The growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel induces apoptosis specifically in BAFF-R–positive CLL cells

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The cytokine B lymphocyte stimulator (BLyS) mediates its effect through cell-surface receptors BAFF-R, TACI, and BCMA. BLyS receptors are expressed only on B cells and not present in other normal cells including normal T lymphocytes. Chronic lymphocytic leukemia (CLL) is a B-cell disease and CLL lymphocytes express BLyS receptors. Gelonin, a type 1 ribosome-inactivating toxin, lacks cell membrane binding domain and hence is nontoxic to intact cells. We generated a construct of recombinant gelonin (rGel) fused to BLyS to specifically target quiescent B-CLL lymphocytes. The construct rGel/BLyS specifically binds and internalizes through BAFF-R into CD19+ B-CLL lymphocytes and induces apoptosis at nanomolar concentrations. In contrast, rGel alone was not able to internalize into these leukemic lymphocytes. Mechanistically, the rGel/BLyS construct inhibits protein synthesis with an IC50 of less than 3 nM compared with more than 5000 nM for rGel toxin alone. This rGel/BLyS-mediated decrease in protein synthesis was associated with a decline in short-lived proteins such as MCL-1 and XIAP, the 2 survival proteins in B-CLL. There was a strong relationship between a decrease in these proteins and the cleavage of PARP, a hallmark feature of apoptosis. Taken together, these data suggest that the rGel/BLyS fusion toxin may have potential therapeutic efficacy for B-CLL patients. (Blood. 2007;109:2557-2564)

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Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is presently an incurable disease representing the most common form of leukemia in North America and Europe.1 This indolent leukemia is a neoplastic disorder, characterized by a relentless accumulation of small, mature B cells with typical B-cell markers CD5, CD19, CD23, and CD20.2

The traditional therapeutic approach to CLL has been to use alkylator-based regimens such as chlorambucil and cyclophosphamide; DNA-directed purine nucleoside analogs such as pentostatin, fludarabine, and cladribine; or the combination of these 2 groups of therapeutic agents.3-6 Although these agents have been used as standard regimens for the treatment of this disorder, unfortunately, the majority of patients either fail to gain a complete response to DNA-directed agents or eventually experiences a relapse. An additional problem with current agents is their therapeutic index. The agents used in the clinic for CLL therapy are nonsel ective and hence induce untoward effect. This emphasizes the importance of focusing upon identification of new and CLL-targeted therapies for the disease.

The B-lymphocyte stimulator (BLyS), also called BAFF (B-cell activating factor belonging to the TNF family)7; TALL-1 (TNF- and Apol-related leukocyte expressed ligand 1)8; and THANK (TNF homolog that activates apoptosis, NF-kB, and JNK),9 is a member of the TNF superfamily of cytokines.7,10 BLyS has been shown to be critical for the maintenance of normal B-cell development and homeostasis.11 The biological (growth stimulatory) effects of BLyS are mediated by 3 cell-surface receptors designated BAFF-R (BAFF receptor)12; TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor)13; and BCMA (B-cell maturation antigen).14

BAFF-R has been identified as the main BLyS receptor responsible for peripheral B-cell homeostasis and specifically binds BLyS, whereas BCMA and TACI can also bind to a proliferation-inducing ligand (APRIL).15,16 Similarities between BLyS- or BAFF-R knock-out mice support the hypothesis that BAFF-R appears to be the predominant receptor for the BLyS ligand.17 Furthermore, the BLyS receptors are expressed only on B cells and are not present in other normal cells including T lymphocytes. Taken together, these observations suggest that BAFF-R could be targeted and exploited for the selective delivery of chemotherapeutic agents and or toxins to B-cell diseases.

Gelonin is a protein toxin originally isolated from the seeds of Gelonium multiflorum plants that predominantly grow in India.18 The recombinant gelonin (rGel) toxin is a single-chain N-glycosidase similar in action to ricin A chain.18,19 rGel is preferred over ricin A chain because it does not appear to generate capillary leak syndrome (CLS), which limits the use of other toxins. Also, clinical trials conducted at MDACC demonstrated that rGel is nonantigenic, while ricin A is known to be antigenic. Immunotoxins and fusion constructs containing rGel specifically kill tumor cells in vitro and in vivo, while rGel alone is ineffective.20 After binding to the cell, these constructs internalize through endocytosis and translocate to the cytosol where rGel inhibits protein synthesis by inactivating ribosomes.21 This toxin requires a carrier to transit the cell membrane and is lethal to mammalian cells within 72 to 96 hours. Previous studies have demonstrated that CLL lymphocytes...
are dependent on protein synthesis for survival,22-24 and in vivo studies have suggested successful utility of immuno-toxins for CLL.25,26 Since clinical studies with rGel suggest that it is less toxic than ricin A chain, we therefore hypothesized that rGel would be an ideal payload to target quiescent CLL lymphocytes.

