Mechanistic studies of a novel, human fusion toxin composed of vascular endothelial growth factor (VEGF)\textsubscript{121} and the serine protease granzyme B: Directed apoptotic events in vascular endothelial cells

Yuying Liu, Lawrence H. Cheung, Philip Thorpe, and Michael G. Rosenblum

1Immunopharmacology and Targeted Therapy Section, Department of Bioimmunotherapy, M. D. Anderson Cancer Center, University of Texas, Houston, TX. 2Hamon Center for Therapeutic Oncology Research, University of Texas, Southwestern Medical Center, Dallas, TX.

Abstract

The serine protease granzyme B (GrB; 25 kDa) is capable of inducing apoptosis through both caspase-dependent and caspase-independent mechanisms. We designed a novel vascular-targeting fusion construct designated as GrB/vascular endothelial growth factor (VEGF)\textsubscript{121}, which is composed of a non-heparin-binding isoform of VEGF and the proapoptotic pathway enzyme GrB fused via a short, flexible tether (G\textsubscript{4}S). The chimeric fusion gene was then cloned into a bacterial vector, and the protein was expressed in Escherichia coli and purified by nickel-NTA metal affinity chromatography. Western blotting confirmed incorporation of both VEGF\textsubscript{121} and GrB proteins into the construct. GrB/VEGF\textsubscript{121} specifically bound (ELISA) to porcine aortic endothelial (PAE)/FLK-1 cells overexpressing the FLK-1/KDR receptor but not to cells overexpressing the FLT-1 receptor. Immunofluorescence studies showed that the GrB moiety of GrB/VEGF\textsubscript{121} was delivered efficiently and rapidly into the cytosol of PAE/FLK-1 cells but not into that of PAE/FLT-1 cells after 4 h treatment with GrB/VEGF\textsubscript{121}. Treatment of cells with GrB/VEGF\textsubscript{121} showed that the IC\textsubscript{50} was ~10 nm against PAE/FLK-1 cells; however, there were no cytotoxic effects observed on PAE/FLT-1 cells at doses up to 200 nm. GrB/VEGF\textsubscript{121} induced apoptotic events specifically on PAE/FLK-1 as assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling assay, DNA laddering, and cytochrome c release from mitochondria. In addition, the fusion construct mediated the cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase in target endothelial cells within 4 h after treatment. In conclusion, delivery of the human proapoptotic pathway enzyme GrB to tumor vascular endothelial cells or to tumor cells may have significant therapeutic potential and represents a potent new class of targeted therapeutic agents with a unique mechanism of action. (Mol Cancer Ther. 2003;2:949–959)

Introduction

Angiogenesis is required for many normal physiological processes and is also important in the pathogenesis of many disorders, particularly in the rapid growth and metastasis of solid tumors (1–4). Neovascularization studies of tumor biopsy specimens have confirmed a direct correlation of vessel count with metastatic potential of both breast and prostate tumors (5–8). In node-negative breast cancer, microvessel count proposed to be an independent prognostic marker of long-term survival (9). This correlation suggests that neovascularization of solid tumors is an essential feature in the determination of an aggressive tumor phenotype, although this issue remains somewhat controversial (10, 11).

Various soluble cytokines have been shown to mediate angiogenesis in vitro and in vivo (12, 13). Vascular endothelial growth factor (VEGF)-A plays a central role in both normal vascular tissue development and tumor neovascularization (14–16). VEGF is primarily an endothelial cell-specific angiogenic protein released by a variety of tumor cells and expressed in human tumors (14, 17, 18). Through alternative splicing of RNA, VEGF may exist as four different molecular species, having 121, 165, 189, or 206 amino acids (19–22). These isoforms differ not only in their molecular mass but also in biological properties such as the ability to bind to cell surface heparin sulfate proteoglycans (22, 24). The lowest molecular mass isoform of VEGF, VEGF\textsubscript{121}, is a soluble, non-heparin-binding variant that exists in solution as a disulfide-linked homodimer. VEGF\textsubscript{121} has been demonstrated in previous studies to contain the full biological activity of the larger variants (23, 24).

