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Chimeric protein ABRaA-VEGF$_{121}$ is cytotoxic towards VEGFR-2-expressing PAE cells and inhibits B16-F10 melanoma growth

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It has been known that VEGF$_{121}$ isoform can serve as a carrier of therapeutic agents targeting tumor endothelial cells. We designed and constructed synthetic cDNA that encodes a chimeric protein comprising abrin-a (ABRaA) toxin A-chain and human VEGF$_{121}$. Expression of the ABRaA-VEGF$_{121}$ chimeric protein was carried out in *E. coli* strain BL21(DE3). ABRaA-VEGF$_{121}$ preparations were isolated from inclusion bodies, solubilized and purified by affinity and ion-exchanged chromatography (Ni-agarose and Q-Sepharose). Finaly, bacterial endotoxin was removed from the recombinant protein. Under non-reducing conditions, the recombinant protein migrates in polyacrylamide gel as two bands (about 84 kDa homodimer and about 42 kDa monomer). ABRaA-VEGF$_{121}$ is strongly cytotoxic towards PAE cells expressing VEGFR-2, as opposed to VEGFR-1 expressing or parental PAE cells. The latter are about 400 times less sensitive to the action of this fusion protein. The biological activity of the ABRaA domain forming part of the chimeric protein was assessed *in vitro*: ABRaA-VEGF$_{121}$ inhibited protein biosynthesis in a cell-free translation system. Preincubation of ABRaA-VEGF$_{121}$ with antibody neutralizing the biological activity of human VEGF abolished the cytotoxic effect of the chimeric protein in PAE/KDR cells. Experiments *in vivo* demonstrated that ABRaA-VEGF$_{121}$ inhibits growth of B16-F10 murine melanoma tumors.

Keywords: chimeric protein, abrin-a A-chain, VEGF$_{121}$, melanoma B16-F10

INTRODUCTION

Vascular endothelial growth factor (VEGF A) is a major regulator of several endothelial cell functions. It plays a central role in tumor angiogenesis and is frequently targeted in anticancer therapies (Ferrara & Kerbel, 2005). Isoforms of this cytokine exert their effect through interaction with receptor tyrosine kinases Flt-1/FLT (VEGF receptor-1 (VEGFR-1)), Flk-1/KDR (VEGFR-2) and isoform-specific coreceptors neuropilin-1 and neuropilin-2 (Hoeben *et al*., 2004; Pan *et al*., 2007). In many tumors vascular endothelium strongly expresses VEGFR-2 and/or VEGFR-1, as opposed to endothelium lining normal vessels (Brown *et al*., 1998; Veikkola *et al*., 2000; Ran *et al*., 2003; Shibuya, 2006). VEGFR-1 binds VEGF A with higher affinity than VEGFR-2, while VEGFR-2, upon binding of the ligand, is phosphorylated much more efficiently; both regulate tumor angiogenesis *via* different mechanisms (Shibuya, 2006). It is generally agreed that VEFGR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF A (Kowanets & Ferrara, 2006).

Among the novel approches to antitumor therapeutic strategy one is based on various engineered chimeric proteins combining toxic agents

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Abbreviations: ABRaA, A chain of abrin-a toxin; DTT, dithiothreitol; hFlt-1, human Fms-like tyrosine kinase receptor (human VEGFR-1); IPTG, isopropyl β-D-1-thiogalactopyranoside; KDR, kinase insert domain receptor (human VEGFR-2); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; VEGF, vascular-endothelial growth factor; VEGFR, VEGF receptor.

linked to a carrier (growth factors, immunoglobulins), the latter allowing specific targeting of various molecules involved in the VEGF pathway (Thorpe, 2004; Szala, 2004). The $VEGF_{121}$ isoform, which is secreted as a freely difusible non-heparin-binding protein, has been successfully used to deliver toxins to the tumor vascular endothelium. Such chimeric toxins using the $VEGF_{121}$ as a targeting domain mediate their cytotoxicity primarily through VEGFR-2 (Arora *et al*., 1999; Backer *et al*., 2001; Veenendaal *et al*., 2002; Liu *et al*., 2003**).**

Abrin is a potent plant toxin belonging to the type II family of eukaryotic ribosome-inactivating proteins (Narayanan *et al*., 2004). Many poisoning features of abrin observed in mammals can be explained by endothelial cell damage leading to increased capillary permeability and vascular leak syndrome (Dickers *et al*., 2003) as well as apoptotic changes in lymphoid tissues and intestine (Griffiths *et al*., 1987). Abrin can inhibit tumor growth and prolong survival of mice in several experimental tumor models (Fodstad *et al*., 1977). Abrin is capable of inducing cell apoptosis by various mechanisms (Shih *et al*., 2001; Ohba *et al*., 2004; Qu & Qing, 2004).

Abrin is a heterodimeric protein comprising enzymatic A chain coupled, *via* a single disulphide linkage, with cell-binding (lectin) B chain (Olsnes & Pihl, 1973; Olsnes *et al*., 1975; Narayanan *et al*., 2004). The A chain of abrin-a isoform (ABRaA) is a specific *N*-glycosidase which causes irreversible inactivation of eukaryotic ribosomes by adenine depurination at position 4324 of 28S rRNA (Endo *et al*., 1987; Wang *et al*., 2004). ABRaA is over 10^5-fold less cytotoxic towards HeLa cells than abrin holotoxin (disulphide-linked A and B chains) (Chow *et al*., 1999) and, following intravenous injection, does not elicit significant toxic effects in Guinea pig (Hwang *et al*., 1984). ABRaA has also been used in developing tumor-targeted toxin-antibody therapeutic conjugates (Hwang *et al*., 1984; Wawrzynczak *et al*., 1990). Compared with immunotoxins containing other ribosome-inactivating proteins (ricin A, gelonin, momortin), ABRaA immunotoxins show longer serum half-life and, when present in the circulation, retain full cytotoxic activity (Wawrzynczak *et al*., 1990). Nevertheless, the use of immunotoxins in cancer treatment is limited because of their poor ability to permeate solid tumors (Pastan *et al*., 2007). Chimeric toxins containing $VEGF_{121}$ thus seem to be good candidates for cancer therapeutic agents as they are able to indirectly destroy tumors by selective disruption of tumor vascular endothelium (Veenendaal *et al*., 2002).