To provide cell targeting and selectivity, we synthesized a growth factor fusion construct composed of rGel linked to human BLyS. Purified rGel/BLyS construct versus rGel were tested against primary CLL lymphocytes. We demonstrated that rGel/BLyS bound specifically to BAFF-R-expressing cells and caused selective cell death of leukemic B cells. This cytotoxic effect was associated with a decline in cellular protein and RNA synthesis resulting in a reduction in proteins that provide a survival mechanism to CLL cells. This cell death was induced only when fusion construct was used, while rGel alone was not cytotoxic to these CLL cells.

### Patients and methods

#### Construction and purification of rGel/BLyS

The construction and purification of the rGel/BLyS fusion construct was accomplished as described previously.26 In brief, the BLyS was first generated by reverse transcription-polymerase chain reaction (RT-PCR) from Jeko cells. Since the C-terminus of this ligand may be involved in receptor recognition, we cloned the rGel molecule upstream (attached to the N-terminus) followed by a G4S flexible peptide tether to the BLyS molecule. The recombiant rGel/BLyS DNA construct was ligated into a bacterial expression vector (pET-32a; Novagen, Madison, WI), expressed in E. coli, and the target protein was purified to homogeneity and used in all the experiments.

#### Patients and healthy donors

Present in vitro studies were carried out in leukemic lymphocytes obtained from patients with CLL (n = 39) or from healthy donors (n = 5). Blood samples obtained from these individuals were used for different pharmacological, biochemical, and molecular end points. All CLL patients signed a written informed consent to participate in this laboratory protocol, which was approved by the M. D. Anderson Cancer Center institutional review board (IRB). Similarly, a separate IRB-approved protocol was used for healthy donors who signed an informed consent form, in accordance with the Declaration of Helsinki.

#### Clinical laboratory end points

The percentage of leukemic lymphocytes (B-cell population) in all patients was measured by flow cytometry using CD19-FITC antibody obtained from BD Biosciences (San Jose, CA). Determination of IgVH gene mutation status and ZAP-70 analysis for the patients in our study were conducted as described previously27 and provided by the Chronic Lymphocytic Leuke-

mia Research Consortium (University of California, San Diego and the University of Texas, M. D. Anderson Cancer Center). Fluorescent in situ hybridization (FISH) analysis data were provided by the Chronic lymphocytic leukemia Research Center, Department of Hematopathology at M. D. Anderson Cancer Center. The FISH technique was used to detect chromosome 17p deletion for the p53 gene in CLL cells. The detailed methodology for the assay has been described previously.27 Chromosomal cytogenetics was determined using CLL lymphocytes at M. D. Anderson Cancer Center.

#### Isolation of leukemic and normal lymphocytes

Whole blood was collected in heparinized tubes, diluted, and layered onto Ficoll-Hypaque (specific gravity, 1.086; Life Technologies, Grand Island, NY). Mononuclear cells were removed from the interphase. A Coulter channelizer (Coulter Electronics, Hialeah, FL) was used to determine cell number and the mean cell volume. Cells were washed and resuspended in medium, supplemented with 10% fetal bovine serum, at a concentration of 5 x 10^6 cells/mL. Fresh cells were used for all experiments.

### Quantitation of CD19, and BAFF-R expression on the surface of CLL lymphocytes

One million blood lymphocytes from each patient or healthy donor were washed briefly with PBS and resuspended in PBS containing 2% BSA. Cells were then stained either anti–CD19-FITC or anti–BAFF-R-PE antibodies, or both antibodies together for 1 hour. Cells were washed 3 times with PBS and analyzed by flow cytometry using the CellQuest software (Becton-Dickinson, Oxford, United Kingdom). As a negative control, lymphocytes from the same individuals were incubated with either FITC- or PE-conjugated secondary antibodies. 7AAD was included in all the samples to eliminate nonspecific binding of antibody to dead cells.