A recent study by Gasparini et al. (25) tested 260 biopsy specimens of node-negative breast cancer for VEGF expression. Tumors from 247 (95%) of the 260 patients had detectable and elevated VEGF levels. More importantly, levels of VEGF were found to be prognostic for both relapse-free and overall survival in both univariate and multivariate analyses. A study by Dirix et al. (26) demonstrated that VEGF levels in serum samples were elevated in 75% of patients during disease progression compared with 15% of patients responding to therapy. Its
prognostic role in patients with node-negative breast carcinoma underlies the importance of this VEGF in tumor development and progression. Follow-up studies examining VEGF expression by semiquantitative PCR confirm its importance in the development of breast cancer metastases and its role during tumor progression, particularly that of angiogenesis-inducing agent (27, 28).

The angiogenic actions of VEGF are mediated via two closely related endothelium-specific receptor tyrosine kinases, FLK-1/KDR and FLT-1. Both are largely restricted to vascular endothelial cells (29–32). Studies by Brown et al. (33) and Plate et al. (34) have shown that both FLK-1/KDR and FLT-1 receptors are overexpressed on the endothelium of tumor vasculature, whereas they are almost undetectable in the vascular endothelium of adjacent normal tissues. Other groups have also found that both VEGF and its receptors are overexpressed in a variety of solid tumor biopsy specimens (35–38). Therefore, the receptors for VEGF appear to be ideal targets for the development of therapeutic agents.

We have selected VEGF121 for our studies, considering it an appropriate carrier to deliver a toxic agent to any tumor endothelium that overexpresses the FLK-1/KDR receptors. Recently, we described a fusion toxin composed of VEGF121 and the plant toxin recombinant gelonin (rGel). This construct was shown to be selectively cytotoxic to vascular endothelial cells overexpressing the KDR receptor for VEGF in both in vitro and xenograft models. We demonstrated that the VEGF121 ligand is an excellent delivery platform with which to target tumor vascular endothelium cells in vivo in PC-3 tumor xenografts (39).

Another possibility for antiangiogenic targeting of tumor cells involves the granule-associated proteases. The granule secretion pathway appears to require the direct intracellular delivery of this family of proteases (granzyme A and granzyme B [GrB]) that activate both caspase-independent and caspase-dependent death programs to ensure that the targeted cell dies (40–42). Perforin, well known for its pore-forming capacity, has long been considered the vehicle that provides the gateway for entry of granzymes through the plasma membrane (41–43). GrB appears to have the most potent apoptotic activity of all granzymes as a result of its caspase-like ability to cleave substrates at key aspartic acid residues. The cell death-inducing properties of GrB have recently been studied in detail (44–48). GrB can cleave and directly activate several procaspases, and it can also directly cleave downstream caspase substrates such as the inhibitor of caspase-activated DNase (49). Overexpression of the antiapoptotic Bcl-2 protein in mitochondria inhibits GrB completely, however, indicating that mitochondrial disruption is an indispensable feature of granzyme-mediated cell death (50). In addition to caspase-dependent mechanisms, there are also caspase-independent pathways: cells in which caspase activity is blocked can also be killed by granzymes, although the caspase-independent mechanisms are poorly understood (51–53).

The current study describes a fusion construct of VEGF121 and the proapoptotic enzyme GrB designed for specific delivery to tumor neovasculature. We cloned the human GrB gene from human cutaneous T-cell lymphoma (HuT-78) cells and then fused the GrB gene to VEGF121 via a short, flexible tether by using a PCR-based construction method. The fusion protein was expressed in Escherichia coli and purified by nickel-NTA metal affinity chromatography. The fusion protein GrB/VEGF121 was characterized and the biological activities and mechanism were determined.

Materials and Methods

Materials

The PCR reagents, RNA isolation, and reverse transcription-PCR kits were obtained from Life Technologies, Inc. (Frederick, MD), and the molecular biology enzymes were purchased from New England Biolabs (Beverly, MA). RNA and DNA purification kits were obtained from Qiagen, Inc. (Valencia, CA). Bacterial strains, pET bacterial expression plasmids, and recombinant enterokinase (rEK) were obtained from Novagen (Madison, WI). Metal affinity resin (nickel-NTA agarose) was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Other chromatography resins were purchased from Amersham (Piscataway, NJ).

