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Anti-angiogenic activities of two recombinant disintegrins derived from the Mohave and Prairie rattlesnakes

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ABSTRACT

Angiogenesis plays a crucial role in the growth and spread of cancer. New vascularization nourishes cancer cells with oxygen and nutrients, allowing these cells to grow, invade nearby tissue, spread to other parts of the body, and form new colonies of cancer cells. Tumor angiogenesis consists of endothelial cell proliferation, migration, and tube formation into the tumor mass. The study of natural and synthetic angiogenesis inhibitors is a promising area for therapeutics since tumors cannot grow or spread without the formation of new blood vessels. Anti-angiogenic activities have been identified in peptides known as disintegrins. Disintegrins are a family of small proteins (45–84 amino acids in length), many which are found in snake venom that function as potent inhibitors of both platelet aggregation and integrin-dependent cell adhesion. This study reports two recombinant disintegrins (r-mojastin 1 and r-viridistatin 2) inhibiting, with similar effectiveness, distinct steps in angiogenesis such as proliferation, adhesion to fibronectin, migration, and tube formation *in vitro* and *in vivo*. Both recombinant disintegrins bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors that are upregulated in tumor endothelial cells, having a higher binding activity to $\alpha_v\beta_3$ integrin.

1. Introduction

Angiogenesis, the formation of new vessels out of the existing vasculature, is involved in numerous processes, such as embryogenesis, wound healing, tissue remodeling, and menstruation. Also, numerous disorders are characterized by either excess or an insufficient number of blood vessels. The best-known disorders are rheumatoid arthritis, psoriasis, restenosis, diabetic retinopathy, and tumor growth (Haubner, 2008). Tumor angiogenesis involves several processes, including endothelial proliferation, migration, invasion, and tube formation all regulated by cell adhesion receptors and specific angiogenesis growth factors produced by tumor cells and the surrounding stroma (Yeh et al., 2001). Integrins of endothelial cells participate in regulating these physiological processes (You et al., 2003).

Tumor cell invasion alone is not sufficient to produce distant metastases; it requires also the transport of malignant cells through blood and/or lymph vessels. Pioneering work by Folkman (1971) showed that avascular tumors could not grow beyond a size of ~1 mm in diameter. At this stage, passive diffusion of nutrients and oxygen becomes rate limiting for the tumor nodule, which is then forced to enter a state of so-called "tumor dormancy." In most cases, tumor vascularization is achieved by sustained angiogenesis, with a significant contribution of bone marrow-derived vascular and hematopoietic progenitor cells. Indeed, tumor angiogenesis is one of the hallmarks of

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cancer (Geiger and Peeper, 2009). Tumor angiogenesis involves increased endothelial cell proliferation and migration, and tube formation into the tumor mass (Silva et al., 2008).

Because angiogenesis is a key process to tumor growth, developing angiogenesis inhibitors with very few side effects is desirable for cancer treatment. Resistance to anti-angiogenesis drugs is also unlikely to occur, or at least at a much lower rate than seen with traditional cytotoxic chemotherapeutics (Cook and Figg, 2010). The study of tumor angiogenesis, therefore, represents a promising area of research for development of anti-cancer therapeutics (Swenson et al., 2007).

Integrins, a family of noncovalently associated heterodimeric transmembrane glycoprotein adhesion molecules, are a major target of interest, as they are known to play a vital role in pathological angiogenesis (Swenson et al., 2007). They comprise an α-subunit, and a β-subunit, which mediate cell–extracellular matrix (ECM) and cell–cell adhesive interactions. Heterodimer composition confers ligand specificity, with most integrins recognizing several ECM proteins and, in turn, most matrix proteins binding to more than one integrin. Endothelial cells express a subset of mammalian integrins including; the fibronectin receptors, $\alpha_4\beta_1$, $\alpha_5\beta_1$; the collagen receptors, $\alpha_1\beta_1$, $\alpha_2\beta_1$; the laminin receptors, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$, the osteopontin receptor, $\alpha_9\beta_1$; and the vitronectin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Silva et al., 2008).

Snake venoms contain many unique components that affect cell–matrix interaction. Disintegrins represent a family of low molecular weight, cysteine-rich polypeptides that bind specifically to integrins $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ expressed on platelets and other cells, including vascular endothelial cells and some tumor cells, leading to inhibition of platelet aggregation, inhibition of cell adhesion, migration and angiogenesis (Calvete, 2013; Huang et al., 2001).

