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Cloning an artificial gene encoding angiostatic anginex: From designed peptide to functional recombinant protein ☆

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Abstract

Anginex, a designed peptide 33-mer, is a potent angiogenesis inhibitor and anti-tumor agent in vivo. Anginex functions by inhibiting endothelial cell (EC) proliferation and migration leading to detachment and apoptosis of activated EC's. To better understand tumor endothelium targeting properties of anginex and enable its use in gene therapy, we constructed an artificial gene encoding the biologically exogenous peptide and produced the protein recombinantly in *Pichia pastoris*. Mass spectrometry shows recombinant anginex to be a dimer and circular dichroism shows the recombinant protein folds with β-strand structure like the synthetic peptide. Moreover, like parent anginex, the recombinant protein is active at inhibiting EC growth and migration, as well as inhibiting angiogenesis in vivo in the chorioallantoic membrane of the chick embryo. This study demonstrated that it is possible to produce a functionally active protein version of a rationally designed peptide, using an artificial gene and the recombinant protein approach.
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Angiogenesis is crucial to a variety of pathological processes, such as tumor growth and metastasis, rheumatoid arthritis, and various inflammatory disorders [1]. In the oncology field, both outgrowth of primary tumors and tumor metastasis are angiogenesis dependent [2–5]. Therefore, the abrogation of angiogenesis makes for an effective anticancer strategy. Recently, we described the design of the angiostatic agent anginex, an antiparallel β-sheet forming peptide 33-mer of which the structure is based on the 3-dimensional folding of the α-chemokines platelet factor 4 (PF4) and interleukin-8 [6]. Anginex has been shown to prevent adhesion and migration of activated endothelial cells (EC), leading to apoptosis induction in these cells. It has also been demonstrated that these effects lead to a significant tumor growth reduction in various mouse models [7–9].

Although a synthetic approach for future clinical applications with anginex has some advantages, large-scale production may be more economical through a recombinant approach. We therefore have constructed an artificial gene of anginex, and cloned it for production of recombinant protein in the *Pichia pastoris* yeast expression system. This eukaryotic system has proven to be suitable for low-cost production of high levels of functionally active recombinant protein of known angiogenesis inhibitors, such as angiostatin [10], endostatin [11],

☆ *Abbreviations:* CAM, chorioallantoic membrane; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; PF4, platelet factor 4; CD, circular dichroism; MS, mass spectrometry.

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and tumstatin [12]. Aside from facilitating large-scale production of the peptide, the anginex gene will be important for use as a molecular biological tool, for example to identify the anginex receptor using yeast-2-hybrid methodology.

Cloning of the anginex gene and isolation from the culture medium yielded recombinant anginex, which has comparable properties as its synthetic form. This was demonstrated both structurally as measured by circular dichroism (CD), and functionally as measured in vitro by inhibition of EC proliferation and migration, and in vivo by inhibition of angiogenesis in the chorioallantoic membrane (CAM)-assay. These results validate the use of the anginex gene in further developing anginex as a useful clinical agent and understanding its mechanism of action. The approach presented here will assist in designing other peptides and their corresponding genes that encode for specific receptor antagonists and the use of these genes in gene-therapy.

Materials and methods

Materials. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Leusden, The Netherlands). Oligonucleotide primers were synthesized by Eurogentec (Liege, Belgium). Nucleotide sequencing was carried out on an Applied Biosystems DNA sequencer, utilizing the ABI prism Big dye terminator reaction mix (Nieuwerkerk aan den IJssel, The Netherlands). All tissue culture reagents, the TA-cloning kit and the *P. pastoris* expression system were purchased from Invitrogen (Breda, The Netherlands). BIAcore equipment and reagent kits and chips were obtained from BIAcore life sciences (Breda, The Netherlands).

Design and cloning of the anginex gene. The gene encoding anginex was made using four primers in a PCR. The DNA codons used to code for the amino acids were chosen in such a way that the primers did not form stable secondary structures in the PCRs. Formation of the artificial 99 bp gene of anginex was a two-step process. Two partial overlapping oligonucleotides were designed to form the gene of anginex: (A) 5′-GCAAACATAAAACTAAGCGTACAA ATGAAACTATTCAAAAGACACCTAAAATGGAAAATA-3′; (B) 5′-GTCTAGGCTTAGTTCTCTTCCGTCGTTTAGTTTTACTATT ATTTTCCATTTTAGGTGTCT. In a secondary PCR, primers (1) 5′-TAT**GAATTC**ATGGCAAACATAAAACTAAGCGTAC-3′ and (2) 5′-TTAT**TCTAGA**CGGTCTAGGCTTAGTTCTCTTCC were used to introduce restriction sites for *Eco*RI and *Xba*I (shown in bold). The coding sequence for endostatin was obtained using PCR on cDNA of a human colon tumor. The following primers were used: 5′-TAT**GAATTC**ATGCACAGCCACCG-3′ and 5′-TAT**TCTAGAT** ACTTGGAGGCAGTCATG-3′. Both amplicons were cloned into the pCR2.1 TOPO-TA cloning kit and the sequence was verified by sequencing. Using the flanking restriction enzymes *Eco*RI and *Xba*I, the anginex and endostatin coding sequence were cloned into the yeast expression vector pPICZα-A. The new expression constructs were sequence verified with primer 5′AOX: 5′-GACTGGTTCCAA TTGACAAGC-3′, confirming an in-frame fusion with the *P. pastoris* α-factor secretion signal sequence at the N-terminal side of and the c-myc and 6× His-tag sequence at the C-terminal side of both genes.