The aim of our study was to generate and purify the ABRaA-$VEGF_{121}$ chimeric protein, to test its properties *in vitro* and to verify the expected antitumor properties in treating experimentally induced B16-F10 murine melanoma.

MATERIALS AND METHODS

Human $VEGF_{121}$ gene synthesis and construction of expression vector encoding ABRaA-$VEGF_{121}$. Human $VEGF_{121}$ coding sequence without a signal sequence was optimized with the help of Prot-2-DNA software (Gustafsson *et al*., 2004) in order to eliminate rare codons and to achieve effective translation in *Escherichia coli*. Synthetic $VEGF_{121}$ gene was constructed from oligonucleotides (BioTez Berlin-Buch GmbH, Berlin, Germany) (Table 1) as described by Mitrus *et al*. (2005), with minor changes. The long oligonucleotides #1–#9 yield a $VEGF_{121}$ coding sequence with *Bam*HI and *Hin*dIII restriction sites and five additional adenines. The oligonucleotides #2–#9 were phosphorylated and oligonucleotide #1 was then added to the reaction mixture and ligated in the presence of the short oligonucleotides #1–2 to #8–9, which should have assured the correct order of linking of the long oligonucleotides #1–#9. The $VEGF_{121}$ cDNA was amplified by PCR using *Taq* DNA Polymerase (Fermentas Inc., Hanover, MD, USA), primer #1 and reverse primer (Table 1) and cloned into pET41a(+) (Novagen, San Diego, CA, USA) using *Bam*HI and *Hin*dIII restriction sites, which generated the pET41/$VEGF_{121}$ construct.

Codon 121, gat (Asp) in the original ABRaA sequence (GenBank accession number AY458627), was replaced with a synonymous codon (gac) in order to remove the *Bam*HI restriction site. A sequence encoding the G_4S linker was added to the 3′ end. The two terminal amino acids of the linker are encoded by the ggatcc sequence, which is also a *Bam*HI restriction site serving to ligate $VEGF_{121}$. A 43-bp fragment of pET41a(+) plasmid, spanning from *Bgl*II site to the enterokinase-recognized site (ER), was added at the 5′ end of the ABRaA coding sequence, and this coding cDNA sequence (ER-ABRaA-G_4S) was synthesized by GenScript Corporation (Piscataway, NJ, USA). The sequence was subsequently cloned, using *Bgl*II and *Bam*HI restriction sites, into the GST-His-S·Tag-EK reading frame of the pET41/$VEGF_{121}$ vector, resulting in the formation of pET41/ABRaA-$VEGF_{121}$+Tag construct. In order to remove additional coding sequences of plasmid origin (GST-His-S·Tag-EK), the ABRaA amino end (223 bp) up to the *Nde*I restriction site was amplified by PCR (Pfx Platinum Polymerase, Invitrogen, Carlsbad, CA, USA) using L(AV) and R(AV) starters (Table 1) and cloned into the pET41/ABRaA-$VEGF_{121}$+Tag plasmid using *Nde*I restriction site present in the plasmid and in ABRaA (construct: pET41/ABRaA-$VEGF_{121}$, without tag). Next, the *Xba*I/*Xho*I fragment from pET41/

Table 1. Oligonucleotides used to obtain synthetic cDNA encoding VEGF$_{121}$ and as PCR primers. *Bam*HI and *Hin*dIII restriction sites, used to clone VEGF$_{121}$ into pET41a(+) vector, are underlined

Primer	Oligonucleotides sequence
#1	aaaaaggatccgccccgatggcggaaggtggtggtcaaaacca
#2	tcacgaggtagtcaaatttatggacgtttaccagcgctcttat
#3	tgccacccgatcgaaacgctggttgatattttccaggaatatc
#4	cggatgaaatcgaatacattttcaaaccgtcttgtgtcccact
#5	gatgcgctgtggtggctgctgcaatgacgagggcctggagtgc
#6	gttccaaccgaagaatccaatattacgatgcaaattatgcgta
#7	ttaaaccgcaccaaggccaacacatcggtgaaatgtctttcct
#8	gcagcacaacaaatgtgaatgtcgcccgaagaaagaccgtgca
#9	cgccaggaaaagtgtgacaagccgcgtcgttaaaagcttaaaaa
#1-2	cctcgtgatggttttg
#2-3	gggtggcaataagagc
#3-4	ttcatccggatattcc
#4-5	agcgcatcagtgggac
#5-6	gttggaacgcactcca
#6-7	cggtttaatacgcata
#7-8	tgtgctgcaggaaaga
#8-9	tcctggcgtgcacggt
reverse	tttttaagcttttaacgacgcggcttgtcacacttttcctggcg
L(AV)	tttttcatatggaagatcgcccgatcaaattt
R(AV)	aataggactgggtgcc

ABRaA-VEGF$_{121}$ plasmid was cloned into pET32b(+) vector (Novagen, San Diego, CA, USA), which gave the pET32/ABRaA-VEGF$_{121}$ plasmid encoding the chimeric protein. Sequencing of the expression plasmid insert was performed using ABI PRISM Big Day Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Expression, isolation and purification of recombinant ABRaA-VEGF$_{121}$ protein. The *E. coli* BL21(DE3) strain (Novagen, San Diego, CA, USA) transformed with pET32/ABRaA-VEGF$_{121}$ plasmid was grown in LB broth (BD Bioscience, Franklin Lakes, NJ, USA) with 100 μg/ml ampicillin (Sigma, St. Louis, MO, USA) to OD_{600}=0.8–1.0. Protein expression was induced using IPTG (Fluka, Buchs, Switzerland) at a final concentration of 0.5 mM and cells were further grown for additional 3 h at 37°C. Bacterial pellets were harvested by centrifugation (45 000 × *g*, 30 min, 4°C) and stored at –20°C.

