

ALTERED APOPTOTIC PROTEIN EXPRESSIONS CHARACTERIZE THE SURVIVAL OF BCR-ABL-INDEPENDENT DRUG-RESISTANT CHRONIC MYELOID LEUKEMIA CELL LINE

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ABSTRACT

Apoptosis is a programmed cellular process that occurs in pathological and physiological pathways and it is one of the most studied topics in cell biology. To understand the underlying mechanism of apoptosis plays an important role in the molecular pathogenesis of many diseases including cancers. Chronic myeloid leukemia (CML) is a clonal myeloproliferative malignancy arising from the neoplastic transformation of the hematopoietic stem cell. Here, we used a Bcr-Abl-independent, imatinib-resistant K562 subpopulation (K562-IR) generated and characterized earlier in our laboratory. We showed that the proteins Bcl-2, Bim, RIP, p-MAPK(Erk1/Erk2) and NF-κB which plays critical roles in cell death pathways are downregulated, Lamin A/C protein expression is upregulated in K562-IR derivative cells compared to K562 ancestral cells. Our data provides new information on the expression of apoptotic molecules in a Bcr-Abl-independent imatinib-resistant CML cell line.

Keywords: Chronic myeloid leukemia, apoptosis, Bcl-2, Bim, RIP, NF-κB

INTRODUCTION

Understanding the cell death and survival mechanisms is crucial for the development of new therapy strategies and novel drugs in malignant diseases. Apoptosis is the first characterized form of programmed cell death. Signaling pathways leading to apoptosis are well conserved between species (1, 2). Apoptosis may be induced in a cell via extrinsic and/or intrinsic pathways, both which converge on the

activation of caspase-dependent proteolysis of thousands of cellular proteins. Membrane blebbing and endonucleolytic cleavage of chromosomal DNA are predominant features. Defects in this process is related to unregulated cell survival and drug resistance. Resistance to apoptosis is one of the main features observed in a malignant transformed cell (3–5).

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease that occurs as a result of the reciprocal translocation between the chromosomes 9 and 22. The resulting derivative chromosome 22 is known as the Philadelphia chromosome. The Bcr-Abl fusion protein resulting from this translocation exhibits an unregulated, continuous tyrosine kinase activity that allows the expansion of the leukemic clones by triggering signaling pathways, leading to cell proliferation, transcriptional activity and resistance to apoptosis. Bcr-Abl oncoprotein phosphorylates various downstream substrates that regulate the apoptotic machinery in the cell (6–8). Imatinib mesylate (STI-571), a tyrosine kinase inhibitor targeting the Bcr-Abl oncoprotein, inhibits the enzymatic activity of Bcr-Abl and as a result has been associated with the induction of apoptosis in leukemic cells (9–11). On the other hand, imatinib resistance is an important problem affecting 30% of CML patients and studies in this area aim to reveal treatment resistance mechanisms. There are several Bcr-Abl dependent resistance mechanisms in which BCR-ABL amplifications/duplications, Bcr-Abl oncoprotein secondary kinase domain mutations are most frequently observed. Bcr-Abl independent mechanisms have been shown in CML cancer stem cells and are still being investigated (12–16). In this study, we aimed to study Bcl-2, Bim, RIP, NFκB, Lamin A/C and Erk1/Erk2 protein expression levels, which may have pivotal roles in the cell death pathway in a Bcr-Abl independent imatinib-resistant CML cell line model.

METHODS

Cell lines and Cell Culture

Human chronic phase myeloid leukemia cell line K562 was obtained from ATCC. A resistant subclone, K562-IR was generated by clonal selection under imatinib mesylate selective pressure (17). Cells were subject to increasing imatinib doses starting at 0.1 μM and the final dose was 10 μM which is two-fold higher than the serum concentration of patients with a daily uptake of 400g imatinib (11). We generated a imatinib resistant sub-population under these conditions. Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 1 unit/mL P/S at 37°C and 5%CO₂. The imatinib resistant K562 clone, K562-IR was continuously cultured with 10 μM imatinib; while

10 μM imatinib was added to K562 cells in appropriate experimental conditions to serve as control.

