Survival of the fittest clone: Pro-apoptotic protein Noxa controls selection of lymphocytes under competitive conditions

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The Noxa, Bim and Mcl-1 axis mediates apoptosis in response to general metabolic stress

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Abstract

Apoptosis provoked by glucose shortage in dividing T cells is mediated via the BH3-only protein Noxa and inhibition of its binding partner Mcl-1. It is unknown how signals from cellular metabolism can affect the balance between Mcl-1 and Noxa and to what extent other Bcl-2 members are involved in this apoptosis cascade. Here, we aim at characterizing the underlying molecular mechanism involved in apoptosis mediated by the Noxa/Mcl-1 axis. First, we established that the Noxa/Mcl-1 balance is regulated by glucose deprivation as well as by general metabolic stress, via changes in proteasome-mediated degradation of both Mcl-1 and Noxa. Second, in contrast with cytokine-deprivation, no transcriptional modulation of Mcl-1, Puma, Bim or Noxa was observed during glucose deprivation. Third, no changes in PKB, GSK-3 activity and surprisingly also no effect on AMPK activity occurred. Fourth, nutrient deprivation-triggered apoptosis was executed without signs of overt autophagy and independent of ROS production or p38 MAP kinase. Lastly, in line with recent insights into interactions and ‘cross-talk’ between Bcl-2 members, apoptosis under nutrient limitation also required Bim and could be blocked by Bcl-2. In conclusion, Noxa functions in a specific apoptotic pathway that integrates overall nutrient stress, independent from attenuated PI3K/PKB signaling or autophagy.

Graphical Abstract
Introduction

Growth factors and nutrients are essential for lymphocyte survival (1). Deprivation of these metabolic resources initiates the mitochondrial apoptosis pathway, which is marked by the release of apoptogenic factors from the mitochondrial intermembrane space, such as cytochrome c. Upstream in this cascade, pro-apoptotic Bcl-2 family members Bax and Bak are directly involved in the permeabilization of the mitochondrial outer membrane. Their function is inhibited by protective Bcl-2 members (Bcl-2, Mcl-1, Bcl-XL, A1 and Bcl-w). The pro-apoptotic BH3-only proteins (e.g. Bim, Bid, Noxa, Puma) constitute the third subgroup of this family and are suggested to function as sentinels that integrate distinct extracellular and intracellular insults (2,3).

Dividing lymphocytes, such as antigen-activated T cells, are highly dependent on nutrients in order to fulfill their metabolic requirements. Triggering of growth factor receptors or of CD28 in conjunction with the T cell receptor promotes glucose uptake by augmenting the expression of cell surface glucose transporter 1 (Glut1) in a PKB-dependent manner (1,4). Previous studies have demonstrated the existence of an apoptotic route coupling nutrient shortage to Bcl-2 family members (5,6). We have demonstrated that during TCR-mediated activation, human T cells upregulate the expression of the BH3-only protein Noxa, and that the balance between Noxa and its pro-survival binding partner Mcl-1 dictates the apoptosis susceptibility of dividing lymphocytes to glucose shortage (7).

Apart from growth factors and glucose, cells require additional essential anabolic building blocks obtained from o.a. amino acids and vitamins. The role of Bcl-2 family members in apoptosis as a result of deprivation of these factors has only recently been subject of study. Obvious candidates such as Bcl-2, Bcl-XL and Bim have been shown to play a role in this apoptotic pathways (8-10), but how cellular metabolic stress signal integrate into the apoptosis executors remains largely unexplored.

Various studies have demonstrated a PI3K-dependent transcriptional and post-translational control of Bcl-2 family members for the induction apoptosis following growth factor withdrawal. De novo expression of BH3-only proteins Bim and Puma mediated by the forkhead transcription factor 3a (FoxO3a) is a key cellular event caused by attenuation of PI3K/PKB signaling as result of the absence/lack of growth factors (11,12). In addition, PI3K/PKB controlled inhibition of glycogen synthase kinase-3 (GSK-3) activity, which targets Mcl-1, the antagonist of Puma, Bim and Noxa, for proteasomal degradation following IL-3 or glucose deprivation (13,14). Alternatively AMPK, the cellular sensor of
energy, has been associated with the control of Mcl-1 protein levels (15). This pro-survival molecule has thus emerged as a common target in apoptosis caused by growth factor and nutrient starvation. However, our understanding on how the intracellular molecules that respond to metabolic stress regulate Mcl-1 still contains significant gaps.

