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PIVL, a snake venom Kunitz-type serine protease inhibitor, inhibits *in vitro* and *in vivo* angiogenesis

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ABSTRACT

Development and homeostasis of the vascular system requires integrin-promoting endothelial cell adhesion, migration and survival. Nowadays, integrins represent potential targets for pharmacological agents and open new avenues for the control of metastatic spread in the treatment of tumor malignancies. We have already reported that PIVL, a serine protease inhibitor isolated from *Macrovipera lebetina* venom, displays an anti-tumor effect through interference with integrin receptor function. Here, we report that PIVL inhibits human vascular endothelial cell adhesion and migration onto fibrinogen and fibronectin in a dose-dependent manner without any cytotoxicity. Furthermore, we show that PIVL increases microtubule dynamic instability in HMEC-1 transfected with EGFP-tagged α-tubulin. Using Matrigel™ and chick chorioallantoic membrane assays, we demonstrate that PIVL exhibits a strong anti-angiogenic effect both *in vitro* and *in vivo*. Interestingly, results herein reveal that the potent anti-angiogenic properties of PIVL are mediated by its RGD-like motif (^{41}RGN^{43}).

Introduction

Angiogenesis, the growth of new blood vessels from existing vasculature, is critical whenever tissues undergo extensive remodeling processes, including during normal growth and development, wound healing (Bao et al., 2009; Folkman and Shing, 1992), the female reproductive cycle (Demir et al., 2010), mammary gland development during pregnancy and lactation (Andres and Djonov, 2010), and the expansion of fat mass in obesity (Cao, 2007; Lijnen, 2008). Excessive vascularization is a hallmark of many diseases including cancer, rheumatoid arthritis, diabetic nephropathy, pathologic obesity, age-related macular degeneration and asthma (Rivera et al., 2011). Angiogenesis is thus required to sustain malignant cells with nutrients and oxygen to assist their growth. The development of new blood vessels in the tumor also facilitates metastasis formation (Alghisi and Ruegg, 2006). Pre-existing endothelial cells must break through the basement membrane, migrate and proliferate in response to pro-angiogenic factors (Drewes et al., 2012). The new outgrowths have to reorganize into a three dimensional tubular structure, which will create the new vessel (Risau, 1997). All these events are supported by the action of the integrin adhesion receptors (Stupack and Cheresh, 2004).

Integrins are heterodimeric transmembrane receptors that mediate cell–cell and cell–ECM interactions. Composed of noncovalently associated α and β subunits, they can assemble into at least 24 known distinct integrins, with heterodimer composition generally conferring ligand specificity (Delon and Brown, 2007). Integrins participate in the complex biological processes of embryonic development and maintenance of tissue integrity (Guo and Giancotti, 2004; Hynes, 2002). During the growth of new blood vessels, adhesion of endothelial cells to the ECM *via* integrins regulates their proliferation, survival, and motility. An array of integrins has been particularly implicated in the control of angiogenesis, including α1β1, α2β1, α3β1, α4β1, α5β1, α6β4, αvβ3, αvβ5 (Akalu et al., 2005; Alghisi and Ruegg, 2006). Changes in integrin expression or function are directly involved in angiogenesis, tumor growth and metastasis, making these receptors promising targets for novel anticancer therapies (Garmy-Susini and Varner, 2008).

Microtubules are highly dynamic cytoskeletal structures assembled from α/β-tubulin heterodimers (Honore et al., 2005). They are regulated both spatially and temporally by numerous microtubule-associated

Abbreviations: bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; ECM, extracellular matrix; MT, microtubule; PIVL, protease inhibitor from *Macrovipera lebetina*; VEGF, vascular endothelial growth factor.

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proteins and this regulation is crucial for mitosis, cell migration, cell signaling and trafficking (Honore et al., 2005; Pasquier et al., 2005; Pourroy et al., 2006). The multiple targeting of focal adhesions by microtubules induces their dissociation to promote cell migration (Connolly et al., 2002; Kaverina et al., 1999). Human endothelial cells have been shown to be very sensitive to microtubule-targeted drugs compared with epithelial cells or other anticancer drugs (Grant et al., 2003; Hayot et al., 2002). In fact, angiogenesis can be inhibited by very low concentrations of microtubule-targeting drugs (Grant et al., 2003; Ribatti et al., 2003) without any mitotic cell block or apoptosis induction (Pasquier et al., 2004) through a disruption of the microtubule-focal adhesion cross-talk (Honore et al., 2008; Pagano et al., 2012). Although current anti-angiogenic strategies have provided promising results in several cancer types, identification of additional anti-angiogenic targets is required to improve the therapeutic response.

Several studies reported that snake venom is a natural source for molecules known as modulators of integrin-mediated functions (Sarray et al., 2007). Among these molecules, disintegrins are the most widely characterized (McLane et al., 2004). Most disintegrins contain an Arg-Gly-Asp (RGD) or RGD-like motif, which is essential to their ability to block integrin interaction with ligands (Calvete et al., 2005). Another family of venom peptides, the C-type lectin proteins (Lu et al., 2005) has also been reported to impair cell–ECM interactions (Pilorget et al., 2007; Sarray et al., 2007). In addition, we recently have reported that snake venom phospholipases A2 are also able to disrupt integrin function. By interacting with integrins, those molecules exert an anti-tumor effect and are able to inhibit angiogenesis both *in vitro* and *in vivo* (Kessentini-Zouari et al., 2010; Limam et al., 2010).