Western blot analysis

Protein lysates were prepared from K562 and K562-IR cells with RIPA buffer. Proteins were quantified by BCA assay. The proteins were separated by polyacrylamide gel electrophoresis and wet transferred to PVDF membranes. Membranes were probed with specific antibodies and visualized by the sensitive X-ray films. Antibodies used are human anti-B-actin HRP (Sigma A3854), human anti-eIF4E (Cell Signaling #2067), human anti-Lamin A/C (Cell signaling #4777), human anti-Bim (ab7888), human anti-RIP (ab106393), human anti-NFκB (ab209795), human anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell signaling #4376)

RESULTS

Cellular characteristics of K562-IR cells were reported in our previous study (17). Pro- and anti-apoptotic protein expressions are altered in ancestral K562 and derived K562-IR cells Western blot analysis was performed for K562 and K562-IR cells with and without imatinib culture conditions (24h). The anti-apoptotic protein Bcl-2 is downregulated in K562-IR resistant cells. On the other hand, pro-apoptotic protein Bim expression is upregulated in K562 cells and imatinib presence increases its expression. Conversely, Bim expression of K562-IR cells is quite low and it is not dependent on imatinib presence. Erk1 and Erk2 phosphorylations are interrupted in K562-IR cells that are cultured continuously in 10 μM imatinib. NF-κB (p100/p50) expression is downregulated in K562-IR cells. RIP expression does not differ between K562 and K562-IR cells but in the presence of imatinib, RIP expression is downregulated in K562-IR cells. In addition, Lamin A/C expression was found to be upregulated in the K562-IR cell line (Fig. 1).

DISCUSSION

To understand the underlying mechanism of apoptosis plays a pivotal role in the pathogenesis of cancers. In this study we used an K562-derived, tyrosine kinase inhibitor resistant cell line K562-IR, obtained by clonal selection under imatinib pressure to investigate apoptotic pathway differences between

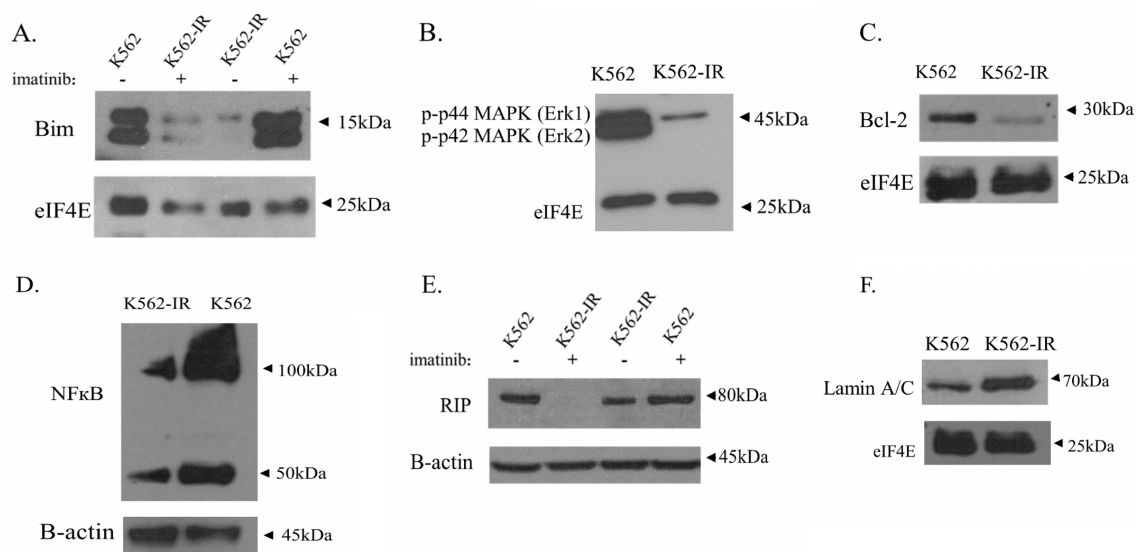


Figure 1. Apoptotic pathway results of ancestral K562 and derived K562-IR cells. Western blot analysis were performed for Bim (A), phosphorilated MAP kinase (B), Bcl-2 (C), NFκB (D), RIP (E) and Lamin A/C (F) proteins. K562 cells were treated with 1μM imatinib for 24h before the experiment (A and E). K562-IR cells were continuously cultured with 10μM imatinib. For without imatinib (-) samples K562-IR cells were washed twice with PBS and cultured for 24h before the experiment (A and E). β-actin and eIF4E proteins are used for loading control.