The pellet (1 g) was resuspended in 2.5 ml of buffer (100 mM Tris/HCl, 25% sucrose, 0.1% sodium azide and 10 mM DTT, pH 8.0) and lysed by sonication. Next, 2.5 ml of buffer (100 mM Tris/HCl, 1% Triton X-114, 1% sodium deoxycholate, 100 mM NaCl, 0.1% sodium azide and 10 mM DTT, pH 8.0) and lysozyme (Sigma, St. Louis, MO, USA) (12 kU/ml lysate) were added and the solution mixed until viscous. DNase I (Fermentas Inc., Hanover, MD, USA) (20 U/ml lysate) and $MgCl_2$ (final concentration=10 mM) were added and the cell lysate was allowed to stand for 30 min at room temperature until no longer viscous. Insoluble material containing inclusion bodies (IBs) with ABRaA-VEGF$_{121}$ was pelleted by centrifugation (30 000 × *g*, 30 min, 20°C). IBs were washed six times in buffer (50 mM Tris/HCl, 1% Triton X-114, 1 mM EDTA, 300 mM NaCl, 0.1% sodium azide, pH 8.0).

The IBs were solubilized in buffer (100 mM Tris/HCl, 2 M urea, 5% sucrose, 1 mM EDTA, pH 8.0) at 4°C for 6 h. Insoluble material was removed by centrifugation (25 000 × *g*, 20 min, 4°C). Denatured recombinant proteins were refolded by gradual dialysis in the presence of reduced and oxidized glutathione. Refolded protein solution was passed through a depyrogenated Ni-NTA column (Qiagen, Valencia, CA, USA) using manufacturer's protocol. Finally, proteins other than ABRaA-VEGF$_{121}$ were removed on a Q-Sepharose column (Sigma, St. Louis, MO, USA) equilibrated with 50 mM Na-phosphate buffer (pH 8.0). Recombinant ABRaA-VEGF$_{121}$ was eluted with 50–200 mM NaCl.

Endotoxin content in ABRaA-VEGF$_{121}$ solutions was measured using the LAL QLC 1000 kit (Cambrex, East Rutherford, NJ, USA). Removal of endotoxin (to <0.0025 EU/1 μg protein) was achieved through affinity chromatography with EndoTrap Red kit components (Profos AG, Regensburg, Germany). Before further use the samples were filter-sterilized and stored at 4°C.

The purified protein was analyzed by 10% SDS/PAGE under reducing and non-reducing conditions and stained by Coomassie blue. Western blots were performed using nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Detection of ABRaA-VEGF$_{121}$ was achieved with a mouse monoclonal antibody against human VEGF (R&D Systems, Minneapolis, MN, USA), horse anti-mouse IgG biotin conjugate (Vector Laboratories, Burlingame, CA, USA) and streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Piscataway, NJ, USA). To perform peroxidase reaction and protein visualization 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) was used as a substrate.

Cell culture. Murine melanoma cells (B16-F10) (American Type Culture Collection, Manassas, VA, USA), bovine aorta endothelial cells (BAEC) (Clonetics, San Diego, CA, USA), lymph node-derived murine endothelial cells (HECa10) (kindly provided by M. Duś from the Institute of Immunology and Experimental Therapy, Wrocław, Poland) were grown in complete RPMI 1640 medium (Gibco BRL, Paisley, United Kingdom) supplemented with 10%

fetal bovine serum (ICN Biomedicals, Costa Mesa, CA, USA). Human umbilical vein endothelial cells (HUVEC) (Clonetics, San Diego, CA, USA) were grown in gelatin-coated flasks using RPMI 1640 medium supplemented with 20% fetal bovine serum, 50 ng/ml basic fibroblast growth factor (ICN Biomedicals, Costa Mesa, CA, USA) and 100 μg/ml heparin. Porcine aortic endothelial (PAE) cells expressing full-length human KDR receptors (PAE/KDR), full-length human VEGFR-1 receptors (PAE/hFlt-1) and nontransfected PAE cells were kindly provided by L. Claesson-Welsh from the University of Uppsala (Sweden). These cells were cultured in Ham's F-12 medium (Gibco BRL, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (ICN Biomedicals, Costa Mesa, CA, USA).

***In vitro* cytotoxicity assay.** Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) colorimetric assay. Briefly, subconfluent cells were seeded in a 96-well plate (3×10^3 cells/well), grown for 24 h and then treated for 72 h with either medium containing various concentrations of ABRaA-$VEGF_{121}$ or medium with equal volume of PBS (pH 7.4) as control. Then, 100 μl MTT solution (0.5 mg/ml) was added to each well and the plate was incubated for 2.5 h at 37°C. Following supernatant removal MTT-formazan crystals formed by metabolically active (viable) cells were dissolved in 100 μl of acidic isopropyl alcohol. Absorbance at λ=570 nm was recorded using an ELX800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Neutralization of ABRaA-$VEGF_{121}$ cytotoxicity by monoclonal antibody. Subconfluent PAE/KDR cells were trypsinized and plated (3×10^3 cells/well) using 96-well plates and allowed to attach for 24 h. The growth medium was then replaced by a medium containing different concentrations of ABRaA-$VEGF_{121}$, previously preincubated (37°C, 2 h) with 2 μg/ml mouse monoclonal antibodies neutralizing the bioactivity of human VEGF (R&D Systems, Minneapolis, MN, USA). As a control, ABRaA-$VEGF_{121}$ preincubated without a monoclonal antibody was used. After 72 h, the effect of ABRaA-$VEGF_{121}$ on cell viability was determined using the MTT assay, as described above.