In this study we questioned how the proteins Mcl-1 and Noxa are coupled to intracellular signaling in response to metabolic stress. Moreover, we investigated whether the engagement of these molecules is restricted to glucose shortage, or may involve other types of nutrient stress. Here, we demonstrate that Noxa is involved in apoptosis caused by general deprivation of nutrients, and that this can occur independently of upstream PI3K components, AMPK and MAP kinases.

**Results**

**Reduction in Mcl-1 protein during glucose shortage is independent of transcriptional regulation or caspase activation.**

Both activated T cells and transformed cells switch their glucose catabolic metabolism from oxidative phosphorylation to glycolysis (the so-called Warburg effect (16)). Cells cultured under low glucose concentrations readily deplete glucose from the medium and induce apoptosis by a gradual decline in Mcl-1 levels (7). Decrease of Mcl-1 might be regulated at the transcriptional level, subsequent to caspase activation after the onset of apoptosis and/or via a post-transcriptional mechanism (15,17,18). First, we investigated whether Mcl-1 or other Bcl-2 family members were transcriptionally regulated in T lymphoblastic leukemia cells (Jurkat cells, clone J16). The relative expression of members of the Bcl-2 family, including Mcl-1, Bim and Noxa, as detected by RT-MLPA (19) did not change significantly despite apoptosis induction. In contrast, levels of survivin, a gene regulated in a cell cycle-dependent manner (20), decreased concomitantly with the reduction in cell division (**Figure 1a** and data not shown).

We next investigated whether the decline in Mcl-1 degradation required caspase activation. Using either the pan-caspase inhibitor zVAD or J16 cells transduced with dominant-negative caspase-9, processing of caspase-3 following glucose deprivation was blocked. Yet, the decline in Mcl-1 protein still occurred under these conditions (**Figure 1b**), indicating that this labile protein is subject to post-transcriptional regulation.
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**Figure 1. Reduction in Mcl-1 protein during glucose shortage is independent of transcriptional regulation or caspases.** J16 cells were cultured under glucose limitation (5mM). (a) At the indicated time points, cell viability was assessed by FACS analysis (left) and mRNA expression of apoptosis genes was measured by RT-MLPA (right). The relative expression for each gene was normalized to the value obtained on day 0. (b) Caspase-independent degradation of Mcl-1 during glucose limitation. Cells were analyzed after 0 and 3 days in culture. Left: J16 cells were cultured in the absence or presence of zVAD, supplemented one day before lysis. Right: Parental J16 cells in comparison with dominant-negative caspase-9 (J16-C9DN). Protein lysates were analyzed by western blotting and probed for the indicated proteins.

**Following glucose deprivation Mcl-1 decline results from increased protein turnover, which is independent of AMPK and GSK3 activity.**

Next we addressed post-transcriptional regulation of Mcl-1 following nutrient depletion. Recently, Pradelli *et al.* described that during acute glucose deprivation, Mcl-1 protein turnover is maintained, but its levels are decreased by a translational block of Mcl-1 transcripts via activation of AMPK (15). To investigate whether Mcl-1 turnover was affected in our culture system of gradual glucose deprivation, its half-life was compared before or after three days of culture in low-glucose medium. Surprisingly, when transcription was blocked, Mcl-1 half-life was reduced approximately four fold. The half-life of Noxa was also reduced two fold, effectively doubling the Noxa to Mcl-1 ratio. Inhibition of proteasome activity by MG132 induced accumulation of both proteins (Figure...
2a, b). No increased AMPK phosphorylation in Jurkat cells with normal or Noxa levels was observed, nor in cells where Noxa was stably knocked down via a shRNA construct (N8). Instead, a consistent decrease (n=7) in activated AMPK occurred after several days of culture (Figure 2c). Together these data indicate that the decrease in Mcl-1 under glucose shortage occurs via increased targeting to the proteasome, and is correlated with a decrease in AMPK activity.