imatinib sensitive and imatinib resistant CML cells. K562-IR cells are resistant to imatinib, dasatinib, ponatinib, nilotinib and bosutinib tyrosine kinase inhibitors (TKIs), they display a semi-adherent phenotype, they are Bcr-Abl independent, and their proliferation rate is 4-fold slower than the K562 cell line (17). They represent cells that have gained an inherent resistance to TKIs when challenged over time, by biologically becoming Bcr-Abl independent; thus not relying on this oncoprotein's signaling as a survival signal.

Bcl-2 protein family have both proapoptotic (e.g., Bim and Bak) and antiapoptotic (e.g., Bcl-2 and Bcl-XL) members which are responsible for the initiation of apoptosis by regulating the release cytochrome c from mitochondria (18). While anti-apoptotic protein Bcl-2 inhibits this process, the pro-apoptotic molecule Bim interacts with Bcl-2 to suppress its activity and drive the cell to apoptosis (19). Bim, Bcl-2 and ERK1/2 phosphorylation plays important roles in Bcr-Abl driven apoptotic pathways (20). Imatinib also suppresses the PDGFR signaling pathway along with the Bcr-Abl pathway. ERK1/2 phosphorylation is a downstream effector molecule of PDGFR signaling and is therefore inhibited in K562-IR cells which are

grown under continuous 10uM imatinib pressure (Fig1.B) The anti-apoptotic Bcl-2 protein expression is expected to be upregulated in K562-IR cells which have high survival capacity, but surprisingly its expression was shown to be lower when compared to K562 cells (Fig.1C).

On the other hand Bim protein expression is abundant in K562 cells and its expression is upregulated by imatinib; whereas Bim is expression is downregulated in K562-IR cells and does not change in the presence or absence of imatinib (Fig.1A).

These result show that apoptosis is triggered and changes of in the Bcl-s family of proteins are observed accordingly in the presence of imatinib, in TKI sensitive wild-type K562 cells. Whereas in the TKI resistant K562-IR cells, presence of imatinib has no influence on Bcl-2 apoptosis signaling. Bim, which blocks the anti-apoptotic activity of the Bcl-2 protein is downregulated leading to a general resistance to apoptotic cell death. On the other hand, Bcl-2 protein expression is not triggered in these Bcr-Abl independent cells due to not sensing a threat by the presence of imatinib. Also it's known that RIP kinase

regulates Bcl-2 protein activity through its interactions with MAPK/NFκB (21); which may explain the the downregulation of Bcl-2 in the K562-IR cells.

RIP kinase usually functions in the programmed necroptosis pathways and participates in extrinsic apoptotic pathways (22–24). Information on the role of RIP kinase in CML literature is quite limited. Studies have shown that RIP overexpression can activate NFκB through TNF receptor-associated proteins. In RIP-deficient cells, TLR3- mediated NF-κB activation is decreased (25–28). Our results show that RIP expression is inhibited in K562-IR cells which are continuously cultured with imatinib and as expected NFκB expression is also downregulated (Fig1.D and E). Whereas in wild-type K562 cells, the presence of imatinib does not effect RIP expression (Fig1.E).

Lamins A and C are structural intermediate filament proteins which provide stability and strength to cells, especially to the nuclear envelope. Lamins are specifically targeted by caspases 3 and 6 that become activated both through the intrinsic and extrinsic apoptotic pathways(29). They are expressed in myeloid leukemia cells (30). There are studies that show normally proliferative or not proliferative cells are devoid of A-type lamin expression, while many neoplastic tissues show remarkable lamin expression levels(30). Our results show that Lamin A/C expressions are downregulated in slow proliferative K562-IR cell (Fig.1F).

Our results provide information on the expression profile of apoptotic molecules in a Bcr-Abl independent imatinib-resistant CML cell line model and contributes to the studies of CML biology and to the drug development technologies. More research is needed to elucidate the precise mechanisms that these molecules exert their effects.

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