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**14. ABSTRACT**

Anastellin is a fragment of fibronectin which inhibits angiogenesis, tumor growth, and metastasis *in vivo*. A structurally similar synthetic peptide anginex also has anti-angiogenic and anti-tumor effects and has been found to resemble anastellin in several biochemical aspects, including dependence on circulating plasma fibronectin to be active *in vivo*. The specific homing of anastellin and anginex to angiogenic blood vessels also requires the presence of fibronectin and is inhibited when an RGD peptide is co-injected. Using NMR structural data we have also produced multiple variants of anastellin that contain mutations in key nucleic acid positions which alter the structure of anastellin. These variants can help elucidate the structure-function relationship of angiogenesis inhibitors.

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## INTRODUCTION

Previous work in our laboratory developed anastellin, which is a 10 kDa fragment of the first type III repeat of fibronectin. It has been shown that anastellin inhibits angiogenesis, tumor growth and metastasis *in vivo* [1-3]. The structure is a β-sheet with an exposed hydrophobic area in the middle [4]. Anastellin polymerizes with fibronectin *in vitro* [1] and requires circulating plasma fibronectin to be anti-angiogenic *in vivo* [5]. Other angiogenesis inhibitors, antithrombin and endostatin, also have been shown to depend on fibronectin and vitronectin to be active *in vivo* [5].

The Beta sheet structural motif is common among angiogenesis inhibitors. Recently, a synthetic 33-amino acid peptide, anginex, was modeled to reproduce the β-sheet structure of anti-angiogenic proteins. Anginex inhibits angiogenesis and tumor growth [6-8], and the bioactive form of anginex has a β-sheet structure [7].

We have found that anginex shares many of the functional properties of anastellin. It has been shown that anginex, similar to anastellin, binds to fibronectin and initiates its polymerization. Moreover, anginex is inactive in mice that lack plasma fibronectin. We have also found that anginex- and anastellin-fibronectin complexes home to angiogenic blood vessels *in vivo*. Anginex and anastellin are both dependent on fibronectin to home to angiogenic blood vessels *in vivo* and are inhibited by an Arg-Gly-Asp peptide.

## BODY

In order to test the hypothesis that it is the RGD sequence of fibronectin that functions as a homing sequence for the anti-angiogenic peptide-fibronectin complexes we added an N-terminal cysteine residue to anastellin and conjugated fluorescein molecules to it using maleimide linkage chemistry. When mixed with fibronectin, anastellin induces the formation of fibronectin fibrils [1]. To check for activity, the conjugated anastellin was mixed with fibronectin and allowed to form fibrils. Turbidity was measured at an optical density of 590 nm to quantify the polymerization. ' An inactive repeat of fibronectin, III-11C, was used as a negative control. The N-terminal fluorescein conjugated anastellin was able to form fibrils when mixed with 0.5 mg/ml of fibronectin in PBS (Fig 1).

Fluorescein-conjugated anastellin and aginex were injected into wild-type and fibronectin knock-out mice that had been implanted with matrigel plugs for 8 days to determine if fibronectin is necessary for the specific homing of these anti-angiogenic peptides (Fig 2). Homing of anastellin and anginex was restored in the fibronectin null mice when 0.5 mg of fibronectin was also injected into the mice (Fig 2 D and H). Our hypothesis that the homing of the peptide-fibronectin complex is due to the RGD sequence of fibronectin was directly tested by co-injecting an RGD peptide with fluorescein-conjugated anastellin and anginex to determine if the homing to angiogenic vessels is blocked. Figure 3 shows that the RGD peptide blocks specific homing by anastellin and aginex to angiogenic vessels *in vivo*.

A β-sheet structural motif is shared by inhibitors of angiogenesis. Variants of anastellin were designed using the NMR structural data [4] and contain specific mutations in the sequence which disrupt the structure of anastellin. The polymerization activity of fibronectin by the anastellin variants and its affect on anti-angiogenic activity and homing *in vivo* will be studied.

