Biochemical and Biophysical Research Communications 391 (2010) 166–169

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

DGDA, a local sequence of the kringle 2 domain, is a functional motif of the tissue-type plasminogen activator's antiangiogenic kringle domain

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ARTICLE INFO

Article history:
Received 15 October 2009
Available online 10 November 2009

Keywords:
DGDA
DGEA
Kringle domain
Integrin α2β1
Angiogenesis inhibitor
Migration

ABSTRACT

Antiangiogenic activity can be elicited by the kringle domains 1 and 2 of tissue-type plasminogen activator (TK1–2), or the kringle 2 domain alone. In a previous report, we showed that the anti-migratory effect of TK1–2 is mediated in part by its interference with integrin α2β1. Since integrin α2β1 interacts with collagen type I through the DGEA (Asp-Gly-Glu-Ala) amino acid sequence, and a similar sequence, DGDA (Asp-Gly-Asp-Ala), exists in the kringle 2 domain, we investigated whether the DGDA sequence has a role in antiangiogenic activity of TK1–2. In an adhesion assay, the DGDA peptide inhibited adhesion of human umbilical vein endothelial cells (HUVECs) to immobilized TK1–2. Pretreatment of the DGDA peptide also blocked anti-migratory activity of TK1–2. When the DGDA peptide alone was tested for antiangiogenic activity, it effectively inhibited VEGF-induced migration of HUVECs and tube formation on Matrigel. In addition, the DGDA peptide decreased differentiation of endothelial progenitor cells on collagen type I matrix. These data suggest that the DGDA sequence presents a functional epitope of TK1–2 and that it can be used as a potential novel antiangiogenic peptide.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting vessels, is required for tumor growth, so it has been suggested to be a promising target for cancer therapy [1,2]. Numerous angiogenesis inhibitors such as angiostatin and endostatin have been identified, and several of them have entered into clinical trials [3–5]. The recombinant protein consisting of kringle domains 1 and 2 of the tissue-type plasminogen activator, TK1–2, has also been shown to inhibit endothelial cell proliferation, migration, tube formation, and *in vivo* tumor growth [6–9]. We have also reported that TK1–2 inhibits adhesive differentiation of endothelial progenitor cells (EPCs) and their contribution to tumor vessel formation *in* vivo [10]. In addition, Reteplase, the thrombolytic therapy drug comprising the kringle 2 domain and the protease domain of t-PA, has been reported to elicit antiangiogenic activity [11]. This observation provides new mechanistic insights into the bleeding complication involved with this drug. In particular, its antiangiogenic activity has been shown to be elicited by the kringle domain 2.

We have previously reported that the anti-migratory effect of TK1–2 is mediated by interference with the extracellular matrix (ECM)–integrin α2β1 interaction [6]. Integrin α2β1 has been reported to play a key role in angiogenesis, and a combination of α2β1-blocking and α1β1-blocking antibodies markedly inhibit VEGF-induced angiogenesis *in vivo* [12]. This combined antagonism also substantially reduces tumor growth and angiogenesis of human squamous cell carcinoma xenografts [13]. In addition, when a small molecular inhibitor disrupts integrin α2β1 function, it causes cell retraction and cytoskeletal collapse, and delays endothelial cell wound healing [14].

The major recognition site of collagen type I by integrin α2β1 has been found to be the DGEA (Asp-Gly-Glu-Ala) sequence [15]. The DGEA peptide is a strong antagonist interfering with collagen–platelet interaction [16], and inhibits endothelial progenitor cell differentiation on collagen type I matrix [17]. Interestingly, we found a DGDA (Asp-Gly-Asp-Ala) sequence, similar to the DGEA sequence, placed in the exposed loop of the kringle 2 domain of TK1–2. The only difference between these two sequences is that an aspartic acid residue, with a shorter side chain, exists at the third position instead of glutamic acid residue. Therefore, we hypothesized that the DGDA sequence may be a functional epitope of TK1–2 related to interference with integrin α2β1. In this study, we tested whether the DGDA sequence could block the activity of TK1–2 as a competitive inhibitor, and whether this peptide alone has antiangiogenic activity *in vitro*.

Materials and methods

Peptide synthesis. The synthetic peptides were purchased from Thermo Fisher Scientific (Ulm, Germany) and Peptron (Taejon,

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doi:10.1016/j.bbrc.2009.11.025

Korea). Peptides were dissolved in distilled water or phosphate-buffered saline (PBS) and stored at −20 °C.

Cell culture. HUVECs were isolated from human cords and were cultured as described previously [6]. Before the experiments, the cells were incubated in serum-free EBM-2 for 4 h.

