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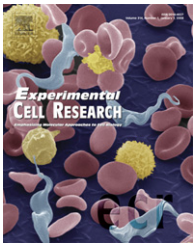

Research Article

# Characterization of the anti-angiogenic properties of arresten, an α1β1 integrin-dependent collagen-derived tumor suppressor

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ABSTRACT

Physiological and pathological turnover of basement membranes liberates biologically active cryptic molecules. Several collagen-derived fragments possess anti-angiogenic activity. Arresten is the 26-kDa non-collagenous domain of type IV collagen α1 chain. It functions as an efficient inhibitor of angiogenesis and tumor growth in mouse models, but its anti-angiogenic mechanism is not completely known. Here we show that arresten significantly increases apoptosis of endothelial cells *in vitro* by decreasing the amount of anti-apoptotic molecules of the Bcl-family; Bcl-2 and Bcl-xL. Although the pro-apoptotic effect of arresten is endothelial cell specific *in vitro*, in mouse tumors arresten induced apoptosis both in endothelial and tumor cells. The tumor cell apoptosis is likely an indirect effect due to the inhibition of blood vessel growth into the tumor. The active site of arresten was localized by deletion mutagenesis within the C-terminal half of the molecule. We have previously shown that arresten binds to α1β1 integrin on human umbilical vein endothelial cells. However, the microvascular endothelial cells (MLECs) are more important in the context of tumor vasculature. We show here that arresten binds also to the microvascular endothelial cells via α1β1 integrin. Furthermore, it has no effect on Matrigel neovascularization or the viability of integrin α1 null MLECs. Tumors implanted on integrin α1 deficient mice show no integrin α1 expression in the host-derived vascular endothelium, and thus arresten does not inhibit the tumor growth. Collectively, this data sheds more light into the anti-angiogenic mechanism of arresten.



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*Abbreviations:* BSA, bovine serum albumin; C-PAE, calf pulmonary aortic endothelial cells; cRGD, cyclic Arg-Gly-Asp; DMSO, dimethylsulfoxide; EC, endothelial cell; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; HIF, hypoxia inducible factor; HMVEC, human microvascular endothelial cells; HSPG, heparin sulphate proteoglycan; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen activated protein kinase; MLEC, microvascular lung endothelial cell; NC1, non-collagenous domain; PBS, phosphate buffered saline; RAD, Arg-Ala-Asp; RT, room temperature; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor

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[3] R.K. and the Beth Israel Deaconess Medical Center (BIDMC) hold patents related to human arresten. R. Kalluri and BIDMC are equity holders in EAI Corp., a company with the option to develop arresten as an anti-cancer drug.

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## Introduction

Angiogenesis, the formation of new blood vessels, is a critical event in tumor growth and metastasis [1]. Solid tumors will remain smaller than a few millimeters in diameter, if they are not able to induce their own blood supply, and thus inhibition of tumor angiogenesis suppresses tumor growth [1,2]. Capillary endothelial cells are supported by vascular basement membranes (VBM), which influence several aspects of endothelial cell (EC) behavior, such as cell proliferation [3,4]. Type IV collagen network is the structural backbone of the VBM [5]. Several cryptic fragments from non-collagenous (NC1) domains of type IV and XVIII collagens, such as arresten, canstatin, endostatin and tumstatin, have been shown to possess anti-angiogenic activity [6–9]. Although all these collagen-derived endogenous inhibitors of angiogenesis have similar molecular sizes, and all of them are proteolytic cleavage products from NC1 domains of VBM collagens, they bind to distinct cell surface receptors and affect different parts of the angiogenic process [10,11].

Arresten is a 26-kDa anti-angiogenic fragment from the α1 chain of type IV collagen. This molecule inhibits endothelial cell proliferation, migration, tube formation and Matrigel neovascularization [9]. Furthermore, it inhibits the growth of human tumors in nude mice and the development of tumor metastasis. Arresten was shown to bind to α1β1 integrin and heparan sulphate proteoglycans (HSPG) [9]. Later, we showed that many of the anti-angiogenic properties of arresten are mediated through α1β1 integrin [11]. Integrin α1β1 is the major collagen and laminin receptor [12], and it is the only collagen receptor that is able to activate the Ras/Sch/mitogen activated protein kinase (MAPK) pathway, thus promoting cell proliferation [13]. Integrin α1β1 is abundantly expressed on microvascular endothelial cells, but the expression is lower or even absent on endothelial cells lining larger blood vessels [14]. Interestingly, when tumors were implanted into the α1 integrin deficient mice, the tumors showed decreased tumor vascularization and growth [13].

