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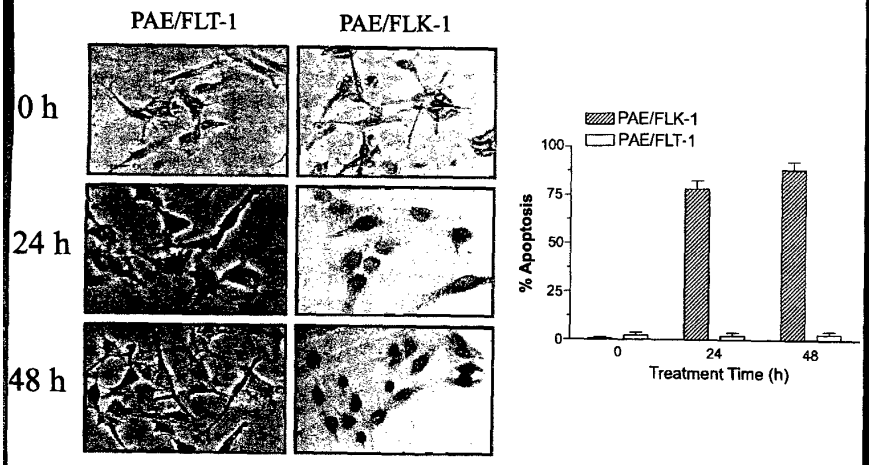

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Development of Novel, Highly Cytotoxic Fusion Constructs Containing Granzyme B: Unique Mechanisms and Functions

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Abstract: Recombinant fusion proteins are an expanding, important class of novel therapeutic agents. The designs of these constructs typically involve a cell-targeting motif genetically fused to a highly toxic class of enzymes capable of ruthlessly attacking critical cellular machinery once delivered successfully to the cytoplasm of the target cell. Initial development of this class of constructs typically contained recombinant growth factors or single-chain antibodies as the cell-targeting motif fused to highly cytotoxic plant or bacterial toxins. This review describes second-generation molecules composed of cell-targeting molecules fused to highly cytotoxic human enzymes capable of generating intense apoptotic response once delivered to the cytoplasm. The human serine protease granzyme B has been shown to be extremely effective as a cytotoxic molecule when incorporated into numerous cell-targeting constructs. The biological activity of GrB-containing constructs rivals that of plant or bacterial toxins and appears to represent a new generation and class of completely human proteins with unique biological activities.

Key Words: Fusion proteins, granzyme B, immunotoxins, serine protease, gp240, VEGF, serpins, H22.

INTRODUCTION

The successful development of targeted therapeutics for cancer applications depends on the identification of ligands and antigens specific for tumor cells (or their microenvironment), generation of molecules capable of targeting those components specifically after systemic administration and, finally, delivery of highly toxic molecules to the tumor (or its surroundings). Immunoconjugates composed of antibodies and small, toxic drugs or radioisotopes have been successfully tested *in vitro*, in animal models and have demonstrated activity in the clinical setting. This field has been the subject of numerous excellent reviews [1-7].

In addition to the use of small molecules for the toxin component, a number of groups have utilized highly cytotoxic protein toxins such as diphtheria toxin, ricin A-chain, Pseudomonas exotoxin, gelonin (rGel) in addition to others [8-16]. However, problems such as capillary leak syndrome, immunogenicity and toxicity continue to limit enthusiasm for long-term or chronic applications of these agents in the cancer setting. Studies by Pastan *et al.* [17] have demonstrated engineered toxin analogs of Pseudomonas exotoxin with reduced antigenicity compared to the original molecule. Studies in our laboratory have also demonstrated rGel analogs with reduced antigenicity and size [18] although immunotoxins containing rGel have demonstrated a low degree of immunogenicity in the clinical setting [19] even after repeated administration.

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Although there are a number of exquisitely cytotoxic payloads as mentioned above which are available for the construction of targeted therapeutic agents, there are a number of considerations which are relevant to the identification of molecules which constitute a class of "perfect" protein payloads. One of the first characteristics we considered was that the payload should be a relatively small human protein in the size range of the current toxins (approximately 25 kDa) or smaller if possible. In addition, this payload should not have a nominal cell-binding and internalization route or at least should have a cell-binding component, which can be engineered out of the molecule. Another characteristic would be that the molecule should be an enzyme, which acts, in a multi-component cellular cascade to reduce the possibility of developing cellular resistance to the delivered therapeutic agent. Finally, the cellular pathways necessary for cellular cytotoxic effects would have to be present in all cells and, in particular, all cancer cells.