Cell adhesion assay. A cell adhesion assay was carried out as described previously [6]. In brief, the TK1–2 protein was coated on 96-well plates for 16 h at 4 °C. Non-specific adhesion sites were blocked by 1% heat-inactivated BSA for 30 min. HUVECs were treated with each peptide and then plated on the TK1–2 coated culture plates for 90 min. Non-adherent cells were washed with PBS, and remaining adhered cells were stained with crystal violet. The stained dye was dissolved in 10% acetic acid followed by absorbance measurements at 560 nm.

Modified Boyden chamber assay. A cell migration assay was performed using a modified Boyden chamber as described previously [6]. HUVECs were treated with each peptide for 30 min and then treated with TK1–2 for another 30 min. After that, the cells were allowed to migrate toward VEGF. After 5 h, the migrated cells were fixed and stained with hematoxylin and eosin. The stained cells were photographed and counted.

Tube formation assay. Chilled Matrigel (150 μl, BD Bioscience) was placed in a prechilled 48-well plate and incubated for 30 min at 37 °C. HUVECs were treated with each peptide for 30 min and then added to the top of the solidified Matrigel. After 19 h of incubation, the tubes formed were photographed. Images were analyzed using Image J (http://rsb.info.nih.gov/ij/) to determine tube lengths.

EPC differentiation assay. Ex vivo cultivation of EPCs from cord blood was carried out as described previously [10]. Mononuclear cells (MNCs) were isolated from cord blood using the Ficoll-Hist-opaque density gradient centrifugation method. Isolated MNCs (5×10^5 cells) were treated with each peptide at various concentrations in serum-free M199 for 30 min at 37 °C. Then, the cells were plated onto a collagen type I (50 μg/ml)-coated 24-well plate, and incubated in a medium supplemented with 10% FBS and 90 μg/ml heparin. After 3 days, fresh media was changed with addition of each peptide, and the cells were incubated further for 4 more days. Then, the cells were incubated in a medium containing 2.4 μg/ml DiI-labeled acetylated Low Density Lipoprotein (DiI-ac-LDL, Invitrogen) for 1 h at 37 °C. After washing with PBS, the cells were fixed with 4% paraformaldehyde and blocked using 1% BSA, followed by staining with 10 μg/ml FITC-conjugated UEA1 (Sigma, St. Louis, MO) and DAPI (Chemicon, Temecula, CA). The cells were photographed using a fluorescence microscope (Calr Zeiss Microimaging, Göttingen, Germany).

Results

DGDA peptide inhibits endothelial cell adhesion to immobilized TK1–2 and blocks anti-migratory activity of TK1–2

The inhibitory mechanism of antiangiogenic TK1–2 has been recently investigated, and it has been elucidated that the anti-migratory effect of TK1–2 is mediated in part by interfering with integrin α2β1 [6]. Integrin α2β1 recognizes the DGEA sequence within collagen type I as a collagen type I receptor. Interestingly, the DGDA sequence, which is similar to the DGEA of collagen type I, exists in the exposed loop of the kringle 2 domain of TK1–2 (Fig. 1A). Since a small molecule derived from a large molecule can function as an inhibitor of the large molecule [18], we carried out the competition assay in cell adhesion. To investigate the competitive effect of the DGDA sequence on cell adhesion to immobilized TK1–2, HUVECs were treated with peptide and plated onto an immobilized TK1–2 plate. Both DGDA and DGEA peptides dose-dependently inhibited adhesion of HUVECs (Fig. 1B). These