The snake Kunitz-type inhibitors have been identified from the venom of Viperidae and Elapidae. These 60 amino-acid long peptides are characterized by 6 conserved cysteine residues engaged in three disulfide bonds contributing to the compact and stable nature of the folded polypeptide. They are considered to act on coagulation, fibrinolysis and inflammation (Chou et al., 2010; Meta et al., 2009). In a previous work, we reported the anti-tumor activity of the Kunitz-type protease inhibitor (PIVL) isolated from *Macrovipera lebetina* venom. Our findings showed that PIVL presents an anti-tumor effect by interfering with integrin receptor function. Moreover, we outlined for the first time the importance of the domain related to amino acid 41–43 of PIVL in this effect (Morjen et al., 2013).

In the present study, we investigated the impact of integrin inhibition by PIVL on vascular endothelial cell behavior. Our results reveal that PIVL inhibits endothelial cell adhesion and migration. We also demonstrate that PIVL increases microtubule dynamics and abolishes angiogenesis both *in vitro* and *in vivo*. Interestingly, we report that the domain related to amino acid 41–43 of PIVL is involved as well in the anti-angiogenic effect.

Material and methods

Cell culture

HMEC-1 cells (Laboratory of Cell Biology, Faculty of Pharmacy, Marseille) were routinely maintained at 37 °C and 5% CO_2 in MCDB-131 medium (Lonza, Levallois-Perret, France) containing 10% heat-inactivated fetal bovine serum, 2 mmol/l glutamine, 1% penicillin and streptomycin (all from Life Technologies, Paisley, UK), 1 mg/ml hydrocortisone (Pharmacia & Upjohn, St-Quentin-Yvelines, France) and 10 ng/ml epithelial growth factor (R&D Systems, Minneapolis, MN). HMEC-1 cells were grown on 0.1% gelatin-coated flasks (Pourroy et al., 2006).

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT solution (500 μg/ml final concentration) was added to the culture medium 4 h before the end of treatment. Subsequently, the MTT solution was removed and replaced with 100 μL of DMSO into each well in order to dissolve the precipitated formazan crystals. Finally, the absorbance was measured at 550 nm.

Cell adhesion and migration assays

Adhesion assays were performed as previously described (Delamarre et al., 2009). Briefly, 96-well plates were coated with purified ECM protein solutions or with PIVL, purified as previously described (Morjen et al., 2013) for 2 h at 37 °C and blocked with 0.5% PBS/BSA. Cells were added to wells and allowed to adhere to the substrata for 1 h at 37 °C. After washing, adherent cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 600 nm. For adhesion assays using antibodies, HMEC-1 cells were treated with 10 μg/ml rat mAb 69.6.5 (anti-αv) (produced in the Laboratory of Cell Biology, Faculty of Pharmacy, Marseille) or mouse mAbs LM609 (anti-αvβ3), Gi9 (anti-α2β1) or Sam-1 (anti-α5β1) purchased from Millipore, for 30 min at room temperature. Cells were then added to 96-well microtiter plates coated with 10 μg/ml of PIVL and allowed to adhere. Adhesion assay was continued as above.

Cell migration was performed using modified Boyden chambers (NeuroProbe Inc., Bethesda, MD) with 8-μm pore polycarbonate Nucleopore membranes (Costar, Cambridge, MA). The undersurface of the membrane was not precoated with ECM components or precoated with 10 μg/ml of fibronectin or 50 μg/ml fibrinogen. After treatment with PIVL for 30 min at room temperature, HMEC-1 cells were seeded into the upper reservoir and allowed to migrate through the filter towards the lower reservoir for 5 h at 37 °C. Cells that migrated to the underside of the filter were stained with 0.1% crystal violet, the colorant was solubilized with 1% SDS and absorbance was measured at 600 nm as previously described (Kadi et al., 1998; Rigot et al., 1998).

Time-lapse microscopy and analysis of microtubule dynamic instability

The transfection of HMEC-1 with the GFP-tagged tubulin plasmid was done as previously described (Pourroy et al., 2006). In brief, 8×10^5 HMEC-1 cells were transfected with 8 μg plasmid pEGFP-Tub (Clontech, Palo Alto, CA) encoding a fusion protein consisting of the human codon-optimized variant of green fluorescent protein (GFP) and the human α-tubulin gene, using transfection buffer solution R and program T-16 of a Nucleofector (Amaxa, Cologne, Germany). DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After transfection, cells were immediately transferred into HMEC-1 culture medium. Cells were seeded in six-well plates onto slides pre-coated with 10 μg/ml fibronectin. Twenty-four hours later, cells were treated for 1 h in the presence or absence of PIVL and microtubule (MT) dynamics measurements were performed as described (Pourroy et al., 2006). Briefly, transfected HMEC-1 cells were placed in a double coverslip chamber maintained at 37 °C ± 1 °C and observed using the 100× objective lens of an inverted fluorescence microscope (Leica). Thirty-one images per cell were acquired at 4-second intervals using a digital camera driven by Metamorph software (Universal Imaging Corporation, Downingtown, PA). Analysis of MT dynamics was done as described previously using the track point function of the Metamorph software (Pasquier et al., 2005). Changes in length ≥0.5 μm were considered growth or shortening events whereas changes in length <0.5 μm were considered phases of attenuated dynamics or pauses. The rates of growth and shortening events were determined by linear regression. Means and SE were calculated per event. The catastrophe frequency based on time was calculated by dividing the number of transitions from growth or pause to shortening by the total time growing and paused for each individual MT. The rescue frequency based on time was inversely calculated, dividing the total number of transitions from shortening to pause or growth by the time spent shortening for each individual MT. Means and SE of transition frequencies were calculated per MT ($n > 30$, for each experimental