DRUG TARGETING SYSTEMS

There are a large number of molecules which potentially fit the considerations mentioned above including several kinases, phosphatases, nucleases [20-23] and proteases [24-26]. One candidate molecule we identified involves the granule-associated serine proteases called granzymes. The serine protease granzyme B (GrB) is integrally involved in apoptotic cell death induced in target cells upon their exposure to cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells (Fig. (**1**)).

The granule secretion pathway appears to require the direct intracellular delivery of this family of proteases (granzyme A and GrB), that activate both caspase-independent

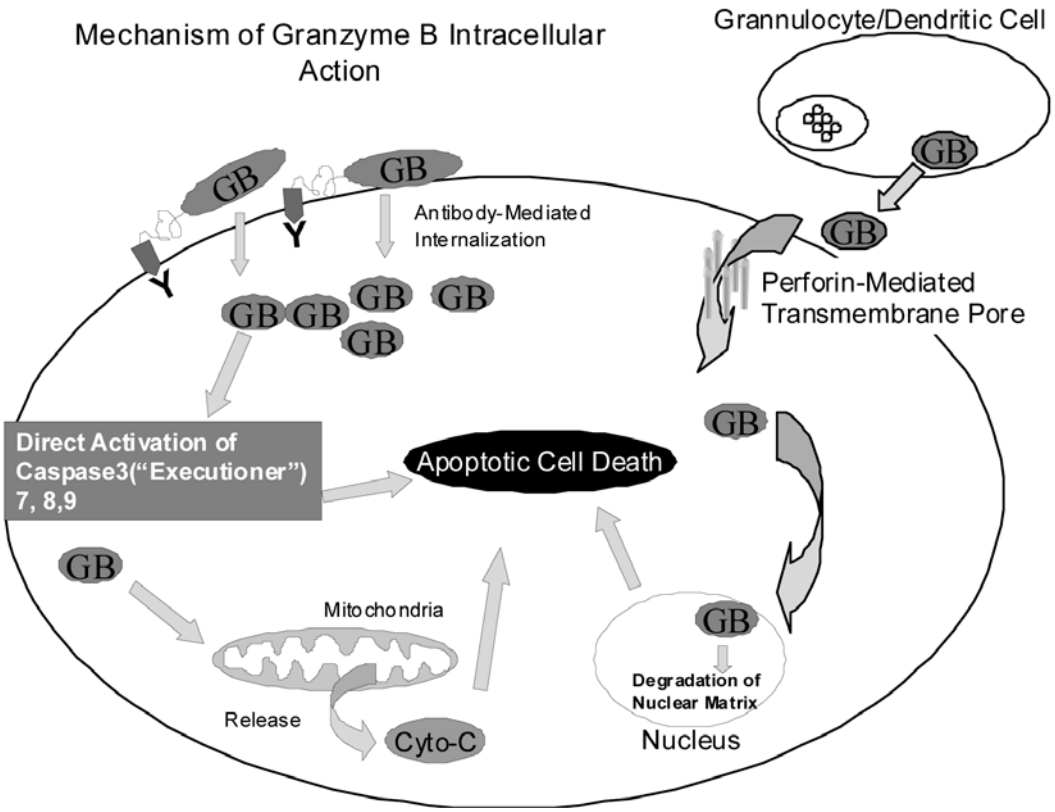


Fig. (1). Intracellular mechanism of action of GrB.