### Binding and internalization of rGel/BLyS into CLL lymphocytes

Leukemic lymphocytes were treated with 100 nM rGel or 100 nM rGel/BLyS for 2 hours, washed briefly with PBS, and incubated with 2% BSA in PBS containing CD19-PE antibodies for 1 hour. Cells were washed briefly 3 times with PBS then fixed in 4% paraformaldehyde (Sigma, St Louis, MO) for 20 minutes at room temperature, followed by a brief rinse with PBS. Cells were then permeabilized for 10 minutes in PBS containing 0.2% Triton X-100, washed 3 times with PBS, and blocked with PBS containing 3% BSA for 1 hour at room temperature. After a brief wash with PBS, cells were incubated with rabbit anti-rGel polyclonal antibody diluted 1:500 in PBS containing 0.1% Tween 20 and 0.2% BSA for 1 hour at room temperature. Cells were washed 3 times in PBS containing 0.1% Tween 20 for 15 minutes and incubated with a 1:100 dilution of FITC-coupled anti–rabbit IgG (Sigma). After 3 washes with PBS containing 0.1% Tween 20, cells were washed once in PBS for 10 minutes. Cells (50,000) were then placed onto slides using cytoSpin (Shandon, Pittsburgh, PA), and mounted in mounting medium containing DAPI. Slides were then analyzed with a Zeiss LSM510 laser scanning microscope (Carl Zeiss, Jena, Germany). Images in Figure 2A-B were obtained using an Olympus Fluoview IX71 confocal microscope system (Olympus, Melville, NY). The objective lens was a 40x/1.30 NA oil lens. Images were viewed and processed using Fluoview FV500 version 4.3 software, and the digital image was enlarged. The remainders of the cells were analyzed using flow cytometry.

### Preparation of stroma for CLL lymphocyte culture

To enhance the survival of B-CLL lymphocytes and to prevent spontaneous apoptosis, during in vitro culturing, we cocultured B-CLL lymphocytes with HS.5/GFP stromal cells. HS.5, a transformed bone marrow stromal cell line, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 100 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin. HS.5 green fluorescent protein (GFP) stromal cells were developed by stably expressing enhanced green fluorescent protein under hygromycin (Invitrogen, Carlsbad, CA) selection (100 μg/mL).

For experimental purpose, HS.5/GFP stromal cells were seeded 0.1 x 10^6/well in 6-well plates and incubated overnight at 37°C. The next morning, stromal cells were washed once with serum-free medium and CLL lymphocytes (1 x 10^6) were cultured on top of 1 x 10^6 stromal cells. After 2 to 3 hours of adhesion, samples were exposed to either control-supplemented media, rGel, or rGel/BLyS for 3 to 5 days. These cells were used for different end points such as RNA and protein synthesis, immunoblot analyses, intracellular nucleotide pools, and cell death assays.

### Inhibition of RNA and protein synthesis

Patient-derived lymphocytes were cocultured with stromal cells as described above under “Preparation of stroma for CLL lymphocyte culture” and incubated with or without rGel or rGel/BLyS at indicated doses for 72 hours, and synthesis of RNA and protein was measured. Prior to removal of the aliquot, 1 μCi (0.037 MBq)/mL [3H]uridine for RNA analysis or 10 μCi/mL (77 Ci/mmol) [3H]leucine was added for protein synthesis analysis and incubated for one hour. The cells were then collected and the
radioactivity in acid-insoluble material was measured by scintillation counting and expressed as the percentage of control (untreated) value of the cells.25

**Immunoblot analysis**

Cells were lysed on ice for 20 minutes in lysis buffer containing 25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM MgCl2, 0.5% sodium deoxycholate, 20 mM glycerophosphate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, pH 8, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, pH 10, and protease inhibitor. Cells were centrifuged at 14 000 rpm. The supernatant was stored at −80°C until use. Protein content was determined using DC protein assay kit according to the manufacturer’s instructions (Bio Rad Laboratories, Hercules, CA). Aliquots (50 μg) of total cell protein were boiled with Laemmli sample buffer and loaded onto 8% to 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (GE Osmonics Labstore, Minnetonka, MN). Membranes were blocked for 1 hour in PBS-Tween containing 5% nonfat dried milk and then incubated with primary antibodies for 2 hours followed by species-specific horseradish peroxidase (HRP)–conjugated secondary antibody (diluted 1:5000) for 1 hour. The blots were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL) and normalized to the β-actin levels (antibody to β-actin was obtained from Sigma) in each extract. Rabbit polyclonal antibody to MCL-1 (sc-819) and mouse monoclonal antibody to PARP and mouse monoclonal antibody to BCL-2 (sc-509) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to XIAP and mouse monoclonal antibody to PARP were purchased from BD Pharmingen (San Diego, CA).

