Regulation of neutrophil apoptosis by Mcl-1

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Abstract
Neutrophils rapidly undergo spontaneous apoptosis, but this process can be considerably delayed by exposure to a variety of agents such as pro-inflammatory cytokines. The anti-apoptotic protein of the Bcl-2 family, Mcl-1, plays a key role in the regulation of neutrophil apoptosis. The protein has some unusual properties compared with other family members, including an extremely high turnover rate. Many factors, such as cytokines and local oxygen concentrations, can regulate cellular levels of Mcl-1 via transcription and post-transcriptional modification, control the survival time of neutrophils within tissues and thereby influence the inflammatory response.

Introduction
Neutrophils have a very short half-life in the circulation (6–18 h) because they constitutively undergo apoptosis [1,2]. However, this short half-life can be considerably extended by exposure to pro-inflammatory agents such as cytokines [e.g. GM-CSF (granulocyte/macrophage (or monocyte) colony-stimulating factor), IL (interleukin)-1β, interferon-γ, IL-2, IL-4, IL-15 and G-CSF] or LPS (lipopolysaccharide), sodium butyrate and glucocorticoids [3–11]. Delay of neutrophil apoptosis can also occur during integrin engagement [12] or when the cells are recruited into tissues during infection or inflammation [13]. This extended lifespan of inflammatory neutrophils is beneficial in that it allows the cells to survive for sufficient time to perform their function effectively. This delay of apoptosis is only transient and inflammatory neutrophils will eventually undergo apoptosis, and then be cleared by tissue macrophages or endothelial cells, to permit the successful resolution of inflammation [14]. In some instances, however, dysfunctional neutrophil apoptosis may be implicated in the tissue damage associated with a number of inflammatory diseases, where non-apoptotic, activated cells may release their cytotoxic products into the local environment. Understanding the mechanisms that are responsible for delaying neutrophil apoptosis may thus provide new ways to treat inflammatory diseases in which neutrophils play a role in the underlying pathology.

Bcl (B-cell lymphocytic-leukaemia proto-oncogene)-2 family expression in human neutrophils
Neutrophils constitutively express a range of pro-apoptotic members of the Bcl-2 family, including Bax, Bad, Bak, Bid and Bik, an observation in accordance with their ability to undergo cell death readily and rapidly [1,15]. The cellular levels of these proteins change very little after treatment of neutrophils with agents that either delay or accelerate apoptosis, and the proteins have relatively long half-lives compared with the lifespan of neutrophils. Neutrophils do not express detectable levels of Bcl-2, and although Bcl-X mRNA can be detected in RNase protection assays, immunoblotting fails to detect significant levels of this latter protein using a variety of commercial antibodies. The very weak signals for Bcl-X, sometimes reported in immunoblots of neutrophil suspensions, arise from signals generated by contaminating mononuclear cells that express high levels of this protein. Similarly, human neutrophils express mRNA for the anti-apoptotic gene A1 (Bfl-1) and levels of this transcript are cytokine-regulated [15,16]. However, no convincing results on the expression of A1 protein are available because no antibody yet exists that can reliably detect the human protein.

The only anti-apoptotic Bcl-2 family member that has been reliably and reproducibly measured at both the mRNA and protein level in human neutrophils is Mcl-1.

Properties of Mcl-1
MCL-1 was originally cloned as an ‘early induction’ gene during differentiation of the myeloid cell line, ML-1 [17]. Sequence analysis revealed that the protein encoded by the cDNA was 37.3 kDa, much larger than Bcl-2 (21 kDa) or Bcl-XL (29 kDa). The mass of the protein detected by Western-blot analysis was 40–42 kDa. Sequence analysis identified three putative BH (bcl-2 homology) domains (BH1–3). Almost all other anti-apoptotic proteins possess a BH4 domain, the only exceptions being Mcl-1 and A1. Since this domain is supposed to be required for molecular interactions with other proteins (such as Bax, calcineurin and Raf-1) [18–20], the absence of BH4 from Mcl-1 and A1 suggests that these anti-apoptotic proteins interact with a different set of proteins as Bcl-2 and Bcl-XL. The BH4 domain may also be involved in the control of the voltage-dependent anion channel of mitochondria [21]. PEST sequences and
Arg:Arg domains are also present in Mcl-1 and these motifs are often present in proteins that are subject to rapid turnover [18]. Indeed, the half-life of Mcl-1 in cells has been estimated to be between 1 and 5 h, depending on the environment in which the cells are exposed [15,22]. Mcl-1 has the fastest turnover rate so far identified in the anti-apoptotic Bcl-2 family members and hence the shortest half-life. Sequence analysis also identified a putative membrane anchor domain at the C-terminus, and this has been confirmed experimentally. Deletion of this domain results in the loss of mitochondrial localization of a green fluorescent protein (GFP–Mcl-1) fusion protein in U-937 cells, resulting in diffuse, cytoplasmic distribution [23].