***In vitro* cell-free inhibition of protein biosynthesis by ABRaA-$VEGF_{121}$.** *In vitro* protein translation was performed using a cell-free rabbit reticulocyte lysate-based system (TNT® T7 Quick Coupled Transcription/Translation System, Promega, Madison, WI, USA) and the non-radioactive luciferase control reaction protocol provided with this system. The reaction mix contained 1 μg/reaction of Luciferase T7 Control plasmid and different concentrations of ABRaA-$VEGF_{121}$ protein. The translation level (bioluminescence emission) at different timepoints was measured and expressed as luciferase activity using the Luciferase Assay Substrate kit (Promega, Madison, WI, USA) and LUMAT LB9501 luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany).

TUNEL assay. Cells were seeded in gelatin-coated 8-well Lab-Tek™ II Chamber Slide plates (NUNC, Rochester, NY, USA). After 24 h either ABRaA-$VEGF_{121}$ in PBS (final concentration 420 ng/ml) or PBS (pH 7.4) was added to the media. The cell cultures were incubated for an additional 24 h and then washed briefly with ice-cold PBS. Cells were fixed for 60 min at room temp. with 4% formaldehyde, rinsed with PBS, permeabilized for 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate, and finally washed twice with PBS. Samples were then incubated for 60 min at 37°C with a TUNEL reaction mixture (*In Situ* Cell Death Detection Kit, TMR red) (Roche Diagnostics, Basel, Switzerland). To ensure positive control, cells were fixed, permeabilized and treated with DNase I (30 U/ml). TUNEL-positive cells were visualized (excitation λ = 540 ± 25 nm, emission max. λ = 580 nm) by fluorescence microscopy (Nicon Eclipse 80 equipped with Lucia v. 4.8 software) and photographed (magn. 400×).

Quantitation of cell death by annexin V and propidium iodide double staining. PAE/KDR cells were seeded (5×10^4 cells/well) in a 6-well plate (NUNC, Rochester, NY, USA) and 48 h after seeding exposed for 16 h to different concentrations of ABRaA-$VEGF_{121}$. Adherent cells harvested by mild trypsinization were pooled together with detached cells and washed with ice-cold PBS. Cells were stained with annexin V (AV) and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, San Diego, CA, USA) according to the manufacturer's protocol. Cells were counted using a BD FACSCanto flow cytometr equipped with BD FACSDiva software (BD Bioscience, San Diego, CA, USA).

Histological analysis. Histological specimens were prepared from tumors or other tissues collected from C57BL/6 mice that had been injected with the ABRaA-$VEGF_{121}$ chimeric protein (1 mg/kg body mass) either intravenously or intratumorally. The protein was administered either once on the 6th day following inoculation with cancer cells, or four times (every other day) beginning from the 6th day from inoculation. Mice were sacrificed at 48 h following final injection of the tested protein, tumor material and various tissues were collected and formaldehyde-fixed. Paraffin-embedded sections (6 μm-thick) were hematoxylin- and eosin-stained (H&E). Specimens were observed under a light microscope

(Nicon Eclipse 80 equipped with Lucia v. 4.8 software).

ABRaA-VEGF$_{121}$ in therapy of B16-F10 melanoma-bearing mice. C57BL/6 mice (six- to eight-week-old females) with their left dorsal side shaved (five animals per experimental group) were inoculated subcutaneously with B16-F10 cells (2×10^5 cells per animal). Starting on the 6th day after inoculation, when tumors reached about 50 mm^3, the mice were injected ABRaA-VEGF$_{121}$ protein, either *via* the tail vein or intratumorally. Injection of ABRaA-VEGF$_{121}$ was repeated (either 1 mg/kg body mass or 0.25 mg/kg body mass) four times, on every other day. Tumor volume was calculated from the formula: Tumor Volume = (Width)^2 × Length × 0.52. Permission for animal studies was obtained from the local Ethics Commission (Medical University of Silesia, Katowice, Poland).

RESULTS

Construction of cDNA encoding ABRaA-VEGF$_{121}$

In order to assure more efficient expression of the chimeric protein in bacterial system, the codons for ABRaA and human VEGF$_{121}$-encoding sequences which are rarely used in *E. coli* host were replaced with more frequent ones (codon optimization). The sequence encoding human VEGF$_{121}$ (GenBank accession No. AF214570), without the signal sequence, was codon-optimized using Prot2DNA software v. 1.0 and an *E. coli* codon usage table. The designed sequence (GenBank accession No. EF424789) was synthesized *de novo* from oligonucleotides (Table 1). The ABRaA synthetic coding sequence, together with a short G_4S spacer, was linked to the cDNA encoding human VEGF$_{121}$ in the pET41a(+) vector. cDNA encoding the ABRaA-VEGF$_{121}$ chimeric protein (GenBank accession No. EF424790) was cloned into pET32b(+) vector. The pET32/ABRaA-VEGF$_{121}$ plasmid was sequenced to verify the coding sequence of ABRaA-VEGF$_{121}$.

Expression in *E. coli* and purification of ABRaA-VEGF$_{121}$

The recombinant ABRaA-VEGF$_{121}$ protein was expressed in *E. coli* BL21(DE3) strain and isolated from bacterial inclusion bodies which were solubilized in 2 M urea buffer. Protein refolding was achieved by eliminating urea by gradual dialysis in the presence of oxidized and reduced glutathione. Under reducing conditions the purified protein migrates in SDS/polyacrylamide gel as a single about 42-kDa band whereas under non-reducing conditions two bands are present: an about 84-kDa one (homodimer) and an about 42-kDa one (monomer) (Fig. 1A). The experimental ABRaA-VEGF$_{121}$ mass agrees well with the theoretical molecular mass. The monomer/dimer ratio observed for SDS/PAGE was about 1:1. ABRaA-VEGF$_{121}$ shows immunoreactivity towards mouse monoclonal anti-human VEGF antibody (Fig. 1B).