Others have shown that during glucose deprivation, attenuated PI3K/PKB signals activate GSK3β, which targets Mcl-1 to the proteasome (13,14). Previously, we demonstrated that the decline in Mcl-1 protein expression and apoptosis during glucose limitation was inhibited in cells with low Noxa levels (Noxa<sup>Low</sup>) versus Noxa-sufficient cells ((7) and Figure 2d). In Jurkat cells, neither PKB nor GSK3β seemed to function as potential upstream regulators in this process, as neither the activity of PKB nor the repressive phosphorylation of GSK-3 was diminished but in fact slightly increased (Figure 2d). This was observed in both control and Noxa-deficient cells, and thus also excluded possible feed-back loops triggered upon apoptosis. Accordingly, we found no difference in apoptosis sensitivity of these cells to inhibition of PKB with Ly294002 or Wortmannin (data not shown).

Since Jurkat cells have excessive PKB activity due to PTEN deletion, we also investigated Mcl-1 degradation in the IL-3-dependent cell line TF-1 under similar conditions as described above. A strong link between cytokine deprivation, GSK3 activity and Mcl-1 stability has been demonstrated (13) and was therefore compared with glucose mediated cell death. TF-1 cells rapidly died following IL-3 deprivation, which could be largely prevented by the GSK3 inhibitor SB216763 (SB). Glucose deprivation also induced apoptosis, but this was not affected by SB (Figure 2e). Both during IL-3 and glucose deprivation, Mcl-1 levels were decreased, which could be reversed by re-addition of IL-3 or glucose, respectively. Mcl-1 levels were maintained when SB was added to IL-3 deprived cells, but not to glucose-deprived cells (Figure 2f), indicating that these processes are mediated via different molecular pathways. Accordingly, IL-3 deprivation resulted in reduced levels of phosphorylated GSK-3, which could be reversed by adding IL-3, but glucose deprivation did not have an effect on phospho-GSK3 levels (Figure 2g). Inhibition of AMPK activity in these cells by Compound C did not lead to reduced apoptosis levels after IL-3 or glucose deprivation (Figure 2h).
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Figure 2. Following glucose deprivation, Mcl-1 decline results from increased protein turnover independent of AMPK and GSK3. (a) Mcl-1 and Noxa are targeted to the proteasome during glucose shortage. On day 0 and day 3, J16 cells were treated with cyclohexamide (CHX) or MG-132 for the indicated time points (hrs). (b) The half-life of Mcl-1 and Noxa was determined by quantification of the protein signals on Western blot, normalized to loading control. Values represent averages of two separate experiments, following CHX treatment on day 0 and 3, normalized to loading control. (c) AMPK phosphorylation. J16 cells transduced with pRetro-control GFP (Mock) or pRetro-Noxi8 GFP (N8) were cultured under glucose-limiting conditions. At designated time points protein lysates were analyzed by western blotting for total (T) and phosphorylated (P) AMPK (Thr172). One representative experiment of seven performed is shown (d) PKB and GSK3 phosphorylation. Mock and N8 cells were cultured under glucose-limiting conditions. At designated time points protein lysates were analyzed by western blotting for Mcl-1, total (T) and phosphorylated (P) PKB (Thr308) and GSK3 (Ser9). Numbers indicate the percentage of viable cells determined by FACS analysis. e-g TF-1 cells were cultured o/n without IL-3 (-IL-3) or 2 days without glucose (-glu) in the presence (+SB) or absence of the GSK3 inhibitor SB216763. (e) Viability measured by FACS. (f) Western blot analysis of Mcl-1 levels. (g) Western blot analysis of total (-T) and phosphorylated (-P) GSK3 levels. (h) TF-1 cells were cultured O/N without IL-3 (-IL-3) or 2 days without glucose (-glu) in the presence (+CC) or absence of the AMPK inhibitor Compound C and viability was measured by FACS.
These data indicate that gradual glucose deprivation resulted in Mcl-1 degradation and apoptosis, independent of AMPK or GSK3 signaling.

