An Evaluation of Bid Phosphorylation Status in Myeloid Malignancies

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BRIEF. An assay was optimized to query a correlation between Bid protein phosphorylation and the development of acute myelogenous leukemia.

ABSTRACT. Leukemia is a blood cancer resulting from the accumulation of abnormal, highly proliferative white blood cells in the bone marrow. Acute myelogenous leukemia (AML) is a subtype of leukemia that may have a poor prognosis upon diagnosis. AML is characterized by translocations on chromosomes 1, 8, 9, 15, 17, 21, and 22; however, the pathophysiology of the disease is not completely understood. As Bid, a crucial protein found in almost all cells, has a known correlation with some leukemia subtypes when deregulated, we hypothesized that Bid may be deregulated in AML. To test this hypothesis, we analyzed the mutation and phosphorylation status of Bid in six human leukemic cell lines. No mutations in *BID*, the gene coding for the Bid protein, were identified. As a result, we optimized a Western blot assay and discovered that the levels of Bid phosphorylation differed between the six leukemic cell lines tested. In future studies, the Bid phosphorylation status of leukemic patient samples will be studied. Further research is necessary to determine the precise effects of misregulation of Bid in AML. The results of these studies may reveal a novel AML drug target in the apoptotic pathway.

INTRODUCTION.

Cancer is a disease that is prevalent around the world, transcending racial, socioeconomic, and environmental barriers. Leukemia is a cancer of the white blood cells and is characterized by an accumulation of abnormal cells in bone marrow [1]. Leukemia is the most common cancer found in children, the most prevalent of which is acute lymphocytic leukemia [2]. Acute myelogenous leukemia (AML) is an aggressive and potentially fatal disease that has the lowest survival rate out of childhood leukemias, a mere 26.4% [2]. Although AML occurs at a higher incidence in adults than in children, the prognosis remains poor with an overall 5-year relative survival rate of 22.6% [3].

Each leukemia type is characterized by certain features, including the speed at which the disease progresses, and the type of cell abnormality resulting from the cancer [4]. For example, chronic myelogenous leukemia (CML) is a slow-onset subtype of the disease [5] characterized by the presence of the Philadelphia chromosome (Suppl. Figure S1). This malformed chromosome, so named due to the city in which it was first discovered, consists of fragments from chromosomes 9 and 22 [6]. This malformation yields a gene that facilitates uncontrolled cell growth and accumulation [7]. Recently, the pharmaceutical, Gleevec, has been approved by the Food and Drug Administration (FDA) to treat CML based on its anti-cell proliferative activity [8]. Unlike CML, a single targetable genetic event does not characterize all AML. In contrast, AML can arise from several chromosomal malformations and other genetic alterations [9, 10]. Therefore, an opportunity exists to identify other drivers of AML that may be targetable.

In addition to chromosomal abnormalities, the absence of certain proteins can also cause diseases, such as the Bid protein. In non-cancerous cells, Bid is key in activating a chain reaction which ultimately leads to programmed cell death (apoptosis) [11]. However, when Bid is phosphorylated (a phosphate molecule binds to the protein), Bid's apoptotic function is inhibited [12, 13, 14]. It is known that Bid can be phosphorylated on three amino acids: threonine 59, serine 65, and serine 78 [11]. When Bid is not functional apoptosis is inhibited, leading to an accumulation of cells that ultimately leads to leukemia development [15].

A study was performed in 2003 that determined known correlations between Bid and chronic myelomonocytic leukemia (CMML) development [11]. CMML is a myeloproliferative neoplasm (abnormal growth in the bone marrow) characterized by a high proliferative rate and the potential to evolve into AML. Thus, a correlation between Bid deregulation and AML was investigated in this study. We hypothesized that the mechanism of Bid deregulation was by either genetic mutation or abnormal protein phosphorylation. To test this, we performed a genetic analysis of *BID* and developed an assay to query Bid protein phosphorylation. If Bid deregulation and AML are correlated, Bid, or the apoptotic pathway, may be an attractive target for therapies to treat AML.

MATERIALS AND METHODS.

Initially, analysis of the *BID* exons was performed to explore if mutations were a possible source of Bid misregulation. Subsequently, we evaluated Bid protein phosphorylation by Western blot analysis. A Western blot is a procedure used to visualize proteins in a sample, as well as compare proteins between samples. Western blots were performed using sodium doceyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1).

PCR (Mutational) Analysis.

In order to query *BID* mutations as a possible source of Bid misregulation, the *BID* exon sequences of six leukemic cell lines were amplified using polymerase chain reaction (PCR), and subsequently analyzed (Suppl. Methods, PCR primers denoted in Suppl. Table S3).

Cell Culture.

Six leukemic cell lines were selected for use in optimizing Western blot conditions due to their availability in the laboratory. The utilized cell lines were: Jurkat (positive control), HL-60, K562, Kasumi-1, KG-1a, and U937 (American Type Culture Collection). The cell lines were derived from patients with leukemia.

