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Actions of the Kunitz-type serine protease inhibitor Amblyomin-X on VEGF-A-induced angiogenesis

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ABSTRACT

Amblyomin-X is a Kunitz-type serine protease inhibitor (Kunitz-type SPI) designed from the cDNA library of the *Amblyomma cajennense* tick, which displays *in vivo* anti-tumor activities. Here, the mechanisms of actions of Amblyomin-X in vascular endothelial growth factor A (VEGF-A)-induced angiogenesis were characterized. Topical application of Amblyomin-X (10 or 100 ng/10 μl; each 48 h) inhibited VEGF-A-induced (10 ng/10 μl; each 48 h) angiogenesis in the dorsal subcutaneous tissue in male Swiss mice. Moreover, similar effect was observed in the VEGF-A-induced angiogenesis in the chicken chorioallantoic membrane (CAM). Additional *in vitro* assays in t-End cells showed that Amblyomin-X treatment delayed the cell cycle, by maintaining them in G0/G1 phase, and inhibited cell proliferation and adhesion, tube formation and membrane expression of the adhesion molecule platelet-endothelial cell adhesion molecule-1 (PECAM-1), regardless of mRNA synthesis. Together, results herein reveal the role of Kunitz-type SPI on *in vivo* VEGF-A-induced angiogenesis, by exerting modulatory actions on endothelial cell proliferation and adhesion, especially on membrane expression of PECAM-1. These data provide further mechanisms of actions of Kunitz-type SPI, corroborating their relevance as scientific tools in the design of therapeutic molecules.

1. Introduction

Serine proteases are essential key enzymes in a broad diversity of physiologic and pathologic processes, and their overexpression is tightly blocked by endogenous inhibitors to maintain homeostasis. The disruption of this equilibrium is the basis for disease genesis, and therefore, serine protease inhibitors (SPI) are targets of the synthetic development of drugs (Cucciloni et al., 2009; Perzborn et al., 2011). The family of Kunitz-type serine protease inhibitors (Kunitz-type SPI) comprise more than twenty members, which include bovine pancreatic trypsin inhibitor, Alzheimer's amyloid precursor protein (APP), and tissue factor pathway inhibitors 1 and 2 (TFPI-1 and 2) (Chand et al., 2005). They are competitive protease inhibitors, with one or more Kunitz-type domains, characterized

Abbreviations: APP, Alzheimer's amyloid precursor protein; Ambly, Amblyomin-X; CFSE, Carboxyfluorescein diacetate succinimidyl ester; CAM, Chick embryo chorioallantoic membrane; FXa, Factor Xa; FACS, Flow cytometry; FITC, Fluorescein isothiocyanate; ICAM-1, Intercellular cell adhesion molecule; Kunitz-type SPI, Kunitz-type serine protease inhibitors; PECAM-1, Platelet-endothelial cell adhesion molecule; PI, Propidium iodide; RT-PCR, Reverse transcription polymerase chain reaction; SPI, Serine protease inhibitors; TFPI-1 and 2, Tissue factor pathway inhibitors 1 and 2; UBC, Ubiquitin C; VEGF-A, Vascular endothelial growth factor; VEGFR-2, Vascular endothelial growth factor receptor 2.

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by intrachain disulfide bonds conserved in all family members (Laskowski and Quasim, 2000).

The relation of Kunitz-type SPI with cancer development and metastases has been shown by reduced levels of endogenous TFPI-2 in some aggressive cancer types (Sierko et al., 2007; Ran et al., 2009) and by reduced tumor cell migration and invasion by TFPI-2 recombinant therapy or TFPI-2 overexpression (Yanamandra et al., 2005; Ran et al., 2009). The proposed mechanisms are related to the inhibition of the expression of matrix metalloproteinase enzymes and activities (MMPs) (Rao et al., 1999; Kong et al., 2004; Ran et al., 2009), tumor cell cytotoxicity (Wong et al., 2007; Kemparah and Kisiel, 2008), reduction of tumor cell lymphatic spread (Sierko et al., 2010), and impairment of angiogenesis (Yanamandra et al., 2005; Provençal et al., 2008; Ran et al., 2009).