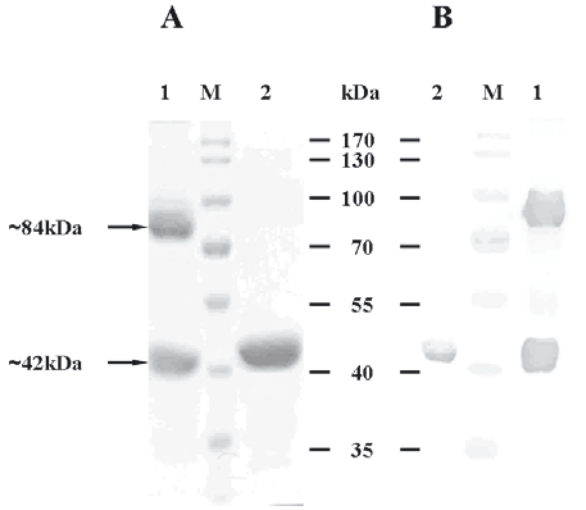

Figure 1. Chimeric ABRaA-VEGF$_{121}$ protein. Recombinant ABRaA-VEGF$_{121}$ was expressed in *E. coli* BL21(DE3) strain and isolated from inclusion bodies. After refolding, purification on Q-Sepharose and endotoxin removal using EndoTrap red columns, the recombinant protein was analyzed by electrophoresis on SDS/PAGE and either stained with Coomassie blue (A) or identified by Western blotting (B) using mouse anti-human VEGF$_{121}$ monoclonal antibodies and diaminobenzidine as color developer. Purified ABRaA-VEGF$_{121}$ was electrophoresed in 10% SDS/polyacrylamide gel under nonreducing (lane 1) or reducing (lane 2) conditions, *M* = molecular mass standard.

ABRaA-VEGF$_{121}$ is selectively cytotoxic against VEGFR-2-expressing PAE cells (PAE/KDR)

The concentration of ABRaA-VEGF$_{121}$ at which 50% of the tested PAE/KDR cells survive (IC_{50}) is ≈ 0.067 μg/ml. The PAE/KDR cells were more than 400-fold more sensitive to ABRaA-VEGF$_{121}$ than either PAE/hFlt1 cells or parental PAE cells (Fig. 2A). No difference in ABRaA-VEGF$_{121}$ toxicity was observed between PAE/hFlt1 and parental PAE cells (for both cell lines IC_{50} ≈ 27.3 μg/ml) (Fig. 2A). These results demonstrate that the chimeric protein is toxic specifically to cells expressing VEGFR-2 (KDR) receptors, but not to those expressing VEGFR-1 (hFlt-1) receptors. The IC_{50} values for ABRaA-VEGF$_{121}$ in the case of HUVEC, BAEC, HECa10 and B16-F10 cells were 16.8 μg/ml, 29 μg/ml, 29.5 μg/ml and 84 μg/ml, respectively.

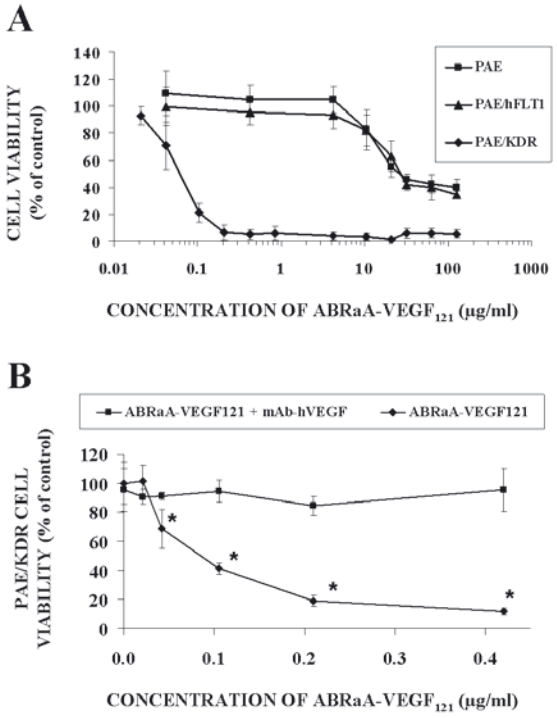

Figure 2. Cytotoxicity of ABRaA-VEGF$_{121}$ towards PAE cells expressing VEGFR-2 (KDR) or VEGFR-1 (hFlt-1) receptors.
A. PAE cells, VEGFR-2-transfected PAE cells or VEGFR-1-transfected PAE cells were treated with different concentrations of ABRaA-VEGF$_{121}$ for 72 h. PAE/KDR cells were more sensitive to ABRaA-VEGF$_{121}$ (IC_{50} about 0.067 μg/ml) than PAE/hFlt-1 or parental PAE cells which were insensitive to ABRaA-VEGF$_{121}$ (IC_{50} about 27.3 μg/ml). Here we show one of three independent experiments. Data points are mean values ± S.D. (n=3). **B**. ABRaA-VEGF$_{121}$ (various concentrations) was incubated with mouse monoclonal antibody neutralizing biological activity of human VEGF. Survival of PAE/KDR cells was checked with MTT test after treatment with ABRaA-VEGF$_{121}$ preincubated with antibody. Preincubation abolished cytotoxicity of the fusion protein towards PAE/KDR cells. Shown is one of two independent experiments. Data points are mean ± S.D. (n=3). Statistically significant differences between groups are denoted with an asterisk (*P<0.05; Mann-Witney U-test).

Neutralization of VEGF$_{121}$ domain abrogates ABRaA-VEGF$_{121}$ toxicity towards PAE/KDR cells

Preincubation of the ABRaA-VEGF$_{121}$ chimeric protein with a monoclonal antibody neutralizes biological activity of human VEGF almost totally abrogates its toxicity towards PAE/KDR cells (Fig. 2B). These data show that the VEGF$_{121}$ domain of ABRaA-VEGF$_{121}$ is essential for recognition of VEGFR-2 (KDR) receptor-expressing cells (PAE/KDR).

