Review

The Oscar-worthy role of Myc in apoptosis

Natalie Meyer, Sam S. Kim, Linda Z. Penn*

Division of Cancer Genomics and Proteomics, Ontario Cancer Institute/Princess Margaret Hospital,
Department of Medical Biophysics, University of Toronto, 610 University Avenue, Rm 9-628,
Toronto, Ont. M5G 2M9, Canada

Abstract

The discovery that the Myc oncoprotein could drive cells to undergo apoptosis in addition to its well-established role in cellular proliferation came in the early 1990s, at the beginning of a period of explosive research on cell death. Experimental evidence revealed that Myc sensitises cells to a wide range of death stimuli and abrogating this biological activity plays a profound role in tumorigenesis. Our understanding of the molecular mechanism and genetic programme of Myc-induced apoptosis remains shrouded in mystery and the focus of much attention. In this review, we will discuss established data, recent advances and future objectives regarding the regulatory processes and the functional cooperators that effect and abrogate apoptosis induced by Myc.

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1. Proliferation versus apoptosis: a good story requires a conflict

Researchers studying the Myc oncogene are celebrating its silver anniversary in 2006. For 25 years, this enigmatic molecule has been the subject of much scrutiny. Ten years of research had shown that Myc certainly was a driving force in the cell cycle and malignant transformation. However, in the early 1990s, some surprising findings arose. In addition to its established roles in growth and proliferation, the Myc oncogene was also a potent inducer of programmed cell death [1,2]. Research into the molecular basis of apoptosis was itself in its infancy, and scientists struggled to explain the new paradox. How could this protein drive such disparate functions? Why would it? How does the cancer programme overcome this obvious hurdle? It was even more puzzling when it was shown that Myc-induced apoptosis required the same regions of the protein as the transformation, proliferation and autosuppression functions [2]. Moreover, the same interaction with its dimerisation partner Max was required [3], ruling out the possibility of an entirely separate mechanism of action.

Two seminal reports in different cell systems first revealed Myc had pro-apoptotic capabilities. When cytokines were withdrawn from 32D myeloid cells with exogenous Myc expression, they underwent rapid cell death, while the normal response was a rapid down-regulation of Myc, G1 arrest and delayed death [1]. It was also discovered that although Rat1 fibroblasts expressing
constitutive Myc were unable to exit the cell cycle upon growth factor withdrawal, the number of cells in culture did not increase significantly over control. The cell number failed to increase over time due to a concomitant increase in cell death, and this death was classified as apoptosis [2].

It was not long before similar reports began surfacing for the viral oncogene E1A [4,5], and the pRB target E2F1 [6–8]. The original observations with Myc were extended and further supported, and a new hypothesis was born: that the proliferation of cells is essentially tied to apoptosis as a built-in safety mechanism to defend against inappropriate proliferation [2].

A “dual-signal” model began to emerge, where it was proposed that sensitisation to apoptosis was a normal function of Myc expression, and this suicide signal was rescued by specific survival factors such as insulin growth factor or platelet-derived growth factor [9]. Apoptosis results if deregulated Myc expression is present in conjunction with an anti-proliferative signal. Ectopic expression of Myc in non-transformed cells sensitises them to apoptosis. Myc was capable of driving cells to die at any stage of the cell cycle, including post-mitotic commitment steps, and in the absence of de novo protein synthesis, indicating that the machinery for sensitisation was already present in a Myc-expressing cell [9]. Furthermore, in the two cell systems where c-myc has been inactivated, the cells are remarkably resistant to death induced by various stimuli [10,11].