Porcine aortic endothelial (PAE) cells transfected with either the human FLT-1 receptor (PAE/FLT-1) or the KDR receptor (PAE/KDR) were a generous gift of Dr. J. Waltenberger, as described (54). Human melanoma A375M, human breast cancer SKBR3-HP, and HuT-78 cells were obtained from the American Type Culture Collection (Rockville, MD). Tissue culture reagents were from Life Technologies. Anti-GrB mouse monoclonal antibody, anti-VEGF receptors (FLK-1/KDR and FLT-1), and anticaspase antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-goat anti-mouse (HRP-GAM) or anti-rabbit conjugate were purchased from Bio-Rad (Hercules, CA). FITC-coupled anti-mouse IgG was obtained from Sigma Chemical Co. (St. Louis, MO). Cytochrome c release apoptosis assay kit was purchased from Oncogene Research Products (Boston, MA). In situ cell death detection kit, AP [terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay], and Fast Red were from Roche Molecular Biochemicals (Indianapolis, IN).

Methods

Cloning the Human GrB Gene and Construct of GrB/VEGF121 Fusion Genes. RNA from HuT-78 cells was isolated and the target premature human GrB cDNA was amplified by reverse transcription-PCR using the following primers: NcoIgb (5′ → 3′): GGTGGCGGTGGCTCCATG–GCCACCGCCTCCCTCGAGCTATTAGTAGCGTTT–GCCACCGCCTCCCTCGAGCTATTAGTAGCGTTT–CATGGT. The PCR product was then cloned into the PCR 2.1 TA vector designated as gbTA. The gbTA was transformed into INVa competent cells, and positive clones were screened by PCR. The DNA from positive clones was isolated and then sequenced. The correct clone was designated gbTA-2.
The fusion construct GrB/VEGF121 was composed of GrB engineered to contain an EK cleavage site upstream of the GrB protein so that EK digestion would leave an isoleucine residue as the initial amino acid of the GrB protein. The GrB was then fused to the VEGF121 coding sequence tethered by a short, flexible tether (G4S). The construction was based on an overlap PCR method. Briefly, GrB coding sequence was amplified from gbTA-2 using the following primers: NgbEK (5’ → 3’): GTTACCCGACGACGACAAGATCATTGGGACATGAG and Cgb (5’ → 3’): GGAGCGACCCACCGTACGTTTCATGGT. These were designed to delete the signal sequence of premature GrB, insert an EK cleavage site at the amino terminus, and add G4S linker sequence to the carboxyl terminus to serve as a short, flexible tether (G4S). The construction was based on an overlap PCR method. Briefly, GrB coding sequence was amplified from gbTA-2 using the following primers: NgbEK (5’ → 3’): GTTACCCGACGACGACAAGATCATTGGGACATGAG and Cgb (5’ → 3’): GGAGCGACCCACCGTACGTTTCATGGT. These were designed to delete the signal sequence of premature GrB, insert an EK cleavage site at the amino terminus, and add G4S linker sequence to the carboxyl terminus to serve as a brief preview of the fusion protein expression. The cells were harvested, resuspended in 10 mM Tris (pH 8.0), and stored frozen at -80°C for later purification.

**Induction and Expression of GrB/VEGF121 Fusion Protein in E. coli.** Bacterial colonies transformed with the constructed plasmid were grown in Luria broth growth containing 400 μg/ml carbenicillin, 70 μg/ml chloramphenicol, and 15 μg/ml kanamycin at 37°C overnight at 240 rpm in a shaking incubator. The cultures were then diluted 1:100 in fresh Luria broth + antibiotics (200 μg/ml carbenicillin, 70 μg/ml chloramphenicol, and 15 μg/ml kanamycin) and grown to an OD600 nm of 0.6 at 37°C; thereafter, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 100 μM at 37°C for 2 h to induce the fusion protein expression. The cells were harvested, resuspended in 10 mM Tris (pH 8.0), and stored frozen at -80°C for later purification.

**Purification of GrB/VEGF121 Fusion Protein.** Thawed, resuspended cells were lysed by addition of lysozyme to a final concentration of 100 μg/ml with agitation for 30 min at 4°C followed by sonication. Extracts were centrifuged at 186,000 × g for 1 h. The supernatant containing only soluble protein was adjusted to 40 mM Tris, 300 mM NaCl, and 5 mM imidazole (pH 8.0) and applied to nickel-NTA agarose resin equilibrated with the same buffer. After washing, the nickel-NTA agarose was washed with 300 mM NaCl and 20 mM imidazole and the bound proteins were eluted with 500 mM NaCl and 500 mM imidazole. Absorbance (280 nm) and SDS-PAGE analyses were performed to determine the presence of the polyhistidine-tagged protein, designated as Pro-GrB/VEGF121. The eluted Pro-GrB/VEGF121 was dialyzed against 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The GrB moiety of Pro-GrB/VEGF121 was activated by the addition of bovine rEK to remove the polyhistidine tag according to the manufacturer’s instructions (1 unit of rEK for cleavage of 50 μg protein incubated at room temperature [RT] for 16 h). The rEK was removed by EK capture agarose. The protein solution was then passed through a column containing Q-Sepharose to remove non-rEK-digested construct and nonspecific proteins. The product was analyzed by SDS-PAGE to determine purity, and Bio-Rad protein assay was used to determine protein concentration. Samples were then aliquoted and stored at 4°C.