**The Noxa-Mcl-1 axis mediates apoptosis provoked by overall nutrient limitation.**

In our culture system, cells are not shifted abruptly from 25 mM to 0 mM glucose as applied in previous reports (14,15), but instead gradually deplete their medium starting from 5 mM glucose, mimicking a more physiological situation of hypoglycemia. Under these conditions, more gradual shifts in glucose metabolism occur, possibly leading to different stress responses. To investigate this notion, we determined whether the signals that engage Mcl-1 and Noxa originated during glycolysis or oxidative phosphorylation. In a competitive outgrowth experiment between WT Jurkat and Noxa\textsubscript{Low} cells under glucose limiting conditions, pyruvate was supplemented to bypass glycolysis and to provide direct substrates for the citric acid cycle. The addition of pyruvate or glucose delayed apoptosis as detected by a drop in MOMP in both populations in a similar fashion (Figure 3a and b). Disruption of the citric acid cycle with the specific aconitase inhibitor SFA (21) induced apoptosis after 48hrs and a survival advantage for cells with reduced levels of Noxa was again observed (Figure 3c). This suggests that despite the high dependence of dividing cells on glycolysis, the responsible stress signals that trigger apoptosis are likely to originate from oxidative phosphorylation.

Second, we observed that prolonged culture under starting conditions of high glucose (25 mM) also provided Noxa\textsubscript{Low} cells with a survival advantage. Importantly, glucose was not fully depleted under these conditions, and lactate was produced from the start of the experiment (Figure 4a). These results suggest that deprivation of other nutrients could also engage the Noxa-Mcl-1 apoptotic axis. To test this, we next cultured cells under conditions of high glucose, but with low levels of anabolic supplements, such as amino acids and vitamins (12.5% of normal values). Apoptosis occurred with clearly different kinetics compared to complete medium but again Noxa\textsubscript{Low} cells displayed a survival advantage (Figure 4b), and the level of Mcl-1 decreased concomitantly with apoptosis induction (Figure 4c). The cells ceased dividing suggesting that inhibition of anabolism triggered apoptosis.

To corroborate a link between deprivation of anabolic building blocks and Noxa, the anabolic pentose phosphate pathway was disrupted by the specific inhibitor 6-AN (22) and a clear survival advantage was observed in Noxa\textsubscript{Low} cells (Figure 4d). Together, these data
suggest that the Noxa-Mcl-1 axis participates in apoptosis initiated by general nutrient scarcity.

Figure 3. Apoptosis under glucose shortage is prevented by addition of pyruvate. Untransduced J16 cells (GFP-) were mixed initially at 1:1 ratio with either Mock or N8.15 cells (GFP+) and cultured in medium supplemented with 5 mM glucose. On day 2, cultures were supplemented with 5 mM glucose (+Glu) or 10 mM Pyruvate (+Pyr). Cells were scored by viable gating and mitotracker positivity. (a) Shown is a representative experiment on day 3, within the viable gate. Numbers indicate the percentages in the corresponding quadrants. (b) Survival of Noxa\textsuperscript{high} (J16 or Mock) and Noxa\textsuperscript{low} (N8.15) cells. The ratio between transduced (GFP+) and untransduced (GFP-) cells was calculated in the viable gate for the conditions indicated. Dashed lines with open or close symbols represent correspondent populations in cultures supplemented with extra 10 mM pyruvate or 5 mM glucose on day 2, respectively. (c) Reduced sensitivity of Noxa\textsuperscript{low} cells to inhibition of the citric acid cycle. Mock or N8 cells were cultured in complete medium for 48hrs in the presence of 2 mM Sodium Fluoro-Acetate (SFA). Viability was assessed by FACS based on mitotracker positivity. Data are averages of 9 separate experiments.

Apoptosis instigated by nutrient shortage occurs in the absence of overt autophagy.

