ZAP-70 tyrosine kinase is constitutively expressed and phosphorylated in B-lineage acute lymphoblastic leukemia cells

Background and Objectives. Zeta-associated protein 70 (ZAP-70), a member of the Syk family of protein tyrosine kinases, is normally expressed in T and NK cells. While little is known about ZAP-70 expression in normal human B cells, it has been reported that ZAP-70 is expressed in a subset of patients with chronic lymphocytic leukemia (CLL) with a poor prognosis. In this study, we examined the expression and phosphorylation status of ZAP-70 in B-lineage acute lymphoblastic leukemia (Blin-ALL).

Design and Methods. First, ZAP-70 protein expression was assessed by Western blotting and flow cytometry and ZAP-70 mRNA transcripts were analyzed by reverse transcription polymerase chain reaction (RT-PCR) on human precursor B cell lines. Experiments were then carried out on cells obtained from 18 patients with Blin-ALL and from normal human bone marrow.

Results. ZAP-70 was constitutively expressed and phosphorylated on tyr319 in human precursor Blin-ALL cell lines as well as in primary B leukemic cells from all examined Blin-ALL patients with pro-B, pre-B and B phenotypes, but not in malignant myeloid cells. Importantly, analysis of normal human bone marrow revealed expression of ZAP-70 transcripts only in the CD34+ cell fraction (either CD19CD10+ or CD19CD10-) but not in the CD34- cell fraction (CD19sIgM+ pre-B cells or CD19 sIgM+ immature B cells).

Interpretation and Conclusions. ZAP-70 was found to be expressed in the CD34+ normal bone marrow compartment including earlier B-cell progenitors, but not in CD34- pre-B and immature B cells. By contrast, ZAP-70 was consistently expressed and phosphorylated in Blin-ALL cells. Further studies are required to determine whether ZAP-70 may play a pathophysiological role in Blin-ALL.

Key words: ZAP-70, SYK, acute lymphoblastic leukemia, precursor B cells, B-lymphocytes.

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gested by the more severe defect in B-cell development in Syk/ZAP-70 double knock-out mice than in Syk−/− mice. In humans, ZAP-70 expression and function in B lineage cells remain largely unknown.

Recently, chronic lymphocytic leukemia cells, from a subset of patients with poor prognosis, expressing non-mutated immunoglobulin V heavy chain genes, were found to contain levels of ZAP-70 protein that are comparable to those detected in T cells of healthy adults. Given the expression of ZAP-70 in mouse B-cell progenitors and in certain human B-cell malignancies, we studied the expression and phosphorylation of ZAP-70 and Syk in B-lineage ALL.

### Design and Methods

#### Patients and samples

Surplus leukemic cells from peripheral blood samples of 18 patients (10 children and 8 adults) with Blin-ALL (Table 1) and 6 patients with acute myeloid leukemia (AML) (FAB types: 4 AML M1, 1 AML M4, 1 AML M5a) were obtained at the time of diagnosis with informed consent and following approval of the local ethics board. All patients were enrolled in induction chemotherapy protocols. Diagnoses were established according to the European Group for the Immunological Characterization of Leukemias (EGIL) and French American British classifications in combination with cytologic and immunophenotypic criteria. Pertinent clinical information for these patients was obtained by reviewing their clinical records.

#### Isolation of patients’ cells and separation procedures

Mononuclear cells were isolated by density-gradient centrifugation over Ficoll-Hypaque. Cells were resuspended in fetal bovine serum containing 10% dimethyl sulfoxide for storage in liquid nitrogen. Before use, cells were thawed and washed twice in cold phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 2 mM EDTA. For Western blot analyses, B-cell blasts from two patients were purified by depleting T cells using anti-CD2 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. RT-PCR studies were performed after sorting CD34 or CD19 positive cells using a Coulter Epics Elite cytometer.

#### Isolation of human bone marrow primary cells

Adult human bone marrow samples were obtained from resected ribs of six healthy renal transplant donors in accordance with policies established by the Institutional Review Board of the University of Alabama at Birmingham. The resected ribs were processed within 24 hours of being obtained and lymphoid cells were isolated by Ficoll-Hypaque gradient centrifugation (Mediatech, Herndon, VA, USA). Non-B lineage cells were selected out of the bone marrow population using a MACS B-cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). The isolated primary B lineage cells were incubated with CD34-APC, CD19-PE and IgM-FITC labeled antibodies for analysis (BD Biosciences, Palo Alto, CA, USA). The cells were sorted into three B lineage populations: CD34+CD19+, CD34-CD19+sIgM− and CD34-CD19+sIgM+ using a MoFlo instrument (Cytomation, Fort Collins, CO, USA). Cell purity, checked by reanalysis following isolation, was 97.8 to 98.9%. To further analyze CD34− subpopulations, CD34− cells were positively selected using a MACS CD34− Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) and incubated with CD34-PE,
CD10-FITC, and CD19-PC5 labeled antibodies. Cells were sorted into CD34+CD19-CD10+ cells and CD34+CD19+CD10+ B-cell precursor fractions.