**Characterization of GrB/VEGF121 by SDS-PAGE and Western Blotting.** Protein samples were analyzed by electrophoresis on an 8.5% SDS-PAGE under reducing conditions. The gels were stained with Coomassie blue. For Western blotting analysis, proteins were transferred from gels into nitrocellulose membranes. The membranes were then blocked with 5% nonfat milk and incubated for 1 h at RT with anti-GrB mouse monoclonal antibody (1.0 μg/ml) or anti-VEGF121 mouse monoclonal antibody (1:2000 dilution). After washing, the membranes were incubated with HRP-GAM (1:5000 dilution). After further washing, the membrane was developed using the Amersham enhanced chemiluminescence detection system and exposed to X-ray film.

**Detection of VEGF Receptors on Porcine Aortic Endothelial Cells by Western Blotting.** PAE/FLK-1 or PAE/FLT-1 cell lysates (30 μg total protein) were separated by SDS-PAGE on 6% gels and transferred to nitrocellulose membranes. After incubation with anti-VEGF-R2 (FLK-1/KDR) or anti-VEGF-R1 (FLT-1), the membranes were incubated with HRP-GAM secondary antibody. The immune complexes were visualized by the Amersham enhanced chemiluminescence detection system.

**Binding Activity of GrB/VEGF121 by ELISA.** Ninety-six-well plates coated with 50,000 cells/well of PAE/FLK-1 or PAE/FLT-1 or A375M and/or SKBR3-HP cells were blocked by 5% BSA and then treated with purified GrB/VEGF121 at various concentrations. After washing, the plates were incubated with either GrB antibody or VEGF121 antibody followed by HRP-GAM IgG. Then, the substrate 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution with 1 μl/ml 30% H2O2 was added to the wells. Absorbance at 405 nm was measured after 30 min.

**Internalization Analysis of GrB/VEGF121 by Immunofluorescence Microscopy.** Cells were plated in 16-well chamber slides (Nunc, Nalge Nunc International, Naperville, IL) at 1 × 104 cells/well and incubated overnight at 37°C in a 5% CO2 air atmosphere. Cells were treated with 100 nM of GrB/VEGF121 for 4 h and then washed briefly with PBS. The cell surface was stripped by incubations with...
glycine buffer (500-mM NaCl, 0.1-M glycine [pH 2.5]) and neutralization for 2 min with 0.5-M Tris (pH 7.4) followed by a wash with PBS. Cells were fixed in 3.7% formaldehyde for 15 min at RT followed by a brief rinse with PBS and then were permeabilized for 10 min in PBS containing 0.2% Triton X-100 and washed thrice with PBS. Samples were incubated with 3% BSA for 1 h at RT to block nonspecific binding sites and then incubated with GrB mouse monoclonal antibody (1:100 dilution) at RT for 1 h followed by three washes with PBS. The samples were incubated with FITC-coupled anti-mouse IgG (1:100 dilution) at RT for 1 h and washed thrice with PBS. The walls and gaskets of the chamber slide were removed carefully. After air drying, the slide was mounted and analyzed under a Nikon Eclipse TS-100 fluorescence microscope. Photographs were taken with a scope-mounted camera.

**In Vitro Cytotoxicity Assays.** PAE cells in Ham's F-12 medium with 10% fetal bovine serum were plated into 96-well plates at a density of 2.5 × 10⁴ cells/well and allowed to adhere for 24 h at 37°C in 5% CO₂. After 24 h, the medium was replaced with medium containing different concentrations of GrB/VEGF₁₂₁ or VEGF₁₂₁/rGel. After 72 h, the effect of GrB/VEGF₁₂₁ or VEGF₁₂₁/rGel on the growth of cells in culture was determined using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT). Plates were read on a microplate ELISA reader at 540 nm.