and -dependent death programs to ensure that the targeted cell dies [27-29]. 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 [30-32]. In CTL-mediated cytolysis, perforin is initially inserted into the target cell membranes and polymerizes to form transmembrane pores which facilitates access of NK or CTL-released GrB to the target cell cytoplasm. 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 [33-37]. 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 [38]. Although many procaspases are efficiently cleaved *in vitro*, GrB-induced caspase activation occurs in a hierarchical manner in intact cells, commencing at the level of "executioner caspases" such as caspase-3, followed by caspase-7 [39]. Overexpression of the anti-apoptotic Bcl-2 protein in mitochondria inhibits GrB completely, indicating that mitochondrial disruption is an indispensable feature of granzyme-mediated cell death [40]. 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 [41]. In addition to the caspase-mediated cytotoxic events, GrB can also rapidly translocate to the nucleus and cleave poly (ADP-ribose) polymerase and nuclear matrix antigen, utilizing different addition, some studies have shown that GrB can direct damage to non-nuclear structures such as mitochondria, subsequently induce cell death through caspase-independent pathways [44-46].

Since almost all cells contain mechanisms responsible for mediating cell death (apoptosis) we propose that targeted delivery of GrB to the interior of cells will result in cell death through apoptotic mechanisms assuming that sufficient quantities of active enzyme can be successfully delivered to the appropriate subcellular compartment (Fig. (**2**)).

In addition to providing a cytotoxic insult directly to target cells, an additional aspect of delivering pro-apoptotic agents is the potential for impacting radio-sensitivity, metastatic spread and sensitivity to chemotherapeutic agents. Numerous studies have suggested that the apoptotic status of cells impacts all three phenomenon and an additional rationale for targeting pro-apoptotic agents is the potential for impacting these cellular events in a unique fashion.

The rationale described above was the impetus for our original studies focusing on developing targeted therapeutic agents targeting tumor vasculature by using vascular endothelial growth factor-A (VEGF) and melanoma-associated antigen gp240 by using the single chain Fv antibody scFvMEL.

GrB/scFvMEL FUSION CONSTRUCT

To target melanoma cells, we chose the recombinant single-chain antibody scFvMEL, which recognizes the high-

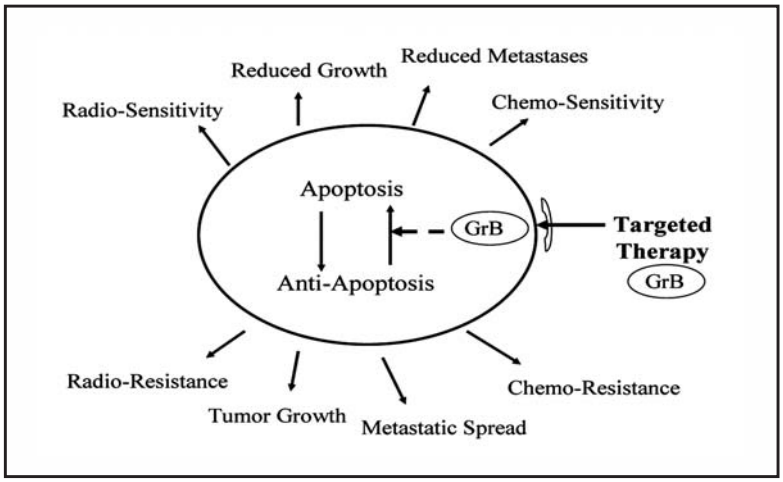
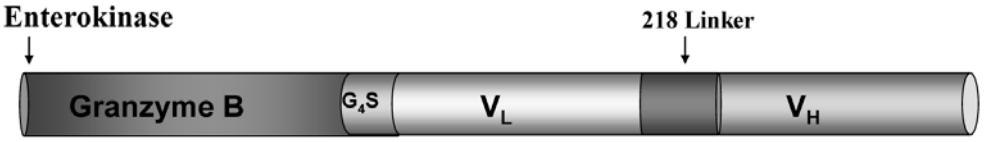


Fig. (2). The impact of apoptosis on various growth and anti-growth signals.



218 Linker: GlySerThrSerGlySerGlyLysProGlySerGlyGluGlySerThrLysGly
Linker G_4S: GlyGlyGlyGlySer

Enterokinase recognition sequence: AspAspAspAspLys

Fig. (3). Schematic Representation of the gene encoding GrB/scFvMEL.

of melanoma cell lines and fresh tumor samples [47,48]. Others and we have demonstrated that this antibody possesses high specificity for melanoma and is minimally reactive with a variety of normal tissues, making it a promising candidate for further study [49-52]. In the present study, we used scFvMEL as a tumor cell-targeting carrier and designed a novel recombinant fusion construct designated GrB/scFvMEL, containing human pro-apoptotic enzyme GrB (Fig. (**3**)). The purpose of these studies was to determine whether an antibody delivery vehicle would be sufficient to deliver active GrB enzyme to drive cellular apoptotic events specifically in melanoma target cells.