Many cell types subjected to starvation undergo autophagy, an evolutionary conserved intracellular degradation process that is instigated via the mTOR complex (23). Since both glucose and nutrient starvation can induce autophagy, we investigated whether this molecular pathway may be the underlying mechanism linked to Noxa and Mcl-1. Compared
Figure 4. The Noxa-Mcl-1 axis mediates apoptosis provoked by overall nutrient limitation. a-b J16 cells (GFP-) were mixed initially at 1:1 ratio with either Mock or N8 J16 cells (GFP+) and cultured in complete medium or medium with reduced levels of amino acids/vitamins (12.5% of normal levels in complete medium) (right). (i) Survival (ii) cell density and (iii) glucose and lactate levels in cultures of Noxa$^{\text{high}}$ (Mock) and Noxa$^{\text{low}}$ (N8) cells. Viability was measured based on the percentage of GFP+ cells found within the total viable population (live and mitotracker positive gating). Glucose consumption (squares) and lactate production (circles) was determined in the supernatants of single cultures cells cultured in (a) complete medium or in (b) medium with reduced amino acids/vitamins (right panel). (c) J16 cells were cultured in medium with 5mM glucose (5mM glu.) or in medium with reduced levels of amino acids/vitamins (Low AA). At indicated time points, protein lysates were analyzed by western blot and probed for the indicated proteins. (d) Reduced sensitivity of N8 cells to inhibition of the pentose phosphate pathway. M or N8 cells were cultured in complete medium for 72hrs in the presence of 1 mM 6-aminonicotinamide (6-AN). Viability (mitotracker$^+$ cells) was assessed by FACS (n=4).
to cell lines that readily induce autophagy during starvation such as the U251 astrocytoma cell line, Jurkat cells appeared less prone to undergo autophagy. This was demonstrated by low levels of cleaved LC3, a well-known autophagy marker (24), when these cells were cultured under low glucose or in the absence of amino acids (Figure 5a). Inhibition of the mTOR complex by rapamycin did induce LC3 cleavage in Jurkat cells. High concentrations of rapamycin resulted in cell death, which was not affected by changes in Noxa levels (Figure 5bi). When cultured under limiting conditions of glucose or amino acids and vitamins, no LC3 cleavage was observed even though Noxa\textsuperscript{Low} displayed enhanced survival (Figure 5bii and data not shown). Thus, in Jurkat cells apoptosis induced by nutrient shortage occurs in the absence of autophagy.

**Figure 5. Metabolic stress does not invoke significant autophagic responses in Jurkat cells.** Control (M) or Noxa\textsuperscript{Low} (N8) J16 cells were cultured for 3 days in complete medium (Hi Glu/AA), medium with 5mM Glucose (Low Glu) or medium with low amino acids/vitamins (Low AA). (a) Protein lysates were analyzed for intact and cleaved LC3 (LC3-I and II, resp.). As a positive control for autophagy, J16 control (M) cells and U251 astrocytoma cells were treated with various concentrations of rapamycin (rapa) or cultured for 1.5hrs in HBSS. (b) Viability of M and N8 cells (i) cultured in complete medium in the presence of increasing concentrations or rapamycin and analyzed after 24hrs (n=8) or (ii) cultured under conditions of metabolic stress and analyzed after 3 days (n=3). Viability was determined based on mitotracker positivity.
Noxa/Mcl-1-dependent apoptosis pathway instigated by nutrient shortage is independent of ROS production.

Glucose deprivation induces oxidative stress and reactive oxygen species (ROS) and this may activate the MAP kinases p38 and/or JNK (25,26). Increased ROS production was indeed observed upon glucose deprivation, which correlated with the induction of apoptosis and this could be delayed by addition of the ROS-scavenger N-acetylcysteine (Figure 6a). A slightly increased phosphorylation of p38 at the onset of apoptosis was detected, and

Figure 6. Noxa/Mcl-1-dependent apoptosis pathway instigated by nutrient shortage occurs is independent of ROS production (a) MAP kinase phosphorylation. Mock and N8 cells were cultured under glucose-limiting conditions. At designated time points protein lysates were analyzed by western blotting for Total (T) and phosphorylated (P) Erk1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182) and JNK 1,2/3 (Thr183/Tyr185). As a positive control, J16 cells were stimulated in parallel with combined PMA (100ng/ml) and ionomycin (1 μM) for the indicated time points. b-c Control (M) or NoxaLox (N8) J16 cells were cultured under glucose limiting conditions in the presence or absence of the ROS scavenger N-acetylcysteine (NAC). (b) Cells were analyzed daily for viability (lower panel). Cells grown in the absence of NAC were assessed for ROS production by FACS (upper panel). (c) Mcl-1 levels were determined by western blot. (d) Jurkat cells were cultured o/n in the presence of the ROS H2O2 or the mitochondrial uncoupler CCCP and Mcl-1 levels were determined by western blot.
phosphorylation of JNK or the pro-survival MAP kinases ERK1/2 was unchanged. Compared to control stimulation with PMA/ionomycin which occurs in minutes, the level of p38 activation was modest after several days of glucose deprivation (Figure 6b). However, no difference in ROS production or MAP kinase activation between control and Noxa\textsubscript{Low} cells was observed under these conditions. In addition, Mcl-1 levels were not influenced by external addition of ROS (H2O2), or by intracellular production of ROS following stimulation with CCCP. Also, addition of NAC during glucose deprivation did not prevent Mcl-1 decline (Figure 6c, d).