**Colony-Forming (Clonogenic) Assay.** The growth inhibitory effects of GrB/VEGF₁₂₁ on the proliferation of PAE cells were evaluated by clonogenic assay. Briefly, 5 × 10⁴ PAE cells/ml were incubated at 37°C and 5% CO₂ for 72 h with different concentrations of either GrB/VEGF₁₂₁ or VEGF₁₂₁/rGel. After 72 h, the effect of GrB/VEGF₁₂₁ or VEGF₁₂₁/rGel on the growth of cells in culture was determined using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT). Plates were read on a microplate ELISA reader at 540 nm.

**TUNEL Assay.** Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular mass DNA fragments as well as single-strand breaks (nicks) in high molecular mass DNA. The DNA strand breaks can be identified by labeling free 3'-hydroxyl termini with modified nucleotides in an enzymatic reaction. Cells (1 × 10⁶ cells/well) were treated with GrB/VEGF₁₂₁ at the IC₅₀ concentration for different times (24 and 48 h) and washed briefly with PBS. Cells were fixed with 3.7% formaldehyde at RT for 20 min, rinsed with PBS, permeabilized with 0.1% Triton X-100, 0.1% sodium citrate on ice for 2 min, and using an inverted light microscope. Growth inhibition was defined as the percentage of cell growth/number of colonies in treated samples in relation to that in the nontreated control sample.

**Figure 2.** Construction of the GrB/VEGF₁₂₁ fusion toxin by PCR and insertion into the pET32a(+) vector. The human GrB sequence with 20-amino acid signal sequence was confirmed and designated as premature GrB. Once the signal peptide was removed, the mature amino-terminal Ile-Ile-Gly-Gly sequence GrB was generated.

**Figure 1.** Cloning the human GrB gene from HuT-78 cells. HuT-78 RNA was isolated, and premature GrB cDNA (~800 bp) was amplified by reverse transcription-PCR and cloned into the PCR 2.1 TA vector. The human GrB sequence with 20-amino acid signal sequence was confirmed and designated as premature GrB. Once the signal peptide was removed, the mature amino-terminal Ile-Ile-Gly-Gly sequence GrB was generated.
washed with PBS twice. Cells were incubated with TUNEL reaction mixture at 37°C for 60 min followed by incubation with Converter-AP at 37°C for 30 min and finally treated with Fast Red substrate solution at RT for 10 min. After the final wash step, the slides were mounted and analyzed for nucleus staining of apoptotic cells under a light microscope with 400× magnification.

**Cytochrome c Release Assay and Bax Translocation.**
PAE cells \( (5 \times 10^7) \) were treated with GrB/VEGF_{121} at concentrations of 0.1 and 20 nM for 24 h. Cells were collected. After cells were washed with 10 ml of ice-cold PBS, they were resuspended with 0.5 ml of 1× cytosol extraction buffer mix containing DTT and protease inhibitors and incubated on ice for 10 min. Cells were homogenized in an ice-cold glass homogenizer. The homogenate was centrifuged at \( 700 \times g \) for 10 min at 4°C. The supernatant was transferred to a fresh 1.5 ml tube and centrifuged at \( 10,000 \times g \) for 30 min at 4°C. The supernatant was collected and labeled as cytosolic fraction. The pellet was resuspended in 0.1-ml mitochondrial extraction buffer mix containing DTT and protease inhibitors, vortexed for 10 s, and saved as mitochondrial fraction.

**Figure 3.** Bacterial expression, purification, and Western analysis of the GrB/VEGF_{121} fusion toxin. A, 8.5% SDS-PAGE and Coomassie blue staining under reducing conditions showed that GrB/VEGF_{121} was expressed as a 55-kDa molecule with tags and the size of the final purified GrB/VEGF_{121} was \( \sim 38 \) kDa. B, Western blotting confirmed that the fusion protein reacted with either mouse anti-VEGF or mouse anti-GrB antibody.