Recombinant disintegrin mojastin 1 (r-mojastin 1) derived from *Crotalus scutulatus scutulatus*, expressed using a prokaryotic host expression system in *Escherichia coli* BL21 cells, was shown to be highly active in inhibiting ADP-induced platelet aggregation using platelet-rich plasma and whole blood, platelet ATP release, and platelet adhesion to fibronectin (Sánchez et al., 2010). r-Mojastin 1 was also tested for its ability to inhibit some cellular function such as adhesion to extracellular matrices, migration and invasion *in vitro* and *in vivo*, using human urinary bladder carcinoma (T24), skin melanoma (SK-MEL-28), and murine melanoma (B16F10) cells (Lucena et al., 2011). r-Mojastin 1 inhibited SK-MEL-28 cells adhesion to fibronectin and SK-MEL-28 cell migration. The invasion studies *in vitro* and *in vivo* shown that r-mojastin 1 inhibited T24 and SK-MEL-28 cells invasion through an artificial basement membrane, and lung tumor metastasis at a dose of 1000 μg/kg, when the r-disintegrin was co-injected with B16F10 cells (Lucena et al., 2011). Recently, we have described the cloning and functional characterization of another recombinant disintegrin derived from *Crotalus viridis viridis* called r-viridistatin 2, which showed potent anti-metastatic activities against five different human tumoral cell lines. r-Viridistatin 2, efficiently inhibited various functions of the tumoral cells such as adhesion, migration, invasion and lung tumor colonization, with different potency depending of the tumoral cell line used (Lucena et al., 2012).

Considering that angiogenesis contributes to the pathogenesis of many disorders, including cancer, in this study we have described the effects of r-mojastin 1 and r-viridistatin 2 on each distinct step of angiogenesis, including proliferation, adhesion, migration, *in vivo* angiogenesis, and *in vitro* tube formation in human umbilical vein endothelium cells (HUVECs).

2. Materials and methods

2.1. Preparation of recombinant disintegrins

Recombinant mojastin 1 and recombinant viridistatin 2 were expressed in *E. coli* and further purified by two-step chromatography using the method of Sánchez et al. (2010) and Lucena et al. (2012), respectively.

2.2. Cell line and culture conditions

Human umbilical vein endothelium cell (HUVEC) line and endothelial cell growth media were obtained from Lonza (USA). The cells were maintained in endothelial cell basal medium (EMB-2) containing 2% fetal bovine serum (FBS), and supplemented with 0.4% bovine brain extract (BBE), 0.1% human epidermal growth factor (hEGF), 0.1% hydrocortisone, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified 5% CO_2 air incubator at 37 °C. HUVECs used in all experiments were from passages 2–6.

2.3. Proliferation assay

Two hundred microliters of HUVECs in EMB-2 medium were plated into the wells of 96-well culture plates at 5×10^4 cells/well in duplicate and incubated at 37 °C in 5% CO_2 for 24 h, then the cells were treated with 20 μL of r-mojastin 1 and r-viridistatin 2 at various concentrations for 24 h. Cells were incubated with 10 μL of 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenltetrazolium bromide (MTT; 5 mg/mL) for 4 h at 37 °C, MTT was aspirated and 100 μL of dimethyl sulfoxide (DMSO) was added to lyse the cells. The absorbance of cell lysate at 570 nm was measured using a Beckman Coulter™ model AD 340 reader. Doxorubicin (4 μL; 2.5 mg/mL), a drug that induced apoptosis in endothelial cells was used as the positive control (Kotamraju et al., 2000). The negative control consisted of cells treated with phosphate buffer saline (PBS), pH 7.4. The percentage of cell proliferation was calculated relative to the negative control, which was defined as 100%. The 50% cytotoxic concentration (CC_{50}) of sample is defined as the venom concentration, which reduced 50% of proliferation. The values of the percentages of cell proliferation inhibition were plotted against disintegrin concentrations, and the CC_{50} was determined.

2.4. Adhesion assay

Recombinant mojastin 1 and r-viridistatin 2 were used to inhibit the binding of HUVECs on fibronectin coated plate (Juliano et al., 1996). Duplicate wells of a 96-well plate (Falcon® Tissue Culture Plate) were coated with 0.1 mL of