The Myc protein exerts its functions as a transcription factor by both activating and repressing target genes. It is a member of a large family of basic-helix–loop–helix-leucine zipper (bHLH-LZ) transcription factors, which dimerise through the HLH-LZ domains and contact DNA in the basic region [12]. The protein contains four additional domains shared by all Myc proteins, known as Myc homology boxes I, II, III and IV. MBII is thought to play a role in protein stability, while MBII encodes both transcriptional activation and repression capabilities essential for transformation and apoptosis [12]. MBIII and MBIV have only been recently characterised [13,14]. The gene regulatory functions require Myc to dimerise with its partner protein Max, a smaller, ubiquitous bHLH-LZ protein that also mutes with several other bHLH-LZ proteins, including the Mxd family (formerly known as Mad/Mxi). It is thought that Myc activates genes at least in part by direct DNA binding and recruitment of transcriptional co-activators, while it represses transcription by binding activators such as Miz-1, interfering with the assembly of active transcription complexes [12,15]. Members of the Mxd family of transcription factors are mainly thought to antagonise Myc function. The details of this transcriptional regulation by Myc are discussed elsewhere in this issue (doi:10.1016/j.semcancer.2006.08.001).

Deregulation of Myc is known to be a common event in cellular transformation, but it is difficult to discern what fraction of human cancers harbour deregulated Myc [12]. This is due to differences in defining the term, and the reality that Myc can be deregulated by several means, not all of them easily detectable. For our purposes, “deregulated Myc” specifies any situation that causes the molecule to be inappropriately active. This includes, but is not limited to, chromosomal translocations and amplifications, activation of upstream growth stimulatory signalling cascades, and increased protein stability.

The Eμ-Myc mouse is a model for the human disease Burkitt lymphoma, where a reciprocal chromosomal translocation to the immunoglobulin locus leads to inappropriate expression of Myc in the B-cell compartment. In the Eμ-Myc mouse, the c-myc transgene is coupled to the immunoglobulin heavy chain μ enhancer, leading to B-cell specific over-expression [16,17]. The lymphomas that develop are consistently clonal, indicating that additional mutations are absolutely necessary to produce tumours. Researchers were given a clue as to why this might be when it was discovered that Myc could drive apoptosis in addi-
tion to proliferation. The BCL2 protein had been characterised by the early 1990s as a potent inhibitor of cell death, although the mechanism was still very much a mystery. A completely novel method of oncogene cooperation was proposed when it was shown that mice doubly transgenic for Eμ-Myc and Eμ-BCL2 mutations displayed a marked decrease in latency of disease, and the disease was a leukaemia of early progenitor cells [18], rather than the lymphoma that develops with Eμ-Myc alone [17]. This was consistent with the identification of BCL2 as an oncoprotein that cooperates with Myc by providing a survival signal in retrovirally transduced cytokine-dependent B cells [19]. The cooperation was further investigated in cell culture, where it was shown that BCL2 could increase cell viability and block apoptosis driven by ectopic Myc in serum-starved Chinese hamster ovary cells [20], Rat1/MycER fibroblasts [21] and Rat1a/Myc fibroblasts [22]. The latter two reports also demonstrated that the inhibition of apoptosis left the proliferation functions of Myc intact. This new method of oncogenic cooperation was markedly different from classical cooperation such as that between Myc and Ras, as the cells typically did not form foci in culture [21]. Enforced BCL2 expression was also capable of blocking Myc-induced cell death following treatment with thymidine or the chemotherapeutic agent etoposide [21], demonstrating a probable mechanism of resistance to cancer treatments. More recently, Letai et al. demonstrated that sustained BCL2 expression is required for maintenance of the Eμ-Myc/BCL2 leukemia by using Eμ-Myc mice harbouring a BCL2 transgene that shuts off expression when doxycycline is administered. When the tetracycline analogue was added to the drinking water, BCL2 expression was down-regulated, massive apoptosis resulted and the tumours regressed [23].

Thus, the initial flurry of research activity clearly showed that the ability of Myc to sensitise cells to apoptosis was an intrinsic property, dependent on Myc:Max interaction, and was somehow controlled by Myc as a regulator of gene transcription. Abrogation of this pro-apoptotic property profoundly contributes to cancer progression. To further define the role of Myc in cell death, a basic understanding of the molecular mechanism of apoptosis is required and follows here (Fig. 1).