**Cell lines**

The RS4;11 pro-B, 697 and Nalm6 pre-B, Jurkat T and K562 cell lines were maintained in culture in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and antibiotics.

**Flow cytometry analysis**

Intracytoplasmic ZAP-70 expression was analyzed by flow cytometry using the Fix and Perm kit (Caltag Laboratories, Hamburg, Germany) according to the manufacturer’s instructions. Cells were then successively incubated with the 2F3.2 anti-ZAP-70 monoclonal antibody and PE-conjugated antibody to mouse immunoglobulins (DakoCytomation, Denmark). Stained cells were analyzed using a Coulter Epics Elite cytometer.

**Western blot analysis**

Cells were lysed in Laemmli’s sample buffer (10 μL/10⁶ cells from patients and 30 μL/10⁶ cells from cell lines) and protein concentrations of each sample were determined by a modified Lowry assay. After boiling, 50 μg of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane, and probed with various antibodies. The blots were developed using appropriate peroxidase-conjugated specific antibodies and an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia Biotech (England) and Santa Cruz Biotechnology (CA, USA). Antibodies used in Western blot and immunoprecipitation experiments

**Immunoprecipitation experiments**

Cells (3x10⁶) were lysed on ice for 30 minutes, in buffer containing 1% digitonin (w/v), 10% glycerol (v/v), 50 mM Tris pH 7.5, 150 mM NaCl and inhibitors (1 mM 4-(2-aminoethyl)-benzen-sulfonyl fluoride, 1 mM Na-orthovanadate, 10 μg/mL leupeptin and apro tinin, 1 μg/mL Bestatin-Antipain-Pepstatin). Cell lysates were clarified by centrifugation for 20 minutes at 15,000 g, 4°C and incubated with the 4G10 anti-phosphotyrosine antibody overnight at 4°C. Immune complexes were collected by incubation with the purification system Pansorbin® (Merck Biosciences, Darmstadt, Germany) for two hours at 4°C, then extensively washed, eluted in Laemmli’s sample buffer and run on SDS-PAGE.

**Antibodies used in Western blot and immunoprecipitation experiments**

The following antibodies were used: antibodies directed to the C-terminus portion (BD Biosciences Farmington, USA), N-terminus (Cell Signaling, Beverly, New England, USA) and two tandem SH2 domains (clone 2F3.2, Upstate Biotechnology, Waltham, MA, USA) of human ZAP-70. Anti-Syk (4D10), anti-actin (C11), anti-TCRβ (G11) (Santa Cruz Biotechnology, CA, USA), anti-phospho-Syk (Tyr352)/anti-phospho-ZAP-70 (Tyr319) (Cell Signaling, Beverly-New England, USA) and anti-phosphotyro sine (4G10) antibodies were also used in this study. Anti-peroxidase-conjugated antibodies specific for rabbit and mouse IgG or for goat IgG were purchased from Amersham Pharmacia Biotech (England) and Santa Cruz Biotechnology (CA, USA), respectively.

**RT-PCR**

Total RNA from cell lines was purified using the RNA Now kit (Ozyme) and RNA from human bone marrow samples was isolated with the RNeasy micro kit (Qiagen). First-strand cDNA was synthesized using oligo(dT) primers and a commercially available kit (Stratagene First-Strand Synthesis System, Stratagene). PCR protocols were performed by using GAPDH or ZAP-70-specific primers (10) or CD3 primers.

**Results**

In this study we conducted a systematic analysis of ZAP-70 expression and phosphorylation in human precursor B-cell lines derived from Blin-ALL patients as well as in primary Blin-ALL samples obtained from 18 patients.