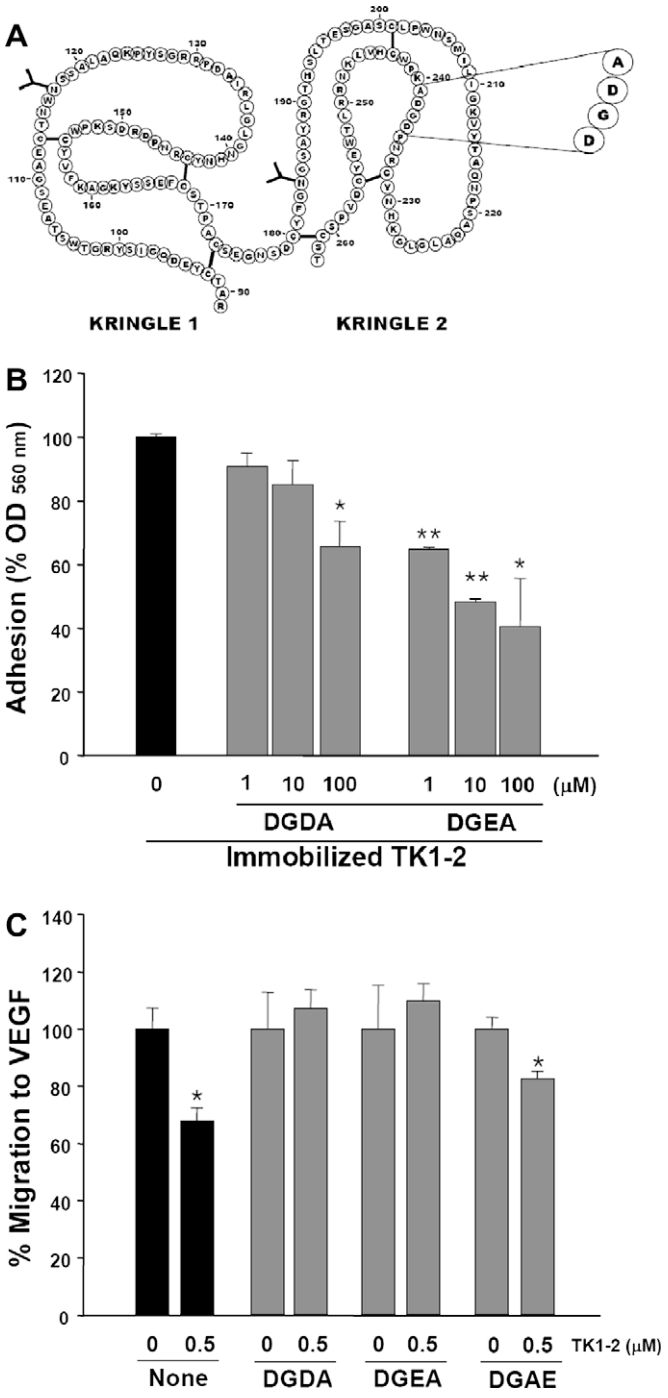

Fig. 1. The blocking effect of DGDA peptide on HUVEC adhesion on immobilized TK1–2 and anti-migratory effect of TK1–2. (A) DGDA is located in the exposed loop of the kringle 2 domain of t-PA. The numbering of amino acid residues is based on the sequence of t-PA. (B) HUVECs were treated with DGDA or DGEA peptide prior to plating onto immobilized TK1–2. Cell adhesion was allowed for 90 min and then attached cells were stained with crystal violet. After dissolving the stained dye, absorbance was measured at 560 nm (mean ± SE). Each value is a percentage relative to non-treated control cells. $^*p < 0.05$, $^{**}p < 0.005$, compared with non-treated control cells. (C) HUVECs were pretreated with DGDA, DGEA, or DGAE peptide (100 μM) prior to treatment of TK1–2. After treatment of TK1–2 for 30 min, the cells were allowed to migrate toward VEGF for 5 h and the migrated cells were counted. $^*p < 0.05$, compared with each relevant control untreated with TK1–2.

results suggest that the interaction between HUVECs and TK1–2 that is mediated by integrin α2β1 is effectively inhibited by DGDA and DGEA peptides.

Next, we tested whether the DGDA peptide could block the anti-migratory activity of TK1–2. To investigate the effect of DGDA peptide on anti-migratory activity of TK1–2, DGDA peptide was treated with ~200 times molar excess prior to TK1–2 treatment in a migration assay. As shown in Fig. 1C, no increased inhibitory effect was observed after post-treatment of TK1–2, indicating that DGDA peptide treatment completely blocked the anti-migratory effect of TK1–2, whereas the control DGAE peptide showed the inhibitory effect elicited by TK1–2. As expected, a similar result was obtained using DGEA peptide. Thus, these data support the conclusion that the inhibition of endothelial cell migration by TK1–2 that interferes with integrin α2β1 is mediated through the DGDA sequence.

DGDA peptide inhibits endothelial cell migration and tube formation

Since the DGDA peptide may function as a potential inhibitor of integrin α2β1, as indicated in the above experimental results, we next investigated the possible antiangiogenic activity of the DGDA peptide. To examine the effect of the DGDA peptide on endothelial cell migration, we carried out a modified Boyden chamber assay. The DGDA peptide dose-dependently inhibited the migration of HUVECs induced by VEGF (Fig. 2). A similar result was obtained using the DGEA peptide, but the control DAGD peptide did not inhibit HUVEC migration. We also examined the effects of the DGDA peptide on tube formation of HUVECs on Matrigel. The DGDA peptide blocked the tube formation by approximately 40% compared with the control DAGD peptide (Fig. 3), showing an inhibitory effect similar to that of RGD peptide (GRGDSP). Thus, these data suggest that the DGDA peptide itself inhibits endothelial cell migration and tube formation as an antiangiogenic agent.

