

Recombinant angioarrestin secreted from mouse melanoma cells inhibits growth of primary tumours

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Angioarrestin is a recently described anti-angiogenic protein whose expression is down-regulated in solid tumours of various origins. It has a sequence identical to angiopoietin related protein-1. In this study we investigated anti-tumour properties of angioarrestin in B16 (F10) melanoma tumour model. We constructed an expression vector encoding human angioarrestin under the control of EF-1 α promoter. This vector was transferred to B16 (F10) cells and recombinant angioarrestin secreted from the transfected cells was tested for anti-angiogenic activity using endothelial cell proliferation assay. Finally, mice were injected subcutaneously with cells that had been transfected with either angioarrestin-encoding vector or empty vector and tumor growth was compared. The obtained recombinant angioarrestin inhibited proliferation of bovine aortic endothelial cells. Tumours derived from an angioarrestin-secreting B16 (F10) cell clone grew *in vivo* more slowly than tumours derived from a cell clone transfected with empty vector. These data show, to our knowledge for the first time, that angioarrestin can inhibit primary melanoma tumour growth.

Keywords: angioarrestin, anti-angiogenic factor, melanoma

Angiopoietins are recently discovered growth factors specific for endothelial cells (Davis *et al.*, 1996). Although the molecular mechanism through which these proteins regulate angiogenesis has not been fully elucidated, there is ample evidence of their participation in tumour angiogenesis. Various angiopoietins share similar molecular structure; they consist of a signal peptide, a coiled-coil domain enabling oligomerisation (Procopio *et al.*, 1999; Suri *et al.*, 1996) and a C-terminal fibrinogen-like domain which binds to Tie2 receptors (Maisonpierre *et al.*, 1997; Valenzuela *et al.*, 1999). A similar structure has been described for angiopoietin related proteins (ARP1, ARP2 and CDT6). However, these proteins do not bind to Tie2 receptors (Kim *et al.*, 1999; 2000).

Angioarrestin is a recently described anti-angiogenic protein (Dhanabal *et al.*, 2002). It is 491 amino acids long and has a sequence identical to angiopoietin related protein-1 found earlier. The mRNA expression profile of angioarrestin is strikingly down-regulated in tumour tissue, in comparison to

matched non-tumour adjacent tissue (Dhanabal *et al.*, 2002). It was also reported that human HT1080 fibrosarcoma cells engineered to ectopically express angioarrestin showed reduced tumorigenicity; after intravenous injection of such cells nude mice showed a marked reduction in the number and size of tumour nodules (Dhanabal *et al.*, 2002). Here, we report an anti-tumour effect of recombinant angioarrestin in syngenic B16 (F10) mouse tumour model. We show that primary melanoma tumours derived from angioarrestin-secreting cells grow more slowly *in vivo* compared to tumours derived from a cell clone transfected with empty vector.

MATERIALS AND METHODS

Plasmid construction. A DNA fragment encoding human angioarrestin was obtained by PCR with liver cDNA as a template. The cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA), oligo-dT primer and

Abbreviations: BAE cells, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; M-MLV, Moloney murine leukemia virus; VEGF, vascular endothelial growth factor.

human foetal liver total RNA template (Stratagene, La Jolla, CA, USA). For PCR *Platinum Pfx* polymerase (Invitrogen) and the following oligonucleotide primers were used: downstream 5'-TTT TTA GAT CTA AAA TGA AGA CTT TTA CCT GGA C-3' and upstream 5'-AAA AAG CTA GCA GGC TTG ATC ATC ATC TGA ACT-3'. The downstream and upstream primers were designed to include *Bgl*III and *Nhe*I sites, respectively. The amplified fragments were digested with *Bgl*III and *Nhe*I and ligated into pEF1/V5-HisA expression vector (Invitrogen, Carlsbad, CA, USA) digested with *Bam*HI and *Xba*I. The pEF1/V5-HisA plasmid contains the promoter from human elongation factor-1 α gene. The resultant recombinant protein is tagged at its C-terminus with both V5 epitope and a 6His fragment.

The inserts' nucleotide sequence was confirmed by sequencing using ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq Polymerase (Applied Biosystems, Foster City, CA, USA). Both insert strands were sequenced and the obtained sequence was compared to the sequence NM_004673 from GenBank. No mutation or polymorphisms were found.