Together, these data indicated that the role of Mcl-1/Noxa axis upon glucose deprivation does not depend on ROS production or p38 phosphorylation.

**Apoptosis provoked by glucose limitation involves the BH3-only member Bim.**

The pro-apoptotic effects of BH3-only proteins depend on the Bcl-2 like proteins they are able to antagonize. Noxa binds exclusively Mcl-1 and A1/Bfl-1 and is therefore an indirect inducer of apoptosis. Other BH3-only proteins, like Bim, (t)Bid and Puma bind all Bcl-2 like family members and therefore directly induce apoptosis once activated (3,27). Hence, apart from Noxa, the apoptosis induced by glucose shortage may involve other BH3-only proteins. As J16 cells express substantial levels of Bim, but not Puma (7), we targeted the expression of Bim protein with Bim-siRNA (28) (Figure 7a). In control experiments, apoptosis induced by known stimuli that require Bim (29-32), such as serum withdrawal, taxol and the proteasome inhibitor velcade, was inhibited in Bim-deficient cells, while apoptosis after CD95 triggering which depends on Bid was unaffected (Figure 7b). Subsequently, we performed competitive outgrowth assays under glucose limiting conditions. Cells deficient in Bim displayed a considerable survival advantage and the degree of protection correlated with the level of Bim knock-down (Figure 7a and ci, left). As expected, based on current knowledge on interactions among Bcl-2 family members (3), overexpression of Bcl-2 could also rescue apoptosis under circumstances of limiting glucose concentrations. (Figure 7ci, right). Together, these data indicate that apoptosis instigated by nutrient shortage involves Bim and is antagonized by Bcl-2.
Figure 7. Bim contributes to apoptosis provoked by glucose shortage. (a) Bim knock-down was obtained using Bim shRNA constructs. Clear knock-down was achieved after limiting dilution from the bulk Bimi population. Bim EL protein, the prominent Bim isoform in Jurkat was monitored in parental J16 and 2 Bimi clones by immunoblotting. (b) M and Bimi cells were treated with the indicated death stimuli. Viability was assessed by FACS analysis 24 hours after culture. Data represent mean ± SD from at least three experiments. (c) Untransduced J16 cells (GFP-) were mixed initially at 1:1 ratio with either M, Bimi (left panels) or Bcl-2 overexpressing cells (right panels) (GFP+). Cells were cultured under limiting glucose concentrations. At the designated time points, populations were analyzed by FACS, first in the live gate and mitotracker positivity and second based on GFP expression. (i) Cell

Discussion

The induction of Noxa sensitizes dividing human T cells specifically to apoptosis induced by glucose withdrawal, but not to other apoptotic stimuli such as serum deprivation, CD95 triggering or staurosporin (7). Here, we further clarify the role of Noxa in the context of metabolic and growth factor deprivation pathways, using Jurkat T cells and erythroid TF-1 cells as a model. We find that Noxa has a specific role upon general metabolic stress in an apoptotic response which does not involve upstream components of the PI3K/PKB/GSK-3 path, AMPK or autophagy. Downstream, the function of Noxa converges into? the common Mcl-1/Bim axis.
Activated T cells drastically shift their catabolism as well as their anabolic requirements. We therefore asked whether Noxa is involved solely in the apoptotic response to glucose deprivation, or also in other metabolic stress situations. Reduced levels of Noxa conferred a survival advantage when cells were supplied with pyruvate as energy source, when the citric acid cycle was disrupted or when the pentose phosphate pathways was blocked. In addition, Noxa levels affected the apoptotic response to general nutrient depletion (amino acids, vitamins). These data indicate that in addition to glucose shortage, Noxa is engaged in a broad metabolic stress response.

