Molecular Cancer Therapeutics

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Preclinical Development

Molecular Cancer Therapeutics

Single-Chain Antibody-Based Immunotoxins Targeting Her2/neu: Design Optimization and Impact of Affinity on Antitumor Efficacy and Off-Target Toxicity

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Abstract

Recombinant immunotoxins, consisting of single-chain variable fragments (scFv) genetically fused to polypeptide toxins, represent potentially effective candidates for cancer therapeutics. We evaluated the affinity of various anti-Her2/neu scFv fused to recombinant gelonin (rGel) and its effect on antitumor efficacy and off-target toxicity. A series of rGel-based immunotoxins were created from the human anti-Her2/neu scFv C6.5 and various affinity mutants (designated ML3-9, MH3-B1, and B1D3) with affinities ranging from 10^{-8} to 10^{-11} mol/L. Against Her2/neu-overexpressing tumor cells, immunotoxins with increasing affinity displayed improved internalization and enhanced autophagic cytotoxicity. Targeting indices were highest for the highest affinity B1D3/rGel construct. However, the addition of free Her2/neu extracellular domain (ECD) significantly reduced the cytotoxicity of B1D3/rGel because of immune complex formation. In contrast, ECD addition had little impact on the lower affinity constructs *in vitro*. *In vivo* studies against established BT474 M1 xenografts showed growth suppression by all immunotoxins. Surprisingly, therapy with the B1D3-rGel induced significant liver toxicity because of immune complex formation with shed Her2/neu antigen in circulation. The MH3-B1/rGel construct with intermediate affinity showed effective tumor growth inhibition without inducing hepatotoxicity or complex formation. These findings show that while high-affinity constructs can be potent antitumor agents, they may also be associated with mistargeting through the facile formation of complexes with soluble antigen leading to significant off-target toxicity. Constructs composed of intermediate-affinity antibodies are also potent agents that are more resistant to immune complex formation. Therefore, affinity is an exceptionally important consideration when evaluating the design and efficacy of targeted therapeutics. *Mol Cancer Ther; 11(1); 143–53. ©2011 AACR.*

Introduction

Immunotherapeutic approaches using antibodies have been widely explored against a variety of tumors, but an effective treatment of solid tumors remains a potential problem because therapeutic antibodies must diffuse into tumors through a disordered vasculature and against a hydrostatic pressure gradient (1, 2). Because low–molecular weight antibody fragments have been shown to have better tumor diffusion properties (3), single-chain variable fragments (scFv) were favored to deliver protein-based toxins to cancer cells (4, 5).

A variety of scFv-based immunotoxins have been engineered that are suitable for diverse therapeutic applications. An anti-CD174 scFv designated SGN-10 fused with *Pseudomonas exotoxin* (PE) was developed for optimal tumor penetration but clinical studies were limited by renal toxicity and gastritis (6, 7). Chaudhary and colleagues (8) and Powell and colleagues (9) generated LMB-2, anti-CD25 scFv-PE immunotoxin and described promising preclinical efficacy on malignant cells from patients with adult T-cell leukemia. However, common toxicities included transaminase evaluation. The therapeutic window for this class of constructs may be optimized by various design changes to lower the efficacious dose, improve specificity by reducing off-target effects, thereby allowing an increase in the maximal tolerated dose (10–12).

Tumor–antigen affinity and specificity of scFvs are important variables that may impact off-target tissue distribution and toxicity *in vivo*. These attributes have led

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to the commonly held concept that scFv must have high affinity to be therapeutically relevant. However, studies by Adams and colleagues (13) and Rudnick and Adams (14) have suggested that high-affinity scFv may be suboptimal vehicles and that lower affinity scFv appear to diffuse more uniformly throughout the tumor interior. In addition, because the presence of shed tumor antigen has the potential to misdirect the targeted constructs through immune complex formation (10, 15), higher affinity scFv could potentially be at risk compared with lower affinity constructs.

Although previous studies primarily focused on the *in vivo* behavior of scFv, few companion studies have been conducted to determine whether scFv-based immunotoxins display the same behavior with regard to the relationship between affinity, tumor penetration, tumor residence, and efficacy. Our present knowledge of the affinity/function relationship of scFv-based immunotoxins is insufficient to afford accurate predictions as to whether a given scFv is appropriate for toxin delivery. A comprehensive head-to-head comparison of recombinant immunotoxins with different affinities targeting the same epitope on an antigen would be useful to guide the developmental strategy for future immunotoxins.

We previously reported the construction and characterization of anti-Her2/neu immunotoxins constructed by fusing scFv C6.5 with the recombinant gelonin (rGel). These constructs showed highly efficient activity against Her2/neu-positive tumor cells (16). In the current study, we generated a series of rGel-containing fusion constructs composed of C6.5 and its mutants with varying affinities to Her2/neu and examined the impact of affinity on *in vitro* cytotoxicity, pharmacodynamics, and antitumor efficacy. In addition, we investigated the effect of antibody affinity on behavior in the presence of soluble antigen, formation of immune complexes, and the coincident development of off-target toxicity.