In the present study, we have further characterized the anti-angiogenic properties of arresten. We show that arresten significantly increases apoptosis of endothelial cells by down-regulating the amount of anti-apoptotic molecules Bcl-2 and Bcl-xL. The active anti-angiogenic site of arresten is located in the C-terminal part of the molecule. We confirm and further explore the role of α1β1 integrin as a functional receptor of arresten on microvascular vessels essential for tumor blood supply. Integrin α1 is required for the anti-survival effect of arresten. Furthermore, the tumors implanted on integrin α1 deficient mice show no integrin α1 positive vasculature, and consequently the growth of tumors and blood vessels in these mice is not inhibited by arresten.

## Materials and methods

### *Production of recombinant arresten and arresten deletion mutants*

Human recombinant arresten was cloned and produced in *Escherichia coli* as previously described [9]. Briefly, arresten was expressed in the pET22b (+) expression vector (Novagen, Madison, WI) in BL21 cells (Novagen). Protein expression was induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After a 3-hour induction, cells were harvested, lysed with a lysis buffer (6 M guanidine, 0.1 M $NaH_2PO_4$, 0.01 M Tris–HCl, pH 8.0), sonicated and centrifuged. The supernatant was passed through a Ni-nitriloacetic acid agarose column (Qiagen, Chatsworth, CA). Arresten was eluted with an increasing concentration of imidazole (10, 25, 40, 125 and 250 mM) in 8 M urea, 0.1 M $NaH_2PO_4$, 0.01 M Tris–HCl, pH 8.0, and refolded by dialyzing twice against PBS. The concentration and purity of the soluble part of arresten was assayed with a bicinchoninic acid assay (Pierce, Rockford, IL) and SDS-PAGE. The production of recombinant human his-tagged arresten resulted in a predominantly soluble 29-kDa protein, consisting of the 26-kDa native arresten and 3-kDa polylinker and 6-histidine tag sequences. In order to localize the active site of arresten, mutants of arresten were generated by deletion mutagenesis, and these were termed Arr-1 (the first 115 amino acids) and Arr-2 (the last 115 amino acids). Both proteins were produced in *E. coli* using the pET-28a vector expression system (Novagen), and purified by their his-tag sequence using a Ni-NTA-agarose column as described above. Polymyxin B (5 μg/ml) (Sigma) was used in all assays to remove the possible endotoxin contamination of arresten purified from bacteria [15].

### *Production of a synthetic T7 peptide*

The synthetic T7 peptide (TMPFLFCNVNDCNFASRNDYSYWL) derived from tumstatin [16] was synthesized at Tufts University Core Facility (Boston, MA), analyzed by mass-spectrophotometer analysis and purified by analytical HPLC.

### *Cell lines*

Calf pulmonary aortic endothelial (C-PAE) cells, HT1080 fibrosarcoma cells, and renal cell carcinoma cells (786-0) were grown in DMEM containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Primary gingival fibroblasts (GF) were isolated and grown as previously [17]. Human prostate adenocarcinoma cells (PC-3) were grown in F12K containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Human tongue squamous cell carcinoma cells (HSC-3) were grown as described previously [18]. Human umbilical vein endothelial cells HUVEC (ATCC CRL-1730) and human primary microvascular endothelial (HMVEC, Lonza) cells were cultured in EGM-2-MV supplemented media (Clonetics/Lonza). CT26 colon carcinoma cells (CRL-2638 from ATCC) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary mouse lung endothelial cell lines (MLEC) from wild type (Charles River) and α1 integrin deficient Balb/c mice [19,20] were prepared as previously [11]. Briefly, the mice were sacrificed with cervical dislocation. The lungs were then perfused with ice-cold PBS-heparin (1 U/ml), and the lung and heart were collected into cold Ham's F-12 (Life Technologies). The lungs were digested with 0.1% collagenase for 1 h at 37 °C, and plated to 0.1% gelatin coated flasks in MLEC media (40% Ham's, 40% DMEM low glucose, 20% FBS, 1% penicillin–streptomycin, 2 mM L-glutamine, 100 μg/ml heparin (H3393, Sigma, St. Louis, MO) and 50 μg/ml endothelial mitogen (Biomedical Technologies)). Negative selection was performed with magnetic beads (Dynabeads M-450 Sheep anti-Rat IgG; Dynal, Oslo, Norway) conjugated with rat anti-mouse FcγII/III (Pharmingen, San Diego, CA), and positive selection was done