The cell cultures grown from each cell line were incubated for 3 hours in either vehicle (H_2O) or hydroxyurea (1 mM or 10 mM). Hydroxyurea (HU) is a DNA damaging agent [16] that is known to increase levels of Bid phosphorylation [17]. Cells were treated with HU to increase Bid phosphorylation, thereby easing detection of Bid phosphorylation levels.

Protein Quantification.

The cell lysates were prepared and quantified using the colorimetric Lowry method (BioRad DC assay) [18]. The Lowry method is a common method of protein quantification utilizing a spectrophotometer [19]; the method is described further in Supplementary Materials.

Protein quantification was performed because equal amounts of protein must be loaded into each lane of the gel to ensure that the compared protein levels are a result of the samples themselves and not attributable to disproportionate gel loading.

Western Blotting: Sample Preparation.

The quantified protein samples were analyzed by 10% Bis-Tris SDS-PAGE (Invitrogen, Carlsbad, CA), the gel with greatest protein separation in the Bid molecular weight range; Bid is 23 kDa [20], phosphorylated Bid is 22 kDa [21], and Actin is 47 kDa [22]. Actin was used as a loading control.

Each sample was diluted in denaturing sample buffer and reducing agents to fully denature the protein. Full denaturing was necessary in order to accurately visualize the proteins contained in each sample by electrophoresis.

Western Blotting: Loading and Running the Gel.

The prepared sample volumes were loaded into the NuPAGE electrophoresis gel, and the proteins were separated by SDS-PAGE.

Western Blotting: Transfer of Proteins from Gel to Membrane.

After electrophoresis, the proteins were then transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA). Proteins were transferred in order to facilitate sample analysis using enhanced chemiluminescence (Suppl. Figure S2).

Western Blotting: Antibody Treatment of Membranes.

For the Western, antibodies phospho-Serine65 (pSer-65), phospho-Serine78 (pSer-78), and total Bid (tBid) were used; Actin antibodies were used as a loading control. In this study, the primary antibody bound to the protein of interest: pSer-65, pSer-78, tBid, or Actin. The secondary antibodies were horseradish-peroxidase (HRP) conjugated in order to facilitate visualization of immunoreactive bands on post-enhanced chemiluminescent films (Suppl. Figure S2).

Several antibodies were used throughout this study: The antibodies were Bid phosphorylation-site specific, total Bid-specific (to verify presence of Bid in the sample), and Actin specific (loading control). As noted above, the secondary antibodies were HRP-conjugated and species-specific.

Following membrane incubation in primary and secondary antibodies, enhanced chemiluminescence (ECL) was performed. ECL was used to visualize immunoreactive bands (Suppl. Figure S2).

The chemiluminescent bands produced by ECL, though not visible to the human eye, can be visualized by exposure to film. Optimized film exposure times were determined (Table 1), as shown in Figure 1.

RESULTS.

BID is not Mutated in a Subset of Leukemic Cell Lines.

As a possible mechanism of Bid deregulation in leukemia, *BID* mutational analysis was performed in 6 leukemic cell lines. Analysis of the *BID* sequences did not yield any mutations (data not shown). As a result, we analyzed total Bid protein and the Bid phosphorylation status in the cell lines as alternative mechanism to Bid deregulation.

The Western blot assay conditions for the following antibodies were optimized: pSer-65, tBid, and Actin. The conditions for pSer-78 will continue to be optimized in ongoing research.

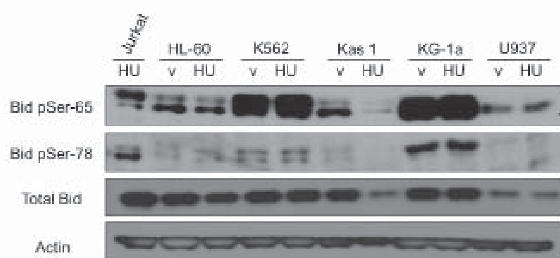

Figure 1. Western blot using Bid phosphorylated Serine-65 (pSer-65), Bid phosphorylated Serine-78 (pSer-78), and total Bid (tBid) antibodies, as well as Actin antibody for a loading control. 6 cell lines: Jurkat (positive control), HL-60, K562, Kasumi 1, KG-1a, and U937. HL-60, K562, Kasumi 1 (Kas 1), KG-1a, U937 cells were treated with vehicle (v) or 10 mM hydroxyurea (HU). Jurkat cells were treated with v or 1 mM HU.

Hydroxyurea Treatment.

Initially, cell cultures from the leukemic cell lines (Jurkat, HL-60, K562, Kasumi-1, KG-1a, and U937) were treated with hydroxyurea for 3 hours [11]. However, the three hour HU treatment did not yield uniform Bid phosphorylation increases (Figure 1).

Ideal Antibody Concentrations Determined.

Antibody concentrations for primary antibodies pSer-65, pSer-78, total Bid, and Actin were determined, as well as optimized enhanced chemiluminescence exposure time, as shown in Table 1.

