

The rGel/BLyS fusion toxin specifically targets malignant B cells expressing the BLyS receptors BAFF-R, TACI, and BCMA

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Abstract

B lymphocyte stimulator (BLyS) is crucial for B-cell survival, and the biological effects of BLyS are mediated by three cell surface receptors designated B cell-activating factor receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B-cell maturation antibody (BCMA). Increased expression of BLyS and its receptors has been identified in numerous B-cell malignancies. We generated a fusion toxin designated rGel/BLyS for receptor-mediated delivery of the recombinant gelonin (rGel) toxin to neoplastic B cells, and we characterized its activity against various B-cell tumor lines. Three mantle cell lymphoma (MCL) cell lines (JeKo-1, Mino, and SP53) and two diffuse large B-cell lymphoma (DLBCL) cell lines (SUDHL-6 and OCI-Ly3) expressing all three distinct BLyS receptors were found to be the most sensitive to the fusion toxin ($IC_{50} = 2-5$ pmol/L and $0.001-5$ nmol/L for MCL and DLBCL, respectively). The rGel/BLyS fusion toxin showed specific binding to cells expressing BLyS receptors and rapid internalization of the rGel component into target cells. The cytotoxic effects of rGel/BLyS were inhibited by pretreatment with free BLyS or with soluble BAFF-R, TACI, and BCMA decoy receptors. This suggests that the cytotoxic effects of the fusion toxin are mediated through BLyS receptors. The rGel/BLyS fusion toxin inhibited MCL cell growth through induction of apoptosis associated

with caspase-3 activation and poly (ADP-ribose) polymerase cleavage. Our results suggest that BLyS has the potential to serve as an excellent targeting ligand for the specific delivery of cytotoxic molecules to neoplastic B cells expressing the BLyS receptors, and that the rGel/BLyS fusion toxin may be an excellent candidate for the treatment of B-cell malignancies especially MCL and DLBCL. [Mol Cancer Ther 2007;6(2):460-70]

Introduction

B lymphocyte stimulator (BLyS), also known by a variety of names, including BAFF, TALL-1, and THANK, is a member of the tumor necrosis factor superfamily of cytokines (1-3). BLyS shares sequence homology with another tumor necrosis factor family member, a proliferation-inducing ligand (4, 5), and these molecules seem to represent a portion of a complex autocrine growth regulatory pathway (6, 7). BLyS seems to be widely expressed by monocytes, macrophages, dendritic cells, and neutrophils (8, 9) and seems to be one of many soluble factors by which these cells regulate human B-cell activation (10). The biological effects of BLyS are mediated by three cell surface receptors designated B cell-activating factor receptor (BAFF-R; ref. 11), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI; ref. 12), and B-cell maturation antibody (BCMA; ref. 13). These receptors are expressed in variable patterns in B-cell chronic lymphocytic leukemia specimens and may describe a subset of patients with inherent resistance to therapeutic agents (14). BAFF-R is expressed in about 80% of mantle cell lymphoma (MCL; ref. 15), a B-cell malignancy capable of developing rapid resistance to conventional chemotherapeutic regimens.

The use of ligands to deliver toxins to target cells overexpressing receptors has also received considerable attention in previous years. Chimeric proteins composed of chemokine ligands, such as interleukin-2, interleukin-3, interleukin-13, or vascular endothelial growth factor (16-20), and fused to various toxins (e.g., gelonin, diphtheria, *Pseudomonas* exotoxin, or granzyme B) have all shown significant and selective cytotoxic effects against target cells at nanomolar concentrations. Our laboratory has been actively developing therapeutic agents containing cytokines, such as tumor necrosis factor (21, 22), and toxins, such as recombinant gelonin (rGel; refs. 20, 23-26). rGel has shown impressive cytotoxic effects when delivered to cells using growth factor ligands (20), as chemical conjugates with monoclonal antibodies (23-25), or fused to single-chain antibodies (26). This toxin is an *n*-glycosidase that disrupts cellular protein synthesis at the ribosomal level in a manner similar to that of ricin A-chain (27). Fusion constructs containing rGel are currently in preclinical

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development, and a chemical conjugate of rGel with an antibody targeting CD33 is currently in phase I development at the M. D. Anderson Cancer Center (25).

Nardelli et al. (28) have suggested that BLyS may be useful as a targeting ligand for the specific delivery of cytotoxic or cytolytic agents to B cells expressing one or more of the receptors for BLyS. Studies by Riccobene et al. (29) have shown that radiolabeled BLyS is capable of specific and rapid localization in B-cell tumors in mice, suggesting its potential suitability for radioimmunotherapeutic applications.

Non-Hodgkin's lymphoma B cells express BAFF-R, which binds BLyS, as well as TACI and BCMA, which bind both BLyS and a proliferation-inducing ligand. This deregulation seems to be at least one autocrine survival pathway to allow cells the ability to evade apoptosis (7). Patients with non-Hodgkin's lymphoma display increased circulating levels of BLyS (30). MCL is an aggressive non-Hodgkin's lymphoma and has one of the poorest prognoses of all non-Hodgkin's lymphoma subtypes (31). Many regimes have been shown to be highly active in producing tumor responses; however, relapse typically occurs, and emergence of chemoresistance is common (32). Therefore, novel therapies are required for relapsed and/or refractory MCL.

In this study, we generated a fusion toxin rGel/BLyS containing rGel linked to the BLyS molecule at its NH₂ terminus for the specific delivery of toxin to B cells expressing BLyS receptors. We characterized the biological activity of this fusion toxin against a variety of human B-cell tumor lines. The rGel/BLyS fusion toxin was highly cytotoxic against MCL and diffuse large B-cell lymphoma (DLBCL) cell lines that express all three BLyS receptors. The fusion toxin internalized rapidly into target cells, and the cytotoxic effects of rGel/BLyS seemed to be receptor mediated. The fusion toxin inhibited cell growth through induction of apoptosis associated with caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage. This study suggests that rGel/BLyS may be an excellent candidate for the treatment of B-cell malignancies, including MCL and DLBCL.

Materials and Methods

Materials

The PCR reagents, RNA isolation kit, and reverse transcription-PCR kits were all obtained from Life Technologies, Inc. (Frederick, MD). The restriction enzymes were purchased from New England Biolabs (Beverly, MA). DNA purification kits were obtained from Qiagen, Inc. (Valencia, CA). Bacterial strains, pET bacterial expression plasmids, and recombinant enterokinase were obtained from Novagen (Madison, WI). Hi-Trap chelating HP resin and other chromatography resins were purchased from Amersham Biosciences (Uppsala, Sweden). The following monoclonal and polyclonal antibodies were used: PARP, β -actin, BAFF-R, TACI, and BCMA (Santa Cruz Biotechnology, Santa Cruz, CA); active caspase-3 (BD Biosciences,

San Jose, CA); BLyS (Upstate, Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Bio-Rad (Hercules, CA). Alexa Flour 594 goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR). Z-VAD-FMK, Z-IETD-FMK, and Z-DEVD-FMK were purchased from R&D Systems (Minneapolis, MN). Latrunculin A, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, 4',6-diamidino-2-phenylindole dihydrochloride, and FITC-coupled anti-rabbit IgG were purchased from Sigma (St. Louis, MI). Cell proliferation kit II (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt) was purchased from Roche (Mannheim, Germany).

