1. ABSTRACT

Multiple myeloma (MM) is a plasma cell dyscrasia characterized by bone lesions and production of a paraprotein. B-lymphocyte stimulator (BlyS) and its receptor (BAFFR) were highly expressed on peripheral blood and bone marrow B cells in MM patients as compared to those with monoclonal gammopathy of unknown significance (MGUS) and healthy donors. Serum BlyS levels in MM patients were significantly higher than those in MGUS patients and healthy controls. BlyS receptor expression was increased in bone marrow specimens from MM patients as ascertained by immunofluorescence. Furthermore, BlyS, together with IL-2 and IL-6, significantly promoted MM cell proliferation and BlyS receptor expression compared with that in the control group. Treatment with bortezomib, a therapeutic proteasome inhibitor induced apoptosis and repressed the proliferation of RPMI8226 and U266 cells through inhibition of NF-κB p65 and IκBα. These findings suggest that BlyS is involved in the immunopathogenesis of MM and may prove to be a hallmark of MM.

2. INTRODUCTION

Multiple myeloma (MM) is a fatal disease characterized by the accumulation of malignant plasma cells in the bone marrow that display a differentiated phenotype and resistance to apoptosis. The B-lymphocyte stimulator (BlyS), also known as B cell activating factor (BAFF), which is a tumor necrosis factor (TNF) family member critical for the maintenance of normal B cell development and homeostasis, promoted the survival of malignant B cells. In this study, we performed a coordinated study of BlyS and its receptors in MM patients and in cell lines.

The drug bortezomib is recommended for single-agent use in the treatment of patients with MM who have received at least 2 prior therapies and are showing improvement in their most recent therapy (1). It is a highly selective reversible inhibitor of the 26S proteasome (2) and may prevent degradation of pro-apoptotic factors and permit the activation of programmed cell death in neoplastic cells dependent upon the suppression of pro-apoptotic pathways.
Blys and its receptors in MM

The results of this study suggest that BLyS and its receptors are expressed on B cells in both the peripheral blood and bone marrow and in the U266 and RPMI8266 MM cell lines. However, the pattern of expression was variable in MM and MGUS (monoclonal gammopathy of unknown significance) patients. Additionally, data from this research suggest that BLyS may modulate the proliferative capacity and survival of MM cells, and that BLyS could promote cell proliferation through NF-κB signaling pathways. However, treatment with bortezomib may inhibit MM growth by reducing the expression of proteins associated with NF-κB signaling. In addition, BLyS, BCMA (B cell Maturation Antigen), TACI (Transmembrane Activator CAML Interactor), and BAFFR (BLYS receptor) were expressed on MM cells. The ability of BLyS to support MM cell growth and survival has exciting implications because it may be potential therapeutic target.

3. MATERIALS AND METHODS

3.1. Samples

Thirteen MM patients were recruited in Xinhua Hospital, which is affiliated with the Shanghai Jiaotong University School of Medicine. The patients were 7 men and 6 women (age, 57–85 years, mean age, 71 ± 8 years). Eleven confirmed MGUS patients (7 men and 4 women; age, 55–87 years; mean age, 71 ± 8 years) were grouped into disease controls. All participants were diagnosed according to international diagnostic criteria; the main symptoms were bone pain, fatigue, extra-medullary plasmacytomas, bacterial infections, renal function impairment, and hypercalcemia. All patients were subjected to monoclonal gammopathy, with IgG accounting for 64.3% of MM; IgA, 28.6%; and lambda, 7.1%. Seven healthy adults (4 men and 3 women; age, 55–80 years, mean age, 71 ± 10 years) were recruited and grouped into healthy controls; these adults were blood donors and normal examiners of Xinhua Hospital.

3.2. Cells and reagents

Mononuclear cells were isolated from the peripheral blood and bone marrow of 3 advanced MM patients (IgA kappa type, kappa light chain type, and IgG lambda type) by density gradient centrifugation using a lymphocyte isolation solution (Shanghai Biochemical Reagent Co., Ltd., Shanghai, China). The cell lines U266 (ATCC No. TIB-196) and RPMI8226 (ATCC No. CCL-155) are plasmacytomas of B cell origin and were obtained from the American Type Culture Collection (ATCC). U266 cells are known to produce monoclonal antibodies and IL-6. RPMI8226 cells only release immunoglobulin light chains. Cells were cultured in RPMI 1640 (Hyclone) containing 10% FBS, and RPMI8226 cells were cultured in RPMI 1640 medium containing 1x antibiotic-antimycotic solution. The U266 cells were cultured in 10% FBS, and the RPMI8266 cells were grown in 20% FBS.