2. Background to the apoptosis story

Programmed cell death is an evolutionarily conserved method of multicellular organisms use to dispose of cells in an orderly fashion. It is characterised by several morphological features, namely nuclear condensation and fragmentation, membrane blebbing and non-inflammatory phagocytosis of the cell fragments [24]. This is in contrast to cell necrosis, where the cell bursts and an inflammatory response follows [25]. Apoptosis studies have obvious applications in cancer research, but they are also important in many other human pathologies, including sepsis, ischemia, neurodegenerative and autoimmune diseases [26]. The importance of apoptosis research was properly recognised in 2002, when Drs. Brenner, Horvitz, and Sulston were awarded the Nobel Prize in Physiology or Medicine for their pioneering work on the genes involved in organ development and programmed cell death. There are two main recognised pathways of apoptosis, the intrinsic and extrinsic pathways, although there is evidence for extensive cross-talk between the two. We will introduce here the features of each pathway that are specifically important to Myc-induced apoptosis; readers are referred to more complete apoptosis-specific reviews for more information [27–30].

The intrinsic pathway of apoptosis can be triggered by various intracellular cues, such as DNA damage, oncogene activation, hypoxia or limited growth factors. The tumour suppressor p53 is a key manager of intracellular death signals, and just how it accomplishes this task is discussed later. Intrinsic pathway signals converge on the mitochondria, where the balance between...
pro- and anti-apoptotic molecules determines whether the cell will survive or proceed inexorably to death. This concept of the “apoptotic threshold” is a recurrent theme, since evidence indicates that healthy cells contain all the necessary molecules for programmed cell death kept in check by survival factors. The BCL2 family of proteins ultimately control the decision on mitochondrial cell death. This family can be broadly classified into two groups. Anti-apoptotic family members such as BCL2 and BCL-XL contain all four BCL2 homology domains (BH1–4). Pro-apoptotic members can be either multi-domain (BH1–3) pore-forming proteins, such as BAX and BAK, or smaller BH3-only proteins, such as BID, PUMA, NOXA and BIM. It is the balance between these pro- and anti-apoptotic proteins that determines if cell death will proceed [29]. “Activator” BH3-only domains such as BID and BIM are thought to be normally sequestered by anti-apoptotic proteins. The “sensitiser” domains, such as those from BAD and BIK, can displace the activators, freeing them to bind and activate the multi-domain pro-apoptotics BAK and BAX [31]. These molecules are thought to homo-oligomerise, forming pores leading to mitochondrial outer membrane permeabilisation (MOMP), loss of membrane potential and respiration, and the escape of various pro-apoptotic factors [32]. Much of the mechanistic evidence discussed in this review shows Myc having profound effects on mitochondrial integrity through the BCL2 family of proteins.

One of the important molecules released from the mitochondria is cytochrome c, which can bind to Apaf-1 and activate caspase-9, which in turn cleaves downstream caspases-3 and -7, for review see [33]. Caspases are a family of proteases that cleave substrates at consensus sites containing asparagine. They must be cleaved from the pro-form to become activated; upstream “activator” caspases cleave downstream “executioners” in a cascade. Caspase activation is antagonised by the inhibitor of apoptosis proteins, IAPs. The executioners cleave a wide variety of cellular substrates, including poly(ADP-ribose)polymerases (PARP), actin and the pRB cell cycle regulator. The fragmentation of the cell that takes place after caspase activation leads to the morphological hallmarks originally used to classify apoptosis [33].