ABRaA-VEGF$_{121}$ inhibits protein biosynthesis *in vitro*

Abrin A chain is known as a "ribosome-inactivating protein" that inhibits protein translation. The ability of the chimeric ABRaA-VEGF$_{121}$ protein to inhibit protein translation was confirmed with a rabbit reticulocyte cell-free system (Promega). Translation was observed to begin 15 min after the start of control reaction. A dose-dependent inhibition of protein translation by ABRaA-VEGF$_{121}$ was found in the 4.2–21.0 ng/ml protein concentration range (Fig. 3A). These data demonstrate that protein translation is indeed inhibited by the chimeric protein.

ABRaA-VEGF$_{121}$ induces death of PAE/KDR cells *in vitro*

A 24-hour incubation with 0.42 μg/ml ABRaA-VEGF$_{121}$ brought positive TUNEL assay results for PAE/KDR cells but not for PAE/hFlt1 or parental PAE cells (Fig. 3B) or untreated cell lines. This indicates that ABRaA-VEGF$_{121}$ specifically induces death in cells displaying VEGFR-2 (KDR) receptors. Additionally, the treated PAE/KDR cells exhibit some morphological changes typical of abrin-caused apoptotic cell death, such as cell rounding, shrinking and detachment from the bottom of culture plate (not shown). A similar observation for abrin-treated HeLa cells was made by Qu and Qing (2004).

The apoptotic death assay based on Annexin V (AV) and propidium iodide (PI) staining followed by FACS analysis showed a dose-dependent apoptotic effect of ABRaA-VEGF$_{121}$ on PAE/KDR cells. FACS analysis with annexin V/PI staining of control cells revealed a large subpopulation of viable cells (marked as AV^-/PI^-) with some staining seen also for early apoptotic (AV^+/PI^-), late apoptotic and/or necrotic (AV^+/PI^+) as well as dead cells (AV^-/PI^+). On the other hand, a 16-hour exposure to increasing ABRaA-VEGF$_{121}$ concentrations (0.21, 0.42, 0.84 μg/ml) decreased the number of live cells, increased the subpopulation of early apoptotic cells and caused no significant changes in the late apoptotic/necrotic or dead cell subpopulations (Fig. 3C).

ABRaA-VEGF$_{121}$ induces necrosis in B16-F10 tumors

Mice harboring B16-F10 tumors were injected (intravenously or intratumorally) with ABRaA-VEGF$_{121}$ protein (1 mg/kg body mass/injection), either once or four times (every other day), starting from the 6th day after inoculation of mice with cancer cells. Forty-eight hours following the final injection mice were sacrificed; tumor material and other tissues were excised and hematoxylin and eosin-stained. Tumor tissue sections revealed tumor necrosis even after a single dose of intravenously administered chimeric protein (Fig. 4A and B). Similar effects were observed following ABRaA-VEGF$_{121}$ intratumoral injection (not shown). In contrast, no necrosis was found in the case of sections obtained

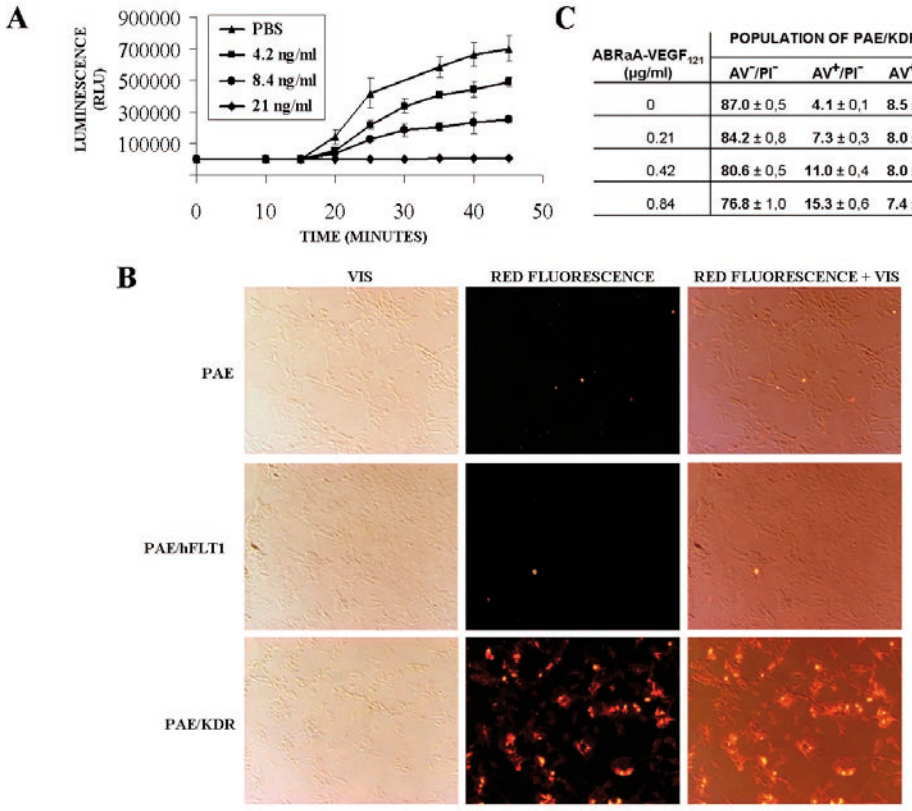

Figure 3. ABRaA-VEGF$_{121}$ inhibits protein biosynthesis and induces apoptosis in VEGFR-2 (KDR) receptor-presenting cells.