**Apoptotic analysis using dual annexin V–PE/7AAD**

The analysis of annexin V binding was carried out with a Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions. Briefly, cells were washed with PBS and resuspended in 200 μL 1x annexin binding buffer obtained from BD Biosciences, at a concentration of 1 x 10^6 cells/mL. Annexin V–PE (5 μL) was added and the cells were incubated in the dark for 15 minutes at room temperature. The labeled cells were then added to 10 μL 7AAD (50 μg/mL) and analyzed immediately with a FACSCALIBUR cytometer (Becton-Dickinson). Data, from at least 100 000 events per sample, were recorded and processed using the CellQuest software. To estimate cell death only in CLL lymphocytes in coculture experiments, gating on GFP-negative populations eliminated stromal cells. Additional staining with anti-CD19 (B cells) or anti-CD3 (T cells) antibodies was used to measure cell death specific to B- and T-cell types. The results were expressed after calculating the percentage of CD19^+ cells in the control untreated sample.

**Results**

**Synthesis of rGel/BLyS conjugate**

We designed a growth factor fusion toxin containing BLYS and the plant toxin rGel as described previously.26 Biologic activity of the toxin component was confirmed also as previously described. On reducing SDS–polyacrylamide gel electrophoresis (PAGE), the purified rGel/BLYS fusion protein migrated at the expected molecular weight (45.5 kDa); no other contaminating bands were observed in the final purified conjugate.

**Expression of CD19 and BAFF-R and apoptosis of normal lymphocytes**

To determine the efficacy of rGel/BLYS for the therapeutic treatment of CLL, we initially examined whether T and B lymphocytes from healthy volunteers express receptors for BLYS (BAFF-R). Peripheral blood mononuclear cells (PBMCs) from 5 healthy donors were isolated and incubated with either antibodies to CD19, BAFF-R, or both antibodies followed by flow cytometric quantitation. 7AAD was used to eliminate nonspecific binding of dead cells. Negative controls stained with only secondary antibody did not have any measurable (background) staining. As shown in Figure 1A and Table 1, PBMCs from healthy donors contained CD19^+ cells ranging from 1.6% to 3%. The BAFF-R expression in the total population is low and ranged between 1.5% and 3.5% of cells, which were all CD19^+. This low expression is due to a low number of B cells in the PBMCs from healthy donors.

To determine if the construct would induce apoptosis in normal B- and T-lymphocytes, we treated PBMCs from 3 donors (I, III, and IV, Table 1) with the construct. Annexin binding assay for these 3 samples demonstrated that only 1.8%, 2.0%, and 1.8% of cells in the total PBMC population were annexin positive. These cells (80% to 90%) were also CD19^+, suggesting that the construct enters normal B cells and induces apoptosis. In contrast, T cells (CD19^-) did not show annexin positivity.

**Table 1. Prevalence of B-cell (CD19^+) population and BAFF-R expression in the peripheral blood of CLL patients or healthy donors**

<table>
<thead>
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<th>BAFF-R^+</th>
<th>CD19^+, BAFF-R^+</th>
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</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>CLL patients</td>
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</tr>
<tr>
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</tr>
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<td>11</td>
<td>56.3</td>
<td>53.4</td>
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</table>

