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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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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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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

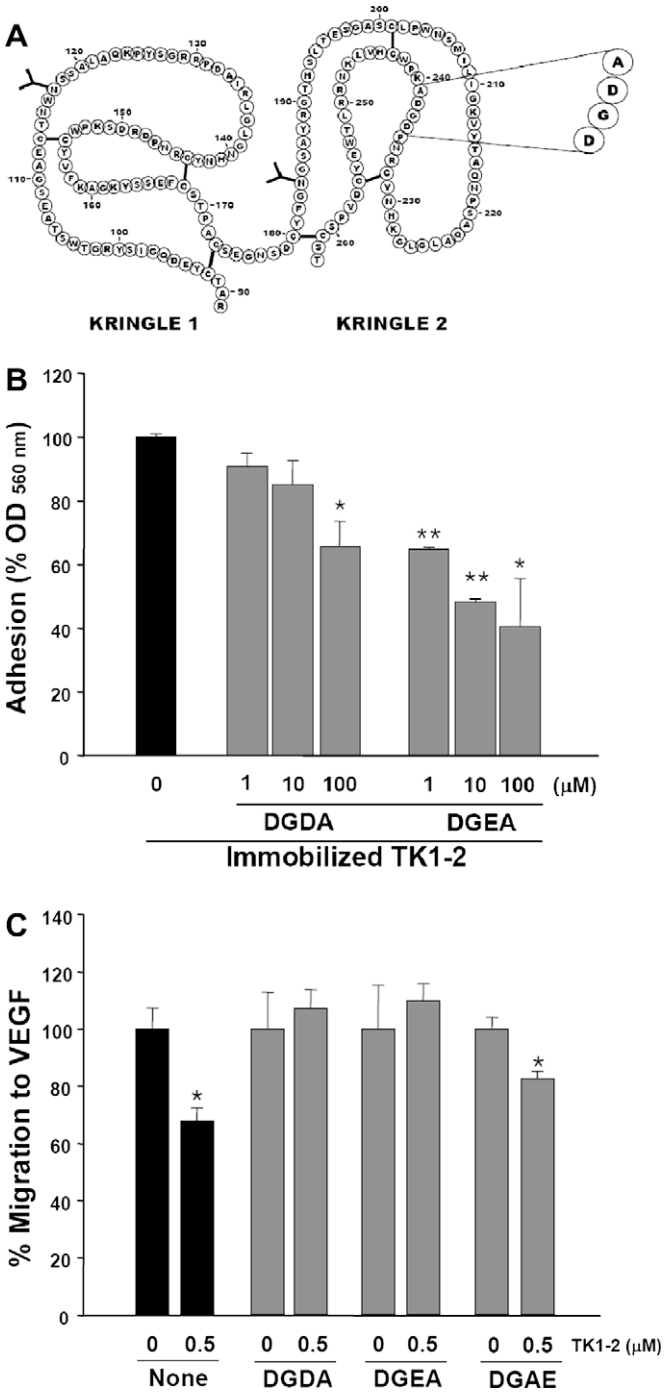

Fig. 1. The blocking effect of DGDA peptide on HUVEC adhesion on immobilized TK1–2 and anti-migratory effect of TK1–2. (A) DGDA is located in the exposed loop of the kringle 2 domain of t-PA. The numbering of amino acid residues is based on the sequence of t-PA. (B) HUVECs were treated with DGDA or DGEA 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.