A. ABRaA-VEGF$_{121}$ inhibits luciferase biosynthesis in a cell-free translation system (TNT® T7 Quick Coupled Transcription-Translation System). Inhibition is ABRaA-VEGF$_{121}$ concentration-dependent. **B**. TUNEL test performed with PAE, PAE/KDR and PAE/hFlt-1 cells cultured for 24 h in the presence of ABRaA-VEGF$_{121}$ (final concentration 0.42 µg/ml). Positive reaction is only seen for PAE/KDR cells. This shows that ABRaA-VEGF$_{121}$ induces apoptosis in VEGFR-2 (KDR) receptor-expressing cells. Magnification 400 ×; VIS, visible light. **C**. Quantitation of PAE/KDR cell death by annexin V (AV) and propidium iodide (PI) double labelling. PAE/KDR cells were treated for 16 h with ABRaA-VEGF$_{121}$ at various low concentrations (0.21 µg/ml, 0.42 µg/ml and 0.84 µg/ml) and stained with AV/PI. Positive staining is marked as (+) and negative as (–). Percentage of labelled subpopulations of cells was calculated. Data points are mean ± S.D. (n=3). Representative results from one of two independent experiments are shown.

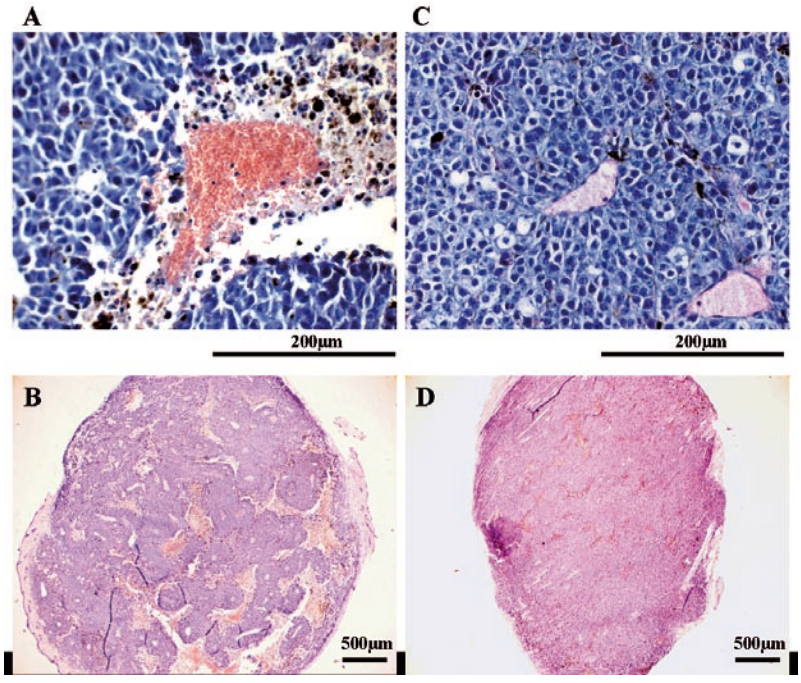

Figure 4. ABRaA-VEGF$_{121}$ induces death of tumor cells adjacent to destroyed blood vessels.

Female C57BL/6 mice (n=3) were inoculated intradermally with B16-F10 cells as described in Materials and Methods. Six days after inoculation, when tumors reached approx. 50 mm^3, ABRaA-VEGF$_{121}$ (1 mg/kg) was injected into the tail vein. Control mice obtained PBS only. Animals were sacrificed 48 h later, tumor material was excised, fixed and paraffin-embedded. Sections were H&E-stained. Tumor sections from mice that received ABRaA-VEGF$_{121}$ reveal extensive areas of eliminated neoplastic cells and extravasated red blood cells (**A** and **B**). No such damage to the vasculature is seen in sections from untreated control tumors (**C** and **D**).

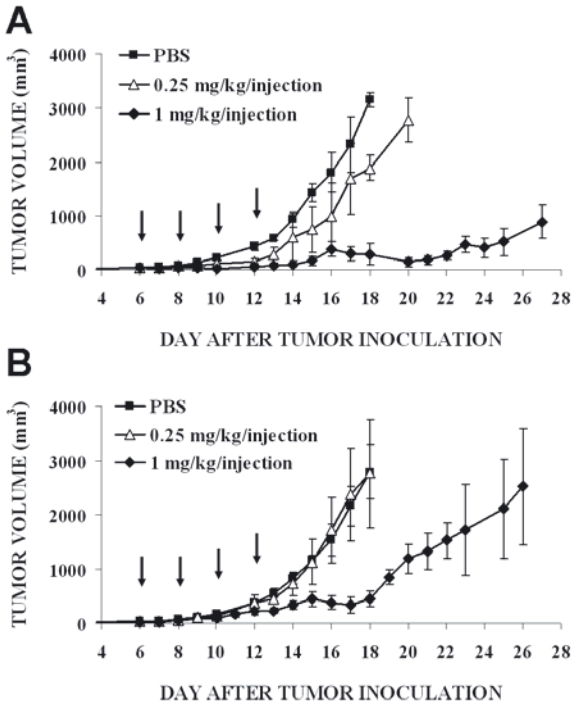

Figure 5. Inhibition of B16-F10 primary tumor growth by ABRaA-VEGF$_{121}$.
Female C57BL/6 mice were inoculated intradermally with B16-F10 cells as described in Materials and Methods. Six days after inoculation, when tumors reached about 50 mm^3, ABRaA-VEGF$_{121}$ was administered intratumorally (**A**) or intravenously (**B**). A total of four injections was given (every other day, see arrows) using either 0.25 mg/kg or 1 mg/kg per injection. Control mice received PBS only. The higher dose of ABRaA-VEGF$_{121}$ inhibited tumor growth irrespective of administration route. For mice receiving 1 mg/kg per injection statistically significant differences in tumour volumes (*vs*. control) were seen from day 8 of the experiment (for intratumoral administration) or from day 13 (for intravenous administration) ($P<0.05$; Mann-Whitney *U*-test). One of two independent experiments is shown. Data points are mean ± S.D. (n=5).

from control tumors (Fig. 4C and D) or normal tissues such as liver, spleen, kidneys and lungs, even after four repetitive injections (total of 4 mg/kg body mass, not shown).