Executioner caspases can also be activated without loss of mitochondrial potential through the death receptor pathway, also known as the extrinsic pathway of apoptosis. Death receptors implicated in Myc-induced apoptosis include CD95/Fas, tumour necrosis factor receptor (TNFR) and death receptor DR5, which responds to TNF-related apoptosis-inducing ligand (TRAIL). Upon death ligand stimulation, receptors oligomerise, bringing intracellular death domains (DD) together so they can interact with adaptor molecules such as FADD and TRADD [28]. Adaptor proteins contain both DD and death effector domains (DED), which in turn interact with the DED domain on procaspase-8, triggering its cleavage and activation. The complex of death receptor, adaptor protein and procaspase-8 is known as the death-inducing signalling complex. Activated caspase-8 can directly activate executioner caspases-3, and -7, triggering cell death without input from the mitochondria. However, the pro-apoptotic BH3-only molecule BID is also a substrate of activated caspase-8. Its cleavage leads to myristoylation and targeting to the mitochondria where it can activate BAX and BAK, amplifying the death receptor signal [34]. This cross-talk between extrinsic and intrinsic pathways provides enough death signal to easily overcome the apoptotic threshold, committing the cell to death. The molecule cFLIP antagonises caspase-8 activation. It has a similar structure to caspase-8, but it lacks the catalytic protease domain and can therefore not cleave executioner caspases or BID [33].

3. Myc structure and signalling add suspense

A number of studies have indicated that Myc repression of target genes is correlated with its apoptosis functions, while transactivation activities tend to segregate with proliferation and growth [35,36]. This is not absolute, however, as mutants shown to be capable of driving transactivation and defective in Miz-1-dependent repression still cause apoptosis [37]. Repression of Miz-1 activity by Myc is important in Myc-induced apoptosis as Miz-1 activates the cell cycle inhibitor p21, leading to growth arrest instead of apoptosis [38]. In addition, MBIV deletion mutants were reported to have reduced function in both transactivation and repression activities, while being largely resistant to serum withdrawal-induced apoptosis. This mutant was capable of transforming Rat1a cells in a soft agar assay, but was defective in Rat1a focus formation [14]. It has also been next to impossible to unlink the structure–function data on Myc-induced death from protein stability studies. Much attention has been paid to mutations found in human Burkitt lymphoma samples and the super-transforming isoforms of the avian v-Myc protein [39]. Many of these mutations centre on the apparent phosphorylation site T58 in MBI, which when mutated to alanine, both increases protein stability [39–41] and leads to reduced apoptosis [36]. It is difficult to discern then, if one or both of these results leads to the increased transforming ability of the mutants. In fact, Tansey and co-workers have proposed a model whereby transcriptional activity is dependent on degradation signals, and suggest a mechanism whereby Myc is “licensed” to regulate transcription for only a limited time [42,43]. Mutations at the T58 phosphorylation site, thought to be a signal for degradation, were shown to have slightly reduced apoptosis and repression at one target reporter gene, and increased colony forming potential [36]. The recent characterisation of the third Myc-homology domain, MBIII, seems to support the repression theory. An MBIII deletion mutant reduced somewhat, but did not abolish, transactivation of four reporter genes; however it was completely incapable of down-regulating any of the repression activities, while being largely resistant to serum withdrawal-induced apoptosis. This mutant was capable of transforming Rat1a cells in a soft agar assay, but was defective in Rat1a focus formation [14]. It has also been next to impossible to unlink the structure–function data on Myc-induced death from protein stability studies. Much attention has been paid to mutations found in human Burkitt lymphoma samples and the super-transforming isoforms of the avian v-Myc protein [39]. Many of these mutations centre on the apparent phosphorylation site T58 in MBI, which when mutated to alanine, both increases protein stability [39–41] and leads to reduced apoptosis [36]. It is difficult to discern then, if one or both of these results leads to the increased transforming ability of the mutants. In fact, Tansey and co-workers have proposed a model whereby transcriptional activity is dependent on degradation signals, and suggest a mechanism whereby Myc is “licensed” to regulate transcription for only a limited time [42,43]. Mutations at the T58 phosphorylation site, thought to be a signal for degradation, were shown to have slightly reduced apoptosis and repression at one target reporter gene, and increased colony forming potential [36]. The recent characterisation of the third Myc-homology domain, MBIII, seems to support the repression theory. An MBIII deletion mutant reduced somewhat, but did not abolish, transactivation of four reporter genes; however it was completely incapable of down-regulating any of the repression reporters tested and led to a greater number of colonies in soft agar [13]. The MycS isoform results from an alternative translation start site located at amino acid 100, and thus entirely lacks MBl. When human c-Myc isoforms were expressed in dmyc-null Drosophila, MycS-expressing flies displayed normal proliferation, but reduced caspase-3 activation and reaper expression, indicating that regions missing from this protein are important for apoptosis induction [44]. More details of the structure and function of Myc are reviewed in [12].