3.3. Cell culture

All human MM cell lines were cultured in RPMI 1640 medium containing 1x antibiotic-antimycotic solution. The U266 cells were cultured in 10% FBS, and the RPMI8266 cells were grown in 20% FBS.

3.4. Preparation of nuclear extracts

Nuclear extracts were prepared according to the method described by Schreiber et al. (3). Briefly, 2 × 10⁶ cells were washed with cold phosphate-buffered solution (PBS) and suspended in 0.4 mL of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were lysed with 12.5 μL of 10% Nonidet P-40. The homogenate was centrifuged, and the supernatant containing the cytoplasmic extract was stored at −80°C. The nuclear pellet was resuspended in 25 μL of ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing the nuclear extracts were secured. The protein content was measured using the Bradford method. Nuclear extract that was not used immediately was stored at −80°C.

3.5. Flow cytometry

Cells (1 × 10⁶) were washed with PBS containing 0.5% BSA and incubated with 20 μL of CD19-PC7, 20 μL of CD45-PC5, 20 μL of PE-BLyS, 20 μL of FITC-BAFFR or 20 μL of FITC-BCMA, and PE-TACI or mouse Ig control alone for 30 min at 4°C. Cells were washed, resuspended in PBS, and analyzed using FACS Calibur and CellQuest software (Becton Dickinson). Isotype and fluorochrome controls were taken for each sample.

3.6. BLyS ELISA

ELISA kits were used according to the manufacturer’s instructions for measuring the BLyS concentrations from the sera of MM and MGUS patients.

3.7. Confocal microscopic analysis

Cells were cytospun onto poly-L-lysine-coated glass slides, fixed with cold methanol for 5 min, and air-dried. Nonspecific protein binding was prevented by blocking the cells with 10% FCS in PBS. Cells were stained with the appropriate primary antibodies (1:200 dilution) for 45 min and washed with PBS. Coverslips were applied with Slow Fade reagent (Molecular Probes, Eugene, OR). The cells were visualized using an Olympus Fluoview 500 (FV500) laser scanning confocal microscope (Olympus America, Melville, NY). Images were captured with a PlanApo oil immersion objective of 40x/1.4 numerical aperture by using the appropriate filter sets. Digital images were obtained using the manufacturer’s Fluoview software.

3.8. Western blot

Cytoplasmic protein extracts (30–50 μg) were prepared as described in the methods section (above) and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies for either Igκ or cyclin D1 (1:3000) for 1 h. The blot was then washed, exposed to the appropriate HRP-
**Table 1.** Mean fluorescence intensity of BLyS and its receptors in MM (±S).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BAFFR</th>
<th>TACI</th>
<th>BLyS</th>
<th>BCMA</th>
<th>cBCMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>13</td>
<td>7.1±2.0</td>
<td>6.1±2.0</td>
<td>20.8±7.8</td>
<td>5.8±1.6</td>
<td>47.3±18.0</td>
</tr>
<tr>
<td>MGUS</td>
<td>11</td>
<td>7.3±1.6</td>
<td>8.5±3.3</td>
<td>7.2±4.2</td>
<td>6.6±1.7</td>
<td>28.9±14.0</td>
</tr>
<tr>
<td>Healthy control</td>
<td>7</td>
<td>7.1±1.7</td>
<td>19.7±5.8</td>
<td>3.9±0.7</td>
<td>5.1±1.1</td>
<td>6.8±2.0</td>
</tr>
</tbody>
</table>

1 P<0.05 compared with healthy control group; 2 P<0.05 compared with MGUS group; 3 P<0.05 MGUS compared with healthy control group

**Figure 1.** Mean fluorescence intensity (MFI) of BLyS and its receptors in MM (±S). MM compared with control group P<0.05 using Student’s t test; MM compared with MGUS group Δ P<0.05 using Student’s t test; MGUS compared with healthy controls Group P<0.05 using Student’s t test.

conjugated secondary antibody for 1 h, and detected by chemiluminescence.

**3.9. Statistical analysis**

Statistical evaluation was performed with STATA 8.0. Values are shown as mean ± SD. Related graphs were drawn using the SigmaPlot software. The comparison between the groups was tested using the Student’s t test. A p value of < 0.05 was considered statistically significant.