Role of Mcl-1 in the regulation of apoptosis
Mcl-1 is expressed in a variety of cells and tissues. Its overexpression in transfection studies results in enhanced cell survival, confirming its role as an anti-apoptotic protein [24], although protection is only short term, unlike the long-term protection afforded by overexpression of Bcl-2 or Bcl-XL.

There is emerging evidence to show that enhanced Mcl-1 expression can confer a malignant phenotype on cells [25]. For example, overexpression of Mcl-1 is considered to be responsible for the impaired apoptosis and resistance to chemotherapy of malignant myeloma cells [26] and in a human oesophageal cell line [27], although the mechanisms responsible for this enhanced expression remain undefined. Overexpression of Mcl-1 has also been proposed to be the underlying mechanism responsible for bile acid-induced carcinogenesis, e.g. in cholangiocarcinomas, which occurs via EGFR/Raf-1 signalling [28]. Even more convincing evidence for the role of overexpression of this protein in inducing a malignant phenotype comes from observations with transgenic mice overexpressing Mcl-1. Aged animals exhibit an extremely high rate of B-cell lymphoma [29] and haematopoietic cells from these animals become immortalized in culture in the presence of IL-3 [30].

In many cell types, it has been demonstrated that expression of Mcl-1 is transient, often occurring at certain stages of development or differentiation. Indeed, in mice, an MCL-1 gene knockout is lethal, with cells not developing beyond the morula/blasta stage of embryogenesis, confirming its key role in development [31]. Its transient expression is required during differentiation of cells, particularly in haematopoietic cells. It is often expressed independently of Bcl-2, and may contribute to transient protection against apoptosis at certain times in differentiation/development. For example, its expression may be triggered before Bcl-2 during differentiation of B cells [32]. Its expression is low in native B cells, high in germinal centre cells and low again in memory cells. This pattern of expression is the opposite of Bcl-2 [33]. It is transiently expressed during differentiation of U-937 cells [34] and specific antisense disruption of Mcl-1 expression in differentiating U-937 cells is sufficient to induce apoptosis in the absence of changes in the expression of any other pro- or anti-apoptotic Bcl-2 proteins expressed in these cells.

Regulation of Mcl-1 expression

Transcriptional control
MCL-1 expression is rapidly stimulated by increases in transcription. The promoter/regulatory regions of the human gene have been isolated and partially characterized by mutagenesis and luciferase reporter gene assays [35]. Transcripts for MCL-1 in neutrophils have been detected by RNase protection assays, and are increased by agents (e.g. cytokines) that delay neutrophil apoptosis [15]. In these systems there is no evidence for the regulation of transcription of MCL-1 by the transcription factor NF-κB, a curious observation in view of the importance of Mcl-1 in cytokine-regulated survival of human neutrophils and the proposed importance of NF-κB in neutrophil survival [36]. Many signalling pathways have been implicated in the regulation of Mcl-1 expression in different cells under different physiological circumstances including MEK/ERK, p38 MAPK, PI3K/Akt and JAK/STAT3 [24]. MCL-1 transcription may also be triggered by hypoxia [37].

Post-transcriptional control of Mcl-1 expression
Mcl-1 expression is also regulated by post-transcriptional events. For example, full-length mRNA for MCL-1 contains three exons but alternative splicing to generate a transcript containing only exons 1 and 3 yields a ‘BH3-only’ protein that has pro-apoptotic properties [38,39].