Materials and Methods

Plasmid construction

The gene encoding human anti-Her2/neu scFv (C6.5 and its affinity mutants, ML3-9, MH3-B1, and B1D3 created by site-directed amino acid substitutions in the CDR3s; ref. 17) were supplied by Dr. James D Marks (University of California, San Francisco, San Francisco, CA; Fig. 1A). Illustrations of the immunotoxin constructs are shown in Fig. 1B. Recombinant immunotoxins containing each scFv and rGel were constructed by overlapping PCR and were designated C6.5/rGel, ML3-9/rGel, MH3-B1/rGel, and B1D3/rGel, respectively.

Protein expression and purification

The immunotoxins were expressed in *Escherichia coli* strain AD494 (DE3) pLysS and purified by immobilized metal affinity chromatography (IMAC) essentially as previously described (16).

Binding affinity of immunotoxins

The binding affinity and specificity of the immunotoxins were tested by ELISA on Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. Rabbit anti-rGel antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG were used as a tracer in this assay as described previously (16).

Internalization and competitive inhibition analysis

Immunofluorescence-based internalization studies were conducted on Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. Immunofluorescence staining and competitive inhibition were analyzed as described in Supplementary Methods.

Cytotoxicity of scFv/rGel and competitive cytotoxicity assays

The cytotoxicity of immunotoxins on log-phase Her2/neu-positive and -negative cell lines were tested with the crystal violet staining method, and competitive assays were conducted as described in Supplementary Methods (18).

Western blot analysis of apoptosis and autophagy

The detection of apoptosis and autophagy on BT474 M1 cells treated with immunotoxins was analyzed as described in Supplementary Methods.

In vivo efficacy studies

BALB/c nude mice bearing subcutaneous BT474 M1 tumors were established and treated (intravenously, tail vein) with immunotoxins, as described in Supplementary Methods.

Tissue distribution study

The MH3-B1/rGel and B1D3/rGel was labeled with IRDye800CW according to the manufacturer's protocol. The tissue distribution assays and the imaging analysis are further described in Supplementary Methods.

Coimmunoprecipitation assay

Liver samples from mice after treatment with MH3-B1/rGel or B1D3/rGel were collected. Samples were examined for the presence of antigen:immunotoxin complexes as described in Supplementary Methods.

In situ immunofluorescent detection

Samples of liver tissues from mice were further prepared for immunofluorescence staining tracing Her2/neu antigen and scFv/rGel immunotoxins as described in Supplementary Methods.

Liver toxicity study

Hepatotoxicity was investigated by measuring activities of alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) in collected serum from treated mice according to an assay kit (Roche). The histologic examination for hepatotoxicity was