**4. RESULTS**

**4.1. Elevated expression of BLyS and BAFFR on B cells of peripheral blood in MM and MGUS patients**

To gain a better understanding of the profile of BLyS levels and its receptors on human B cells, we first gated CD19+ B cells together with CD45 and then analyzed the expression of BLyS, BAFFR, TACI, and BCMA by flow cytometry (Figure 1). Soluble biotinylated BLyS was used to determine the BLyS-binding capability (Table 1). The results showed that BAFFR was generally expressed in MM and MGUS patients, as well as in the healthy controls. More importantly, MM patients expressed higher levels of BLyS than MGUS patients, but lower levels of TACI; BCMA was lacking on the cell surface of MM patients. The level of BCMA in the cytoplasm was significantly higher in MM patients than in MGUS patients. The lack of membrane BCMA on different subsets of peripheral blood B cells was noted, which suggests that BCMA was an intracellular protein. The different expression of BLyS and BCMA in MM and MGUS patients suggests that these proteins might be involved in the pathogenesis of MM.

**4.2. Expression of BLyS in bone marrow specimens of MM patients**

The expression of BLyS in bone marrow specimens was evaluated using a confocal immunofluorescence microscope. As shown in Figure 2, the lymphoplasmacytic cells infiltrated the bone marrow of MM patients (n = 3) with BLyS expression, while minimal diffuse expression was seen in the bone marrow specimens from healthy controls. We observed that BLyS expression in the B cell membrane of MM patients sample was independent of the immunotype.

**4.3. Elevated free BLyS levels in MM patients**

Because BLyS plays a crucial role in B cell development, survival, and maintenance, we postulated that serum BLyS levels in patients with MM would be significantly elevated. Therefore, we analyzed the ELISA data of serum BLyS from MM and MGUS patients and healthy controls. As shown in Figure 3, serum BLyS levels in MM patients (mean, 6.0 ± 1.88 ng/mL) was significantly higher (p < 0.05) than that in healthy controls (mean, 2.25 ± 0.71 ng/mL) and in MGUS patients (mean, 3.24 ± 0.28 ng/mL) (p < 0.05). These data support the hypothesis that BLyS is important in MM pathogenesis. The average value of BLyS detected in the healthy controls (2.25 ng/mL) was similar to the previously reported values.

**4.4. BLyS promoted the expression of BAFFR in the RPMI8226 cell line**

To further investigate the biological function of BLyS, we co-cultured RPMI8226 cells with BLyS protein (Active) and found that BLyS significantly promoted MM cell proliferation when compared with the control group (Figure 4).
Figure 2. Expression of BAFFR, TACI, BLyS and BCMA analyzed in B cell of bone marrow of MM by confocal immunofluorescence microscopy. A: BAFFR clustered around B cell membrane; B: TACI expressed equally in B cell membrane; C: CD19 PC7-positive B cell in BM; D: Both BAFFR and TACI were expressed in B cell membrane; E: BCMA expressed lightly in B cell membrane; F: BLyS equally located in B cell membrane; G: CD19 PC7-positive B cell in BM; H: Both BLyS and BCMA were expressed in B cell membrane.

Figure 3. Expression levels of BLyS protein in MM and MGUS groups. MM compared with control Group *P<0.05 using Student’s t test; MM compared with MGUS Group P<0.05 using Student’s t test.

4.5. Bortezomib induced apoptosis in MM cells

U266 cells were treated with 20 nM bortezomib for different time intervals to evaluate the level of bortezomib-induced apoptosis. Results in Figure 5 show Annexin V positive cells increased in a time-dependent manner, indicating the onset of apoptosis in bortezomib-treated cells after 24 h. These results suggest that bortezomib had the ability to inhibit the growth of U266 cells and induce apoptosis against high levels of BLyS.

4.6. Bortezomib down-regulated the expression of NF-κB p65

Previous studies have shown that after MM cell activation, the p65 subunit of NF-κB containing the transactivation domain was translocated to the nucleus (4). While in an inactive state, the p65 subunit of NF-κB is combined with p50 and retained in the cytoplasm. To evaluate the suppressing effect of bortezomib on the nuclear retention of p65, U266 and RPMI8226 cells were treated with 20 nM bortezomib for 24 h. Western blotting results show that the untreated U266 and RPMI8226 cell lines constitutively expressed Ser32-phosphorylated IκBα. However, after bortezomib treatment, the phosphorylation of IκBα and p65 decreased rapidly (Figure 6A–B).

5. DISCUSSION

BLyS is mainly expressed in the monocytes, macrophages, dendritic cells, and activated neutrophils (5–8). BCMA and BAFFR are expressed by B lymphocytes, while TACI is expressed in B cells and active T cells. BAFFR is a specific receptor of BLyS (9–11). Data from this study suggest that BLyS and its receptors are expressed by B lymphocytes in the peripheral blood and the bone marrow of MM patients. A higher expression level of BLyS in MM patients suggests that B lymphocytes in MM patients have a stronger ability to bind with BLyS. BCMA has typically been found to be an intracellular protein (12); therefore, it was not surprising that the expression of BCMA in the cytoplasm was higher than that on the cell surface. Previous studies have suggested that elevated BLyS levels are found in B-cell inflammatory disorders and malignancies (13–18). Therefore, we used an immunofluorescence confocal microscope to detect the presence of BLyS in bone marrow specimens from MM patients, and we found increased BLyS expression when compared with the bone marrow of the controls. Currently, we do not know what component in the tumor micro-environment is responsible for BLyS up-regulation; it will be of interest to investigate this issue in future studies.

