used for tumor therapy or those in clinical trials.

Angiogenesis is required for the activation of diverse signal mediators and the production of various pro-angiogenic mediators. In particular, activation of ERK is important for endothelial cell proliferation and angiogenesis [16]. Our data shows that RLYE effectively inhibited VEGF-mediated ERK phosphorylation, whose inhibitory effect was the most potent of the 10 peptides used in this study. In addition, endothelial cell-derived NO is an important stimulator of angiogenesis, as neovascularization was defective in eNOS-deficient mice [19]. Here, we found that RLYE strongly suppressed VEGF-induced NO production in HUVECs. These results suggest that RLYE inhibits angiogenesis by blocking VEGF-induced signaling events.

Our current study provides a possible clue by which RLYE exerts potent anti-angiogenic activity, as assessed by the inhibitory effects of RLYE on VEGF-induced endothelial cell proliferation, migration and tube formation *in vitro* and the suppression of developmental blood vessel formation in a zebrafish model. Therefore, RLYE could be applied as a new therapeutic approach for angiogenesis-associated human diseases. Although the exact anti-angiogenic mechanism of RLYE has not yet been elucidated, we are currently undertaking a mechanistic investigation of the anti-angiogenic activity of RLYE and attempting to identify its cellular target.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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