The fusion protein was generated by PCR, sequenced and cloned into a bacterial expression system (pET-32, Novagen) containing a thioredoxin tag upstream of the coding sequence for the final protein. The material was purified from bacterial paste using immobilized metal affinity chromatography and the final product was generated by enterokinase cleavage to uncover the N-terminal Ile of the GrB molecule, which is essential for enzymatic activity[53]. An ELISA was performed to determine the binding specificity of the GrB/scFvMEL fusion construct to antigen-positive A375-M and to antigen negative SKBR3 cells. As shown in Fig. (**4**), GrB/scFvMEL specifically bound to antigen-positive A375-M cells but we were able to detect little bind-

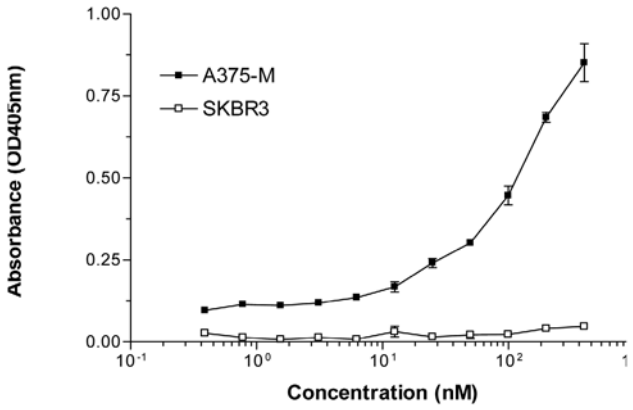


Fig. (4). ELISA binding of the GrB/scFvMEL fusion construct to antigen-positive A375-M and antigen-negative SKBR3 cells.

ELISA of GrB/scFvMEL on gp240 Ag-positive A375-M versus gp240 Ag-negative SKBR3 cells detected using an anti-GrB mAb. Ninety-six-well plates containing adherent A375-M or SKBR3 cells (5 x 104 cells/well) were blocked by addition of 5% BSA and then treated with purified GrB/scFvMEL at various concentrations. After washing, the cells were incubated first with anti-GrB mAb, and then with HRP-GAM. Then, substrate solution (ABTS plus 1 µl/ml

To assess the functionality of the GrB component of the fusion construct, the ability of the enzyme to cleave a BAADT substrate was assessed and compared to a known GrB standard (Table **1**). The fusion construct GrB/scFvMEL was shown to have intact GrB enzymatic activity with a specific activity comparable to that of the unmodified enzyme, (SA = 2.6×10^5 units/µmole for the GrB/scFvMEL compared to 4.8×10^5 units/µmole for native GrB). As expected, the fusion construct which had the thioredoxin tag on the molecule (non-rEK cut) had no activity since the N-terminal Ile of the GrB was hindered.

The GrB moiety of GrB/scFvMEL was delivered into the cytosol of A375-M cells after treatment with GrB/scFvMEL for 1 h or 6 h assessed by analysis of confocal microscope imaging as detected by anti-GrB antibody (Fig. (**5**)). GrB was found in the cytosol after treatment for 1 h, and the signals were stronger after treatment for 6 h than that after 1 h demonstrating localization and concentration of the construct over time. Antibody ZME-018 is the parental murine antibody for the scFvMEL recombinant fragment. Both agents recognize the same antigenic domain on the gp240 target antigen present on the cell surface of human melanoma cells. When cells were pre-treated with ZME-018, GrB fluorescent signal could not be detected in the cytosol after treatment with the construct, demonstrating that the uptake of the construct is dependant on specific interaction with gp240 on the cell surface.