Transfection and preparation of cell-conditioned medium. B16 (F10) murine melanoma cells (from American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (ICN Pharmaceuticals, Irvine, CA, USA). The plasmid pEF1/V5-HisA/ARR or pEF1/V5-HisA parental plasmid were transfected into B16 (F10) cells using *Lipofectamine 2000* (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfectants were selected and grown in medium containing G418 (1 mg/mL). One of the clones transfected with the angioarrestin-encoding vector and one clone with empty vector were used in further experiments. Cells were plated at a density of 5×10^3 /well of a 24-well culture dish. At various time-points after plating (24, 48 and 72 h) cells were incubated with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) from Sigma (St. Louis, MO, USA). The resulting formazan was solubilised with 0.04 M HCl in isopropanol and absorbance was determined at 570 nm using a microplate absorbance reader. Conditioned medium was collected from parental B16 (F10) cells and stably transfected cell clones grown in T-75 bottles (NUNC, Wiesbaden, Germany). Sub-confluent cells were washed with RPMI 1640 without serum and were incubated in this medium for 48 h. Cell-conditioned medium was collected, centrifuged to remove cell debris and stored at -70°C until use. The medium was then concentrated and desalted with de-ionized water using Ultrafree-15 centrifugal filter device 10000 MWCO (Millipore Co., Bedford, MA, USA). Protein concentration was determined using

Bio-Rad protein microassay kit (Bio-Rad, Hercules, CA, USA).

Western blot. Samples of concentrated media conditioned by parental and stably transfected B16 (F10) cells were electrophoresed in 8% polyacrylamide gel containing SDS and transferred onto nitrocellulose membrane (Schleicher & Schuell, Germany). The membrane was incubated in a blocking buffer (TBS/3% skim milk) for 1 h at room temperature and then incubated at 4°C overnight with monoclonal antibodies against V5 epitope (Invitrogen, Carlsbad, CA) diluted 1:5000 with TBS/3% skim milk. On the following day the membrane was washed four times with TBS/0.1% Tween 20 and then incubated for 75 min at room temperature with horse anti-mouse IgG biotin conjugate (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 with TBS/1% skim milk. After four washes in TBS the membrane was incubated for 60 min at room temperature with streptavidin biotinylated horseradish peroxidase complex (Amersham, Aylesbury, UK) diluted 1:1000. The peroxidase substrate used for band staining was 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA).

Endothelial cell proliferation assay. The assay was performed as described previously (Wilczyńska *et al.*, 2001). Briefly, 5×10^3 BAE cells per well of gelatinized 24-well plate were plated in RPMI/5% fetal calf serum (FCS) and incubated for 2 h. The medium was replaced with 0.5 ml fresh RPMI containing 2% FCS and samples of concentrated conditioned media. After 1-h incubation 0.5 ml medium containing 2% FCS and 10 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA) was added. After 72 h the number of living cells was quantified using MTT assay.

Animal studies. Six- to eight-week-old C57Bl/6 mice (females) had their left dorsal side shaved and were inoculated subcutaneously with B16 (F10) cells. To assess the *in vivo* growth of stably transfected B16 (F10) cell clones, mice were injected with 5×10^5 cells (per animal) containing either the pEF1/V5-HisA/ARR plasmid or the pEF1/V5-HisA parental plasmid. Cells were suspended in 100 μl of phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} . Tumours were measured with a caliper gauge three times a week. Tumour volume was calculated from the following formula: Tumour Volume = (Width)² \times Length \times 0.52.

RESULTS

A cDNA fragment encoding angioarrestin was cloned into pEF1/V5-HisA expression vector under the transcriptional control of elongation factor-1 α promoter (Mizushima & Nagata, 1990; Salomon *et al.*, 2000). The recombinant protein was tagged with

V5 epitope and 6xHis fragment. Using Western blot we obtained two protein bands about 60 and about 64 kDa under reducing conditions and 3 bands about 130, about 180 and about 220 kDa under non-reducing conditions (Fig. 1). Our data suggest that recombinant angioarrestin is secreted from transfected B16 (F10) cells as disulphide-linked multimers.