Primary PBMCs from either the healthy donors (1-5) or from the patients (1-11) were stained either with CD19-FITC, or BAFF-R-PE antibodies, or both of the antibodies and analyzed by flow cytometry.
Gel/BLyS was found to internalize in all of the CD19 flow cytometry (Figure 2C-D). As illustrated in Figure 2B, the gelonin toxin using a rabbit antigelonin polyclonal antibody. After were then fixed, permeabilized, and stained for intracellular stained with surface markers for B cells (ie, CD19-PE). The cells with 100 nM of either rGel/BLyS or rGel for 2 hours and then peripheral blood of CLL patients. We treated Ficoll buoyant cells of the rGel/BLyS compared with rGel into cells obtained from the 2560 NIMMANAPALLI et al BLOOD, 15 MARCH 2007 have a high percentage of CD19 compared with healthy donors, B-CLL patient–derived PBMNCs 1). Again, these were all CD19. Since we know that all patient-derived cells express BAFF-R. Quantitative expression of CD19 and BAFF-R on leukemic lymphocytes As done for normal lymphocytes, we also examined whether patient-derived cells express receptors for BlyS (BAFF-R). PBMCNs from 11 CLL patients were isolated and incubated with either antibodies to CD19, BAFF-R, or both antibodies followed by flow cytometric quantitation. CLL patient–derived PBMCNs consisted of 23% to 85% CD19+ cells (Figure 1B; Table 1). In contrast to PBMCNs from healthy donors, 27% to 89% of the PBMCNs derived from patients with CLL were positive for BAFF-R (Table 1). Again, these were all CD19+. These data demonstrate that compared with healthy donors, B-CLL patient–derived PBMCNs have a high percentage of CD19+ cells in the population. Because BAFF-R expression was found to be specific to CD19+ cells, we reasoned that the rGel/BLyS construct has significant potential as specific targeted therapy for B-CLL patients. Binding and internalization of rGel/BLyS and rGel on CLL cells Since we know that all patient-derived cells express BAFF-R specifically on CD19+ cells, we next evaluated the internalization of the rGel/BLyS compared with rGel into cells obtained from the peripheral blood of CLL patients. We treated Ficoll buoyant cells with 100 nM of either rGel/BLyS or rGel for 2 hours and then stained with surface markers for B cells (ie, CD19-PE). The cells were then fixed, permeabilized, and stained for intracellular gelonin toxin using a rabbit antigelonin polyclonal antibody. After staining with FITC-conjugated secondary antimouse antibody, cells were analyzed by either confocal microscopy (Figure 2A-B) or by flow cytometry (Figure 2C-D). As illustrated in Figure 2B, the rGel/BLyS was found to internalize in all of the CD19+ cells. Because CLL lymphocytes have a major volume of nucleus with very little cytoplasm on the periphery of nucleus, the Gel-FITC appears as a peripheral stain. When cells were treated similarly with rGel alone, no internalization was observed (Figure 2A). Consistent with these microscopy data, only 2.2% of the cells showed internalization of rGel (Figure 2C), while 91.5% demonstrated internalization of rGel after exposure to rGel/BLyS (Figure 2D) when analyzed using a flow cytometer. These data indicate that internalization and binding of rGel/BLyS is not only specific to CD19+ cells (B cells) but is also efficient (91% vs 2%) compared with the toxin alone. Effect of rGel/BLyS on macromolecule synthesis Our data using B-CLL cells demonstrated that all CLL patient B cells express BAFF-R and that rGel/BLyS internalizes specifically into B cells. To understand the mechanism of action, we then treated leukemic lymphocytes from 7 CLL patients. The median white blood cell (WBC) count was 139 × 10^9/L (range, 54 × 10^9/ L-360 × 10^9/L). Although, most of these patients (n = 5) were previously untreated, the biology of the disease was heterogeneous. Their disease status (Rai stage) ranged from 1 to 4, and their leukemia cells were mostly (n = 5) negative for ZAP-70 and IgVH mutation. Three patients (nos. 3, 4, and 7) had deletion of chromosome 17, and 2 patients (nos. 5 and 6) had deletion in chromosome 13. Two patients (nos. 1 and 2) were negative for cytogenetic abnormalities. Our data indicate that rGel/BLyS internalizes into patient-derived CLL lymphocytes. To determine the consequences of toxin internalized in to these cells, leukemic lymphocytes from 7 patients were treated either with 1, 2.5, and 5.0 μM rGel or 1, 2.5, and 5.0 nM rGel/BLyS for 72 hours, and the inhibition of protein synthesis was assessed by measuring [3H]leucine incorporation. Generally, a dose-related decrease in protein synthesis was observed in CLL cells of all patients when treated with nanomolar levels of the rGel/BLyS construct (Table 2). At 5 nM, the inhibition was between 30% and 80%. In contrast, a micromolar level of rGel alone was needed for protein synthesis inhibition in the majority of patient samples, and 3 were still resistant (nos. 2, 5, and 6). Based on these 7 patient samples, the IC50 for protein synthesis inhibition by rGel was more than 5000 nM, whereas the IC50 for rGel/BLyS was 2.7 nM. Because RNA synthesis is linked to protein synthesis, a decrease in protein synthesis should have an indirect effect on RNA production. Hence, we also measured the inhibition of RNA synthesis by measuring [3H]uridine incorporation in to nascent RNA. In the samples from 7 patients tested, generally RNA synthesis was not altered by rGel treatment, but there was significant inhibition in all samples treated with rGel/BLyS (Table 3). These data suggest that protein inhibition by rGel/BLyS was significantly high when compared with RNA inhibition, indicating that inhibition of protein was the primary action of the toxin in these cells. Effect of rGel/BLyS on CLL survival proteins The gelonin-mediated inhibition of protein synthesis would have an effect on short-lived, rapid turnover proteins such as MCL-1 and XIAP, which are known to be the most important survival proteins in CLL. To investigate this postulate, the immunoblot analysis was performed for antiapoptotic proteins such as MCL-1, BCL-2, PUMA, and XIAP. For illustrative purposes, an immunoblot from one patient’s sample is shown (Figure 3A). The levels of MCL-1
protein was unaffected with rGel at micromolar concentration, while nanomolar level of conjugate decreased MCL-1. Similarly, XIAP expression level was unchanged with rGel alone but declined with the rGel/BLYS construct. In concordance with the decline in MCL-1 and XIAP, only the rGel/BLYS treatment resulted in cleavage of PARP protein, a hallmark feature of apoptosis (Figure 3A). There was not a significant change in PUMA (data not shown) or BCL-2 (Figure 3A) protein levels with either agent in all samples tested.