Table 1. Enzymatic Activity of GrB and GrB Fusion Constructs

Samples	ΔmOD/min	Units (U)	U/µg	MW (kDa)	Specific Activity (U/µM)
Native GrB	48.2	1.0	19.2	25	4.8×10^5
GrB/scFvMEL (Un-rEK cut)	2.0**	-	-	70	-
GrB/scFvMEL (rEK-cut)	68.6	1.42	4.7	53	2.6×10^5

* BAADT: N-α t-butoxycarbonyl-L-alanyl-L-alanyl-L-aspartyl-thiobenzyl ester.

** The rate of non-enzymatic hydrolysis of BAADT at 0.2 nM, in 0.3 nM Ellman's Buffer at 25 °C is ≤ 5 Δ mOD/min.

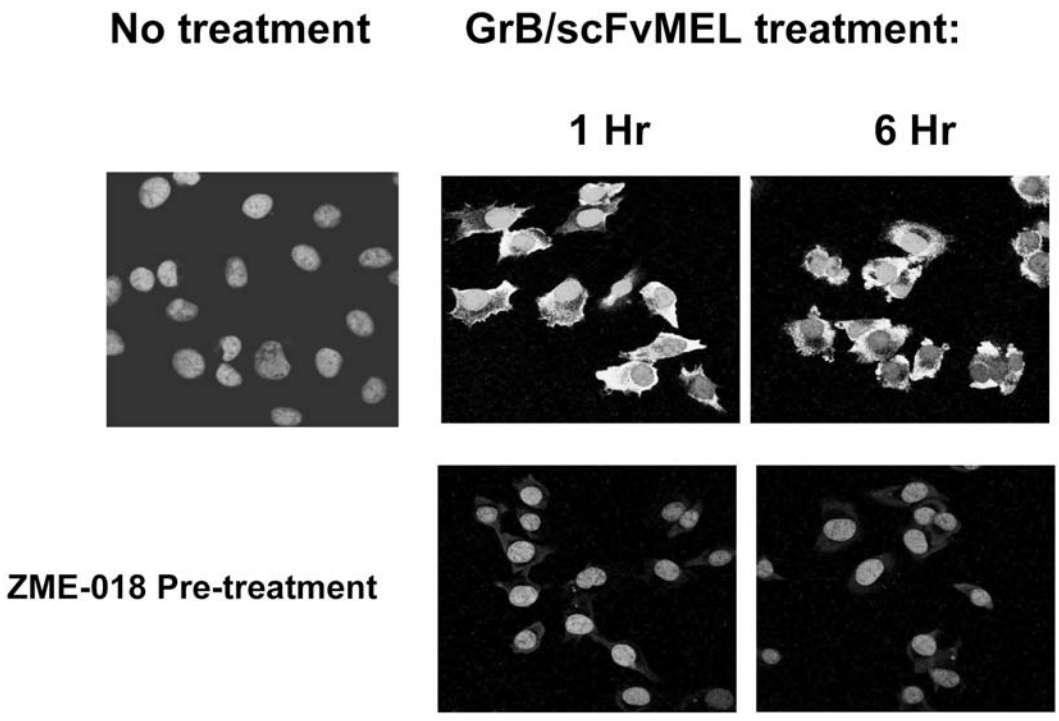


Fig. (5). Rapid internalization of GrB/scFvMEL fusion construct into target cells is blocked by pre-treatment with an anti-gp240 antibody.

Internalization of GrB/scFvMEL into A375-M cells assessed by confocal microscopy. A375-M cells were pretreated with ZME-018 (3 µM) for 2 h, and the cells were then treated with 40 nM GrB/scFvMEL for 1 or 6 h. Molecules bound to the cell surface were removed by brief treatment with glycine buffer (pH 2.5). Cells were fixed in 3.7% formaldehyde and permeabilized in 0.2% Triton X-100. Samples were blocked with 3% BSA, incubated with goat anti-GrB mAb, and then incubated with FITC-coupled anti-goat IgG and PI. The slides were mounted with DABCO containing 1 µg/ml of PI and analyzed by Zeiss LSM 510 confocal laser scanning microscopy. A, no GrB/scFvMEL treatment control. B, pretreatment with ZME-018 (3 µM), then GrB/scFvMEL treatment for 1 h. C, pretreatment with ZME-018, then

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