**ZAP-70 is constitutively expressed and phosphorylated in human B-cell precursor lines**

To verify the approaches used to assess ZAP-70 expression, we utilized Blin-ALL cell lines before examining samples from patients. Using an antibody that specifically recognizes the N-terminus portion of ZAP-70, total RNA from cell lines was purified using the RNA Now kit (Ozyme) and RNA from human bone marrow samples was isolated with the RNeasy micro kit (Qiagen). First-strand cDNA was synthesized using oligo(dT) primers and a commercially available kit (Stratagene First-Strand Synthesis System, Stratagene). PCR protocols were performed by using GAPDH or ZAP-70-specific primers (10) or CD3 primers. Interestingly, and as previously reported for B-CLL cells, intracellular ZAP-70 was easily detected by flow cytometry for all permeabilized pro-B and pre-B cell lines. Again, the amounts of ZAP-70 were much lower in these cells than in Jurkat T cells. The ratio of the mean fluorescence intensity for ZAP-70 compared to the isotypic immunoglobulin control was 4.6 for the Jurkat cell line, 1.5 for the RS4;11 cell line and 2.1 for the 697 cell line (Figure 2). ZAP-70 was not detected in the K562 chronic myelogenous leukemia cell line. The
presence of ZAP-70 transcripts in the B-cell precursor lines and in the Jurkat T cells was clearly demonstrated by RT-PCR, using specific primers (Figure 3). Syk, a closely related tyrosine kinase sharing a high homology of sequence with ZAP-70, was also detected by Western blotting using antibodies directed against ZAP-70, Syk and their phosphorylated forms. Actin levels were assessed to verify protein loading. Importantly, in all B-cell lines expressing ZAP-70 and Syk, both molecules were found to be constitutively phosphorylated at variable levels (Figure 1A). Indeed, analyses of unstimulated as well as FCS-starved cells (data not shown) by Western blot indicated that both molecules were phosphorylated on tyrosine residues 319 and 352, respectively. The phosphorylation state of ZAP-70 was further confirmed by immunoprecipitation experiments with the 4G10 antibody followed by immunoblotting with polyclonal anti-ZAP-70 antibody (Figure 1B). Altogether our findings clearly demonstrate that human leukemic pro-B and pre-B lines constitutively express ZAP-70 and Syk molecules and their phosphorylated forms.

**ZAP-70 is constitutively expressed and phosphorylated in primary Blin-ALL cells**

Next, we conducted these analyses on primary Blin-ALL cells collected from 18 patients. The patients comprised 10 children and 8 adults aged from 1 to 73 years, with a male to female ratio of 10/8. According to the EGIL criteria, the phenotypes of these cases of acute lymphocytic leukemia were pro-B (BI, 2 cases), pre-pre-B/common (BII, 11 cases), pre-B (BIII, 3 cases) and mature B (BIV, 2 cases). The main karyotypic changes
Table 1 presents results for seven cases of ALL. Since the purity of ALL blasts ranged between 93 to 100% by flow cytometry after Ficoll purification, we wished to verify that ZAP-70 expression was not due to the presence of contaminating T cells. Under these conditions, NK cells represent less than 1% of the cells. T cells were present as TCRβ chain was detected in seven of the 18 samples. T cells were therefore depleted by means of anti-CD2 antibody coupled to magnetic beads and Blin-ALL cells were re-examined for the expression of ZAP-70 (Figure 4B). Even upon removal of contaminating T cells, as assessed by flow cytometry (not shown) and the loss of TCRβ detected by Western blot, ZAP-70 levels were not modulated in two ALL samples. ZAP-70 could not be detected in six AML samples even if there were contaminating T cells as shown by TCRβ detection (Figure 5). Notably though, this tyrosine kinase was recently reported to be expressed in the blasts of some Syk-negative AML cells. Moreover RT-PCR analysis confirmed the presence of ZAP-70 transcripts in Blin-ALL and not in AML cells (Figure 6). As in leukemic cell lines, the tyrosine phosphorylated form of ZAP-70 was detected in all 18 Blin-ALL samples and these findings were not altered by the removal of contaminating T cells (Figure 4A and B). It was previously reported that

**Figure 4.** Expression and phosphorylation of ZAP-70 and Syk in primary acute leukemia cells. (A) Cellular extracts of primary blast cells from acute leukemia were analyzed by Western blotting for expression and activation of ZAP-70 and Syk proteins. The presence of contaminating T cells was evaluated with an anti-TCRβ antibody. Peripheral blood mononuclear cells (PBMNC) from a healthy donor and Nalm6 pre-B cells were used as positive controls. Cellular extracts from seven patients with B phenotype ALL (1-7) are presented. (B) Blast cells from an additional ALL patient were purified by depleting CD2+ cells with magnetic beads. ZAP-70 expression and ZAP-70/Syk phosphorylation were analyzed in total (before sorting) and purified (after sorting) lysates. TCRβ levels were assessed as a measure of the presence of T cells.

**Figure 5.** ZAP-70, Syk, phospho-ZAP-70/Syk, TCRβ and actin levels were monitored by Western blotting in cellular extracts from patients with acute myeloid leukemia (AML 1-6).
pediatric CD19-CD10+ pro-Blin-ALL have markedly reduced Syk activity, associated with aberrant mRNA sequences encoding abnormal Syk molecules. Here, we detected Syk expression and phosphorylation in all Blin-ALL samples. It would therefore be of interest to assess ZAP-70 expression in the reported pro-Blin-ALL cells. Moreover, it has been recently suggested that the level of Syk phosphorylation in B-CLL is enhanced by the presence of ZAP-70. Whether ZAP-70 could potentially interfere with Syk phosphorylation or vice-versa in Blin-ALL is difficult to assess because both kinases were expressed in all our Blin-ALL cells. However, it is notable that the relative fractions of tyrosine-phosphorylated ZAP-70 and Syk proteins in the leukemic cells were very low.