There was heterogeneity among patients regarding decline in MCL-1, XIAP, and induction of apoptosis. Data from 7 patients’ samples (nos. 14-18, 21, and 44) were quantitated by densitometry. In 6 of 7 samples, the levels of MCL-1 protein declined with the nanomolar levels of rGel/BLYS construct; while even at micromolar concentration, rGel alone was not effective in reducing this protein (Figure 3B-C). The data were similar for XIAP protein levels (Figure 3D-E). The decrease in these protein levels was not due to apoptosis and cleavage of protein as no lower molecular bands were detected.

When analyzed for PARP cleavage, of 7 patients’ samples, 3 (nos. 14, 18, and 44) did not show a significant amount of cleaved PARP, while in other samples PARP protein was completely cleaved with the construct. To determine whether there was an association between changes in MCL-1 and XIAP protein levels and cell death, these data were plotted and statistical values were calculated (Figure 3F-G). The levels of these antiapoptotic molecules were inversely and strongly correlated with induction of apoptosis (for MCL-1: r = 0.79, P = .001; for XIAP: r = 0.85, P = .001).

rGel/BLYS causes apoptosis in CD19⁺ CLL cells

Although PARP protein expression immunoblot suggested induction of apoptosis, the analyses reflected cell death of all subsets (CD19⁺ and CD19⁻) from PBMCs from patients and also stroma cells. To clearly define the cytotoxic effect on only CD19⁺ CLL cells, we analyzed cell death by flow cytometry. A total of 20 samples from previously treated (n = 9; Figure 4A) or untreated (n = 11; Figure 4B) patients was incubated in the presence of stromal cells with either rGel/BLYS or rGel alone. Specific cell death of leukemic cells was analyzed by gating the GFP-positive stromal cells and staining with CD19-APC, annexin V-PE, and 7AAD. CD19⁺ cell death was assessed by staining a parallel sample with CD19/PE and CD3/APC and calculating the percentage of live T and B cells. The percentage of B cells in the untreated sample and the percentage of cell death after treatment with rGel alone or rGel/BLYS construct demonstrated that in 16 of 20 patient samples, there was an induction of apoptosis (>20% annexin positivity) with the fusion construct treatment (Figure 4). In contrast, only 3 of the patient samples demonstrated increased apoptosis (>20% annexin positivity) after treatment with rGel alone. These data suggest that the rGel/BLYS construct but not rGel itself is specifically cytotoxic to leukemic lymphocytes, and there is heterogeneity among patient samples regarding the response to the construct. This was due neither to the WBC count nor the Rai stage of the disease (compare WBC counts or Rai stage with the percent positivity) with the fusion construct treatment (Figure 4).

Table 2. Effect of rGel or rGel/BLYS on the protein synthesis

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>% untreated control (dpm/10⁷ cells)</th>
<th>Protein synthesis, % of control</th>
</tr>
</thead>
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<tr>
<td></td>
<td>1.0 μM</td>
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<tr>
<td>1</td>
<td>100 (15 389)</td>
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<td>2</td>
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</tr>
<tr>
<td>7</td>
<td>100 (173 473)</td>
<td>103</td>
</tr>
</tbody>
</table>

Primary CLL cells from patients 1 to 7 were isolated and incubated with either 1.0, 2.5, and 5.0 μM rGel or 1.0, 2.5, and 5.0 nM rGel/BLYS for 72 hours and pulsed with [³H]leucine for 1 hour. At the end of incubation, the leucine incorporation into protein was measured. The values are expressed as percentage of control (untreated) cells.