**Figure 4.** VEGF receptors on PAE cells. PAE/FLT-1 or PAE/FLT-1 cell lysates \( (30-50 \text{ total protein}) \) were separated by SDS-PAGE on 6% gels and transferred to nitrocellulose membranes for standard Western blotting using either anti-VEGFR-1 \( (\text{FLT-1}) \) or anti-VEGFR-2 \( (\text{FLK-1/KDR}) \) antibody. VEGF-R1 \( (\text{homodimer, molecular mass} \sim 180 \text{ kDa}) \) expressed on PAE/FLT-1 cells and VEGF-R2 \( (\text{homodimer, molecular mass} \sim 220 \text{ kDa}) \) expressed on PAE/FLT-1 cells.

**Figure 5.** GrB/VEGF_{121} bound to PAE/FLT-1 cells but not to PAE/FLT-1 cells or A375M or SKBR3 cells. Binding of GrB/VEGF_{121} to cells was assessed by 96-well ELISA plates coated with 50,000 cells/well of PAE/FLT-1 or PAE/FLT-1 or A375M or SKBR3 cells were blocked with 5% BSA and then treated with purified GrB/VEGF_{121} at various concentrations and then incubated with either anti-GrB antibody \( (A) \) or anti-VEGF antibody \( (B) \) followed by HRP-GAM IgG. We then added ABTS solution with 1-μl/ml 30% H₂O₂ to the substrate. Absorbance at 405 nm was measured after 30 min.

**Anti-FLT-1 Antibody**

![Anti-FLT-1 Antibody](image)

**Anti-FLK-1 Antibody**

![Anti-FLK-1 Antibody](image)
Protein concentrations were determined by using Bio-Rad Bradford protein assay. Aliquots of 30 µg from each cytosolic and mitochondrial fraction isolated from nontreated and treated cells were loaded on a 15% SDS-PAGE, standard Western blot procedure was performed, and the blot was probed with mouse anti-cytochrome c antibody (1 µg/ml) or mouse anti-Bax antibody (1 µg/ml).

**DNA Laddering**. PAE cells were plated onto six-well plates (2 × 10^5 cells/well). Twenty-four hours later, cells were shifted to fresh culture medium containing 20 nM of GrB/VEGF121 (1.5 ml/well). After 24 h of incubation at 37°C, DNA was extracted and purified with DNA ladder kit (Roche) and fractionated on 1.5% agarose gels.

**Assays for Caspase Activation**. Western blotting analysis was used to identify activation of caspases including caspase-8, caspase-3, and poly(ADP-ribose) polymerase (PARP) cleavage.

**Results**

**Human GrB Gene Cloning from HuT-78**

The RNA from HuT-78 cells was isolated, and we successfully obtained the premature GrB gene by reverse transcription-PCR. The 1% agarose gel electrophoresis showed that human premature GrB cDNA was ~800 bp. The gene and amino acid sequences identified the first 20 amino acids as signal sequence (Fig. 1). The human GrB sequence with signal sequence was designated as premature GrB. In cytotoxic cells, active GrB was generated from a zymogen by dipeptidyl peptidase I-mediated proteolysis (55), which removes the two residue (Gly-Glu) propeptide and exposes Ile21. The amino-terminal Ile-Ile-Gly-Gly sequence of GrB is necessary for the mature, active GrB.

**Construction of GrB/VEGF121 Fusion Genes**

We used PCR to amplify the coding sequence of GrB from Ile21, effectively deleting the signal sequence and Gly-Glu domain. At the same time, we inserted a cleavage site for EK (DDDDK) upstream and adjacent to Ile21. Mature GrB was attached to the recombinant VEGF121 carrier via flexible tether (G4S). The fused gene fragment was then introduced into the XbaI and XhoI sites of the pET32a(+) to form the expression vector pET32GrB/VEGF121 (Fig. 2). This vector contains a 17 promoter for high-level expression followed by a Trx.tag, a His.tag, a thrombin cleavage site, and an EK cleavage site for final removal of the protein purification tag. Once protein tag is removed by rEK digestion, the first residue (isoleucine) of mature GrB is exposed, thereby activating the GrB moiety of GrB/VEGF121 construct. The sequence of the active GrB/VEGF121 was confirmed by DNA sequencing.

**Purification of GrB/VEGF121 Fusion Protein**

The recombinant protein GrB/VEGF121 was expressed as polyhistidine-tagged protein designated as pro-GrB/VEGF121 and then purified by nickel-NTA metal affinity chromatography. The His.tag was cleaved by addition of rEK to form GrB/VEGF121. After rEK digestion and removal of rEK, Q-Sepharose ion exchange resin was used for further purification. One liter of the culture typically yielded ~100 µg of the final purified GrB/VEGF121 product. SDS-PAGE analysis showed that the final purified GrB/VEGF121 fusion construct migrated under reducing conditions as a band at the expected molecular mass of 38 kDa (Fig. 3A). Specificity of the cleaved fusion protein was confirmed by Western blot using either VEGF121 mouse monoclonal antibody or GrB mouse monoclonal antibody (Fig. 3B).