To confirm the anti-angiogenic properties of recombinant angioarrestin we performed endothelial cell proliferation assay. Statistically significant inhibition of BAE cell proliferation was observed when cells were treated with concentrated media from angioarrestin-secreting cells when compared to media conditioned by empty vector transfected cells (Fig. 2). The effect is dose-dependent. The highest dose (350 µg of protein/ml medium) gives a 61% inhibition. These data indicate that the obtained recombinant angioarrestin significantly inhibits bFGF-stimulated proliferation of endothelial cells.

Tumours derived from an angioarrestin-secreting B16 (F10) cell clone grew more slowly *in vivo* than tumours derived from a cell clone transfected with empty pEF1/V5-HisA vector (Fig. 3A). However, the *in vitro* proliferation rate of these clones was equal (Fig. 3B). A statistically significant difference in primary tumour growth was observed starting from the 15th day of the experiment ($P < 0.04$; Mann-Whitney *U*-test). There were no statistically significant differences between the control groups. These data indicate that human angioarrestin se-

creted from transfected B16 (F10) cells significantly inhibits growth of primary melanoma tumours.

DISCUSSION

We show that recombinant human angioarrestin tagged with V5 and 6xHis epitopes is secreted from transfected B16 (F10) cells as disulphide-linked dimers. Similar results were shown for angioarrestin expressed in human HT1080 fibrosarcoma cells (Dhanabal *et al.*, 2002). Under reducing conditions we also obtained two bands of monomer protein (about 60 and about 64 kDa). This can be a result of alternative posttranslational modification of angioarrestin. Analysis of angioarrestin amino-acid sequence using NetNGlc 1.0 software revealed that angioarrestin contains two sites of potential N-glycosylation but only one of these sites is predicted to be N-glycosylated with high (77%) probability. Under non-reducing conditions we observed three high molecular mass bands (about 130, about 180 and about 220 kDa). Most probably, the about 130 kDa protein is a disulphide-linked dimer (Dhanabal *et al.*, 2002). The bands about 180 and about 220 kDa may be tri- and tetramers (Procopio *et al.*, 1999; Peek *et al.*, 2002) or may result from angioarrestin binding by other proteins present in conditioned media.

Dhanabal *et al.* (2002) have reported that angioarrestin blocks VEGF/bFGF-mediated endothelial cell proliferation, migration, tube formation and adhesion to extracellular matrix proteins. Using the pooled transfected HT1080 cells they also demonstrated that ectopic expression of angioarrestin inhibited tumour nodule formation. In our animal study tumours derived from angioarrestin-transfected B16 (F10) cells were significantly smaller (in-

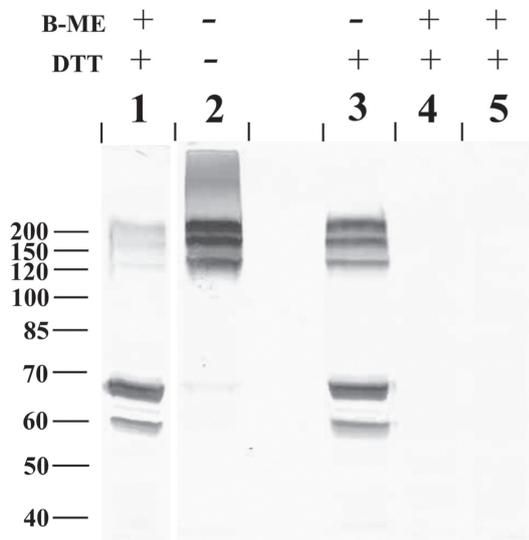


Figure 1. Identification of angioarrestin secreted by stably transfected B16 (F10) cells (Western blot).

An aliquot of concentrated conditioned medium (10 µg of total protein) was electrophoresed under non-reducing conditions or in loading buffer containing 75 mM dithiothreitol (DTT) and/or 2.5% β-mercaptoethanol (B-ME). Proteins were electrophoresed and immunoblotted with monoclonal antibodies against V5 epitope. Lanes 1, 2, 3, B16 (F10)/pEF1/V5-HisA/ARR; lane 4, B16 (F10)/pEF1/V5-HisA; lane 5, non-transfected B16 (F10).