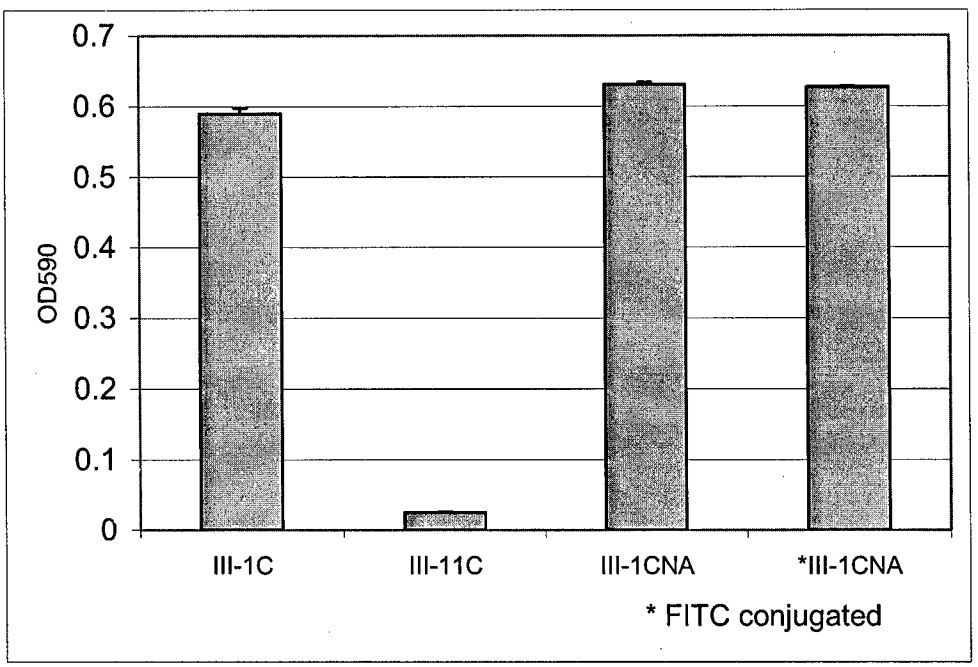


**Fig 1.** Activity of fluorescein conjugated anastellin. Peptides at a concentration of 1.5 mg/ml were mixed with 0.5 mg/ml of fibronectin and polymerization was monitered by measuring optical density at 590 nm. (III-1C-Anastellin, III-11C-Inactive repeat of fibronectin, III-1CNA-anastellin with an N-terminal cysteine, and *III-1CNA-FITC conjugated to the N-terminal cysteine of anastellin).