Following the detection of serum BLyS levels in
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Figure 4. Expression of BAFFR in RPMI8226 after treatment with BLyS protein. A: control mouse IgG1; B: RPMI8226 + IL-2 + IL-6 BAFFR 68.65%; C: RPMI8226 + BLyS + IL-2 + IL-6 BAFFR 81.6%.

Figure 5. Effect of Bortezomib on U266 and RPMI8226. 20 nM/ml Bortezomib inhibits the growth of U266 and RPMI8226 cells and induces apoptosis after 24, 48, and 72 hours. U266 and RPMI8226 cells were incubated without Bortezomib after 24, 48, and 72 hours.
patients with MM, we found substantially elevated BLyS levels when compared with both the MGUS group and the healthy controls. Elevated levels of BLyS in either the serum or the bone marrow micro-environment may support survival and growth of malignant MM cells. Therefore, BLyS could be a useful therapeutic target. However, regardless of the mechanism, the ability of BLyS to bind to B cells and the elevation of serum soluble BLyS levels suggest that BLyS may be a hallmark of this disease.

Data from this study also indicate that BLyS promotes the survival and proliferation of MM cells. Although our study focused on the role of BLyS in MM, APRIL may also have an important role in this disease. APRIL, a homologue of BLyS, binds to TACI and BCMA and has been shown to stimulate malignant B cells from MM patients (19–23). Studies are currently under way to better understand the significance of APRIL in MM patients. In this study, we provide evidence that BLyS co-cultivation with IL-2 and IL-6 may up-regulate the expression of BAFFR in RPMI8226 cells. Furthermore, BLyS promoted the survival and proliferation of RPMI8226 cells.

Based on the results of our study, we predict that bortezomib, which plays an agonistic role to BLyS, will have significant potential therapeutic efficacy in MM patients. We found that bortezomib suppressed constitutive activation in RPMI8226 and U266 cell lines. The major mechanism of bortezomib acting as a growth inhibitor might be by blocking the inhibitor –IκB, thereby abrogating NFkB signaling. BLyS blockade in MM patients may help decrease the amount of paraprotein in the serum. The degradation of IκBα and subsequently NF-κB (p65 to p50) requires prior phosphorylation at Ser32 and Ser36 residues. IKK can be regulated by several upstream kinases (24–26). Western blot analysis also showed an inhibition of the downstream NF-κB signaling pathway. Furthermore, receptor-ligand pairs of the TNF-R/TNF superfamily play critical roles in humoral immunity by regulating the responses of activated B cells.

This research has revealed a potential mechanism underlying MM that could deregulate the expression of BAFFR. Furthermore, bortezomib down-regulated expression of p65 and IκBα that led to the apoptosis of MM cells. We hypothesize that abnormal BLyS signaling and NF-κB activity form a positive feedback loop. NF-κB activation induces BLyS expression, while the upregulated BLyS ligand is in turn involved in maintaining constitutive NF-κB activation after binding to the cognate receptor. Delineation of the specific molecular MGUS mechanisms involved in generating this positive feedback loop will be crucial in elucidating further mechanisms in MM and could provide future therapeutic targets for MM.

In summary, our findings confirm that BLyS and its receptors are expressed by B lymphocytes in the peripheral blood and the bone marrow of patients with MM. In addition, the ability of BLyS to bind to B cells and the evidence of elevated serum-soluble BLyS levels suggest that BLyS may be seen as a hallmark of MM. We also note from this study that bortezomib, a new proteasome inhibitor, induced apoptosis in MM cells; MM cell proliferation and survival were inhibited because of the inhibition of the NF-κB pathway (27), thereby suggesting that it might be involved in the mechanism of BLyS regulation. We hypothesize that abnormal BLyS signaling and NF-κB activity form a positive feedback loop that may be crucial in elucidating further mechanisms in MM pathogenesis and could provide future therapeutic targets for MM.

6. ACKNOWLEDGMENTS

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7. REFERENCES

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**Abbreviations:** MM: Multiple myeloma; BLyS: B-lymphocyte stimulator; TNF: tumor necrosis factor; ATCC: American Type Culture Collection; PBS: phosphate-buffered saline; BSA: bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum

**Key Words:** Multiple myeloma, B-lymphocyte stimulator, NFkappaB

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