**VEGF Receptors Exist on Porcine Aortic Endothelial Cells**

Western blotting confirmed that the transfected cell line PAE/FLK-1 cells expressed VEGF-R2 (FLK-1/KDR, FLT-1), which was consistent with the presence of VEGF-R2 as determined by Western blotting (Fig. 4).

**Treatment:**

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<th>GrB/VEGF121 4 h</th>
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molecular mass ~220 kDa) and expressed virtually no VEGF-R1. In addition, the transfected PAE/FLK-1 cells expressed VEGF-R1 (FLT-1, molecular mass ~180 kDa) and expressed no VEGF-R2 (Fig. 4).

**ELISA of GrB/VEGF121 Fusion Protein Binding Activity**

GrB/VEGF121 specifically bound to PAE/FLK-1 cells. However, the protein did not bind to PAE/FLT-1 cells or to human melanoma A375M or human breast cancer SKBR3-HP cells, as detected by either an anti-GrB mouse monoclonal antibody (Fig. 5A) or an anti-VEGF121 mouse monoclonal antibody (Fig. 5B).

**Internalization of GrB/VEGF121 into Porcine Aortic Endothelial Cells as Assessed by Immunofluorescence Microscopy**

Immunofluorescent staining clearly showed that the GrB moiety of GrB/VEGF121 was delivered into the cytosol of PAE/FLK-1 but not into that of PAE/FLT-1 after treatment with GrB/VEGF121 for 4 h (Fig. 6). Analysis of PAE/FLT-1 cells treated for 24 and 48 h demonstrated no further increase in immunofluorescent staining over that observed at 4 h.

**Cytotoxicity of GrB/VEGF121 against PAE/FLK-1 versus PAE/FLT-1 Cells**

The cytotoxicity of GrB/VEGF121 was assessed against log-phase PAE/FLK-1 and PAE/FLT-1 cells in culture. An IC50 effect was found at a concentration of ~10 nM on PAE/FLK-1 cells. However, no cytotoxic effects were found on PAE/FLT-1 cells at doses up to 200 nM (Fig. 7A). By comparison, the cytotoxic effects of another fusion toxin, VEGF121/rGel (39), were relatively greater (on a molar basis) against target cells in culture and demonstrated specific cytotoxicity against PAE/FLT-1 cells at an IC50 of ~1 nM. In the clonogenic assay (Fig. 7B), the concentration of GrB/VEGF121, which suppressed cell colony growth by 50% (IC50), was determined to be ~20 nM on PAE/FLK-1 cells. In contrast, there was no effect on colony growth of PAE/FLT-1 cells at concentrations of GrB/VEGF121 up to 100 nM. There was also no effect of irrelevant fusion protein GrB/scFvMEL targeting human melanoma cells (56) on colony growth of PAE cells at concentrations of 100 nM.

**In Situ Cell Death Detection (TUNEL Assay)**

TUNEL assay produced positive results on GrB/VEGF121-treated PAE/FLK-1 at 24 h (75%) and 48 h (85%) but not on GrB/VEGF121-treated PAE/FLT-1 cells (10%) (Fig. 8), indicating that GrB/VEGF121 induced apoptosis in PAE/FLK-1 cells.

**Cytochrome c Release and Bax Translocation**

Western blot studies using anti-cytochrome c and anti-Bax antibodies demonstrated that cytochrome c was released from mitochondria into the cytosol at a concentration of 20-nM GrB/VEGF121 on PAE/FLK-1 cells, but this effect was not observed on PAE/FLT-1 cells. Bax was found to be normally present in both cytosol and mitochondria of untreated PAE cells. However, when PAE/FLT-1 cells were treated with 20 nM of GrB/VEGF121, Bax levels decreased in cytosol and increased in mitochondria. This effect was not observed on PAE/FLT-1 cells (Fig. 9).

**GrB/VEGF121 Induces DNA Laddering**

DNA laddering indicative of apoptosis was observed after a 24-h exposure with GrB/VEGF121 on PAE/FLK-1 cells. As expected, there was no DNA laddering detected on PAE/FLT-1 cells after treatment with the fusion construct (Fig. 10).