Cell Lines and Cell Culture

The multiple myeloma doxorubicin-sensitive and doxorubicin-resistant cell lines MM1.S and MM1.R were kindly provided by Dr. Varsha Gandhi (M.D. Anderson Cancer Center, Houston, TX). The plasmacytoma myeloma melphalan-resistant cell line 8226/LR-5 was kindly provided by Dr. William Dalton (Arizona Cancer Center, Tucson, AZ; ref. 33). The four MCL cell lines (JeKo-1, Mino, SP53, and Granta 519) were kindly provided by Dr. Hesham Amin (M.D. Anderson Cancer Center; ref. 34). The two DLBCL cell lines (SUDHL-6 and OCI-Ly3) were kindly provided by Dr. Ricardo Aguiar (UT Health Science Center, San Antonio, TX; ref. 35). With the exception of Granta 519, JeKo-1, SUDHL-6, RPMI 8226, IM-9, MM1.S, MM1.R, HL-60, KBM-5, THP-1, and Jurkat cell lines were grown in RPMI 1640 (American Type Culture Collection, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin. For Granta 519 cell line, RPMI 1640 was substituted by DMEM (Invitrogen, Grand Island, NY). Melphalan (5 μ mol/L) was included in the RPMI 1640 for 8226/LR-5 cell line. Twenty percent heat-inactivated fetal bovine serum was included in RPMI 1640 for Mino, SP53, and OCI-Ly3 cell lines.

Construction of rGel/BLyS Fusion Toxin

We constructed the rGel/BLyS fusion toxin by first isolating RNA from JeKo-1 cells (which express BLyS) and then generating the cDNA encoding human BLyS, which was amplified by reverse transcription-PCR using the following primers: BLyS forward (5'→3'), GGA-GAAGGCAACTCCAGTCAGAAC and BLyS reverse (5'→3'), GTCCATGTCCTTGGGGATGAATTG (2). A rGel/BLyS fusion DNA construct was generated by the splice overlap extension PCR method (36) using the entire coding region of the BLyS and rGel as DNA templates to amplify individual gene fragments. To construct the rGel/BLyS fusion DNA, an upstream overlap extension PCR fragment encoding the enterokinase and the restriction enzyme *Kpn*I digestion site was amplified from the NH₂-terminal portion of rGel using the oligonucleotide primers PETgelfor (5'-AGCCCAGATCTGGGTACCGACGACGACGA-CAAGGGCCTGGACACCGTGAGC-3') and rGelbaclink (5'-GCTCCCGCCTCCCCCTTAGGATCTTT-3'). An adjoining downstream overlap extension PCR fragment encoding a G₃ linker and a restriction enzyme *Xho*I site was amplified

from the *BLyS* gene using the oligonucleotide primers *BLyS* forward (5'-GGGGGAGGCGGGAGCGCCGTT-CAGGGTCCA-3') and *BLyS* reverse (5'-GCCGTCGACCTC-GAGTCATTACAGCAGTTTCAATGC-3'). The upstream and downstream overlap extension PCR fragments were then reassembled as a full-length *rGel/BLyS* fusion gene by an additional PCR step using a pair of oligonucleotide primers PETgelfor and *BLyS*rev flanking the 5'- and 3'-end (see above). The final PCR fragment was purified and cleaved with *KpnI* and *XhoI* restriction endonucleases and then cloned into the pET-32a bacterial expression vector, which uses the T7 promoter for the transcriptional control of the inserted fusion gene. The fusion toxin *rGel/BLyS* gene constructs were verified by DNA sequencing, and correct fusion toxin constructs were transformed into *Escherichia coli* strain AD494 (DE3) for protein expression of the fusion toxin.

Expression of rGel/BLyS Fusion Toxin in *E. coli*

Bacterial colonies transformed with the plasmid carrying the *rGel/BLyS* insert were cultured in Luria broth medium containing 400 µg/mL ampicillin and 30 µg/mL kanamycin at 37°C overnight in a shaker incubator at 235 rpm. The bacterial cultures were then diluted 1:100 with fresh Luria broth medium containing antibiotics and grown to $A_{600} = 0.8$ at 37°C. Thereafter, the cultures were diluted 1:1 with fresh Luria broth medium plus 400 µg/mL of ampicillin and 30 µg/mL of kanamycin, and expression of the fusion toxin *rGel/BLyS* was induced at 23°C by addition of 100 µmol/L isopropyl-1-thio-β-D-galactopyranoside overnight. The cells were collected by centrifugation, resuspended in 40 mmol/L Tris-HCl (pH 8), and frozen (-80°C).

Purification of rGel/BLyS Fusion Toxin

Frozen bacterial cells were thawed and then lysed by physical disruption (Bead Beater, Biospec Products, Bartlesville, OK) at 4°C. The bacterial lysates were ultracentrifuged at 40,000 × *g* for 1.5 h. The final concentration of NaCl in supernatant was adjusted to 500 mmol/L NaCl and then loaded onto Hi-Trap chelating HP resin charged with 200 mmol/L Ni₂SO₄. The column was washed with wash buffer [40 mmol/L Tris-HCl (pH 8), 500 mmol/L NaCl, and 30 mmol/L imidazole] and eluted with elution buffer [40 mmol/L Tris-HCl, 500 mmol/L NaCl, and 300 mmol/L imidazole]. The *rGel/BLyS* containing fractions were pooled and dialyzed into dialysis buffer [20 mmol/L Tris-HCl (pH 7.4) and 150 mmol/L NaCl]. The *rGel/BLyS* fusion toxin containing the polyhistidine tag was digested overnight at room temperature with recombinant enterokinase to remove the tag. Contaminating proteins and the free histidine tag were removed by ion exchange chromatography (Q-Sepharose Fast Flow) and by affinity chromatography (Blue-Sepharose CL-6B). The purified *rGel/BLyS* fractions were dialyzed into PBS, filter sterilized, aliquoted, and stored at 4°C.

Analysis of rGel/BLyS Fusion Toxin

To confirm the presence of the *rGel* toxin and *BLyS* components in the fusion toxin, aliquots (2 µg) were analyzed by 12% SDS-PAGE under reducing conditions and stained with Coomassie blue. The immunoreactivity of

rGel toxin and *BLyS* components of the *rGel/BLyS* fusion toxin was confirmed by Western blot analysis using an anti-gelonin antibody or anti-*BLyS* antibody. The molecular weight of *rGel/BLyS* was assessed by gel-permeation fast-performance liquid chromatography as described previously (21).

Cell-Free Protein Synthesis Inhibitory Activity of rGel/BLyS Fusion Toxin

The enzymatic (*n*-glycosidase) activity of the *rGel* component of *rGel/BLyS* was assayed by using a cell-free protein translation assay (Promega, Madison, WI) as described previously (37).

Detection of BLyS, BAFF-R, TACI, and BCMA Expression on Various Tumor Cell Lines

The expression of *BLyS*, *BAFF-R*, *TACI*, and *BCMA* mRNA in 13 different human tumor cell lines was assessed by reverse transcription-PCR analysis using a panel of leukemia, myeloma, and MCL cell lines. Total isolated RNA from these lines was used to synthesize the first-strand cDNA that in turn was amplified by PCR by using specific primers designed to amplify human *BLyS* (313 bp; ref. 2), *BAFF-R* (256 bp; ref. 7), *TACI* (196 bp; ref. 38), and *BCMA* (285 bp; ref. 38). Glyceraldehyde-3-phosphate dehydrogenase was used as a control. The mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase.

The expression of *BAFF-R*, *TACI*, *BCMA*, and *BLyS* protein in 10 cell lines was further assessed by Western blot analysis.