Mcl-1 is the predominant pro-survival binding partner of Noxa, and its expression is essential for lymphocyte survival (33). Previously, we described that the decrease in Mcl-1 protein upon nutrient deprivation renders dividing lymphocytes susceptible to apoptosis (7). Here we established in more detail that this decline in Mcl-1 was regulated post-transcriptional via proteasomal degradation, independent from caspase activation. Although Mcl-1 is generally considered as an intrinsically labile protein (34), our findings emphasize that its half-life is subject to regulation depending on the metabolic status of the cell. In parallel, the half-life of Noxa was also reduced during nutrient deprivation. Since it is reported that the engagement with Noxa in fact controls the half-life of Mcl-1 (35), this implies that signals conveying metabolic stress may indeed converge on Noxa. A recent indication that Noxa itself is also subject to regulation by ubiquitination (36) is in agreement with the observed changes in its half-life.

Increased proteasomal targeting of Mcl-1 and subsequent apoptosis is also observed upon growth factor deprivation (13,14). This process is mediated by GSK3, which is activated following decreased PKB phosphorylation. However, in different cell lines we observed no decrease in PKB or GSK3 phosphorylation upon glucose deprivation and Mcl-1 decline or apoptosis could not be prevented with GSK3 inhibitors. IL-3 deprivation on the other hand did result in GSK3 mediated Mcl-1 degradation. This suggests that growth factors and nutrient starvation use two different signaling pathways that converge at the level of Mcl-1 degradation to induce apoptosis.

In seeming contrast with a with a recent report (15), we observed decreased AMPK activity instead of the expected increase in glucose–deprived Jurkat cells, which correlated with a decrease in Mcl-1 protein amount. Various observations may account for this apparent discrepancy. First, AMPK is involved in a broad range of metabolic stress responses and intersects with signaling pathways such as PKB and mTOR (37,38).
Importantly, in primary T cells and leukemic Jurkat cells, TCR-mediated Ca\(^{2+}\)-signaling also activates AMPK, and this occurs independently of increased AMP/ATP ratios (39). Second, the often used activator of AMPK, 5-aminoimidazole-4-carboxamide riboside (AICAR), induces apoptosis in Jurkat cells which is reportedly independent of AMPK (40). Third, since we applied a gradual decline of glucose levels, rather than an abrupt loss of glucose or inhibition of glycolysis as often used (15,39), previously unappreciated compensatory mechanisms may take over. These mechanisms may be related to the same general metabolic stress response that mediates cell death after amino acid and vitamin deprivation.

Likely candidates to regulate this metabolic stress response are the MAPK signaling pathway, autophagy or reactive oxygen species. All of these are related to nutrient starvation-induced cell death and all have been shown to activate mitochondrial apoptosis (38,41-43). However, in this study we could exclude an overt role for these candidates signaling mediators in Noxa-dependent apoptosis. Future studies are required to dissect the molecular connection between sensors of cellular energy and metabolism and apoptosis involving the Noxa-Mcl-1 axis. In conclusion, it appears that both primary and leukemic T cells posses both TCR/Ca\(^{2+}\)- and ATP/AMP-dependent routes to AMPK activation, which may be reflect their intrinsic capacity for rapid cell division and adaptation to metabolic changes.

Lastly, in accordance with current knowledge of the interactions among Bcl-2 family members (44,45), we found that reduction of Bim in Jurkat cells showed a similar though more pronounced survival advantage, compared to the Noxa-deficient cells. Together, our data indicate that Noxa expression sensitizes cells to metabolic stress in a response that eventually targets Mcl-1 to the proteasome, and also requires the concert action of Bim.
Materials & Methods

Cell lines
The J16 clone derived from the human T acute lymphoblastic leukemia cell line Jurkat was used (46). The Noxa-deficient cells J16-N8 and J16-N8.15, and J16 cells transduced with Caspase-9 dominant-negative were described previously (7,47). The J16 cells over-expressing Bcl-2 (48) were kindly provided by Dr. Jannie Borst (NKI, Amsterdam). TF-1 cells were provided by Dr. P Coffer (UU, Utrecht) and maintained with 3µM of human rIL-3 (R&D research).