Table 3. Effect of rGel or rGel/BLYS on the RNA synthesis

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>% untreated control (dpm/10⁷ cells)</th>
<th>RNA synthesis, % of control</th>
</tr>
</thead>
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<tr>
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<td>2.5 μM</td>
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<td>5</td>
<td>100 (197 562)</td>
<td>108</td>
</tr>
<tr>
<td>6</td>
<td>100 (178 934)</td>
<td>116</td>
</tr>
<tr>
<td>7</td>
<td>100 (172 653)</td>
<td>111</td>
</tr>
</tbody>
</table>

Primary CLL cells from the patients 1 to 7 were isolated and incubated with either 1.0, 2.5, and 5.0 μM rGel or 1.0, 2.5, and 5.0 nM rGel/BLYS for 72 hours and pulsed with [³H]uridine for 1 hour. At the end of incubation the uridine incorporation into RNA was measured. The values are expressed as percentage of control (untreated) cells.
induction. Similarly, positivity for ZAP-70 (nos. 17, 36, 40, 44, and 16) did not affect the extent of apoptosis observed in these samples. Taken together, these data, albeit in a small number of patients, suggest no clear correlation between CLL prognostic factors and sensitivity/resistance to the toxin construct.

Discussion

Because of the disseminated nature of the disease, chemotherapy is the only effective approach to treat leukemia. Based on the causal molecular defects, targeted therapeutics have been successfully used for some leukemias; however, CLL, the most prevalent hematologic malignancy in the western world, does not provide a molecular or biochemical signature to attack therapeutically.

Studies dealing with the biology of this tumor have identified this neoplastic disorder as a gradual accumulation of small, mature lymphocytes with typical B-cell markers. Although a slowly proliferating pool of cells has been described in lymph nodes and in bone marrow that might feed the accumulating pool in the blood, in general lack of proliferative properties makes these cells inherently quiescent. Even though replications dormant, the accumulation of leukemic cells both in the bone marrow and the peripheral blood may be due to intrinsic defects in their apoptotic machinery or dysregulated production of survival signals from their microenvironment. To maintain a pool of protein, these replicationally quiescent cells are active in mRNA transcription and protein translation. Hence, these physiological processes could be targeted to induce deleterious effects in CLL.

The survival advantage of CLL lymphocytes may be due to the presence of antiapoptotic proteins of the BCL-2 family. Several lines of evidence now support the oncogenic nature of the Mcl-1 protein as it appears critical for the existence of CLL cells. First, MCL-1 is essential during early lymphoid development and then later for the maintenance of mature T and B lymphocytes. Second, the level of expression of antiapoptotic proteins in normal and malignant lymphocytes is in concordance with its role in survival. Third, and related to the present work, high levels of MCL-1 and BCL-2 mRNA and protein have been found in most cases of B-CLL. Additionally and specifically, the levels of MCL-1 in CLL cells are inversely correlated with in vitro response to chemotherapeutic agents. Fourth, high levels of MCL-1 are observed in relapsed leukemias and are associated with the failure of B-CLL patients to achieve complete responses (CRs) to initial therapy with fludarabine. Fifth, consistent with these reports, down-regulation in the expression of MCL-1 protein by direct measures such as antisense oligonucleotides or through indirect MCL-1 transcription and translation inhibitor approaches results in cell death during in vitro culture or in vivo therapy. Conversely, overexpression of MCL-1 prolongs the survival of cells exposed to a variety of apoptosis-inducing stimuli, including...
cytokine withdrawal, c-Myc overexpression, and treatment with chemotherapeutic and radiotherapeutic insults.\textsuperscript{39,40} Taken together, this evidence establishes MCL-1 as a key survival factor for CLL. Hence, we reasoned that the optimal approach for this disease would be to target CLL cells, based on cell surface receptor, to deliver toxin that inhibits protein synthesis.

The use of ligands to deliver toxins to target cells overexpressing receptors has received considerable attention in previous years. Chimeric proteins composed of chemokine ligands, such as IL-2,\textsuperscript{41} IL-3,\textsuperscript{42} IL-4,\textsuperscript{43} IL-13,\textsuperscript{44} or VEGF\textsuperscript{45} fused to various toxins (eg, gelonin, diphtheria, pseudomonas exotoxin, and more recently, granzyme B),\textsuperscript{45} have all demonstrated significant and selective cytotoxic effects against target cells at nanomolar concentrations and below. Studies in our laboratory have suggested that the rGel toxin has impressive cytotoxic effects when delivered to cells using ligands or fused to single-chain antibodies.\textsuperscript{20}

