Pediatric Acute Lymphoblastic Leukemia: Role of BIM Protein in Prednisolone-Induced Apoptosis

12

Nan Jiang, Grace Shimin Koh, and Allen Eng Juh Yeoh

Contents

Abstract

Acute lymphoblastic leukemia (ALL) is a heterogeneous cancer characterized by abnormal accumulation of immature blasts in the bone marrow. Glucocorticoids such as prednisolone (PRED) have been widely used in the treatment of pediatric ALL and the resistance to PRED is associated with unfavorable outcome in patients. We have identified BIM to be an important regulator of PRED-induced apoptosis, and its expression level may have prognostic value. By understanding the molecular basis of PRED-induced apoptosis, we hope that improved treatment strategies can be defined.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer, accounting for more than 30% of the newly diagnosed childhood cancer cases annually throughout the world (Pui et al. 2004). The intensity of modern chemotherapy for children with ALL is tailored according to risk groups defined by both clinical and laboratory features, in order to minimize long-term toxicity from overtreatment on one hand and relapse from under treatment on the other hand. Currently, the event-free survival (EFS) rates

N. Jiang • G.S. Koh
Department of Paediatrics, Tower Block, Level 12, National University Health System, 1E Kent Ridge Road, Singapore 119228

Division of Hematology and Oncology, Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
e-mail: nan_jiang@nuhs.edu.sg; grace.koh@nus.edu.sg

A.E.J. Yeoh (✉)
Department of Paediatrics, Tower Block, Level 12, National University Health System, 1E Kent Ridge Road, Singapore 119228

Division of Hematology and Oncology, Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Viva-University Children's Cancer Centre, University Children's Medical Institute, National University Hospital, Singapore

National University Cancer Institute of Singapore, National University Health System, Singapore

Cancer Science Institue, Singapore, National Univeristy of Singapore, Singapore
e-mail: allen_yeoh@nuhs.edu.sg

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for childhood ALL has reached 80% in most developed countries. However, a significant remaining 20% relapse despite current intensive chemotherapy regimens.

Several study groups have evaluated a variety of early response estimates as prognostic factors for treatment stratification in childhood ALL. This includes Day 8 prednisolone (PRED) response, Day 15 bone marrow blast count and minimal residual disease levels. Conventional cytogenetic abnormalities like t (9;22) and new genetic alterations identified by whole genome technology such as the deletion/mutation of IKZF1 present at diagnosis predicts a poorer outcome in ALL.

Since 1986, the widely adopted German Berlin-Frankfurt-Munster (BFM) clinical trials have consistently shown that patient early response to PRED is one of the most important prognostic factors (Schrappe et al. 2000).In the BFM protocols, therapy for all patients starts with 7 days of PRED monotherapy and one intrathecal dose of methotrexate on Day 1. The absolute number of leukemic blasts in the peripheral blood on Day 8 is then determined. PRED poor responders had a total blast count of ≥1,000/μl on Day 8; and patients who exhibit a PRED good response have favourable outcome (Schrappe et al. 2000). This provided a powerful yet simple tool to pick up a group of high risk patients, comprising of 10% of all patients, who had a significantly poorer outcome. More importantly, this suggested that resistance to therapy is already present at initial diagnosis and detection of early resistance to therapy allow us to reliably stratify patients so as to improve eventual outcome.

PRED is a form of glucocorticoid (GC). Although GCs has been the most effective drug used in the treatment of ALL for more than 50 years, the molecular basis of GC sensitivity and resistance remains largely unknown. Resistant mechanisms have been postulated to involve mutations affecting glucocorticoid receptors (GR), defects in the GC response genes or their cross talks distal to the binding of GR. Besides drug resistance, GC therapy also has severe side effects like severe weight gain especially with high doses. Therefore, there is a need for a deeper understanding of GC-induced apoptosis and its signal transduction pathways in order to optimize its value in the treatment of ALL.

Prednisolone-Induced Apoptosis

The process of GC-induced apoptosis can be arbitrarily divided into three stages: initiation, decision and execution stages (Fig. 12.1) (Distelhorst 2002).

First, GC enters the cell by passive diffusion and binds to glucocorticoid receptors (GR). The GC-GR complex then undergoes conformational changes and translocates into the nucleus. In the nucleus, the GC-GR complex dimerizes and binds to glucocorticoid response elements (GREs) causing transactivation and transrepression.