Table 1. Optimized Concentration and Exposure Time for Antibody-Incubated Blots

Antibody	Concentration	ECL* Exposure
pSer-65	1:1,000	45 minutes
pSer-78	1:1,000	45 minutes
Total Bid	1:5,000	1 minute
Actin	1:10,000	10 seconds
Secondary (gαr)	1:10,000	—
Secondary (gαm)	1:10,000	—

*Enhanced Chemiluminescence

At each of the concentrations and exposure times noted in Table 1, the resulting bands on film were clearly visible and with little to no background.

Western Blot Experimentation and Phosphorylation Levels in Cell Lines.

Numerous variations on the Western blot were performed. Such variations included protein concentration, sample composition, and total sample size. These conditions are catalogued in Suppl. Table S4. Bid phosphorylation was visualized using a Western blot; as shown in Figure 1, when phosphorylated and unphosphorylated Bid are present in a sample, two bands appear. The top band is phosphorylated Bid because phospho-Bid has a greater molecular weight, and will therefore migrate through the gel more slowly than unphosphorylated Bid.

Initial analysis of the six leukemic cell lines demonstrated a variation in the Bid phosphorylation levels. Jurkat, the positive control, had the greatest phosphorylation levels, though phosphorylation was visible in every other cell line, excepting U937. With pSer-78 incubation, phosphorylation was not as visually apparent as with pSer-65 incubation, but was present in every cell line. Total Bid (tBid) treatment verified that each protein sample did contain the Bid protein, and Actin treatment ensured that the gel was loaded with equal protein concentrations in each lane. These trends are depicted in Figure 1.

DISCUSSION.

The aim of this study was to determine the optimal Western blot assay conditions in which leukemic patient samples could be studied. In order to conserve the limited patient samples, the assay optimization was performed utilizing cell lines. In further research, application of the optimized conditions will permit analysis of the leukemic patient samples in a highly effective, non-wasteful manner.

In initial assay optimization, Bid phosphorylation levels varied between cell lines. Such variation in the levels of Bid phosphorylation and total Bid between cell lines indicates that Bid phosphorylation levels may fluctuate between patient samples, which may indicate even more specific correlations between certain patient characteristics and Bid phosphorylation levels.

As shown in Figure 1, the phosphorylation levels differed the most between pSer-65 and pSer-78 incubated cell lines, while tBid incubated cell lines were somewhat more consistent. Actin regularly displayed even protein levels, thereby verifying that the variation in phosphorylation levels between the cell lines was indeed due to differences between the cell lines, and not due to disproportionate gel loading.

Hydroxyurea treatment of three hours did not yield uniform increases in Bid phosphorylation, as was expected. This indicates a possible difference between cell lines in the efficacy of DNA damaging agents, and therefore Bid functionality as well. Alternatively, this could indicate that HU treatment was too long; future studies will include HU treatment of cell cultures with shortened exposure times.

Possible causes of Bid protein misregulation have been postulated: mutations in the *BID* gene, hypermethylation of the promoter, rapid protein degradation, and irregular phosphorylation. As genetic mutations were not detected in the cell lines tested, continuing the analysis of Bid was necessary in order to determine the impetus, if any, of possible Bid misregulation. This study developed an optimized assay to investigate Bid misregulation by way of phosphorylation.

This study sought to elucidate the correlation between Bid phosphorylation and acute myelogenous leukemia (AML) through development of an optimized assay. Through extensive experimentation, fully optimized assay conditions were determined for three antibodies: phospho-Ser65, tBid, and Actin.

Successful determination of ideal assay conditions to query Bid phosphorylation correlation with acute myelogenous leukemia (AML) is instrumental in deepening the understanding of acute myelogenous leukemia. Due to the successful development of an optimized assay, the phosphorylation mechanism can now be further investigated with leukemic patient samples.

CONCLUSION.

Leukemia is a disease that has affected individuals across the world, and will continue to do so unless highly effective and safe treatment methods are developed. Research directed at fully comprehending the disease mechanism is critical in order to more effectively pursue treatment methods that target specific disease mechanisms. Deepened understanding of the disease will facilitate development of treatments, and possibly even preventative measures for the affliction.

This study sought to elucidate the correlation between phosphorylation of Bid, a protein instrumental in facilitation of apoptosis [12], and acute myelogenous leukemia. In order to achieve this end, a Western blot assay was optimized that would allow for eventual analysis of leukemic patient samples. Four antibodies were selected: phosphorylation site-specific Serine 65 and Serine 78, total Bid, and Actin. For all but the pSer-78 specific antibody, assay conditions were successfully determined.

In ongoing research, conditions for the pSer-78 antibody will be optimized. Furthermore, patient samples will be analyzed using the optimized assay conditions to further query the presence of a correlation between AML and Bid phosphorylation. If a positive correlation is determined, Bid phosphorylation may be an attractive target for therapies, such as anti-phosphorylation pharmaceuticals.

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SUPPORTING INFORMATION.

Supplementary Methods Section

Figure S1. Structure of the Philadelphia Chromosome

Figure S2. Enhanced Chemiluminescence

Table S3. PCR Primers used for Bid Amplification

Table S4. Protein Concentrations and Antibodies Used to Optimize Western Blot Conditions

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