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14. ABSTRACT

Anastellin is a fragment of fibronectin which inhibits angiogenesis, tumor growth, and metastasis in vivo, but the mechanisms behind these effects were previously unknown. We have shown that anastellin co-localizes with Annexin V, a marker for anionic phospholipids on the surface of tumor blood vessels in vivo. Anastellin potentially uses the increased levels of phosphatidylserine on the surface of proliferating endothelial cells in angiogenic blood vessels to bind to the membrane and lyse the cell, leading to its anti-angiogenic effect in tumors.

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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], but the mechanisms by which it causes its affects are unknown. The structure is a -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 structure-function relationship of the beta-sheet in bacterial pore forming peptides is well established [6]. Amphiphilic antimicrobial peptides bind to membranes and disrupt lipid bilayers by micellization or pore formation [7]. The net positive charge of the peptide gives it the ability to bind to bacterial membranes, which contain a large amount of anionic phospholipids. In mammalian cells, anionic phospholipids are normally located at the inner leaflet of the cell membrane, whereas the outer leaflet is rich in neutral and zwitterionic lipids [8]. Apoptosis, cell stress, and cell activation can cause this membrane asymmetry to be reversed and result in anionic phospholipids, such as phosphatidylserine, becoming available on the cell surface. Based on the structural similarity to antimicrobial peptides we hypothesized that anastellin can target and cause disruption of the lipid bilayer of cell membranes through a similar mechanism.

BODY

In order to determine if anastellin targets the lipid bilayer of endothelial cells, FITC-anastellin and Alexa 594-conjugated Annexin V, a marker for anionic phospholipids were co-injected into mice containing prostate cancer xenograft tumors. As seen in Figure 1 anastellin co-localizes with Annexin V on the surface of the tumor blood vessels. This interaction with anionic phospholipids such as phosphatidyl serine was also confirmed *in vitro.*