The gene that encodes for the GR is located on chromosome 5q31 and is a transcription factor. Much research has provided evidence that GCs induce apoptosis by the transrepression of the GR interaction with transcription factor AP-1 (activating protein-1), which regulates expression of genes involved in cell growth, differentiation and transformation. There are also evidence that GC-induced apoptosis involves the transactivation of the GR through NF-κB. This differs from other apoptosis inducers as GC-induced apoptosis is initiated at a transcriptional level involving the multi-catalytic proteasome and calcium. In addition, the cross talks between GR and other signaling pathways add to the complexity of apoptosis induction. However, the specific genes that mediate cell death in response to transactivation and transrepression have not been clearly identified. It is also unclear if both pathways are required in mediating cell death in ALL cells (Helmberg et al. 1995; Chapman et al. 1996).

The decision stage in GC-induced intrinsic apoptosis involves the mitochondria and caspase cascade. This is also where the regulatory role of Bcl-2 family members is indispensable. GC-induced apoptosis has been reported to be both positively and negatively regulated by the Bcl-2 family members. The balance between pro- and anti-apoptotic signals decides if the cell survives or dies.

The most well-studied anti-apoptotic Bcl-2 family member is BCL-2. Although the inhibitory

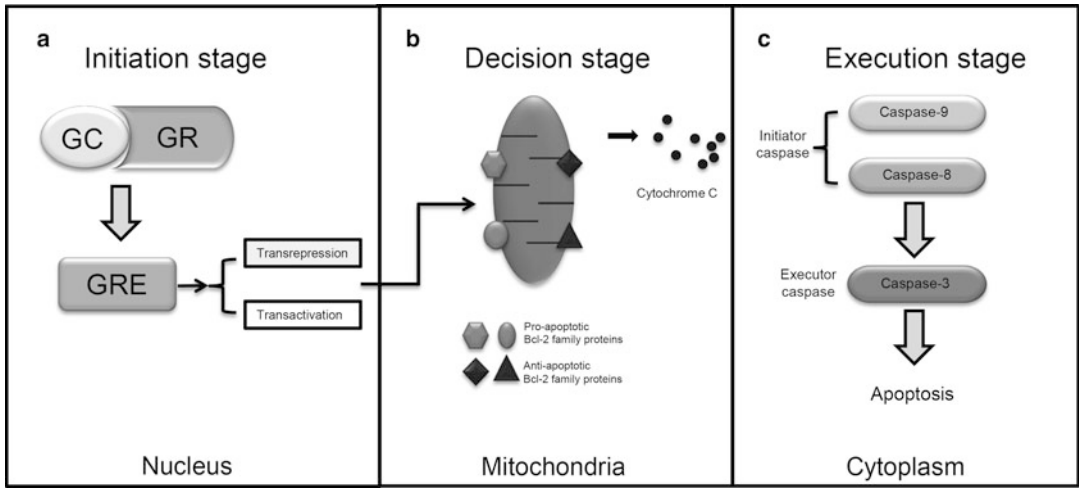

Fig. 12.1 Schematic of glucocorticoid (*GC*) induced apoptosis. GC-induced apoptosis can be arbitrarily divided into three stages: initiation stage, decision stage and execution stage. (**a**) In the initiation stage, GC enters the cell and binds to glucocorticoid receptors (*GR*). The GC-GR complex is activated and translocates into the nucleus. In the nucleus, the GC-GR complex binds to the glucocorticoid response elements (*GRE*), initiating transactivation and transrepression. (**b**) GC-induced apoptosis is reported to be positively and negatively regulated by the Bcl-2 family members. In the decision stage, the balance between pro- and anti-apoptotic signals of Bcl-2 family members decides if the cell survives or dies. Cytochrome c is released and further activates caspases. (**c**) In the execution stage, the activated caspases will execute the final step of apoptosis. There are two types of caspases, initiator and effector caspases. Initiator caspases including caspases -8 and -9, are first activated; they will further cleave effector caspases such as caspase-3. A number of morphologic changes will happen due to the cleavage of caspase-3 such as cell shrinkage, membrane blebbing, and the formation of apoptotic bodies

mechanisms of BCL-2 are not clear, it is believed to act via the outer mitochondrial membrane to affect mitochondrial function (Kroemer and Reed 2000). Experiments showed that after dexamethasone treatment, the apoptosis induced in *bcl-2*$^{-/-}$ knockout mice is accelerated (Veis et al. 1993). In contrast, BCL-2 overexpression was reported to inhibit apoptosis (Camilleri-Broet et al. 1998). *Bcl-2* antisense oligonucleotides also induce apoptosis in drug-resistant cells in various hematological cell lines (Keith et al. 1995). Another important anti-apoptotic member in the Bcl-2 family, MCL-1(which was identified to have putative GREs within the promoter regulatory regions), has also been well studied for its anti-apoptotic roles in GC-induced apoptosis (Lynch et al. 2010).