DGDA peptide inhibits differentiation of EPCs on collagen matrix

EPCs play a role in vascularization in tumor growth or repair of ischemic injury [19], and the endothelial cell commitment of circulating EPCs is promoted by ECM [20]. It has previously been reported that collagen type I matrix promotes the differentiation of EPCs such as fibronectin and vitronectin, and that the DGEA peptide inhibits differentiation of EPCs on collagen matrix [17]. Therefore, we tested whether the DGDA peptide has the same effect on collagen-induced EPC differentiation. As shown in Fig. 4, the DGDA peptide decreased the number of Ac-LDL-DiI/UEA1 double positive EPCs to a level similar to that of the DGEA peptide. On the other hand, the DGAE control peptide did not. Thus, these data suggest that DGDA peptide inhibits collagen-induced EPC differentiation. This result is also consistent with the previous observation that TK1–2 inhibits EPC differentiation *in vitro* [10].

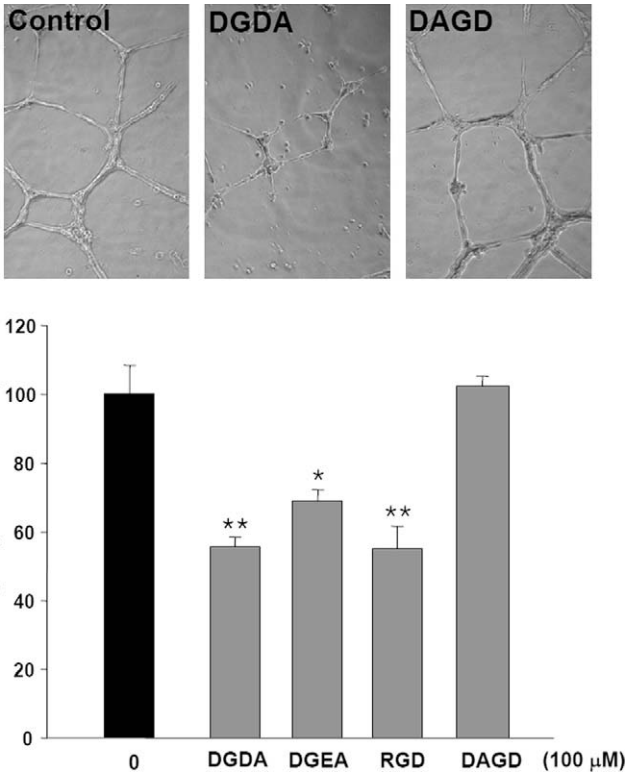

Fig. 3. The inhibitory effect of DGDA peptide on tube formation. HUVECs were incubated on solidified Matrigel with or without each peptide (100 μM) for 19 h. The upper panel is the representative field of each peptide (magnification 100×). The lower panel presents the quantitative graph of the relative percentage of tube length obtained from each peptide treatment. $^{*}p < 0.05$, $^{**}p < 0.005$ compared with non-treated control.

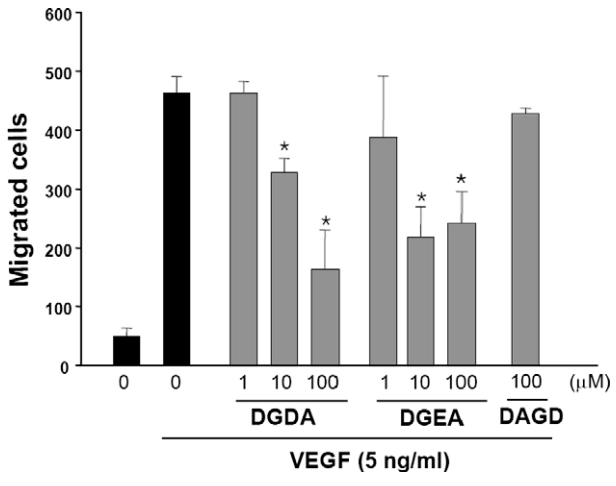

Fig. 2. The inhibitory effect of DGDA peptide on endothelial cell migration. HUVECs were treated with DGDA, DGEA, or DAGD peptide at various concentrations for 30 min, and then allowed to migrate toward VEGF. Migrated cells were stained with hematoxylin and eosin, photographed, and counted. $^{*}p < 0.05$, compared with VEGF alone-treated control.