Detection of apoptotic cells
The viability of cells was analyzed by FACS following culture with various death stimuli. Cells were incubated with Mitotracker Orange (200 nM; Molecular Probes) for 30 min at 37°C. Populations were analyzed daily by FACS, first in the live gate and mitotracker positivity and secondly based on GFP expression.

Reagents
Phorbol myristate acetate (PMA 100ng/ml), ionomycin (1 µM) and N-acetylcysteine were purchased from Sigma (St Louis, MO). A panel of different chemical drugs was used to induce apoptosis: LY294002 and wortmannin (from Calbiochem., San Diego, CA), rapamycin (Wyeth Laboratories, Philadelphia, PA), velcade (50 ng/ml) (Janssen-Cilag (Tilburg, The Netherlands), taxol (5mM) (Sigma) and Fas10 mAb (5 µg/ml) (agonistic antibodies to the CD95 receptor) was a kind gift from Pr of. Dr. L. Aarden (Sanquin, Amsterdam, The Netherlands). Z-VAD (100 µM) was obtained from (Alexis). Cyclohexamide (5 µg/ml), MG-132 (5 µM) and Compound C (100 nM) were purchased from Calbiochem. CCCP was acquired from Sigma. The inhibitor SB216763 (5 µM) was purchased from Enzo Lifesciences. DMEM without glucose was purchased from Gibco (Paisley, Scotland, U.K.), and supplemented by addition of glucose (Merck) or pyruvate (Sigma). HBSS (BioWhittaker, Verviers, Belgium) was used as source of amino acid/vitamin depleted medium.
**Chapter II**

**ROS detection**

Cells were transferred to phenolred-free medium (Gibco) and incubated with DHE (14 µM; Molecular Probes) for 20min at 37°C. The MFI of cells within the viable gate, caused by DHE oxidation, was measured over 1 min per sample on a FACScalibur.

**SDS-Page and Western Blotting**

Western blotting was performed as described (7). Proteins were visualized by staining immuno-blots with anti-Noxa (Biocarta Carlsbad, CA, USA), anti-Mcl-1 (BD Pharmingen), anti-Bim (Stressgen, Canada), anti-β-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA); Anti-cleaved caspase-3 (Asp175), anti-phospho-PKB(Thr308), anti-phospho-PKB(Thr306), anti-phospho-Erk1/2(Thr202/Tyr204), anti-Erk1/2, anti-phospho-p38(Thr180/Tyr182), anti-AMPK, anti-phospho-AMPK (Thr172), anti-p38, anti-phospho-Jnk1, 2/3 (Thr183/Tyr185), and anti-JNK 1,2/3 were obtained from Cell Signaling Technology; anti-phospho-GSK-3β (Ser9) and anti-GSK-3β were purchased from Santa Cruz. Anti-LC3 (Sanbio BV, The Netherlands) was a kind gift from F.B. Mullauer.

**Reverse transcriptase multiplex ligation-dependent amplification (RT-MLPA) procedure and analysis**

The apoptotic gene expression profile measured by RT-MLPA was performed as described previously (19).

**Glucose and Lactate measurements**

Glucose and lactate levels in the supernatants were measured according to standard diagnostic Hexokinase and lactate oxidase methods, respectively.

**Retroviral constructs & retroviral Infection**

To knock-down Bim, we used the pSuper-Bim vector kindly provided by Dr. Andreas Villunger (Innsbruck, Austria), containing the human Bim targeting sequence (referred to as Bimi), described previously (28). Retroviral particles and transductions were done as described (7). The efficiency of transduction was estimated by determining the percentage of green fluorescence protein (GFP)-positive cells, 2 to 3 days later, by flow cytometry. GFP-positive cells were sorted on a FACS sorter (Moflow; Dako-Cytomation, Carpinteria, CA) to >90% purity for further experiments. To improve knock-down of Bim or Noxa in
J16 cells, a limiting dilution of transduced population was performed according to standard procedures. Resulting clones (N8.15, Bimi18, Bimi19) were selected based on GFP, and further characterized by Western blot.

Reference List


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