The involvement of pro-apoptotic members of the Bcl-2 family in GC-induced apoptosis have also been investigated including BAX, BAK, BID, BAD, and BIM (Gross et al. 1999). These pro-apoptotic members transmit death signals generated from the initiation stage to the execution stage during apoptotic signaling. Mice that lack BAX and BAK, BIM, PUMA or NOXA are GC resistant. However, the relative importance of each Bcl-2 family member in GC-induced apoptosis is still unknown as they appear to be cell type dependent.

In the execution stage, the cleavage of caspases will induce the final step of apoptosis leading to the cell's death in a programmed manner. Caspases represent a family of proteases that cleave substrates at aspartate residues. There are two types of caspases: initiator and effector caspases. The initiator caspases include caspase-8 and -9, which when activated, will further cleave effector caspases such as caspase-3 and -7. A number of morphologic changes will result from the cleavage of caspase-3 and -7 such as cell shrinkage, membrane blebbing and the formation of apoptotic bodies. The caspase cascade can be activated in two ways; (1) caspase-9 dependent and caspase-3 independent and (2) caspase-9 independent and caspase-3 dependent. The dominant pathway of GC-induced apoptosis in ALL

cells is caspase-9 dependence. Although the activation of caspases during apoptosis is a down-stream effector event, the signal that triggers this caspase cascade remains unclear. GC may induce either the extrinsic or intrinsic apoptotic pathways or both. In the extrinsic apoptotic pathway, the initiator caspase-8 is activated and will further activate effector caspase, caspase-3, by either a mitochondria-dependent or a mitochondria-independent pathway. However, the role of the extrinsic apoptosis pathway in GC-induced apoptosis remains inconclusive.

The Mechanism of Prednisolone Resistance

GC resistance has deleterious impact on treatment outcome of ALL patients. Although many mechanisms of resistance have been proposed, most of them remained uncertain. To arbitrarily classify the mechanism of GC resistance, it can be separated into "upstream" and "downstream" mechanisms (Sionov et al. 2008). The factors involved in "upstream" mechanisms include: GR, its ligand and GR-associated proteins. The "downstream" mechanisms involve the genes or proteins that respond to GC-GR signals and the ones that can regulate this signal.

Regarding the upstream GC resistance mechanism, several studies have tried to link the resistance of GC with the low expression or mutation of GR proteins (Sionov et al. 2008). The nuclear-transfer-deficient receptor was also reported to contribute to GC resistance (Moalli and Rosen 1994). It is proposed that these receptors may have mutations in nuclear localization signals. Some other resistance mechanisms include the modification of GR by mono-ADP ribosylation or phosphorylation, overexpression of GRβ and genetic polymorphisms. Until now, no conclusive data has shown a clear correlation between GR expression or modification to GC resistance in ALL.

In our study (Jiang et al. 2011a), we have analyzed the presence of GR mutations in 72 ALL patients and none of them carried any GR mutation. This may indicate that GR mutation is not the main cause of PRED resistance observed in clinical settings, but it may be more frequently observed *in vitro*. It also highlights the possibility that defects down-stream of GR activation could be the major cause of GC resistance at the initial induction phase. Defects in downstream signaling components such as GC-regulated genes will cause resistance to GC. Using gene microarray, the GC-treatment induced genes were identified to be involved in many important regulation processes such as transcription, mRNA splicing and protein synthesis (Obexer et al. 2001).

The role of Bcl-2 family members in GC resistance has been extensively investigated (Kfir-Erenfeld et al. 2010) and several clinical studies have established that Bcl-2/Bax ratio is associated with GC resistance. However, a clear correlation in the expression levels of BCL-2 or Bcl-2 family members with GC resistance is still lacking in childhood ALL. Since Bcl-2 family members play indispensable role in GC-induced apoptosis, further investigation is needed to increase current understanding on the mechanism of GC-induced resistance in ALL cells.

BIM, the Main Regulator of Bcl-2 Family Members in Prednisolone-Induced Apoptosis

GC-induced apoptosis involves many genes and proteins, making it difficult to elucidate the mechanism of GC-induced apoptosis. The role of key proteins from the Bcl-2 family has been the major focus of many investigations in the last few decades.

