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Anti-angiogenesis and anti-tumor activity of recombinant anginex ☆

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Abstract

Anginex, a synthetic 33-mer angiostatic peptide, specifically inhibits vascular endothelial cell proliferation and migration along with induction of apoptosis in endothelial cells. Here we report on the *in vivo* characterization of recombinant anginex and use of the artificial anginex gene for gene therapy approaches. Tumor growth of human MA148 ovarian carcinoma in athymic mice was inhibited by 80% when treated with recombinant anginex. Histological analysis of the tumors showed an approximate 2.5-fold reduction of microvessel density, suggesting that angiogenesis inhibition is the cause of the anti-tumor effect. Furthermore, there was a significant correlation between the gene expression patterns of 16 angiogenesis-related factors after treatment with both recombinant and synthetic anginex. To validate the applicability of the anginex gene for gene therapy, stable transfectants of murine B16F10 melanoma cells expressing recombinant anginex were made. Supernatants of these cells inhibited endothelial cell proliferation *in vitro*. Furthermore, after subcutaneous injection of these cells in C57BL/6 mice, an extensive delay in tumor growth was observed. These data show that the artificial anginex gene can be used to produce a recombinant protein with similar activity as its synthetic counterpart and that the gene can be applied in gene therapy approaches for cancer treatment.
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The development of several angiogenesis inhibitors has been hindered by difficulties in their production and pharmacokinetics [1]. Some of these limitations might be solved using a gene therapy approach whereby the angiostatic proteins are expressed *in vivo*. The major advantage of this approach is that the therapeutic agent is produced locally in a high concentration and for a sustained period, thereby avoiding the problems encountered with administration of recombinant proteins, antibodies, or drugs [2].

We have previously described the designed angiostatic peptide anginex [3]. The structure of this synthetic peptide is based on the 3-dimensional β-sheet structures of the α-chemokines platelet factor 4 (PF4) and interleukin-8 (IL-8). Anginex inhibits angiogenesis through preventing proliferation and migration of activated endothelial cells and by inducing apoptosis in these cells [4,5]. Recently, we designed an artificial gene encoding anginex and validated the production and isolation of the recombinant protein from *Pichia pastoris* culture supernatant. The recombinant protein was shown to have comparable angiostatic properties as the synthetic peptide [6].

In the current study we set out to determine the therapeutic effect of recombinant anginex in an *in vivo* tumor model and to analyze the potential of the anginex gene for gene therapy applications. Treatment of an ovarian

☆ *Abbreviations:* CAM, chorioallantoic membrane; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; PF4, platelet factor 4; r-anginex, recombinant anginex; IL8, interleukin 8.

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carcinoma model in mice with recombinant anginex resulted in a strong inhibition of tumor growth and a similar angiostatic activity compared to synthetic anginex. Introduction of the anginex gene in mouse melanoma cells induced an extensive delay in tumor growth following injection into mice.

Taken together, our data show that the artificial anginex gene can be used to produce a recombinant protein with similar activity as its synthetic counterpart and that the gene can be applied in gene therapy approaches for cancer treatment.

Materials and methods

Expression and isolation of recombinant anginex. Expression and isolation of recombinant anginex was performed as described previously [6]. In short, 1L BMGY was inoculated with a 50 ml overnight culture of *P. pastoris* strain GS115 (Invitrogen) transformed with pPICZα-A-anginex and cultured for 24 h at 30 °C with shaking. These cells were harvested and resuspended in 2 L BMMY in order to induce expression. After 72 h, supernatant was collected and concentrated using Centricon-plus 80 biomax5 concentrators (Millipore). His-tagged recombinant anginex was isolated from the concentrated supernatant with His-select beads (Sigma). After dialysis against water and concentrating the recombinant anginex, protein concentration was measured using the micro BCA protein assay reagent kit (Pierce).

MA148 ovarian carcinoma xenograft mouse model. The ovarian carcinoma xenograft model was used as described previously [7]. Anti-angiogenesis therapy using either synthetic anginex or recombinant anginex was started 25 days after subcutaneous injection of 2×10^6 MA148 cells in the flank of 21 athymic (nu/nu) mice. Mice were randomly divided into three groups. Synthetic anginex (10 mg/kg), recombinant anginex (equimolar to synthetic anginex), and PBS (control) were IP injected every three days. Tumor growth was measured throughout the experiment and tumor volumes were calculated as $\text{width}^2 \times \text{length} \times 0.52$. Mice were sacrificed 15 days after start of treatment. Tumors were quickly dissected and snap-frozen in liquid nitrogen. Animal experiments were approved by the local Ethical Committee.

B16F10 melanoma mouse model .The B16F10 melanoma mouse model was used as described previously [5]. Five C57BL/6 mice were subcutaneously injected on the right flank with 100,000 stably transfected B16F10 melanoma cells (see below). Tumor growth was measured throughout the experiment and tumor volume was calculated as $\text{width}^2 \times \text{length} \times 0.52$. The experiment was stopped at day 25 when only two mice had palpable tumor. Tumors were dissected and quickly snap-frozen in liquid nitrogen. Tumor growth was compared to historical growth curves of B16F10 and B16F10 treated with synthetic anginex, since these tumors and their response to treatment with synthetic anginex are very comparable between different experiments.