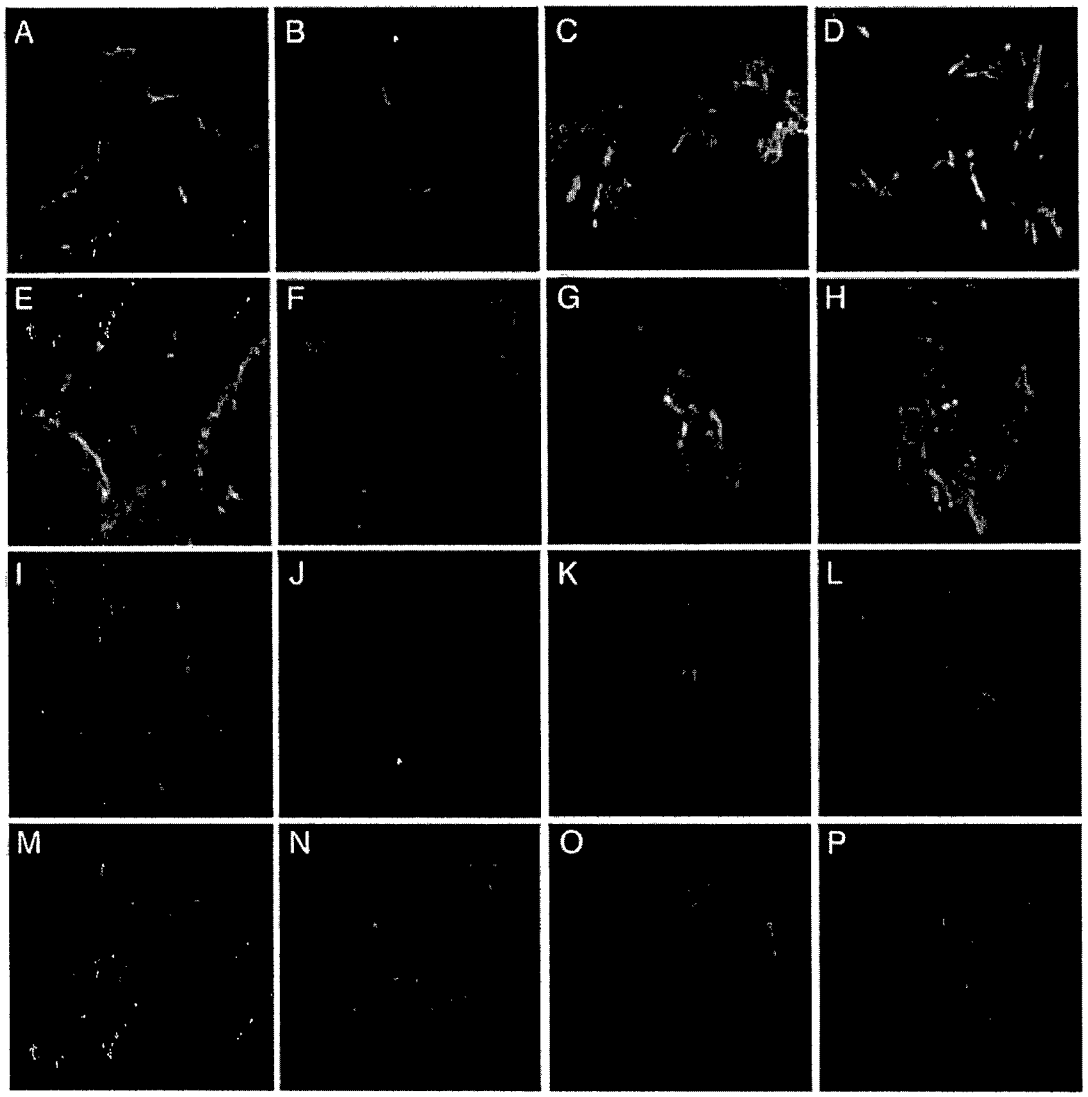


**Fig 2.** Anastellin and anginexhometo angiogenic vasculature *in vivo*. Normal or plasma fibronectin-deficient mice implanted with Matrigel plugs 8 days earlier were i.v. injected with fluorescein-labeled anastellin (750μg; *A–D* and *I–L*) or anginex (250μg; *E–H* and *M–P*), and the mice were killed 2 h later. Endothelial cells in the plugs and in various tissues were visualized with anti-CD31 staining. Yellow reveals colocalization of the injected fluorescent protein (green) and endothelium (red). Nuclei were counterstained with DAPI (blue). Anastellin and anginex fluorescence is observed in the plugs from wild-type mice (*A* and *E*). The vessels in the plugs of $pFN^{-/-}$ mice showed no significant anastellin (*B*) or anginex (*F*) fluorescence, whereas the plugs of the $pFN^{+/+}$ littermates of the $pFN^{-/-}$ mice were positive (*C* and *G*). i.v. injection of 0.5 mg of fibronectin into the $pFN^{-/-}$ mice restored the homing of anastellin (*D*) and anginex (*H*) to the plugs. No anastellin or anginex fluorescence was detected in the s.c. tissue (the site of the Matrigel plug implantation; *I* and *M*), the heart (*J* and *N*), or the brain (*K* and *O*). Faint green fluorescence was present in the liver (*L* and *P*). (Original magnification: 400X.)