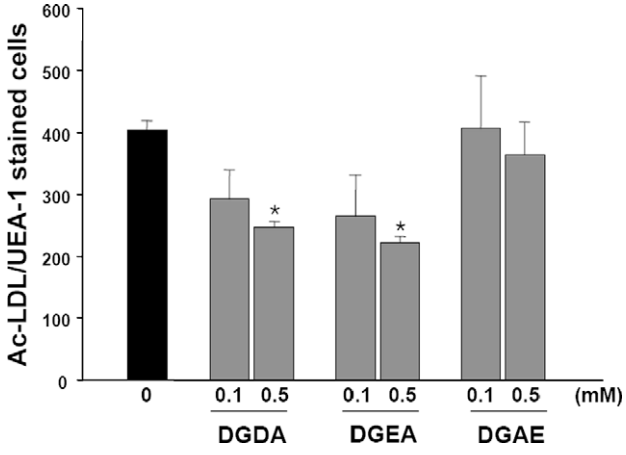

Fig. 4. The inhibitory effect of DGDA peptide on EPC differentiation. Isolated MNCs were treated with DGDA, DGEA, or DGAE peptide and cultured on collagen-coated plate in an endothelial cell culture medium containing 10% FBS. On day 7, the adherent cells were incubated with Ac-LDL-DiI followed by staining with FITC-conjugated UEA1 and DAPI. The graph presents the number of differentiated EPCs double positive for Ac-LDL-DiI uptake and UEA1 lectin binding upon peptide treatment. $^{*}p < 0.05$, compared with non-treated control cells.

Discussion

Integrin–ECM ligation is important for cell survival, proliferation, and migration [21,22]. In previous reports, we have demonstrated that TK1–2 has antiangiogenic activity and that its inhibitory activity is mediated in part by interfering with integrin α2β1 [6–9]. Integrin α2β1 is a receptor of collagen type I, II, IV, and laminin [23,24], and recognizes the DGEA sequence of collagen type I [15]. An immobilized DGEA peptide has been shown to promote the differentiation of MSC via interaction with integrin α2β1 as collagen does [25], but on the other hand, soluble DGEA peptide inhibits the differentiation of EPCs on collagen I [17]. These results suggest that a non-immobilized, soluble DGEA peptide, a partial sequence of collagen, may function as an integrin α2β1 inhibitor via interference of interaction of ECM with integrin α2β1.

In this study, we tested whether the DGDA motif, corresponding to residues 236–239 within the kringle 2 domain of t-PA, is required for the anti-migratory effect of TK1–2, and assessed whether DGDA itself elicits antiangiogenic activity. Indeed, the DGDA peptide treatment blocked HUVEC adhesion to immobilized TK1–2, and prevented the anti-migratory effect of TK1–2. The DGDA peptide also inhibited HUVEC adhesion to collagen type I matrix as the DGEA peptide does (data not shown). These results indicate that the DGDA sequence derived from TK1–2 can act as a competitive inhibitor of TK1–2, and suggests a possibility of the DGDA sequence as a functional epitope of TK1–2. Therefore, the results support the idea that TK1–2 acts as an angiogenesis inhibitor in part by interfering with integrin α2β1 through the DGDA local sequence on the exposed loop of the kringle domain 2. Consistent with an idea that DGDA acts as an integrin α2β1 antagonist, the DGDA peptide alone effectively inhibits the VEGF-induced migration and tube formation on Matrigel. Moreover, the DGDA peptide inhibits EPC differentiation on collagen type I matrix, and this data corresponds well with the data obtained from DGEA treatment by other group [17]. This result may also suggest a possible mechanism how TK1–2 effectively inhibits EPC differentiation *in vitro*. Therefore, we strongly suggest that the antiangiogenic activity of TK1–2 is mediated in part by the DGDA local sequence of TK1–2.

Although many angiogenesis inhibition proteins have potent antiangiogenic activity, they are limited by such as poor bioavailability, antigenicity, unfavorable pharmacokinetics, and inconsistency in bioactivity from batch to batch [26,27]. To overcome these disadvantages, antiangiogenic small peptides such as Anginex [28], Flt2-11 [18], T7 [29] have been developed. In this context, the DGDA peptide can be studied further to explore the potential of a novel antiangiogenic peptide which can be used more efficiently.

Acknowledgments

We thank Sang-Bae Lee and Eunju O for assistance in manuscript preparation. This work was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family affairs, Republic of Korea (0820210).

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