Plant toxins currently used or envisioned in pharmaceutical formulations are predominantly either type I or type II toxins. The type II toxins are composed of 2 disulfide-linked polypeptides (A and B). The B chain binds to the plasma membrane and facilitates entry of the catalytic A chain into the cytosol where it inhibits protein synthesis by irreversibly depurinating the 28 S rRNA in the 60 S subunit of the host cell’s ribosome.\textsuperscript{46} Type I toxins are devoid of the B chain and hence they lack mechanism for efficient entry into the cytosol.\textsuperscript{18} Gelonin, a 30-kDa type I ribosomal inactivating toxin from the seeds of Gelonium multiflorum, is an ideal candidate for therapeutic delivery due to its minimal toxicity when added extracellularly.\textsuperscript{47} Gelonin, when delivered intracellularly, has been shown to be a potent inhibitor of cell growth, but one that is relatively inefficient in gaining entry to the cytosol.\textsuperscript{47} Because of this latter property, one major benefit for using gelonin is its relatively low toxicity when applied extracellularly or when liberated from dying cells in plasma. This general property is consistent with our experiments in which recombinant gelonin showed no detectable cellular entry, mechanistic consequences, or apoptosis when incubated with primary patient-derived PBMNCs at concentrations up to 5000 nM.

In the current study, we characterized the growth factor fusion construct rGel/BLyS for its ability to inhibit protein synthesis in replicationally quiescent CLL cells. These inhibitory actions were preceded by internalization of the rGel/BLyS construct specifically in leukemic lymphocytes expressing BAFF-R and followed by apoptosis of the CD19\textsuperscript{+} cells in 16 of 20 patient samples. In contrast, 2 of the patient samples showed cytotoxicity with rGel alone, although at micromolar levels of the toxin. These data suggest that rGel/BLyS construct is necessary for cell death of these leukemic lymphocytes, albeit there is heterogeneity regarding cellular response to the construct. The heterogeneity seems to stem from differential effect on the expression levels of 2 survival proteins MCL-1 and XIAP (Figure 4).

Recently, Tai et al\textsuperscript{48} reported that in multiple myeloma bone marrow stromal cells are the main source of BAFF or BLyS, and this expression is enhanced when myeloma samples were adhered to patient-derived stromal cells. In the present study, neither bone marrow–derived stromal cells (HS.S) nor CLL cells secrete BLyS protein, but when CLL cells were in contact with stromal cells a significant amount of BLyS secretion was seen (data not shown). This indicates that the rGel-conjugated BLyS is able to compete with extracellular BLyS, implying that our model system mimics the in vivo situation where BLyS is circulating in plasma.

The levels of antiapoptotic and survival factors are dependent on the intrinsic stability of the individual transcripts and proteins. Because the MCL-1 mRNA contains the adenylate/uridylate-rich elements (AREs) in the 3’ untranslated region, its rapid degradation is expected after inhibition of global mRNA synthesis.\textsuperscript{49} In addition, the protein contains 2 PEST regions, rich in proline (P), glutamic acid (E), serine (S), and threonine (T), the signature feature that dictates fast turnover of a protein molecule.\textsuperscript{50} Bcl-2, which does not have a PEST region, is a long-lived protein with a half-life of 10 to 24 hours.\textsuperscript{51,52} Decrease in Mcl-1 and XIAP protein without a change in Bcl-2 protein levels is consistent with the intrinsic stability of these molecules.

These studies demonstrate that the rGel/BLyS fusion construct has impressive and specific cytotoxic effects on B-CLL cells that overexpress the BAFF-R, the receptor for the BLyS ligand. Assuming animal model efficacy studies now ongoing demonstrate effective cytotoxic effects in vivo,\textsuperscript{53} these data suggest that rGel/BLyS construct may have potential utility as a therapeutic agent.

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Authorship

Contribution: R.N. was responsible for designing and conducting all the experiments for this research project and writing the paper; she participated in interpretation and analysis of data with V.G.; M.-A.L. was responsible for synthesis of growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel; she purified the construct and after quality control analysis provided it for experimental use; M.D. was responsible for obtaining and processing peripheral blood samples and maintaining patient database; M.J.K. was responsible for identifying patients, explaining to them the nature of experiments from their peripheral blood samples, and getting consents from patients with CLL; M.R. was responsible for providing the construct and background information about growth factor fusion constructs in B-cell lines, and supervising M.-A.L. in synthesis and purification of the construct; V.G. was responsible for conceptualizing this investigation and use of construct for B-cell CLL; she participated in study design, interpreted and analyzed data, and wrote the paper with R.N.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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The growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel induces apoptosis specifically in BAFF-R–positive CLL cells

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