Angiogenesis or neovascularization is a highly complex pathophysiological process, where pre-existing endothelial cells must break through the basement membrane, migrate and proliferate in response to angiogenic factors. The new outgrowths have to reorganize into a patent three-dimensional tubular structure, which will create the new vessel (Risau, 1997). All steps of the process are influenced by a strongly controlled balance of positive or negative modulators, secreted by different cell types, and by the expression of cell membrane adhesion molecules, which allows the perfect cell–cell and cell–extracellular matrix interactions (Ramjaun and Hodivala-Dilke, 2009). In this context, vascular endothelial growth factor A (VEGF-A) is one of the most important mediators of angiogenesis, which interacts with a specific membrane receptor (VEGFR-2) expressed in most adult vascular endothelial cells and with circulating endothelial progenitor cells as well (Bergers and Benjamin, 2003; Coultas et al., 2005). Agonist activation induces conformational changes within VEGFR-2, followed by receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domains, which activates several intracellular pathways, displaying endothelial cell proliferation, migration, differentiation, tube formation, and vascular permeability increase and integrity (Hicklin and Ellis, 2005; Kerbel, 2008).

Amblyomin-X is a Kunitz-type SPI recombinant protein of 15 kDa, obtained from the cDNA library of *Amblyomma cajennense* salivary glands (Batista et al., 2008), which shares similarities with TFPI (Salemink et al., 1999) and inhibits Factor Xa (FXa) and consequently delays the time of blood coagulation *in vitro* and *ex vivo* (Batista et al., 2008, 2010). Recent evidence has extended our knowledge of the actions of Amblyomin-X, as Amblyomin-X treatment in C57BL6 mice reduced tumor mass and the number of metastatic events caused by intravenous injection of murine melanoma B16F10 cells. In addition, *in vitro* Amblyomin-X treatment caused apoptosis in melanoma (SK-Mel-28) and pancreatic adenocarcinoma (Mia-PaCa-2) cells, and the proposed mechanisms are increased expression of the proteasome b2 catalytic subunit gene (PSBM2), decreased proteasomal activity and increased pool of polyubiquitinylated proteins (Chudzinski-Tavassi et al., 2010).

Considering the *in vivo* anti-tumor effects of Amblyomin-X and the role of SPI in neovascularization, the present work investigated the effects of the Amblyomin-X on VGEF-A-induced *in vivo* angiogenesis and its actions on endothelial cell functions during the process. The findings highlight the effects of Amblyomin-X on endothelial cell proliferation and adhesion, mainly on VEGF-A-endothelial PECAM-1 expression, which may contribute to its modulatory effect on *in vivo* angiogenesis.

2. Methods

2.1. Animals

Male Swiss mice (25–30 g) were fed on standard pellet diet and water *ad libitum*, and anesthetized with a combination of ketamine (20 mg/kg) and xylazine solution (2 mg/kg, i.p) before each experimental procedure. All procedures were performed according to protocols approved by the Brazilian Society of Science of Laboratory Animals (SBCAL) for proper care and use of experimental animals.

2.2. Amblyomin-X

The Amblyomin-X protein (15 kDa) was obtained from a cDNA library of the salivary glands of the *A. cajennense* tick (GenBank accession AAT68575; Batista et al., 2008). Amblyomin-X was initially expressed in prokaryotic system (BL21(DE3) *Escherichia coli*) using the pAE vector. This kind of production inserts 6 histidin residues in the molecule (Batista et al., 2010), becoming easier the protein purification process. However in the present study, it was used Amblyomin-X cloned and expressed in methylotrophic yeast system (*Pichia pastoris*) employing the pPIC9K vector (Faria et al., personal communication). In this system Amblyomin-X was produced without histidin-tag, excluding unexpected effects of the fusion protein. The recombinant protein was maintained at −80 °C and diluted in sterile phosphate buffered saline (PBS).

2.3. Cell culture

Polyoma middle T oncogene-transformed mouse endothelioma cells derived from thymus (t-End) (Willians et al., 1988) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2, humidified atmosphere, at 37 °C. Cells were used in the 3rd passage. t-End cells were used to carry out the *in vivo* and *in vitro* studies in mice, and the expression of PECAM-1 was determined before the beginning of assays, which provided data about the responsiveness of the cell strain to be employed.

2.4. In vivo *angiogenesis*

2.4.1. Dorsal skinfold chamber

The dorsal skinfold chamber was implanted in male Swiss mice under anesthesia, as previously described by Harder et al. (2004). Amblyomin-X (1, 10 or 100 ng/10 μl) or PBS was topically applied and in sequence VEGF-A (10 ng/10 μl) or PBS (10 μl) was also locally applied. This treatment schedule was carried out on the 3rd, 5th and 7th days after chamber implantation. Animals were immobilized in a polycarbonate tube and the microcirculatory network in