Ras signalling has been found to play a dual role in Myc-induced apoptosis. On the one hand, when the signal from
Ras flows primarily through the PI3K and PKB/AKT pathway, Myc-potentiated apoptosis is abrogated. However, when Ras stimulates the Raf, MAPK and ERK pathway, the net effect is to increase the level of apoptosis [45,46]. A number of parallel observations have been made for the stress-activated protein kinase p38/c-fos pathway [47–49]. More light was shed on this subject when it was shown that phosphorylation of the protein-stabilising site S62 was likely controlled by ERK, leading to Myc stabilisation and apoptosis; while PKB/AKT downstream target GSK3 was likely phosphorylating Myc at T58, leading to protein degradation [50,51]. The small GTPase RhoB has also recently been shown to shorten Myc half-life by increasing nuclear accumulation of GSK3 [52]. Another group found that cells transformed with Myc and Ras required MEK activity to abrogate apoptosis when growth factors were present, consistent with the observation that the MEK pathway can confer survival in certain cell types [53].

Curiously, one report showed that the most common mutation in Burkitt lymphoma, T58I, showed comparable apoptosis levels to wild-type in reconstituted Myc-null rat cells, and a reduced transformation ability in a Ras/Myc rat embryo fibroblast co-transformation assay [54]. This indicates that abrogation of apoptosis through mutation of T58 may not be a major contributor to cancer progression. Further support is found in human neoplastic disease, which commonly shows deregulation of Myc by various methods, and rarely reveals changes to the protein sequence [55]. More often seen are genetic changes in cooperating pathways, some of which are the focus of this review.

4. Myc and p53: co-starring roles

If Myc can cooperate with the over-expression of a strong anti-apoptotic molecule, it follows that cooperation would also be seen with loss of a pro-apoptotic molecule. The p53 tumour suppressor is widely known as the “guardian of the genome” [56]. p53 acts to sense and integrate intrinsic cellular damage signals and leads the cell down a path towards either growth arrest, where the damage can be repaired, or into apoptosis [57,58]. The small GTPase RhoB has also recently been shown to shorten Myc half-life by increasing nuclear accumulation of GSK3 [52]. Another group found that cells transformed with Myc and Ras required MEK activity to abrogate apoptosis when growth factors were present, consistent with the observation that the MEK pathway can confer survival in certain cell types [53].

A number of recent reports have demonstrated that p53 can also promote apoptosis in a transcription-independent manner, with the p53 protein itself translocating from the nucleus to directly function at the mitochondria. It has been reported that p53 can bind to either BCL2/BCL-XL, BAK, or promote BAX activity [65]. Recent evidence suggests that transcription-dependent and -independent mechanisms of p53-induced apoptosis are linked, as p53 at the mitochondria can cooperate with its target gene product PUMA to induce apoptosis [66].

Although p53 protein is present in cells at all times to act as a sentinel against damage, protein levels must be kept low in unstressed cells since it can so potently induce cell death. Mdm2 is the best characterised negative regulator of p53. Mdm2 binds to and mono-ubiquitinates p53, leading to its translocation from the nucleus to the cytoplasm where it can be then further polyubiquitinated and degraded by the proteosome [67]. The embryonic lethality of Mdm2−/− mice can be rescued when crossbred to p53−/− mice, demonstrating that the Mdm2−/− mice die in utero due to high levels of p53 [68]. Mdm2 and its human homolog Hdm2 are commonly found to be amplified or over-expressed in tumours, suggesting that evasion of p53-mediated apoptosis can be achieved through increased Mdm2 expression [69]. Mdm2+/−/Eµ-Myc mice live twice as long as their Mdm2−/−/Eµ-Myc littermates [70]. This phenotype is rescued in the triple mutant Mdm2−/−/ARF+/−/Eµ-Myc mouse [71].