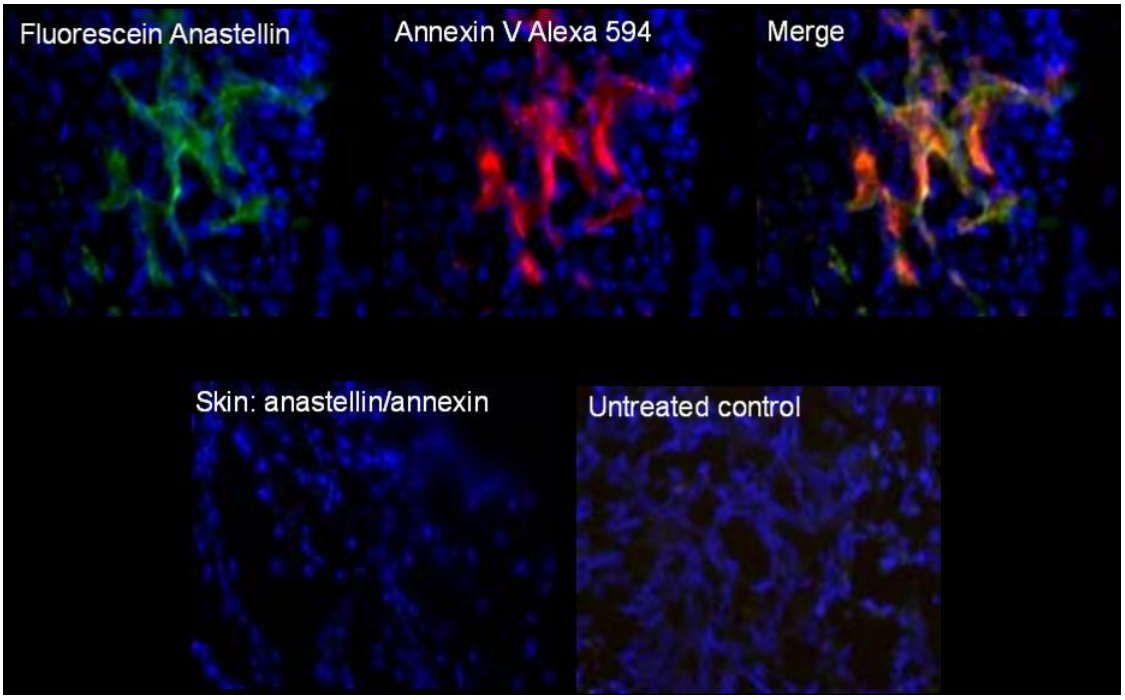

Fig 1. Co-localization of anastellin and Annexin V. FITC conjugated anastellin and alexa 594-conjugatated annexin V were co-injected into mice containing PPC-1 prostate xenograft tumors and analyzed for co-localization.

Activation of cells with hydrogen peroxide (H_2O_2) has been shown to disturb membrane asymmetry [9]. CHO cells deficient in proteoglycans and activated with H_2O_2 have an upregulated phosphatidylserine expression on the outer membrane, which is evidenced by increased Annexin V binding to cells using FACS analysis (Fig 2). These cells were

tested for a change in the interaction with anastellin after activation. As seen in Figure 2, anastellin and its variants have increased binding to cells activated by H_2O_2.

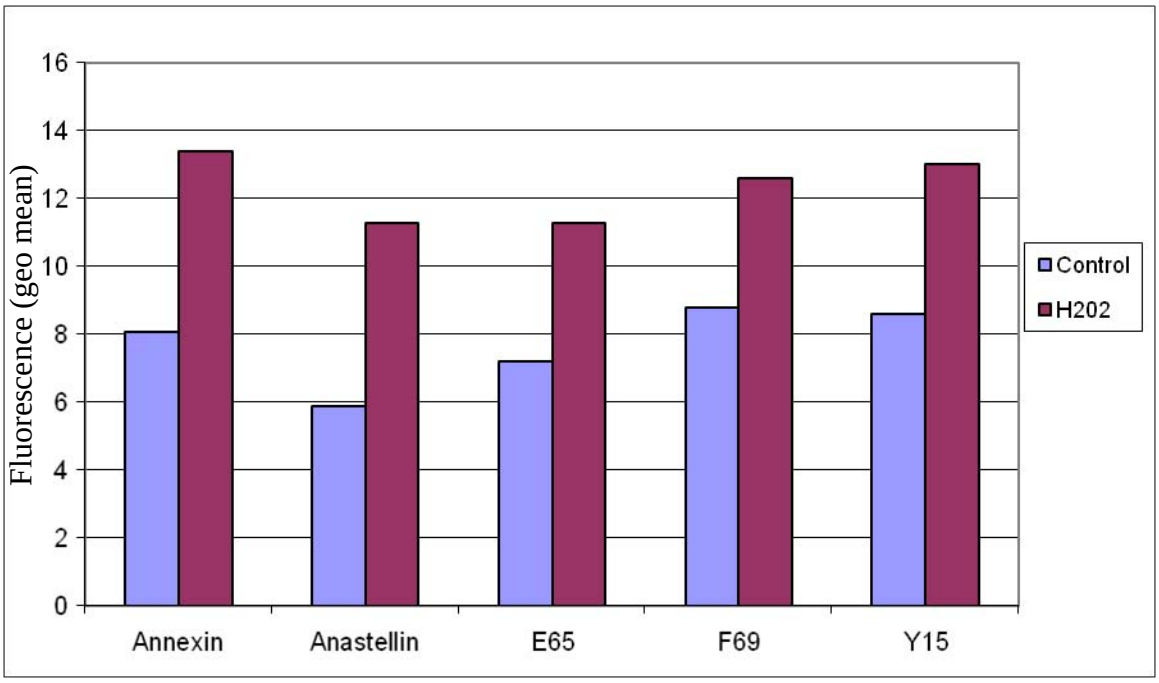

Fig 2. Anastellin and its variants preferentially bind cells that have anionic phospholipids on their outer membrane. CHO cells activated with H_2O_2 were analyzed with FACS analysis for peptide binding. There is an increase in the number of cells that bind Annexin V (a marker for anionic phospholipids) or anastellin and its variants after activation with H_2O_2.