Binding Activity of rGel/BLyS Fusion Toxin to BLyS Receptor-Expressing Cells

HL-60, SUDHL-6, Jurkat, Granta 519, MM1.S, RPMI 8226, OCI-Ly3, JeKo-1, KBM-5, and IM-9 cells were immobilized onto poly-L-lysine-coated 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 1×10^5 per well. The plates were blocked with 3% bovine serum albumin and then incubated with different concentrations of *rGel/BLyS* or *rGel*. After washing, plates were incubated with rabbit polyclonal anti-gelonin antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. Then, plates were developed with 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid. Absorbance at 405 nm was measured with ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT).

Internalization of rGel/BLyS Fusion Toxin into Cells Expressing BLyS Receptors

The JeKo-1, KBM-5, IM-9, and RPMI 8226 cell lines were added to polylysine-coated 16-well chamber slides (Nunc, Rochester, NY) at 1×10^4 per well and treated with 100 nmol/L *rGel* or 100 nmol/L *rGel/BLyS* for 4 h. Cells were then concentrated onto slides using a cytospin centrifuge (Shandon, Pittsburgh, PA), and then proteins bound to the cell surface were removed by incubation with glycine buffer [500 mmol/L NaCl and 0.1 mol/L glycine (pH 2.5)] and neutralization for 5 min with 0.5 mol/L Tris (pH 7.4) followed by a wash with PBS. The cells were fixed in 3.7% formaldehyde for 20 min at room temperature followed by a brief rinse with PBS and then permeabilized

for 10 min in PBS containing 0.2% Triton X-100 and washed thrice with PBS. To block nonspecific binding, slides were incubated with 3% bovine serum albumin for 1 h at room temperature. After a brief wash with PBS, cells were incubated with rabbit polyclonal anti-rGel antibody at room temperature for 1 h followed by three washes with PBS containing 0.1% Tween 20. Slides were incubated with FITC-coupled anti-rabbit IgG containing 1 $\mu\text{g}/\text{mL}$ of propidium iodide or Alexa Flour 594 goat anti-rabbit IgG containing 1 $\mu\text{g}/\text{mL}$ of 4',6-diamidino-2-phenylindole dihydrochloride and washed thrice with PBS. After air-drying, slides were mounted and analyzed with a Zeiss LSM510 laser scanning microscope or Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany).

To determine whether internalization of the fusion toxin involved cytoskeleton-dependent endocytosis, JeKo-1 cells were pretreated with 5 $\mu\text{mol}/\text{L}$ latrunculin A for 1 h and then treated with 100 nmol/L rGel or 100 nmol/L rGel/BLyS for 4 h. The cells were then processed immunofluorescence staining as described above.

Cytotoxic Activity of rGel/BLyS and Competitive Inhibition by Free BLyS or BAFF-R, TACI, or BCMA Decoy Receptors

To examine the comparative IC_{50} values of rGel/BLyS against various human tumor lines, cells were seeded (5×10^3 per well) in flat-bottomed 96-well microtiter plates (Becton Dickinson Labware), and various concentrations of either rGel itself or rGel/BLyS were added in quadruplicate wells. To assess the specificity of rGel/BLyS against BLyS receptor-expressing cells, we treated JeKo-1 cells with various concentrations of BLyS itself, free rGel, CTP/rGel (a non-B cell-targeting chemical conjugate), or rGel/BLyS and which were added to quadruplicate wells. After 96 h, cell viability was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay as described previously (22). Absorbance was measured at 450 nm using an ELISA reader (Bio-Tek Instruments).

For competitive inhibition assays, JeKo-1 cells were pretreated with 1 nmol/L BLyS, 50 nmol/L BLyS, 10 $\mu\text{g}/\text{mL}$ BAFF-R:Fc, 10 $\mu\text{g}/\text{mL}$ TACI:Fc, or 10 $\mu\text{g}/\text{mL}$ BCMA:Fc for 2 h, and then cells were treated with various concentrations of rGel/BLyS in quadruplicate wells. The cells were incubated for a further 96 h, and then cell viability was assessed using the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay.

To examine the effects of caspase inhibitors on the cytotoxicity of rGel/BLyS, JeKo-1 cells were pretreated with or without 200 $\mu\text{mol}/\text{L}$ Z-VAD-FMK, Z-IETD-FMK, or Z-DEVD-FMK for 2 h and then treated with 20 pmol/L rGel/BLyS for a further 96 h. Cell viability was then assessed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay.

Detection of Apoptosis

To assess apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, JeKo-1 cells were added to polylysine-coated 16-well chamber

slides at 5×10^3 per well and then treated with 100 pmol/L BLyS, 100 pmol/L rGel, or 100 pmol/L rGel/BLyS for 4 days. Floating cells were then collected and affixed to slides using a cytospin centrifuge. The slides were air-dried, fixed in 3.7% formaldehyde followed by a brief rinse with PBS, and then permeabilized in PBS containing 0.2% Triton X-100 and 0.1% sodium citrate. After washing, fixed cells were then stained with an *in situ* cell death detection kit (Roche). Cells undergoing apoptosis were identified by fluorescence microscopy (Nicken, Tokyo, Japan).

Western Blot Analysis

To examine the effects of rGel/BLyS on caspase-3 and PARP cleavage, JeKo-1, Granta 519, Mino, or SP53 cells were seeded at 5×10^5 per 24-well plate and then treated with 100 pmol/L BLyS, 100 pmol/L rGel, or 100 pmol/L rGel/BLyS. After 96 h, cells were washed twice with PBS and lysed on ice for 20 min in 0.3 mL of lysis buffer [10 mmol/L Tris-HCl (pH 8), 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.2% NP40]. Cell lysates (50 μg) were separated by SDS-PAGE (8–15%), electrotransferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and probed with different antibodies. After washing, the membrane was developed using ECL detection reagent (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Data are presented as the relative density of protein bands normalized to β -actin. Relative protein quantitation was done using Flour Chem 8900 (Alpha Innotech Co., San Leandro, CA).

Results

Construction, Expression, and Purification of rGel/BLyS Fusion Toxin

The design of the fusion toxin was assisted by structural studies (39) suggesting that the trimeric nature and the COOH terminus of the natural BLyS molecule may be of critical importance to receptor recognition. We therefore designed this fusion toxin with the rGel component fused to the NH_2 terminus of the BLyS molecule. The purified rGel/BLyS migrated on SDS-PAGE as a monomer at the expected molecular weight of 45.5 kDa under reducing conditions. The rGel/BLyS was immunoreactive with antibodies to BLyS and rGel, thus showing the presence of both toxin and BLyS components in the fusion toxin (Fig. 1A). Molecular weight analysis as assessed using gel-permeation fast protein liquid chromatography of the fusion toxin indicated that the molecule migrated at an approximate size of 136,500 Da (data not shown), consistent with a trimeric structure for the molecule.

The calculated IC_{50} values for rGel/BLyS and rGel were found to be 10 and 61 pmol/L, respectively (Fig. 1B). Therefore, the enzymatic activity of the rGel component of rGel/BLyS was slightly higher than that of free rGel.