Transformation of P. pastoris, determination of the mut phenotype, and expression of recombinant anginex. Both expression vectors were linearized using the restriction enzyme *Sac*I in order to facilitate integration at the *AOX1* locus of the yeast genome. The linearized vector was transformed into the *P. pastoris* strains GS115 and X33 by using the *Pichia* Easycomp kit (Invitrogen) according to the manufacturer's instructions. For each construct, several zeocine-resistant clones were selected and tested for a Mut^+ phenotype by patching the colonies, respectively, on MMH (minimal methanol with histidine) and MDH (minimal dextrose with histidine) plates. From the clones that had a Mut^+ phenotype, 10 colonies were selected for a small-scale expression test in order to select the best expressing strain. Using these clones, 25 ml BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol) was inoculated. The culture was grown with shaking (300 rpm) at 30 °C until the culture reaches an $OD_{600\ nm}$ of 2.0. Cells were harvested and resuspended in 50 ml BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol). For continuous expression, methanol was added every 24 h to a final concentration of 0.05%. After 3 days, the cells were harvested and the supernatant was tested for recombinant protein using standard 15% SDS–PAGE.

Large scale expression and His-tag affinity isolation of recombinant anginex. Clones that secreted the highest amount of recombinant protein were selected and used to inoculate 50 ml BMGY at 30 °C and shaking (300 rpm) for 24 h to create biomass. This culture was added to 1 L BMGY and cultured for another 24 h. Cells were harvested and resuspended in 2 L BMMY in order to induce expression. After 24 h, the supernatant was collected and concentrated using Centricon-plus 80 biomax 5 concentrators (Millipore Amsterdam, The Netherlands) to approximately 50 ml. Recombinant anginex/His-tag-related fusion proteins were isolated using 1 ml His-select beads (Sigma; Zwijndrecht, The Netherlands) according to the native batch purification method described by the manufacturer. Eluted recombinant proteins were dialyzed three times against 5 L water for at least 3 h at 4 °C. The dialysates were concentrated using a centricon YM-3 (Millipore) ultrafiltration device. Concentration of recombinant proteins was measured using the micro BCA protein assay reagent kit (Pierce; Etten-Leur, The Netherlands) according to the instruction manual.

Mass spectrometry and amino acid analysis of recombinant anginex. A MALDI-TOF Voyager DE-PRO mass spectrometer (Applied Biosystems) was used to analyze the mass of recombinant anginex. In this MALDI technique, recombinant anginex was mixed with a matrix (α-cyano-4-hydroxycinnamic acid). The crystallized sample was irradiated by a laser beam for desorption and ionization of the peptide. Automated N-terminal Edman degradation consisted of repetitive cycles of Edman chemistry, followed by PTH analysis on a HPLC column according to standard procedures. The first 11 N-terminal amino acids were determined.

Biacore analysis. Real time monitoring of protein interactions was performed at 25 °C using the BIAcore 1000 biosensor according to the manufacturer's instructions. Synthetic anginex was mobilized on a CM5 sensor chip using the Amine Coupling Kit with a target resonance level of 4000 RU. For interaction analysis, proteins diluted in HBS-EP (0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant p20) were injected at a flow rate of 10 μl/min after which the flow cells were regenerated by injection of regeneration buffer (10 mM glycine–HCl, pH 2.0) at a flow of 10 μl/min. Data were analyzed using the BIAevaluation software (version 3.0).

Circular dichroism. For CD measurements, freeze-dried synthetic or recombinant anginex was dissolved in 10 mM potassium phosphate buffer, pH 5.2, at a concentration of 0.1 mM. CD spectra were recorded on a Jasco J-710 spectropolarimeter (Jasko, Easton MD) from 190 to 250 nm using a 0.1 mm path-length thermally jacketed quartz cuvette maintained at room temperature. Acquisition was performed using a 0.1 nm step resolution, 100 nm/min scan speed, and a 1.0 nm bandwidth. The response time was 2 s, and the sensitivity was 100 mdeg. Reported spectra are averages of six scans.

Cell proliferation. Human umbilical vein ECs (HUVECs) were harvested from normal human umbilical cords and cultured in

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