Post-translational modification of Mcl-1 can also modify its function. Sequence analysis of Mcl-1 reveals several potential phosphorylation sites and it has been shown that hyperphosphorylation can be detected as a band shift on SDS/PAGE [40]. Indeed, it has been shown that in HEK-292 cells (human embryonic kidney 292 cells), Mcl-1 can be phosphorylated at Ser121 and Thr163 via a JNK-dependent pathway in response to oxidative stress [41]. This phosphorylation resulted in the inactivation of Mcl-1, thereby negating its anti-apoptotic effects. It has also been shown in Burkitt lymphoma cells, BL41-3, that Mcl-1 may be phosphorylated at two levels: phorbol ester can induce a phosphorylation via Erk that does not result in a band shift, whereas agents such as taxol and okadaic acid induce Mcl-1 hyperphosphorylation that does result in a mobility shift on SDS/PAGE [40].

The function of a large number of Bcl-2 family members is controlled by their phosphorylation status. For example, Bcl-2 is phosphorylated at Ser70 to enhance its anti-apoptotic activity, but its hyperphosphorylation by drugs such as taxol at Thr69 and Ser67 within the flexible loop region may inhibit its function and enhance proteolysis via the proteasome [42,43]. Bcl-X1 function is also regulated via its phosphorylation status [44], whereas Akt/PI3K-dependent phosphorylation of Bad on Ser112, Ser136 and Ser155 residues may promote association with 14-3-3 protein within the cytoplasm, thereby releasing it from Bcl-X1, and allowing Bcl-X1 to hetero-dimerize with pro-anti-apoptotic proteins to promote survival [45,46]. Thus phosphorylation events can exert profound effects on the cell death machinery of cells by activating, inactivating or enhancing the turnover of anti- or pro-apoptotic proteins.
Regulation of Mcl-1 expression in neutrophils

Cellular levels of Mcl-1 in human neutrophils closely correlate with their survival kinetics. Non-apoptotic cells have high levels of this survival protein, whereas levels are low in apoptotic cells. In view of the absence of expression of Bcl-2 and Bcl-X in neutrophils, we have proposed that Mcl-1 levels play a crucial role in determining cell fate: if levels of the protein are high (above a threshold level), the neutrophil will survive, whereas if they fall below this protective threshold, then the activity of the pro-apoptotic proteins predominates and the cells become apoptotic. Confirmation of this hypothesis comes from antisense experiments to specifically disrupt Mcl-1 expression, which have shown that Mcl-1 depletion accelerates constitutive or GM-CSF regulated apoptosis.

The biological properties of Mcl-1 thus make it an ideal molecule to allow for acute and dynamic regulation of the kinetics of neutrophil apoptosis. Blood neutrophils express moderate levels of this protein, but in the absence of continued de novo expression, cellular levels will become rapidly depleted due to the short half-life and rapid turnover of the protein. Extension of neutrophil lifespan (e.g. during inflammatory activation) can trigger enhanced Mcl-1 expression (via stimulated gene expression) to allow the cell to survive for extended periods. Again, the naturally short half-life of Mcl-1 affords a mechanism to permit apoptosis for the resolution of inflammation when transcriptional signalling stops and the protein is naturally degraded. Such acute and fine control of neutrophil apoptosis would be much more difficult and require other regulatory mechanisms if neutrophil survival was regulated by a more stable survival protein such as Bcl-2.

Role of Mcl-1 in neutrophil survival during inflammation

As discussed above, a variety of physical (integrin engagement) and soluble factors (e.g. cytokines) can trigger delayed neutrophil apoptosis during extravasation and inflammatory activation. However, another important factor within the tissue local environment that affects neutrophil fate is the local oxygen tension. Hypoxia (decreased oxygen tensions) has been shown to delay neutrophil apoptosis [37,47], and antioxidants such as catalase (but not superoxide dismutase) added to neutrophil cultures in vitro, can similarly delay apoptosis. There are also reports that neutrophils from CGD neutrophils (that have defective ability to generate reactive oxidants via an impaired NADPH oxidase) have delayed apoptosis in vitro [48]. Within tissues, local oxygen tensions may play an important role in neutrophil survival: low tensions may promote survival, but reactive oxidants generated via the respiratory burst following the reduction of $O_2$ to $O_2^-$, may be pro-apoptotic. Thus neutrophil fate will be finely balanced by the local conditions of oxygenation. These considerations are extremely important because infected or inflamed tissues are invariably relatively low in oxygen tensions, compared with oxygen tensions found in the circulation. The levels of reactive oxidants that neutrophils generate at these inflamed sites will also be important in determining their fate and hence survival time within the tissue.

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References


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