**GrB/VEGF121 Activates Caspases on Porcine Aortic Endothelial Cells**

PAE cells were treated with GrB/VEGF121, total cell lysates were loaded onto 12% SDS-PAGE, and standard Western blotting was performed. The results showed that...
treatment with GrB/VEGF_{121} cleaved caspase-8, caspase-3, and PARP on PAE/FLK-1 cells but not on PAE/FLT-1 cells (Fig. 11). These data indicate that the GrB/VEGF_{121} construct activated caspases involved in the apoptosis pathway.

**Discussion**

The discovery of unique factors present at higher levels on tumor endothelium as opposed to normal vasculature provides an excellent opportunity to develop targeted therapeutic agents with specificity for tumor vasculature. Receptors for VEGF (FLT-1 and KDR) have been shown to be overexpressed in tumor vasculature compared with normal endothelium and appear to be excellent candidates for targeted approaches.

Molecular engineering has enabled the generation of numerous recombinant targeted therapeutic agents (57, 58). Studies by Arora et al. (59) described fusion constructs of VEGF isoforms and truncated diphtheria toxin. These agents were highly active against proliferating endothelial cells and against Kaposi’s sarcoma cells expressing KDR. Our group recently demonstrated (39) that a fusion toxin composed of VEGF_{121} and the plant toxin (rGel) had excellent specificity for vascular endothelial cells over-expressing the KDR receptor *in vitro*. In addition, this agent demonstrated impressive and prolonged growth inhibitory activity against human melanoma and prostate xenograft tumors. These studies clearly demonstrated the capability of the VEGF growth factor to provide both *in vivo* tumor endothelium localization and cellular entry of attached toxins.
The current study confirms and extends these observations. VEGF was able to specifically delivered GrB to KDR-expressing cells but not to FLT-1-transfected cells. This was confirmed by ELISA binding studies, by Western analysis of cell homogenates after treatment with the construct, and by immunofluorescence studies of treated cells, all demonstrating intracytoplasmic delivery of the fusion construct. The IC$_{50}$ of the GrB/VEGF$_{121}$ fusion toxin was 10 nM compared with the IC$_{50}$ of VEGF$_{121}$/rGel for 1 nM and suggested that these agents are similar in overall biological activity despite the fact that the toxic components of the constructs are markedly different in their mechanism of action.

The GrB/VEGF$_{121}$ fusion construct induced apoptosis in target cells as assessed by TUNEL and DNA laddering. In contrast, the VEGF$_{121}$/rGel fusion construct, in contrast, although also cytotoxic to target cells, did not kill cells through an apoptotic process (39). Further analysis of the intracellular mechanism of action of GrB/VEGF$_{121}$ indicated that treatment with this agent releases cytochrome c from mitochondria into the cytosol. Cytochrome c can be considered a key signal because it initiates the irreversible events in apoptotic cell death. Bax can form transmembrane pores across the outer mitochondrial membrane, which leads to a loss of membrane potential. The localization of Bax has been shown to change from the cytosol to the mitochondria during apoptosis. We also demonstrated that the apoptotic mechanism included activation of caspase-3 and caspase-8 and cleavage of PARP within 4 h after drug treatment.

This appears to be the first description of the use of the proapoptotic, serine protease GrB in targeted therapeutic constructs. This agent is the culmination of our search for a human therapeutic protein that is both highly active and potentially universal. In addition, as a critical consideration, we demonstrated that the GrB enzyme was effective even while it remained covalently attached to an assembled targeting vehicle. This advance avoids problems with linker technology that can result in premature release of the active component prior to target internalization or loss of biological activity of the active component due to permanent attachment to the targeting molecule. Importantly, the demonstration of biological activity with a GrB-containing fusion protein does not appear to be confined to the current approach using VEGF$_{121}$ as a vehicle to target tumor endothelium. Recent studies in our laboratory have demonstrated impressive cytotoxic activity of GrB-containing fusion constructs composed of single-chain antibodies targeting numerous human tumor cell types. The unique mechanism of action of GrB-based fusion-targeted therapeutic agents may also provide for novel interactions with other types of therapeutic agents or with other modalities such as hyperthermia or radiation therapy. Further in vitro and animal studies with this agent are clearly warranted.

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