ABRaA-VEGF$_{121}$ inhibits B16-F10 tumor growth in mice

ABRaA-VEGF$_{121}$ (1 mg/kg body mass/injection) inhibited B16-F10 primary tumor growth after intratumoral (Fig. 5A) or intravenous (Fig. 5B) injection. A stronger inhibitory effect was obtained with the intratumoral mode of administration. The therapeutic effect, at the dose of 0.25 mg/kg of body mass/injection, was visible only in the case of intratumoral ABRaA-VEGF$_{121}$ injection. Treated mice showed no noticeable weight loss (not shown). These data suggest that the inhibitory effect of ABRaA-VEGF$_{121}$ on tumor growth depends on both the dose and mode of administration.

DISCUSSION

This report concern design, purification and properties of ABRaA-VEGF$_{121}$, a novel chimeric protein. We constructed a cDNA encoding a fusion protein containing the VEGF$_{121}$ domain linked to the A-chain of abrin-a (ABRaA). The latter is a plant toxin which irreversibly inactivates eukaryotic ribosomes (Endo *et al*., 1987; Wang *et al*., 2004). To ensure efficient expression of chimeric ABRaA-VEGF$_{121}$ protein in a bacterial host, codons rarely used in *E. coli* and naturally occurring in the ABRaA and human VEGF$_{121}$ sequences were replaced (codon optimization) with the frequently used ones (Wang *et al*., 2004; Gustafsson *et al*., 2004).

We found that ABRaA-VEGF$_{121}$ is specifically cytotoxic towards cells overexpressing VEGFR-2 (PAE/KDR), but not VEGFR-1 (PAE/hFlt-1) receptors (Fig. 2A). The PAE/KDR cells, which express about 1.5×10^5 VEGFR-2 receptors/cell (Waltenberger *et al*., 1994) were more than 400-fold more sensitive to ABRaA-VEGF$_{121}$ than PAE/hFlt-1 cells, which express about 0.5×10^5 VEGFR-1 receptors/cell. No difference in toxicity was observed between PAE/hFlt-1 cells and the parental PAE cell line which lacks VEGFRs. These data demonstrate that the ABRaA-VEGF$_{121}$ protein is highly toxic only to those cells that overexpress VEGFR-2 receptors. The cytotoxicity of ABRaA-VEGF$_{121}$ towards PAE cells, which do not have VEGFR-2 surface receptors (Waltenberger *et al*., 1994), as well as towards human endothelial cells (HUVEC) with low expression of VEGFR-2 receptors (1.7×10^3 VEGFR-2/cell) (Brogi *et al*., 1996) did not differ significantly. The low cytotoxicity of ABRaA-VEGF$_{121}$ towards HUVEC cells may suggest that this particular protein requires the presence of a sufficiently large number of VEGFR-2 receptors in order to trigger its toxic effect.

When the VEGF biological activity was blocked with a specific monoclonal antibody, it strongly abrogated the cytotoxicity of ABRaA-VEGF$_{121}$ towards PAE/KDR cells (Fig. 2B). Such result indicates that the VEGF$_{121}$ domain of ABRaA-VEGF$_{121}$ is responsible for specific interactions with cells presenting VEGFR-2 and thus mediates toxicity of the chimeric protein.

Since the mode of action of ABRaA is based on irreversible inactivation of eukaryotic ribosomes (Endo *et al*., 1987; Wang *et al*., 2004), we investigated whether such activity would also be displayed by ABRaA-VEGF$_{121}$ chimeric protein. Using a cell-free system we found a concentration-dependent inhibition of *in vitro* protein translation by ABRaA-

$VEGF_{121}$ (Fig. 3A). We also found that ABRaA-$VEGF_{121}$ induces death of PAE/KDR cells expressing VEGFR-2 receptors (TUNEL test, Fig. 3B). Treatment of PAE/KDR cells with increasing concentrations of ABRaA-$VEGF_{121}$ resulted in a dose-dependent distribution shift from a population of live cells to that of early apoptotic cells (Fig. 3C).

Histological staining of sections from B16-F10 tumors (Fig. 4A, C) excised 48 h after a single intravenous administration of ABRaA-$VEGF_{121}$ (1 mg/kg body mass) documented necrotic tumor areas with extravasated red blood cells. Treatment of animals with ABRaA-$VEGF_{121}$ caused neither a weight loss nor destruction of normal tissues at least as seen for liver, spleen, kidneys and lungs, even after four repetitive administrations (total of 4 mg/kg body mass, not shown).

We also show that ABRaA-$VEGF_{121}$ inhibits growth of B16-F10 murine melanoma tumors, following intratumoral or intravenous administration (Fig. 5A and B). The therapeutic effect is dose- and mode of injection-dependent. The best results were obtained following intratumoral administration of the fusion protein, presumably because of its accumulation within the tumor. Because our *in vitro* experiments demonstrate that B16-F10 cells are less sensitive to ABRaA-$VEGF_{121}$ ($IC_{50} \approx 84$ μg/ml) than other cell lines (HUVEC (16.8 μg/ml), PAE (27.3 μg/ml), PAE/KDR (0.067 μg/ml), BAEC (29 μg/ml), HECa10 (29.5 μg/ml)), it is quite possible that the antitumor effect of the fusion protein results mainly from the disruption of an established tumor vascular network. However, we cannot exclude the possibility of ABRaA-$VEGF_{121}$ directly eliminating B16-F10 cells, especially after intratumoral administration.

The best therapeutic effects were observed a few days after administration of the chimeric protein (between day 15th and 21st). At that time, over 90% of tumor mass was necrotized (not shown). Despite that, cancer cells surviving the ABRaA-$VEGF_{121}$ therapy and still present at the periphery of destroyed tumors caused of their subsequent regrowth. The appearance of necrosis in tumors has been well described for numerous drugs known as Vascular Disrupting Agents (Thorpe, 2004; Szala, 2004).

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