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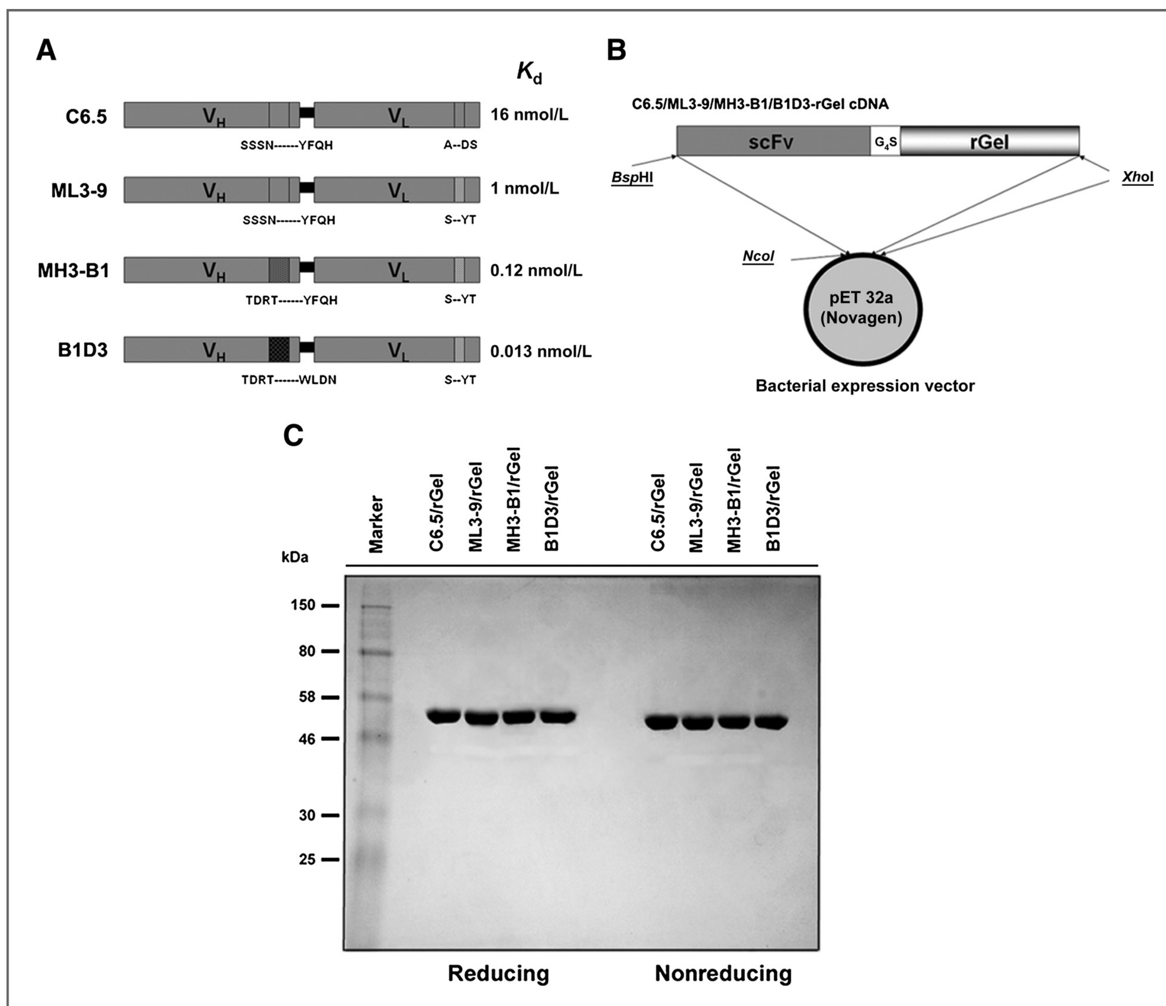

Figure 1. Construction and preparation of scFv/rGel immunotoxins. A, amino acid mutations and affinity parameters of the C6.5 and its mutants, ML3-9, MH3-B1, and B1D3. The listed amino acids for each scFv indicate mutations to the sequence and the substituting amino acids. Dashes indicate no changes from the original sequence. B, schematic diagram of immunotoxin constructs containing scFv (C6.5, ML3-9, MH3-B1, or B1D3) and rGel. C, SDS-PAGE analysis of purified immunotoxins.

assessed by hematoxylin and eosin staining. Further details are presented in Supplementary Methods.

Statistical analysis

Statistical analyses were conducted with SPSS version 17.0.2 software (SPSS Inc.). Data were presented as mean ± SD, and significance was determined using a 2-sided Student *t* test, unless otherwise noted. A value of $P < 0.05$ was considered statistically significant.

Results

Preparation of scFv/rGel fusion constructs

The scFv/rGel constructs were created from human anti-Her2/neu scFv C6.5 and various affinity mutants (designated ML3-9, MH3-B1, and B1D3, in increasing affinity order). The affinities of the scFv ranged from 10^{-8} to 10^{-11} mol/L (Fig. 1A; refs. 17, 19). The immunotoxin genes were cloned into vector pET-32a(+) separately (Fig. 1B). Sequenced DNA clones were subsequently transformed into *E. coli* AD494 (DE3) pLysS for protein expression. As shown in Fig. 1C, after purification, all the immunotoxins migrated on SDS-PAGE at the expected molecular weight of 55 kDa under both reducing and nonreducing conditions.

Binding and cellular internalization of the fusion constructs

To ensure that immunotoxins retained antigen-binding ability, the fusion proteins were compared in an ELISA-

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based–binding assay using Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells. All the scFv/rGel constructs showed specific and significant ELISA binding to Her2/neu-positive cells with negligible binding to negative cells (Fig. 2A). The equilibrium dissociation constants (K_d) were calculated (GraphPad Prism), and the affinities of immunotoxins for BT474 M1 cells were found to be 53.13 nmol/L (C6.5/rGel), 1.45 nmol/L (ML3-9/rGel), 0.18 nmol/L (MH3-B1/rGel), and 27 pmol/L (B1D3/rGel). The correlation between the K_d values of the scFvs and fusion constructs was found to be significant with a correlation coefficient of 0.939 ($P < 0.01$), indicating that introduction of the rGel component did not affect the binding affinity of the scFv.

We next examined whether the various affinity scFv/rGel fusions could specifically internalize into target cells. Immunofluorescence staining was conducted on Her2/neu-positive and -negative cells. As quantified by relative fluorescence (Fig. 2B), the internalization efficiency exhibited a moderate increase with increasing binding affinity in Her2/neu-positive cells. For BT474 M1 cells, the relative fluorescence intensities were 56.30 (C6.5/rGel), 73.69 (ML3-9/rGel), 86.29 (MH3-B1/rGel), and 90.41 (B1D3/rGel). There was a good correlation of between increases in apparent affinity and internalization efficiency ($r^2 = 0.8289$; $P < 0.01$) indicating that efficient binding to the cell surface appears to be primarily responsible for rapid internalization after cell exposure.