Expression of ARF inhibits Mdm2 function. ARF is a tumour suppressor, whose transcript is formed from the alternative reading frame of the Ink4a locus [72]. The locus encodes two important tumour suppressors, ARF and p16INK4a, and is deleted in a variety of human cancers. p16INK4a functions as a tumour suppressor by inhibiting cell cycle progression through binding to cyclin-dependent kinases-4 and -6 [73]. The second gene product identified from the Ink4a locus was ARF. This protein was suspected to be in a common genetic pathway as p53 when ARF loss abrogated the requirement for p53 inactivation to immortalise mouse embryo fibroblasts [74]. Furthermore, ARF was able to inhibit transformation of MEFs by Mdm2 and Ras, but had no effect on transformation in cells lacking p53 [75]. Mechanistically, ARF inhibits Mdm2 in two ways. ARF can bind to Mdm2 directly and inhibit its p53 ubiquitination function [76], and it can sequester Mdm2 in the nucleolus and prevent it from binding to p53 [77,78].

ARF is sensitive to the activation of many oncopgenes, including Myc, E2F1, E1A, v-abl, and Ras [73]. Myc activation leads to increased ARF expression [79], although the exact mechanism is unknown. So far we are not aware of any evidence that Myc can transcriptionally regulate ARF by binding to Ink4a locus. The missing link might be E2F1, since Myc induces E2F1 expression in primary cells [80,81], and E2F1 can induce ARF directly [82], although this was not evident in a mouse model [83]. It remains though, that Myc can induce p53-dependent apoptosis through activation of ARF, which inactivates Mdm2, in turn leading to activation of p53.

p53 mutations have been found in at least 30% of Burkitt lymphoma biopsies [84,85] and 60–70% of Burkitt lymphoma cell lines [86–89]. The great majority of Burkitt lymphoma
cell lines and Ep-Myc mice have either Ink4a locus deletions, Mdm2 over-expression, or p53 mutations [90,91]. Both suggest that inhibition of the ARF-Mdm2-p53 pathway is necessary for tumours to evade Myc-induced apoptosis and maintain tumorigenicity.

Although there is strong evidence supporting the Myc-ARF-Mdm2-p53 axis, emerging evidence proposes more complex regulation of this pathway. It has long been known that Mdm2 and p53 have a dynamic feedback loop where Mdm2 negatively regulates p53, while p53 transcriptionally up-regulates Mdm2. Recently though, two independent groups have reported a novel feedback mechanism involving Myc and ARF. Although the details differ, both groups demonstrated that when ARF and Myc are over-expressed, they interact, translocate to the nucleolus, and Myc activity is reduced [92,93]. These findings advocate that ARF can function as tumour suppressor and inhibit cell proliferation through two distinct mechanisms, inactivation of Mdm2 and interference with Myc transcriptional activity.

p53 activation leads to either growth arrest or apoptosis, and evidence shows the decision between these two possible outcomes might be critically regulated by Myc. p53 mediates cell cycle arrest partly through direct transcription of the cell cycle inhibitor p21 [94], which is also a transcriptional target of Miz-1 [95]. Myc mediates repression of p21 [96] through Miz-1 [37,95], and the outcome of daunorubicin-mediated p53 activation in HCT116 colorectal cancer cells is dependent on expression of Myc [38]. Surprisingly, ectopic expression of Myc did not alter the expression of p53 pro-apoptotic target genes PUMA, PIG3, and BAX. Myc not only activates p53, but it also causes p53 to induce apoptosis instead of growth arrest by repressing p21, while leaving pro-apoptotic targets unaffected [38]. Another related feedback mechanism that has been characterised is between Myc and p53, with the recent report that activated p53 can repress c-myc in mouse tissues and human tumour cell lines [97]. The authors demonstrated p53 binding to the c-myc promoter in vivo through chromatin immunoprecipitation. Ectopic expression of Myc reduced p53-mediated G1 arrest and differentiation and increased the ability of p53 to promote apoptosis. These data suggest that for the p53 growth arrest program it is essential to not only transactivate p21, but also repress c-myc. Together, the Myc and p53 feedback loop is fascinating. These findings suggest it is the state and level of both Myc and p53 that are important in determining whether cells will undergo apoptosis or cell growth arrest.