Cellular Expression of BLyS, BAFF-R, TACI, and BCMA mRNA

We examined the mRNA expression profile of the BLyS ligand and its three receptors by reverse transcription-PCR

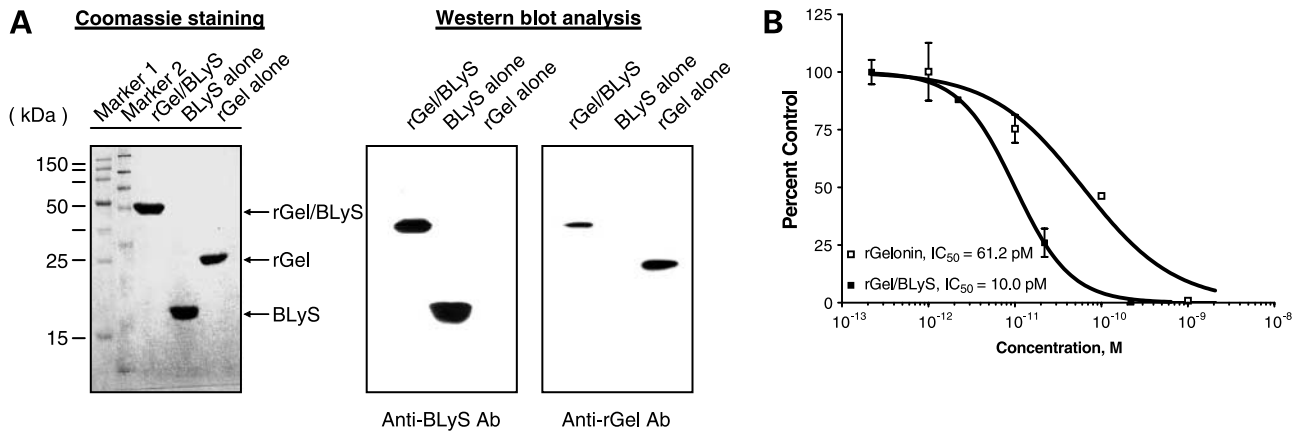


Figure 1. Purification of the rGel/BLyS fusion toxin. **A**, to confirm the presence of rGel toxin and BLyS component in the rGel/BLyS fusion toxin, the final samples were analyzed by 12% SDS-PAGE under reducing conditions. As shown in the Coomassie-stained SDS-PAGE analysis, rGel/BLyS monomer migrated at 45.5 kDa (left). Western blot analysis using an anti-gelonin antibody or anti-BLyS antibody showed that the fusion toxin was reactive with both antibodies, confirming the presence of the rGel toxin and the BLyS component (right). **B**, the enzymatic (*n*-glycosidase) activity of the rGel component of the fusion toxin was assessed using a cell-free protein translation assay (37). Inhibition curves for the rGel/BLyS and native rGel are presented.

analysis. All tested cell lines were found to express BLyS and BAFF-R mRNA, whereas TACI and BCMA mRNA were expressed in nine cell lines tested. Four leukemia cell lines (HL-60, KBM-5, THP-1, and Jurkat) showed no or undetectable TACI or BCMA mRNA. The expression of BLyS and BAFF-R mRNA was found to be variable on all the cell lines tested. As a group, the MCL cell lines (JeKo-1, Mino, SP53, and Granta 519) were all found to express high levels of BAFF-R mRNA. Compared with all lines tested, the MM1.S and MM.1R multiple myeloma cell lines and the

KBM-5 myeloid leukemia cell lines expressed relatively low levels of BAFF-R mRNA (Table 1).

Sensitivity to rGel/BLyS Fusion Toxin and Correlation with BAFF-R, TACI, and BCMA Protein Expression

We examined the IC₅₀ values for rGel and rGel/BLyS against these various tumor cell lines. The ratio of IC₅₀ values of rGel to rGel/BLyS was calculated for each cell type. This ratio (targeting index) represents the ability of the BLyS component of the rGel/BLyS to mediate the delivery of the toxin component to the target cell cytoplasm.

Table 1. mRNA expression of BLyS, BAFF-R, TACI, BCMA and comparative IC₅₀ values of the rGel/BLyS fusion toxin against various types of tumor cell lines

Cell line	Cell type	BLyS (313 bp)	BAFF-R (256 bp)	TACI (196 bp)	BCMA (285 bp)	IC ₅₀ (nmol/L)		Targeting index*
						rGel	rGel/BLyS	
BCL-1	Mouse B lymphoma	ND	ND	ND	ND	150	0.0008	187,500
JeKo-1	MCL	+	+++	++	+++	400	0.005	80,000
Mino	MCL	+	+++	+	++	70	0.002	35,000
SP53	MCL	++	+++	++	+++	110	0.004	27,500
Granta 519	MCL	+	+++	++	++	1,000	150	6.7
OCI-Ly3	DLBCL	ND	ND	ND	ND	10	0.001	10,000
SUDHL-6	DLBCL	ND	ND	ND	ND	700	5	140
RPMI 8226	Plasmacytoma myeloma	+	++	+	+++	280	10	28
8226/LR-5	Plasmacytoma myeloma	+	++	+	+++	200	110	1.8
IM-9	Multiple myeloma	+	+++	++	++	700	200	3.5
MM1.S	Multiple myeloma	+	+	++	++	600	220	2.7
MM1.R	Multiple myeloma	+	+	+	+++	1,000	280	3.6
HL-60	Acute promyelocytic leukemia	+	++	–	–	1,000	300	3.3
KBM-5	Myeloid leukemia	+	+	–	–	250	70	3.6
THP-1	Acute monocytic leukemia	++	++	–	–	110	30	3.7
Jurkat	Acute T-cell leukemia	+	++	–	–	3,000	1,500	2.0

NOTE: The mRNA levels were normalized to GAPDH. +, low expression (relative density = 0.3–0.7); ++, intermediate expression (relative density = 0.8–1.3); +++, high expression (relative density >1.4).

Abbreviation: ND, not determined.

*Targeting index represents IC₅₀ of rGel / IC₅₀ of rGel/BLyS.

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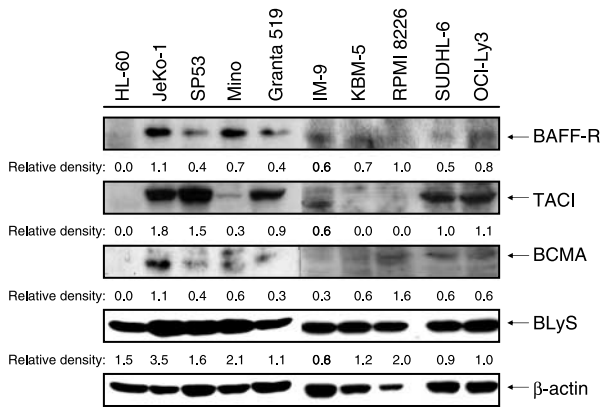


Figure 2. Expression of BLYS, BAFF-R, TACI, and BCMA protein in various tumor cell lines. Expression of BAFF-R, TACI, BCMA, and BLYS protein was analyzed by Western blot analysis in 10 different human tumor cell lines. Relative density of protein bands normalized to the β-actin protein loading control.

This ratio normalizes for the inherent cellular sensitivity to the rGel toxin. As summarized in Table 1, Granta 519, 8226/LR-5, IM-9, MM1.S, MM1.R, HL-60, KBM-5, THP-1, and Jurkat all showed a targeting index of between 1 and 7. Of

all the tumor lines tested, three MCL cell lines (JeKo-1, Mino, and SP53) were found to be the most sensitive to the fusion toxin (targeting index > 27,500), whereas nontargeted rGel itself showed no specific cytotoxic activity. Interestingly, the OCI-Ly3 DLBCL cell line (activated B cell-like DLBCL) was very sensitive to rGel/BLYS (targeting index = 10,000). We found that dexamethasone-sensitive (MM1.S) and dexamethasone-resistant (MM1.R) cell lines (40) were equally sensitive to rGel/BLYS (targeting index = 2.7 versus 3.6, respectively). This result suggests that cellular resistance to dexamethasone does not seem to result in development of cross-resistance to the rGel/BLYS fusion toxin.