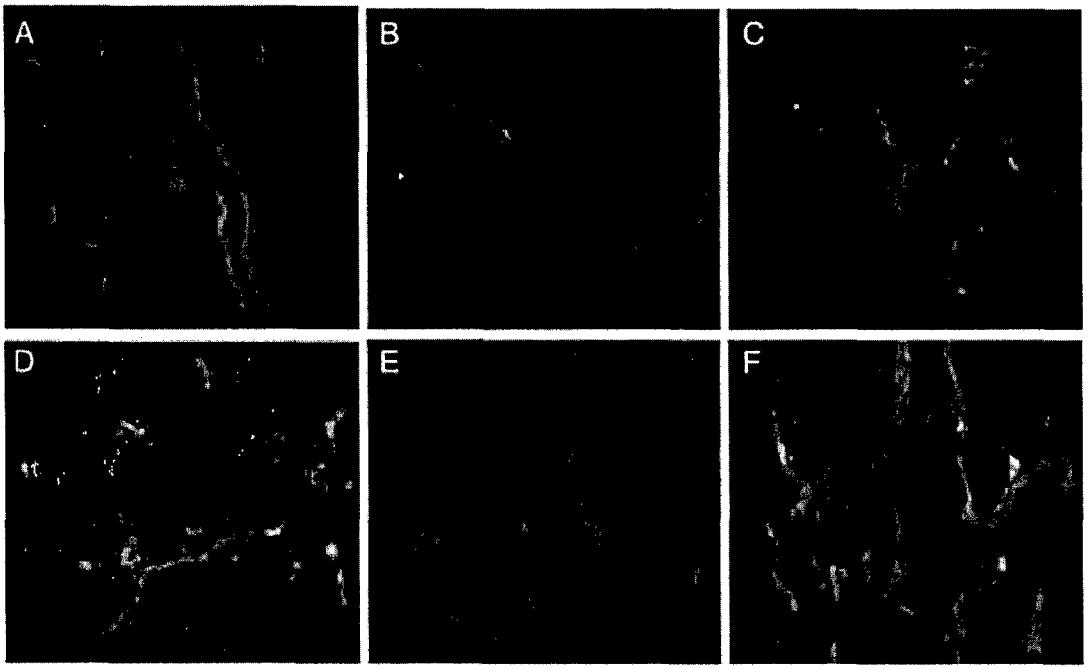


**Fig 3.** The homing of anastellin and anginex to angiogenic vasculature is inhibited by an RGD-containing peptide. Mice bearing Matrigel plugs were i.v. injected with fluorescein-labeled anastellin (*A–C*) or anginex (*D–F*) as in Fig. 2 together with PBS (*A* and *D*), 10mgof GRGDSP (*B* and *E*), or an equal molar amount of an unrelated peptide (*C* and *F*). Little or no fluorescence is observed in the Matrigel plugs of mice coinjected with the RGD-containing peptide.

## RESEARCH TRAINING AND ENVIRONMENT

I have benefited greatly from doing my pre-doctoral research at the Burnham Institute for Medical Research in the lab of Dr. Erkki Ruoslahti. There is a weekly seminar series with invited speakers on the campus and the institute is within a two-mile radius of The Scripps Research Institute, The Salk Institute and UCSD which also each host numerous seminars on a wide range of topics.

The lab also has daily group meetings in which members of the lab present papers for discussion and also present their ongoing progress on their projects. Outside speakers are also invited to the meetings once a week to present their research and introduce different

techniques to the lab. Guidance is also given through individual discussions to give suggestions on the direction of projects and future experiments.

## KEY RESEARCH ACCOMPLISHMENTS

- Fluorescein was conjugated to an N-terminal cysteine of anastellin, which is still active
- Fibronectin is necessary for the specific homing of anastellin and anginex to angiogenic vessels *in vivo*
- Homing of anastellin and anginex to angiogenic vessels is blocked by the co-injection of an RGD peptide
- Variants of anastellin containing mutations at key sites affecting the structure of anastellin have been produced

## REPORTABLE OUTCOMES

Akerman, M.E., Pilch, J., **Peters, D.**, Ruoslahti, E. (2005) Angiostatic peptides use plasma fibronectin to home to angiogenic vasculature. PNAS, 102:2040-2045.

## CONCLUSIONS

We have made significant progress toward understanding the mechanism of anastellin and anginex *in vivo*. The original goal of this project was to target anastellin to specific vascular sites *in vivo* by fusing anastellin with homing peptides that home to the vasculature of breast cancers. However, this did not improve the efficacy of anastellin. Anastellin and anginex appear to be naturally targeted to angiogenic vasculature *in vivo* by co-aggregating with fibronectin and utilizing the RGD-sequence of fibronectin to

home to angiogenic vasculature. The presence of fibronectin is necessary for the homing of anastellin and anginex to angiogenic vessels *in vivo* and is specifically blocked by the co-injection of an RGD peptide. Variants of anastellin have also been developed using the NMR structural data which contain point mutations affecting the structure of the peptide. The can be studied to further clarify the structure-function relationship of inhibitors of angiogenesis.

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