5. Myc cooperation, the climax of apoptosis

Despite almost heroic efforts, we are still uncertain about the specific pathways by which Myc sensitises cells to apoptosis. The target genes activated and suppressed by Myc are offering clues, but in many cases the wide range of targets mainly clouds the issue [98–101]. Powerful new technologies, discussed in the next section, will hopefully shed light on the genetic programme key to the apoptotic pathway. In the meantime, strong functional evidence has emerged from the discovery of proteins, and more recently non-coding RNAs, that cooperate with Myc in cellular transformation. This is helping to unravel the molecular mechanism by which Myc can drive apoptosis.

Because Myc can sensitise cells to such a broad range of apoptotic stimuli, it is very likely that it affects a common node in the apoptosis pathway. Much of the evidence, whether in p53-dependent or -independent systems, points to the signal amplification step at the mitochondria, where Myc seems to play a role in altering the balance between pro- and anti-apoptotic BCL2 family members, moving the cell closer to the so-called apoptotic threshold. A summary of the molecules discussed in this section is shown in Fig. 2.

An early report from Juin et al. indicated that a robust release of cytochrome c from the mitochondria lies downstream of Myc activation [102], and many articles have followed to try to determine how Myc exerts this effect. Cytochrome c has been proposed to be a direct transcriptional target of Myc [103]. A number of studies performed in the Cleveland lab have demonstrated Myc-dependent, yet likely indirect, suppression of BCL2 and BCL-XL [104–106], and indicate that suppression of these anti-apoptotic family members must be averted for lymphoma development in the Ep-Myc mouse [104]. Reports reveal that suppression of BCL-XL, rather than BCL2, is especially important in the p53-independent apoptosis pathways driven by Myc [104,106], but that this preference might be cell-type specific [10,105].

The pro-apoptotic molecule BAX is thought to follow a multi-step path to become fully activated, form a pore, and mediate the release of cytochrome c and other apoptotic factors from the mitochondria [107]. The presence of BAX increased annexin-V staining in serum-starved MEFs expressing ectopic Myc [108]. BAX loss was clearly shown to cooperate with Myc in driving lymphomagenesis in the Ep-Myc model, and interestingly, 29% of tumours analysed did not have detectable mutations in the ARF-Mdm2-p53 pathway [109], indicating that this pathway might operate independently or downstream of p53. Further evidence for this hypothesis has been found in the observation that Myc can functionally cooperate with BAX in fibroblasts in vitro [110]. Although BAX was found to be capable of translocating to the mitochondria in the absence of Myc, it did not undergo the conformational change thought to be required for oligomerisation and pore formation [10,107]. This Myc-dependent conformational change might operate through BID [111]. It is clear that BAX expression levels are not affected by the presence or absence of Myc [10,38,104,109]. Despite the functional overlap seen in most cases for BAX and BAK, cooperative data on Myc and BAK loss has been conspicuously absent. This issue was addressed definitively in a study showing that Myc-induced apoptosis is abrogated in pancreatic β cells of Bax−/−Bak+/− animals, while Bax+/−Bak−/− animals undergo apoptosis normally when Myc is driven by the insulin promoter [112]. Further strengthening the argument for BAX involvement in Myc-sensitised apoptosis is the glycoprotein Clusterin, which was shown to block Myc-induced apoptosis by binding to conformation-altered BAX and inhibiting its oligomerisation. Over-expression and knock-down experiments demonstrated that Clusterin cooperates with Myc in the presence of BAX to form colonies in soft agar and tumours in nude mice.
Fig. 2. Deregulated Myc can sensitise cells to apoptosis through many different pathways. Many pro-apoptotic tumour suppressor proteins, the “Ambassadors”, have been shown to act downstream of deregulated Myc to induce the apoptotic safety mechanism. Much of the evidence is from functional data, with no suggestion of direct transcriptional effects by Myc. Shown below are oncogenes that have demonstrated cooperation with deregulated Myc, the “Abrogators”. These oncogenes block Myc-induced apoptosis, allowing the full proliferative potential of Myc to be realised. Pathways in the image are linear for simplicity, but much evidence for cross-talk exists.