We next examined whether a correlation existed between receptor protein expression and sensitivity to rGel/BLYS using 10 cell lines. The fusion toxin showed specific cytotoxic activity against three MCL cell lines (JeKo-1, Mino, and SP53) that express all three BLYS receptors. The most sensitive MCL line, JeKo-1 (targeting index = 80,000), was found to express the highest levels of BAFF-R, TACI, BCMA, and BLYS protein of all lines tested. The OCI-Ly3 (activated B cell-like DLBCL) cell line was also very sensitive to rGel/BLYS (targeting index = 10,000) and also expressed levels of BAFF-R, TACI, and BCMA protein in a

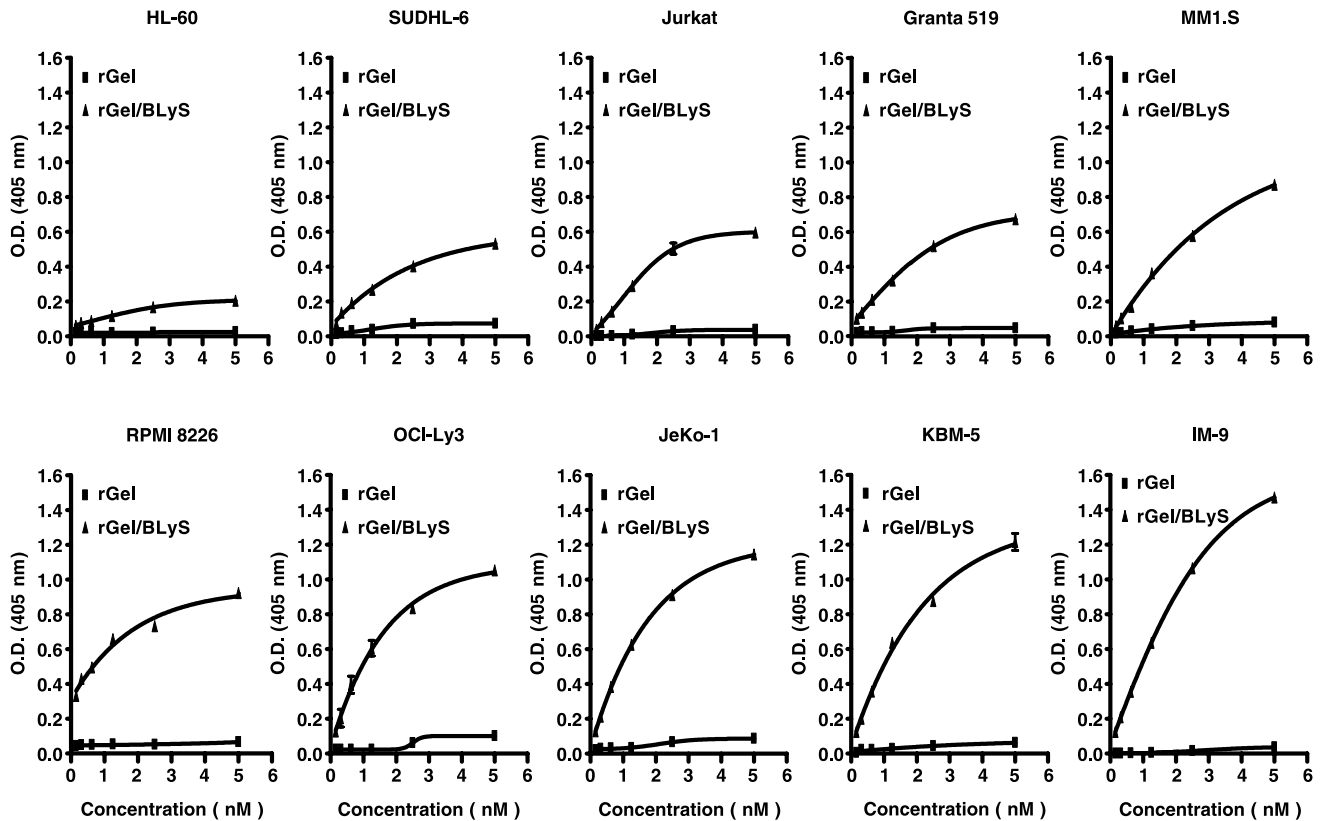


Figure 3. ELISA binding activity of the rGel/BLYS fusion toxin to BLYS receptor-expressing cells. The receptor-binding activity of the rGel/BLYS fusion toxin was determined using intact HL-60, SUDHL-6, Jurkat, Granta 519, MM1.S, RPMI 8226, OCI-Ly3, JeKo-1, KBM-5, and IM-9 cells adherent to 96-well plates and incubated with fusion toxin followed by addition of rabbit polyclonal anti-rGel antibody and visualization as described in Materials and Methods. The spectrophotometric absorbance was measured at 405 nm using an ELISA reader.