GC-induced apoptosis could be positively and negatively regulated by members of the Bcl-2 protein family, commonly termed as "Bcl-2 rheostat" (Schmidt et al. 2004). Currently, the widely accepted hypothesis is that anti-apoptotic members such as BCL-2, BCL-XL and MCL-1 act on the outer mitochondrial membrane to preserve the mitochondrial integrity by inhibiting pro-apoptotic members such as BIM. This inhibits BAX and BAK activation and subsequent apoptosis (Kfir-Erenfeld et al. 2010).

In our recent study (Jiang et al. 2011b), we validated five members of the Bcl-2 family

(BCL-2, MCL-1, BCL-XL, BID and BIM) and an inhibitor of apoptosis family member, SURVIVIN, in four ALL cell lines that respond differentially to PRED treatment (three PRED-sensitive and one PRED-resistant). These genes were identified to be differentially expressed between PRED poor response and PRED good response patients based on the microarray data generated by 45 paired ALL bone marrow samples at diagnosis (D0) and after 7 days of PRED treatment (D8) (35 PRED good responders and ten PRED poor responders) among all the other Bcl-2 family members (Yeoh et al., unpublished data). Only BIM protein was validated to be upregulated in PRED-sensitive cells but not in PRED-resistant cells. Co-treatment with Ru486, a GR antagonist, inhibited the elevation of BIM, indicating that the upregulation of BIM was GR-dependent. Transfecting specific siRNAs to silence the expression of BIM showed that when BIM was silenced, PRED-induced apoptosis was significantly inhibited. Similarly, the upregulation of BIM by GCs treatment has been confirmed in other types of leukemic cells (Kfir-Erenfeld et al. 2010). Taken together, the role of BIM in PRED-induced apoptosis is indispensable. BIM may play the main regulatory role in the "decision stage" of GC-induced apoptosis that control the downstream "execution stage" which involves the release of cytochrome c and caspase cascade activation.

GC-induced BIM upregulation may be a GC-specific pro-apoptotic process in the signaling pathways of a cancerous cell. The exact mechanism by which GC activates BIM is not clear. It is known that the promoter of the *bim* gene does not contain a consensus GRE (Wang et al. 2003). Thus, it is hypothesized that the presence of a GR-dependent upstream regulator could control BIM activation and the subsequent apoptotic signals. Many studies suggest that BIM could be regulated by a dynamic "Kinome" which is influenced by GC and may be cell type dependent (Kfir-Erenfeld et al. 2010). Future studies focusing on this area to elucidate the upstream regulation mechanism of BIM will shed light on the understanding of GC resistance mechanisms and have the potential to reverse the GC-resistance in the clinic.

BIM Is Identified as a Prognostic Gene Using Whole Genome Expression Studies

To improve the quality of life for pediatric patients with ALL, there is a requirement for prognostic biomarkers that can reflect the activation or inactivation of certain molecular pathways in treatment regiments which will increase our chances to understand the disease progression and to enhance our ability to predict outcome. Currently, we are able to identify potential biomarkers systematically using modern technologies such as gene microarray, proteomics tools or transcriptome analysis.

Using global gene profiling (GEP), several prognostically important subtypes of childhood ALL such as *ETV6-RUNX1*, *BCR-ABL*, *TCF3-PBX1*, *MLL* rearrangement, hyperdiploidy could be identified and differentiated from each other by their gene expression signatures (Yeoh et al. 2002). Large panels of genes that respond to GC treatment have been revealed, providing valuable information regarding resistance mechanisms and involvement of pathways for further functional investigations.

Many studies have found that BIM has prognostic value in clinical settings. Schmidt et al. (2006) identified that BIM was a frequently upregulated Bcl-2 family member in 13 pediatric ALL patients after 24 h GC treatment using GEP, while Bachmann et al. (2010) reported that GC resistance attributes to epigenetic silencing of the *bim* gene in pediatric ALL biopsies and xenografts established in immune-deficient mice from direct patient explants. Based on gene microarray data using our patients' bone marrow samples, we have shown that only *bim* was found to be upregulated at both gene and protein expression levels among all the Bcl-2 family members screened. BIM expression was found to be highly predictive of PRED response (ROC area under the curve = 0.81; $p = 0.032$) in paired bone marrow samples and is independent of molecular subtype. Patients whose BIM protein expression levels fail to be upregulated at Day 8 compared to Day 0 have significantly poorer EFS (60%) than

those patients whose BIM protein expression levels were upregulated (92%). Despite a relatively small sample size, we have demonstrated that BIM stands out as a promising prognostic biomarker of PRED response at Day 8 in B-lineage ALL patients (Jiang et al. 2011b). Further validation is needed on larger cohorts of patients to confirm the prognostic value of BIM protein within the first 7 days of GC induction therapy. This promises earlier diagnosis to identify slow responders or drug-resistant patients.