Anastellin and its structural variants; E65, Y15, and F69, have been shown to cause decreased angiogenesis in vivo. Using a carboxyfluorescein dequenching assay, we showed that binding of anastellin or its variants causes disruption of the lipid bilayer and is more effective in releasing the contents from anionic vesicles made of dioleoyl-glycerophosphocholine (DOPC) and dioleoylglycerophosphoglycerol (DOPG, molar ratio 8:2) than those made with DOPC alone (Fig 3).

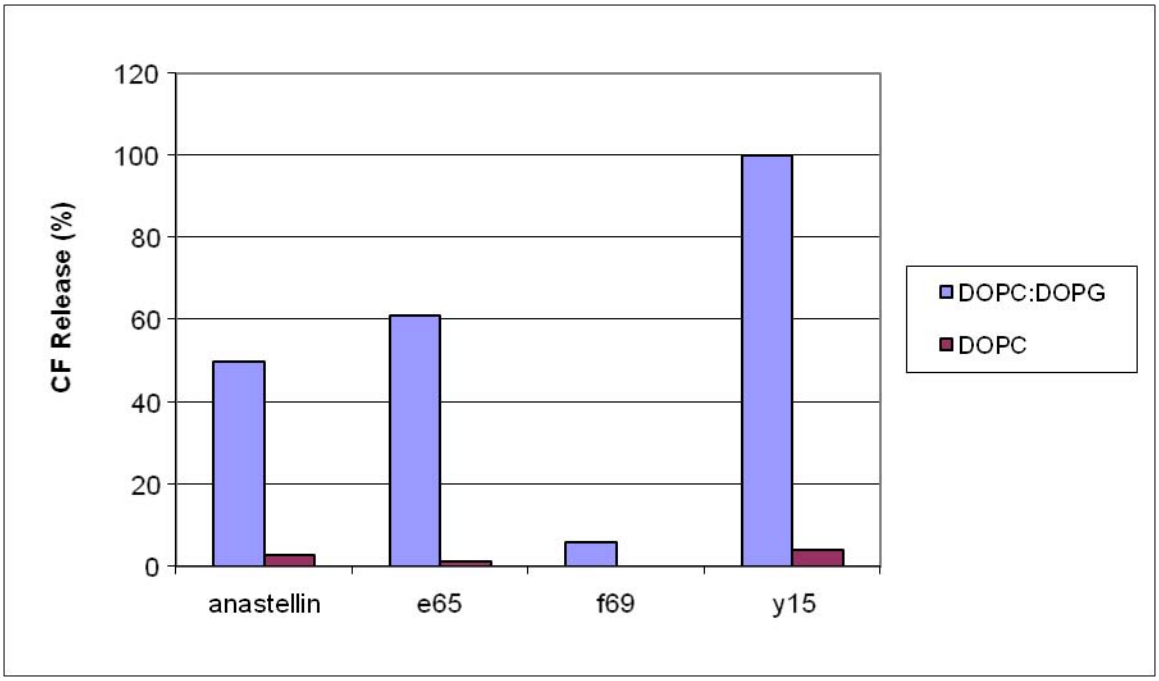

Fig 3. Anastellin and its variants lyse anionic vesicles. Carboxyfluorescein (CF) dequencing was measured after addition of anastellin or one of its structural variants to vesicles made with DOPC and DOPG (molar ratio 8:2) or those made with only DOPC. The peptides were significantly more effective at lysing the anionic vesicles.