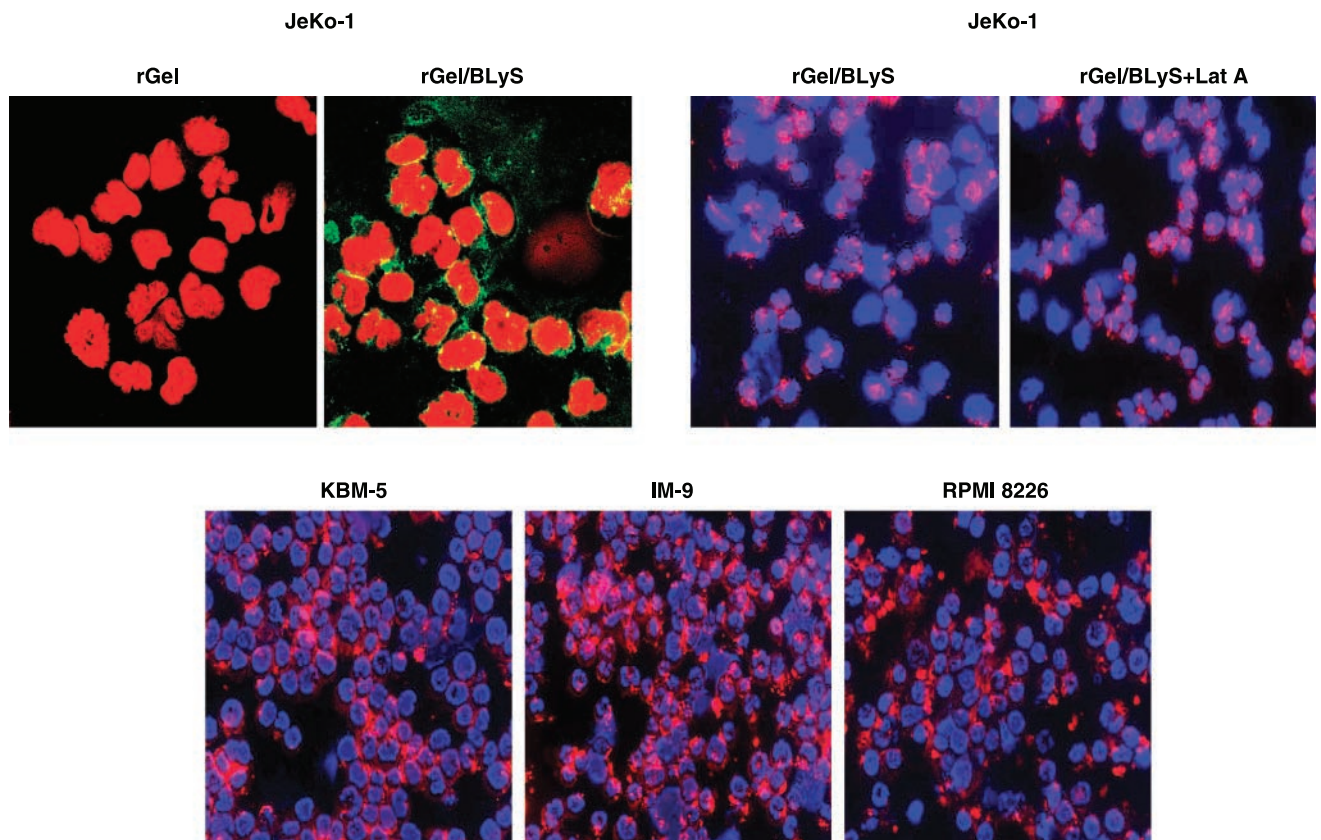


Figure 4. Internalization of the rGel/BLyS fusion toxin to BlyS receptor-expressing cells. For the cellular internalization assay, JeKo-1, KBM-5, IM-9, and RPMI 8226 cell lines were treated with 100 nmol/L rGel or 100 nmol/L rGel/BLyS for 4 h. To determine whether cytoskeleton-dependent endocytosis is required for cellular uptake of rGel/BLyS into target cells, JeKo-1 cells were pretreated with 5 μ M latrunculin A for 1 h and then treated with 100 nmol/L rGel or 100 nmol/L rGel/BLyS for 4 h. The cells were then concentrated onto a microscope slide; cell surface proteins were stripped by glycine buffer; and the cells were then fixed in 3.7% formaldehyde followed by a brief rinse with PBS. Cells were then permeabilized and incubated with rabbit polyclonal anti-rGel antibody. After a brief wash with PBS, cells were incubated with FITC-coupled anti-rabbit IgG containing 1 μ g/mL of propidium iodide or Alexa Fluor 594 goat anti-rabbit IgG containing 1 μ g/mL 4',6-diamidino-2-phenylindole dihydrochloride. After washing with PBS, slides were mounted in mounting medium and then analyzed with a Zeiss LSM510 laser scanning microscope ($\times 630$) or a Zeiss Axioplan 2 microscope ($\times 200$).

manner similar to that of JeKo-1 cells. The SUDHL-6 line was ~ 70 -fold less sensitive to rGel/BLyS than the OCI-Ly3 line and expressed comparatively less BAFF-R protein, although expression of the other receptors and BlyS itself were similar between the two cell lines. HL-60 cells expressed BlyS; however, we could not find evidence of cognate receptor protein expression, although the cells have BAFF-R mRNA. The KBM-5 and RPMI 8226 cell lines expressed BAFF-R and BCMA protein but not TACI protein (Fig. 2). These data suggest that cells that express high levels of BAFF-R protein were the most sensitive to rGel/BLyS, and that coexpression of TACI and BCMA protein seemed to result in cells that exhibited the highest degree of response to the fusion toxin.

Binding Activity of rGel/BLyS Fusion Toxin

The rGel/BLyS fusion toxin showed specific binding activity to BAFF-R-expressing cells, whereas rGel did not bind to these cell lines (Fig. 3). The binding of rGel/BLyS to HL-60 cells was found to be lowest (<0.2 optical density) for this group, and this finding was consistent with Western

blot analysis showing that there was no demonstrable expression of BlyS receptors. Intermediate binding (0.5–1.0 optical density) activity was found with SUDHL-6, Jurkat, Granta 519, MM1.S, and RPMI 8226 cells. The highest binding (>1.0 optical density) was found for OCI-Ly3, JeKo-1, KBM-5, and IM-9 cells. This result suggests that the cytotoxic activity of rGel/BLyS seems to be mediated through binding to BlyS receptors, but not all cells that were able to bind high levels of rGel/BLyS were sensitive to its cytotoxic effects (KBM-5 and IM-9). This suggests that there may be other factors beyond receptor binding that can influence the cytotoxicity of this fusion toxin.

Cellular Uptake of rGel/BLyS Fusion Toxin

We examined whether the rGel/BLyS fusion toxin can internalize into target cells. The rGel component of rGel/BLyS fusion toxin was observed in BAFF-R-expressing cells after a 4 h exposure to the fusion toxin, thus showing that rGel/BLyS is capable of efficient internalization through binding to cellular BlyS receptors (Fig. 4). In addition, we could detect no differences in the observed

internalization between cells that were highly sensitive to the fusion toxin (JeKo-1) and cells that were less sensitive (KBM-5, IM-9, and RPMI 8226).

We next determined whether the BAFF-R-directed cellular internalization of the fusion toxin involved an actin-dependent endocytotic process. Immunofluorescence study showed that internalization of rGel/BLyS was not affected by treatment with latrunculin A (Fig. 4), a drug that sequesters actin monomer. Dose-response curves of rGel/BLyS with and without latrunculin A pretreatment were superimposable (data not shown). Because neither cellular entry of the rGel/BLyS fusion toxin nor cytotoxic effects of the fusion toxin seem to be affected by pretreatment with latrunculin A, this suggests that the internalization of rGel/BLyS does not seem to involve an actin/cytoskeleton-dependent endocytotic process.

Specificity of rGel/BLyS Fusion Toxin

The specificity of rGel/BLyS against the BLyS receptor-expressing cells was assessed against JeKo-1 cells. BLyS itself has no effect on cell growth at tested concentrations, whereas non-B cell-targeting conjugate CTP/rGel showed a similar IC_{50} of free rGel. However, rGel/BLyS was very cytotoxic to JeKo-1 cells expressing the BLyS receptors BAFF-R, TACI, and BCMA (Fig. 5).

The biological effects of BLyS are mediated by three cell surface receptors designated BAFF-R, TACI, and BCMA (11–13). Therefore, pretreatment with BLyS might partially block the binding of rGel/BLyS fusion toxin to BLyS receptors. We found that pretreatment of BLyS showed a shift in the dose-response curve in rGel/BLyS-treated JeKo-1 cells but not in rGel-treated JeKo-1 cells. A dose-dependent inhibition of rGel/BLyS-induced cytotoxicity was observed (Fig. 6A). BAFF-R:Fc, TACI:Fc, or BCMA:Fc decoy receptor binds BLyS and blocks the binding of BLyS to their receptors BAFF-R, TACI, and BCMA, inhibiting

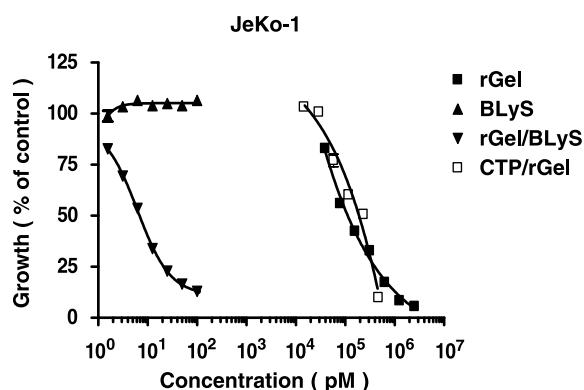


Figure 5. Specificity of the rGel/BLyS fusion toxin. To assess the specificity of rGel/BLyS against the BLyS receptor-expressing cells, JeKo-1 cells in log-phase culture were seeded (5×10^3 per well) in flat-bottom 96-well microtiter plates, and various concentrations of BLyS, rGel, CTP/rGel, or rGel/BLyS were added in quadruplicate wells. After 96 h, 50 μ L of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt labeling mixture was added to each well, after which the cells were incubated for another 6 h or overnight. The spectrophotometric absorbance was measured at 450 nm using an ELISA reader.