In the normal bone marrow B-cell compartment, expression of ZAP-70 transcripts is restricted to the CD34+ cell fraction. One major issue was to determine whether the expression of ZAP-70 in Blin-ALL cells just reflected conserved potentialities of normal precursor B cells or whether it had any link with the leukemogenesis process. To address this issue we analyzed the expression of ZAP-70 transcripts in the different compartments of bone marrow B-cell progenitors. RT-PCR analysis of highly purified cells revealed the presence of ZAP-70 transcripts only in the CD34+ compartment, which includes the CD19-CD10+ (mostly committed myeloid cells) and the CD19-CD10+ (pro-B and to a lesser extent early pre-B) cell fractions. The detection of ZAP-70 was not due to contaminating T cells as CD3 transcripts could not be detected by RT-PCR in either fraction. By contrast, ZAP-70 transcripts were totally absent in the CD34 CD19+IgM- pre-B and the CD34 CD19+IgM+ immature B cells (Figure 6). Analysis of the phosphorylation status of ZAP-70 in normal B cell progenitors could not be assessed due to the very low number of cells harvested after sorting.

**Discussion**

A number of signaling molecules, including protein kinases, transcription factors and cell cycle regulators, have been shown to have altered expression and/or activation in Blin-ALL cells. In this paper, we report the expression and activation of ZAP-70 in pro-B and pre-B cell lines as well as in samples from patients with Blin-ALL of different stages. Previously described altered expression and/or activation of signaling molecules in Blin-ALL cells are not consistent and may vary depending on the karyotypic changes. Various genetic alterations contribute to the leukemic transformation of hematopoietic stem cells or precursor B cells, by interfering with cell proliferation, differentiation and survival. A recent report has pointed to the possible role of the BLNK adaptor molecule in the pathogenesis of Blin-ALL; BLNK was not detected in 16 of 34 ALL patients, and as such was proposed to act as a tumor suppressor gene. However, using microarray data of gene expression, Imai et al. have shown widespread BLNK expression in Blin-ALL. In our study, ZAP-70 was constitutively expressed in all patients’ samples irrespectively of the karyotype status (Table 1) or BLNK expression (unpublished observations). To understand the role of ZAP-70 in the pathophysiology of Blin-ALL cells, we analyzed ZAP-70 in normal precursor B cells. We found that ZAP-70 transcripts were expressed in CD34+CD19+CD10+ cells and CD34+CD19+CD10+ normal precursor B cells but not in the CD34+CD19+IgM- or IgM+ cells of the B lineage. These data strengthen the idea that this tyrosine kinase does not constitute per se a basic alteration of the leukemic process. Previous studies found that leukemia frequently combines features of earlier and subsequent differentiation stages of B-cell development and therefore represents phenotypic transient stages. It is nowadays well established that the phenotypic repertoire of ALL blasts is never an exact replica of physiological expression regulation. Our observations allow ZAP-70 expression in Blin-ALL of pro-B phenotype to be interpreted as conservation of physiological properties. However, its expression in Blin-ALL of pre-B and B phenotypes as well as its activation together with Syk in all cases, might result in an alteration of the proliferative capacity of the leukemic cells. In this regard, some studies suggest that Syk may...
suppress the growth of AML cells\textsuperscript{19} or breast tumor cells\textsuperscript{20,21} whereas others suggest that Syk may contribute to the leukemic process.\textsuperscript{22} The mechanisms that lead to the activation of ZAP-70 and Syk in these cells also remain unclear. In B cells, BCR engagement results in Syk activation in a Src independent manner.\textsuperscript{23} In T cells, ZAP-70 is activated following TCR or CD8 crosslinking, probably via the Lck Src kinase that subsequently allows its recruitment to the ITAM motifs of the zeta chain. In B-CLL cells ZAP-70 was found to be activated upon BCR crosslinking and to associate with the Ig\textalpha/Ig\textbeta heterodimers.\textsuperscript{4} Moreover it has been recently shown that ZAP-70 may directly enhance BCR signaling in such cells a feature that could contribute to the relatively aggressive clinical behavior generally associated with CLL cells that express unrearranged immunoglobulin V heavy chain genes.\textsuperscript{24}

In conclusion, the unexpected presence of ZAP-70 in all Blin-ALL as well as in normal CD34+ precursor B cells is potentially the sign of a basic function of ZAP-70 in human B-cell development and in this context might constitute a target for new therapeutic agents in ALL. Experiments are currently underway to analyze growth and survival of cells following specific inhibition of the ZAP-70 tyrosine kinase in order to evaluate the role of ZAP-70 in Blin-ALL and in normal B-cell development.

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