In vitro cytotoxicity of scFv/rGel fusion constructs

All the scFv/rGel constructs and rGel were tested against a number of different tumor cell lines (Table 1). As expected, there appeared to be a good correlation ($r^2 = 0.7812$; $P < 0.01$) between apparent affinity and IC_{50} values. Targeting indices were found to be highest for the highest affinity B1D3/rGel construct. This study showed that for the scFv/rGel immunotoxins, binding affinity appears to mediate internalization efficiency and this appeared to directly impact the overall cytotoxic effects observed. Furthermore, against Her2/neu-negative cells, there was little or no specific cytotoxicity of the constructs compared with rGel itself.

Effects of various fusion constructs on cytotoxic mechanisms

The cytotoxic effects mediated by scFv/rGel immunotoxins were analyzed in BT474 M1 cells. As shown in Fig. 3A, scFv/rGel fusions did not activate caspase-dependent apoptosis in target cells, showing no cleavage of the caspase substrate PARP. We next assessed LDH release and found that exposure of BT474 M1 cells to immunotoxins did not induce necrotic cell death (data not shown).

Then, we examined whether the cytotoxic effects of these immunotoxins activate autophagic signaling. As shown in Fig. 3B, the ratio of LC3-II formation to the β-actin control was shown to be increased after treatment with the fusion constructs, showing that autophagic flux

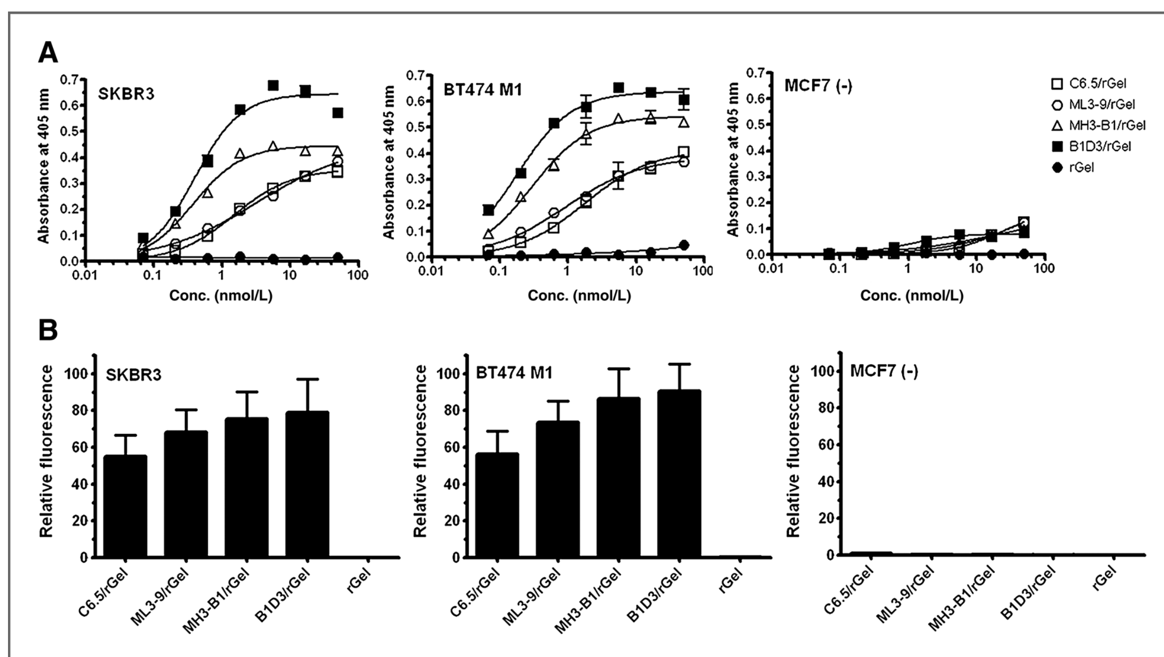

Figure 2. Characterization and comparison of scFv/rGel immunotoxins. A, evaluation binding activity of the scFv/rGel to Her2/neu-positive (SKBR3, BT474 M1) and -negative (MCF7) cells by whole-cell ELISA. B, quantification of internalization rate of the immunotoxins on Her2/neu-positive and -negative cells. Cells were subjected to immunofluorescent staining with anti-rGel antibody (fluorescein isothiocyanate–conjugated secondary antibody). The bar graphs were calculated from relative fluorescence estimation, and the values are expressed as mean ± SD ($n > 50$).

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