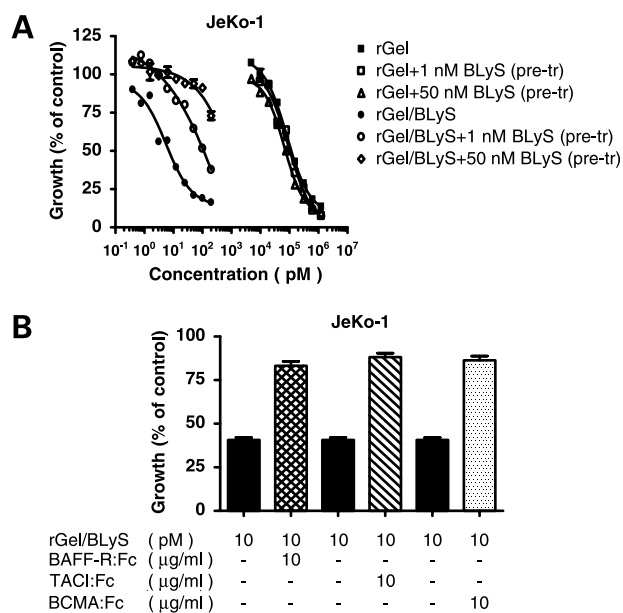


Figure 6. Competitive inhibition of rGel/BLyS binding to JeKo-1 cells. For competitive inhibition assay, JeKo-1 cells were seeded (5×10^3 per well) in flat-bottomed 96-well microtiter plates and pretreated with 1 nmol/L BLyS, 50 nmol/L BLyS (A), 10 μ g/mL BAFF-R:Fc, 10 μ g/mL TACI:Fc, or 10 μ g/mL BCMA:Fc (B) for 2 h, and then rGel or rGel/BLyS was added in quadruplicate wells. After 96 h, 50 μ L of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt labeling mixture was added to each well, after which the cells were incubated for another 6 h or overnight. The spectrophotometric absorbance was measured at 450 nm using an ELISA reader.

BLyS-mediated B-cell activation. We found that pretreatment of BAFF-R:Fc, TACI:Fc, or BCMA:Fc decoy receptors blocked the rGel/BLyS-mediated cytotoxicity in JeKo-1 cells (Fig. 6B). These data are consistent with our studies that show that the cytotoxic effects of rGel/BLyS seem to be mediated through direct binding to cellular BLyS receptors.

Effects of rGel/BLyS on Apoptotic Pathways

We determined whether the cytotoxic effect of rGel/BLyS was associated with an apoptotic mechanism. The rGel/BLyS-treated JeKo-1 cells showed 34% of apoptotic cell death, whereas treatment with rGel showed no induction of cellular apoptosis (Fig. 7A).

To determine whether caspase-3 was activated in MCL cells during rGel/BLyS-induced cell death, we investigated the cleavage of caspase-3 and its substrate PARP. Treatment with BLyS or rGel had no effect on caspase-3 and PARP cleavage, whereas treatment with rGel/BLyS resulted in cleavage of caspase-3 and PARP at 96 h (Fig. 7B). To determine whether the rGel/BLyS-induced apoptosis was dependent on activation of the caspase pathways, we next examined the effect of caspase inhibitors on the cytotoxicity of rGel/BLyS against JeKo-1 cells. The rGel/BLyS-induced cytotoxicity was found to be inhibited by treatment with the general caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK), or

caspase-3 inhibitor (Z-DEVD-FMK; Fig. 7C). This result shows that rGel/BLyS elicits an apoptotic response that seems to be mediated, at least in part, through a caspase-8- and caspase-3-dependent cascade.

Discussion

MCL and DLBCL are aggressive B-cell non-Hodgkin's lymphomas with very poor prognosis (31, 41). Many clinical therapeutic regimens have been shown to be highly

active in producing tumor responses; however, relapse typically occurs, and emergence of chemoresistance is common (32). Therefore, novel therapies are required for relapsed and/or refractory MCL and DLBCL.

Successful development of tumor-targeted therapeutic agents is dependent, in part, on the site-specific delivery of therapeutic agents and also on the biological activity of the delivered agent. BLyS is crucial for B-cell survival, and the biological effects of BLyS are mediated by three cell surface receptors designated BAFF-R, TACI, and BCMA (11–13).