Gene expression profile should also be considered as a diagnostic tool to provide a clearer picture of early response genes. Great effort has been dedicated to understand the genetic profiles and significant progress has been made in this area recently. Flotho et al. (2007) have investigated the correlation between gene signature and minimal residual disease. Low expression of CASP8AP2 has been identified to be able to predict a low event-free survival and a higher rate of leukemia relapse (Flotho et al. 2006). MCL-1 may also be an important regulator of GC-induced apoptosis. Rapamycin, an mTOR inhibitor, can modulate MCL-1 to reverse GC resistance in lymphoid malignancies (Wei et al. 2006). Therefore, finding a solution to overcome steroid resistance and directly using genomic information at the "bench" will facilitate diagnosis and patients stratification at the "bedside" for childhood ALL management. It also opens up the opportunities for novel drug development based on the biomarkers identified through a hypothesis-driven approach to make personalized therapy possible.

Targeting Bcl-2 Family Members to Induce Apoptosis in ALL Cells

It is well known that resistance to GC therapy is associated with unfavorable outcome in ALL. Moreover, acquired resistance towards GC is also a huge challenge for relapsed ALL. To re-sensitize GC-resistant cells to therapy requires novel therapeutic strategies.

BIM can bind and sequester all anti-apoptotic Bcl-2 family members with high affinity and has greater potential to be an apoptosis-inducer compared to other BH3-only proteins, such as BAD and NOXA that bind to limited number of anti-apoptotic members (Youle and Strasser 2008). However, it is observed that upregulation of BIM alone was not sufficient to induce apoptosis because it requires posttranslational activation or interaction with other proteins (Puthalakath and Strasser 2002).

A novel group of chemicals commonly termed "BH3-mimetics", that mimic the BH3 domain and has been used in the treatment of various cancers (Chonghaile and Letai 2008). Several chemicals have been identified to be BH3-mimetics including Obatoclax (GX15-070), ABT-737 and Gossypol. ABT-263, a member of this family, is currently used in Phase I clinical trials for many cancer types. Studies showed that BH3-mimetics may have a unique way to induce cell death by mimicking BH3-only proteins such as BIM or PUMA and regulating the interaction between Bcl-2 family members (Chonghaile and Letai 2008). It is proposed that the high expression of anti-apoptotic members of the Bcl-2 family in malignant cells is the main reason why BH3-mimetics are effective (Wei et al. 2006). Indeed, Bonapace et al. (2010) reported a complete re-sensitization of multidrug-resistant childhood ALL cells to GCs with subcytotoxic concentrations of Obatoclax, a putative antagonist of Bcl-2 family members, through induction of autophagy-dependent necroptosis. Mason et al. (2009) reported that ABT-737 is highly effective as a single agent against most primary chronic lymphocytic leukemia (CLL) samples, and is synergistic with a range of cytotoxic chemotherapy agents.

However, there are conflicting evidence concerning the correlation of Bcl-2 family members expression level and sensitivity to BH3-mimetics; several cell types were found to be less sensitive to the treatment using BH3-mimetics. The reason for their resistance is largely unknown. In addition, some BH3-mimetics may have other apoptotic induction mechanisms besides regulating Bcl-2 family members alone. Studies have shown that known prognostic factors cannot predict the responsiveness of leukemic cells to BH3-mimetics (Mason et al. 2009). Therefore,

it would be greatly beneficial if the synergism between different BH3-mimetics and BH3-mimetics with conventional chemotherapeutic drugs is tested before use in the clinic.

In conclusion, glucocorticoids, like PRED, are important medications in the treatment of pediatric ALL and resistance to GC is associated with unfavorable outcome. Among all the Bcl-2 family members, BIM, a pro-apoptotic BH3-only protein, plays an essential regulatory role in PRED-induced apoptosis in pre-B ALL cells. BIM was up-regulated after PRED treatment in PRED-sensitive cell lines and primary bone marrow samples of PRED good response patients both at gene and protein expression levels. This has potential prognostic value in clinical settings. BH3-mimetics may re-sensitize resistant patients by rational combination in the future.

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