Immunohistochemistry. Approximately 50% of each tumor was fixed in paraformaldehyde and subsequently paraffin embedded. Deparaffinized tissue sections were immunostained with monoclonal antibodies from the 9F1 rat monoclonal cell-line (Generous gift from A. Duijvestijn) specific for mouse EC [8]. Before application of the primary antibody, sections were blocked for endogenous peroxidase activity by 0.3% hydrogen peroxide in methanol for 20 min. For antigen retrieval, sections were microwaved with 10 mM Tris/1 mM EDTA buffer, pH 8.0. Primary antibody was incubated for one hour, and secondary (donkey anti-rat-Ig-biotin; Dako) and tertiary antibody (Streptavidin ABC complex HRP; Dako) were incubated for 30 min. Antibody localization was visualized by incubation with diaminobenzidine. Sections were counterstained with hematoxylin. Quantification of microvessel density (MVD) was determined in the complete section, corrected for the size of the section.

RNA isolation and cDNA synthesis. Total RNA was isolated from homogenized tumor samples using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer manual. To prevent contamination with genomic DNA, the on-column DNase treatment as suggested by the supplier was applied. RNA concentration and purity were determined using a Nanodrop spectrophotometer. cDNA synthesis was performed using 1 μg total RNA and the iScript cDNA synthesis kit (Bio-Rad) as described in the supplier's manual.

Real-time quantitative PCR. For real-time quantitative PCR, the I-cycler IQ system (Bio-Rad) was employed using Sybr Green chemistry. All tumors were analyzed for 16 angiogenesis factors (bFGF, PLGF, VEGF-A/B/C/D, VEGFR1/2/3, Tie1/2, Nrp1/2, and Ang1/2/3) with primers (Eurogentec) specific for human as well as mouse transcripts as described previously [9]. Cyclophilin A was used as reference gene. Conditions for PCR were 10 min at 95 °C, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The PCR was performed in a 25 μl reaction volume containing 1 × IQ Sybr green supermix (Bio-Rad), 25 ng cDNA, and 800 nM forward and reverse primers. To compare the different relative expression levels between treatments, the 'delta–delta Ct method' was applied. All experiments were performed in triplo.

Cloning of pcDNA3.1-anginex. Anginex was PCR amplified from the previously described anginex gene [6] using the following primers: forward TAGGTACCACCATGGCAAACATAAAACTAAGCGTAC, reverse TA TCTAGACTAGTCTAGGCTTAGTTCTCTTCC. The amplified anginex fragment was digested with *Kpn*I and *Xba*I (NEB), purified and cloned into the expression vector pcDNA3.1+-hygro (Invitrogen). The obtained pcDNA3.1-angx vector was sequence verified using a T7 promoter universal primer.

Generation of a stable anginex expressing B16F10 melanoma cell line. B16F10 melanoma cells were cultured as described previously [5]. Empty vector was used as control. Before transfection, vectors were linearized using restriction enzyme *Fsp*I (NEB). Cells were transfected in a 24-well format with a DNA:lipid ratio of 2:3 using Fugene (Roche) according to the manufacturer instructions. The next day, selective pressure was applied using 150 μg/ml hygromycin B (Roche). After confirmation of the identity of the cell line, using RT-PCR with primers specific for the vector (Forward TAGAAGGCACAGTCGAGG, reverse TAATACGACTCACT ATAGGG), a clonal cell line was made by limited dilution.

Cell proliferation. Human umbilical vein EC (HUVEC) were harvested from normal human umbilical cords and cultured in fibronectin-coated flasks in HUVEC culture medium (RPMI1640 with 20% (v/v) human serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured at 37 °C and 5% CO_2. For the proliferation assay, HUVEC were seeded at 5000 cells/well in fibronectin-coated flat-bottomed 96-well tissue culture plates and grown for three days in supernatant of the cell lines supplemented with 1 ng/ml bFGF. On the third day, 0.3 μCi/well [^{3}H]thymidine was added and incorporation was allowed to occur for 6 h. After harvesting the cells, thymidine incorporation was quantified by liquid scintillation counting.

Statistical analysis. Tumor volumes are given as mean values ± SD. The effect of treatment in the mouse tumor model was analyzed by 2-way ANOVA . Microvessel density and proliferation data were analyzed using the Student's *t*-test. Correlations were determined using Spearman's rho. p-values < 0.05 were considered significant and all statistical calculations were performed using Prism 3.0 software.

Results and discussion

Tumor growth is strongly dependent on the formation of new blood vessels, i.e., angiogenesis [10]. The discovery of angiogenesis inhibitors has confirmed the feasibility of angiogenesis inhibition for cancer treatment as postulated by Dr. Folkman in the early 1970s [11]. Unfortunately, the development of several promising angiogenesis inhibitors has been hampered by difficulties in production and activity [1]. Some of these limitations might be solved using a gene therapy approach whereby the angiostatic proteins

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