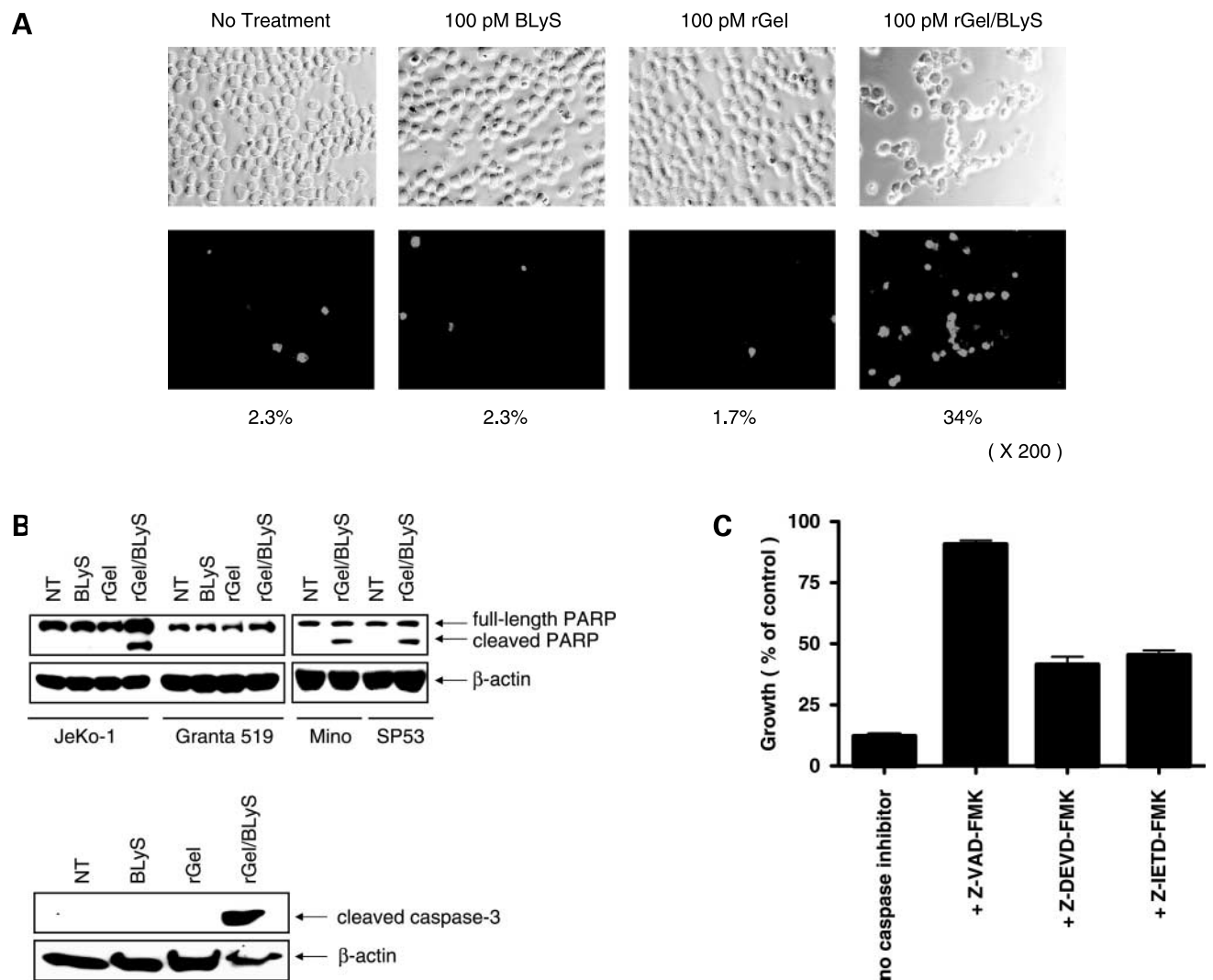


Figure 7. Effects of rGel/BLyS on apoptotic pathways. **A**, microscopic appearance of JeKo-1 cells after treatment. JeKo-1 cells were treated with 100 pmol/L BLyS, 100 pmol/L rGel, or 100 pmol/L rGel/BLyS. After 96 h, JeKo-1 cells were assayed for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining. Apoptotic cells were counted in randomly selected fields ($\times 200$) and expressed as a percentage of total cells counted. **B**, to examine the effect of rGel/BLyS on apoptotic pathways, JeKo-1, Granta 519, Mino, or SP53 cells were seeded at 5×10^5 per 24-well plate and then treated with 100 pmol/L BLyS, 100 pmol/L rGel, or 100 pmol/L rGel/BLyS. After treatment, cells were collected, washed, and lysed in 0.2 mL of lysis buffer. Cell lysates (50 μ g) were fractionated by 8% to 15% SDS-PAGE and electrophoretically transferred to Immobilon-P nitrocellulose membranes. Membranes were blocked and then probed with various antibodies. Secondary antibodies conjugated with horseradish peroxidase were used to visualize immunoreactive proteins using ECL detection reagent. Actin was used as a control for protein loading. **C**, influence of caspase inhibitors on the viability of rGel/BLyS-treated JeKo-1 cells. JeKo-1 cells pretreated with or without 200 μ mol/L general caspase inhibitor (Z-VAD-FMK), 200 μ mol/L caspase-8 inhibitor (Z-IETD-FMK), or 200 μ mol/L caspase-3 inhibitor (Z-DEVD-FMK) for 2 h and then treated with 20 pmol/L rGel/BLyS. After 72 h of exposure, viability was determined using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt assay.

BAFF-R is expressed in about 80% of MCL and in about 44% of DLBCL (15). It therefore seems that targeting BlyS and its receptors may disrupt this important autocrine growth loop in B-cell malignancies.

In this study, we generated a fusion toxin designated rGel/BlyS for the specific delivery of the rGel toxin to neoplastic B cells expressing the BlyS receptors, and we evaluated the biological activity of this molecule against a variety of human B-cell tumor lines. Of all the tumor lines tested, three MCL cell lines (JeKo-1, Mino, and SP53) expressing all three distinct BlyS receptors were found to be the most sensitive to the fusion toxin (targeting index = 27,500–80,000), whereas nontargeted rGel itself or CTP/rGel (non-B cell–targeting chemical conjugate) showed no specific cytotoxic activity against these three cell lines. As our previous findings (20), this result suggests that the rGel has impressive cytotoxic effects when delivered to cells using growth factor ligands. Interestingly, BCL-1 a mouse B lymphoma showed the highest targeting index (187,500) of all lines examined so far. Kanakaraj et al. (42) reported that BCL-1 cells have 4,800 binding sites per cell, whereas IM-9 cells have 3,200 binding sites per cell. The responsiveness of BCL-1 cells to rGel/BlyS may be related to the total number of BlyS receptors.

In this study, we were able to find a direct correlation between the expression levels of BAFF-R protein and sensitivity to rGel/BlyS, whereas no correlation was observed between sensitivity to rGel/BlyS and the expression levels of TACI protein among three MCL cell lines and two DLBCL cell lines. On the other hand, expression of BlyS receptors, cell surface binding of rGel/BlyS, and internalization of the fusion toxin into target cells were not alone sufficient to guarantee sensitivity to the cytotoxic effects of the fusion toxin because at several cell lines shown, all three characteristics but were relatively insensitive to rGel/BlyS. This may suggest that there is differential binding activity of the BlyS component of the rGel/BlyS to different BlyS receptors, and it may suggest that some cells have a different intracellular fate for internalized fusion toxin. Differences in subcellular localization could be too subtle to detect by conventional confocal imaging.

Gene profiling studies by Martinez et al. (43) have shown that the genes involved in the tumor necrosis factor and nuclear factor- κ B signaling pathways are overexpressed in MCL and may contribute to the resistant phenotype. Our preliminary studies examined the expression levels of antiapoptotic proteins in these four MCL cell lines to identify mechanisms that may account for the divergent cytotoxic effects. We found that Granta 519 cells expressed the highest levels of Akt, phosphorylated Akt, Bcl-2, and cyclin D1 among four MCL cell lines (data not shown). This result suggests that up-regulation of antiapoptotic mechanism in Granta 519 cells may lead to resistance to rGel/BlyS, although Granta 519 cells showed relatively high level expression of all BlyS receptors and relatively intermediate binding activity of the fusion toxin. Alternatively, He et al. (44) reported that

neutralization of endogenous BlyS and a proliferation-inducing ligand by the presence of soluble TACI and BCMA decoy receptors may allow cells to evade apoptosis through down-regulation of antiapoptotic proteins Bcl-2 and Bcl-xL. Kern et al. (7) reported that BlyS expression seems to be involved in resistance to apoptosis of B-cell chronic lymphocytic leukemia through an autocrine pathway. All of these results suggest that BlyS through numerous pathways may contribute to resistance to rGel/BlyS treatment in Granta 519 cells and, perhaps, in other cell lines.

Our experiments show that the cytotoxic effects of rGel/BlyS seem to be BlyS receptor mediated, and the rGel/BlyS fusion toxin inhibits cell growth through induction of apoptosis associated with caspase-3 activation and PARP cleavage. The proapoptotic nature of this fusion toxin seems to be related to the cell targets rather than the rGel toxin itself. In previous studies (20, 26), we have found that rGel-containing fusion constructs targeting solid tumor cells and tumor vascular endothelium seem to be cytotoxic through a nonapoptotic (necrotic) mechanism that involves inhibition of protein synthesis. The protein synthesis inhibitory properties of rGel are similar to that of ricin A-chain and depend on ribosomal inactivation through enzymatic (*n*-glycosidase) activity of the toxin molecule (27). Studies by Keppler-Hafkemeyer et al. (45) have suggested that both RTA and *Pseudomonas* exotoxin may exert their effects on cells through both inhibition of protein synthesis and by activation of caspases.

Taken together, our data suggest rGel/BlyS may be a good candidate for the treatment of MCL or DLBCL, and that BlyS has the potential to serve as a useful targeting ligand for the specific delivery of toxin to neoplastic B cells that express receptors for BlyS.

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