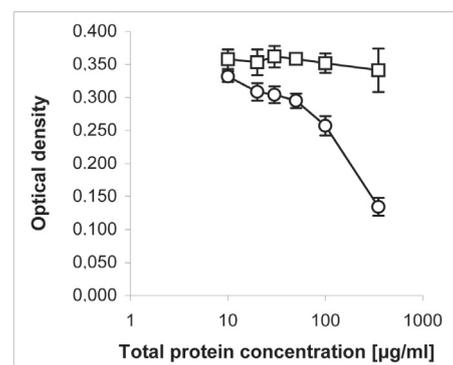


Figure 2. Inhibition of bFGF-induced proliferation of bovine aortic endothelial (BAE) cells.

BAE cells were grown in media containing concentrated proteins secreted by B16(F10) cell clones transfected with pEF1/V5-HisA/ARR (○) or pEF1/V5-HisA empty vector (□). Cell proliferation was quantified by the MTT assay. Results are shown as means of triplicate wells ±S.D. ($P < 0.05$ Mann-Whitney *U*-test).

hibition of tumour growth was from 40% up to 60% when compared with control). The proliferation rate *in vitro* of both transfected cell clones was identical. In addition, we did not observe increased survival time in the group of mice injected with angioarrestin-secreting cells, compared to control. However, *in vitro* transfer of angioarrestin cDNA into B16 (F10) cells reduces tumour growth but does not produce dormancy of the primary melanoma tumours. We obtained similar results previously using renal carcinoma cells transfected with mouse endostatin (Szary & Szala, 2001). Probably, the level of angioarrestin

expression is not sufficient to completely arrest primary tumour growth; this might be related to the loss of the transgene during tumour growth and/or promoter methylation. It is also possible that as primary tumour grows VEGF secretion from tumour cells and macrophages (Brown *et al.*, 1997; Damert *et al.*, 1997) induced by hypoxia may oppose the anti-angiogenic effect of angioarrestin. Hypoxia induces also overexpression of VEGF receptor gene (Forsythe *et al.*, 1996) and this phenomenon is important for growth and metastases of melanoma tumours (Rofstad & Danielsen, 1998).

In summary, our data confirm the anti-angiogenic and anti-tumour activity of angioarrestin.

Acknowledgements

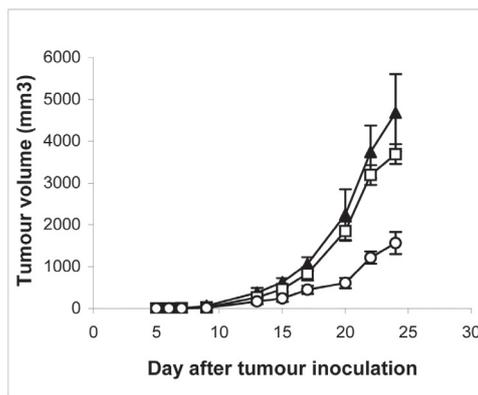
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A



B

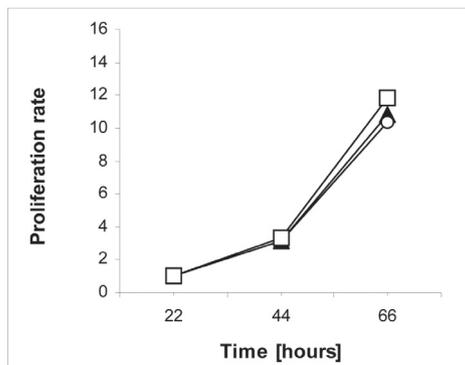


Figure 3.

A. Tumour growth in syngenic mice injected with angioarrestin-transfected cells (○), empty vector-transfected cells (□), or parental B16 (F10) cells (▲).

All experimental groups consisted of six mice. Values represent mean tumour volume \pm S.D. The difference between tumour volumes in both groups was statistically significant from day 15 of the experiment ($P < 0.04$ Mann-Whitney *U*-test). These are representative results from one out of two experiments.

B. *In vitro* proliferation rate of B16 (F10) cell clone stably transfected with empty pEF1/V5-HisA (□), pEF1/V5-HisA/ARR vector (○) or parental B16(F10) cells (▲).

The number of cells after 22 h was taken as one unit. The proliferation rate shown is the mean of quadruplicate samples. The S.D. never exceeded 5% of the reported values. There were no statistically significant differences between these groups (Mann-Whitney *U*-test).

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