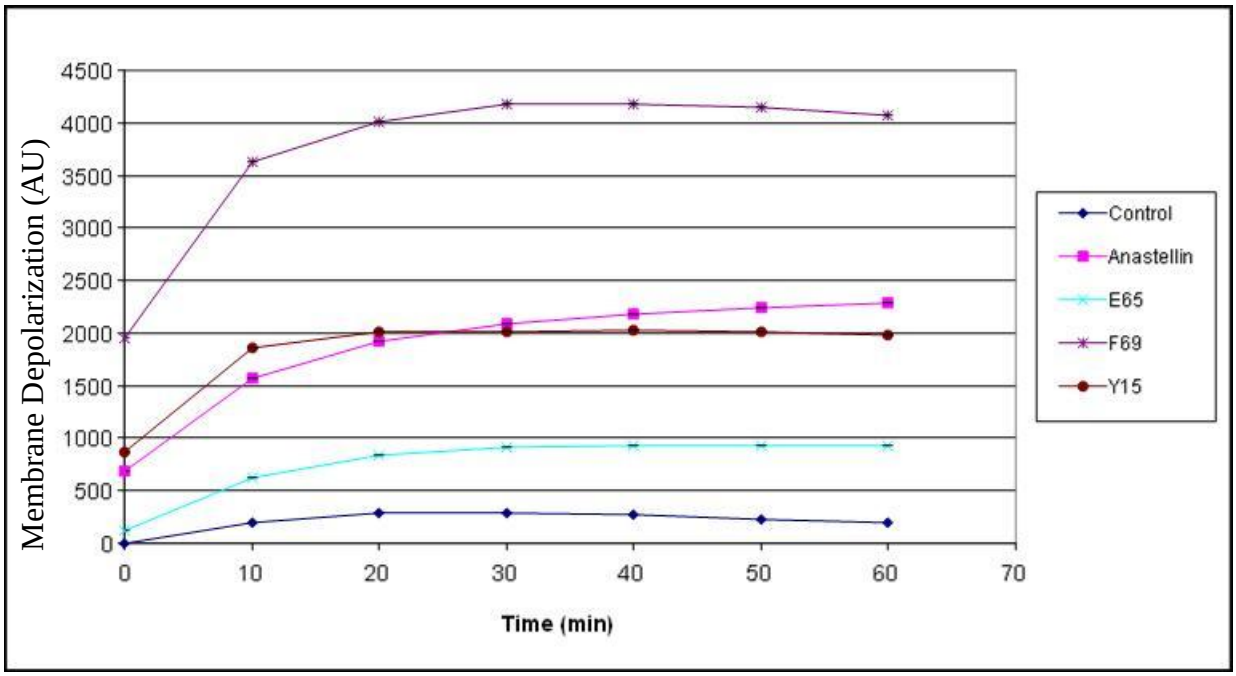

Fig 4. Anastellin and its variants cause depolarization of HUVEC cells compared to a vitronectin control peptide.

Anastellin and its variants were also shown to destabilize endothelial cell membranes. We observed membrane depolarization in response to anastellin in HUVEC cells, but not in response to a vitronectin control peptide (Fig 4). Depolarization is a sign of leakage because sodium ions can freely cross the cell membrane.

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 of their individual 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 determine future experiments.

KEY RESEARCH ACCOMPLISHMENTS

Anastellin co-localizes with Annexin V staining on the surface of tumor blood vessels *in vivo*

Anastellin and its variants bind to H_2O_2 activated CHO cells were have increased phosphatidylserine expressed on their surface

Anastellin and its variants preferentially lyse liposomes containing anionic phospholipids

Anastellin and its variants destabilize the membrane of HUVEC cells causing depolarization

REPORTABLE OUTCOMES

There are no reportable outcomes during this period.

CONCLUSIONS

We have made significant progress toward understanding the mechanism of anastellin's affects *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 appears 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. Once there anastellin causes decreased angiogenesis in tumors, but the mechanism behind this action was unclear. It has now been shown that anastellin and its structural variants preferentially bind to anionic phospholipids and cause disruption of the lipid bilayer. This cell permeabilization provides a potential explanation for anastellin's anti-angiogenic effects.

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