[113]. To be clear, none of these studies is indicating a mitochondrial role for Myc, rather that Myc is transcriptionally up- and down-regulating other factors that control BAX oligomerisation at the mitochondria.

PUMA (p53-up-regulated modulator of apoptosis) is another pro-apoptotic BH3-only protein linked to Myc and p53. PUMA localises to the mitochondria to induce cell death, and experiments in Puma−/− mouse thymocytes and myeloid stem cells suggest it is essential for p53-mediated apoptosis as its loss renders cells resistant to death induced by gamma irradiation or deregulated Myc [114]. These findings imply that PUMA functions as a pro-apoptotic molecule downstream of both Myc and p53. The mechanism as to how PUMA can function as a pro-apoptotic molecule at the mitochondria has been recently elucidated. PUMA was found to be important in releasing mitochondrial p53 from its complex with Bcl-xL presumably allowing p53 to activate Bax [66].

It is always prudent to remember that while experiments in culture can reveal details of molecular mechanism, they may not always be representative of conditions in animals. For instance, Soengas et al. clearly demonstrated that loss of Apaf-1 and caspase-9, which are thought to operate downstream of MOMP, reduced p53-dependent, Myc-induced apoptosis in cultured fibroblasts, and led to colony formation in soft agar and tumours in nude mice [115]. However, when Apaf-1−/− and caspase-9−/− mice were crossed to the Eµ-Myc mouse, no cooperative effect was seen in transplanted foetal liver cells [116]. Other reports hint that Apaf-1 and caspase-9 might not be obligate mediators of apoptosis in mouse models [117,118]. Similarly, NF-κB activation was shown to abrogate Myc-induced apoptosis in cell lines [119–121], while its loss was not found to have any effect on Eµ-Myc lymphomas [122].
or tumour- or cell-type specificities before we can move our knowledge into the clinic.

It has long been known that Myc can drive apoptosis through at least one mechanism that is entirely p53-independent, but the molecular mechanism has not yet been solved. Some exciting research into this problem has been reported in the last two years, beginning with the observation that haploinsufficiency or loss of the BIM protein, which promotes apoptosis by binding to and sequestering BCL2 [31], decreased the latency of disease in Eμ-Myc transgenic mice, and that this loss actually relieved the transformed cells of the necessity to disable the p53 pathway [123]. It was also recently shown that the common Burkitt lymphoma alleles T58A and P57S were not capable of up-regulating Bim and thus showed a decreased latency of disease [124].

Functional cloning is a powerful method of identifying novel cooperators, and two recent examples demonstrate how frequently researchers are led back to the p53 pathway. In the paper classifying the bHLH-LZ protein Twist as a potential oncogene, the authors showed that Twist could block Myc-induced apoptosis and cooperate with Ela and Ras to drive colony growth in soft agar, and that Twist expression interfered with p53 transcriptional activity by down-regulating ARF expression [125]. siRNA-mediated knock-down of Twist in human neuroblastoma cells led to increased Myc- and p53-dependent apoptosis. Interestingly, Ink4a-null fibroblasts were still transformed by N-Myc and Twist, indicating that the effect on ARF is not the only method of cooperation [126]. Similarly, we recently identified CUL7, a member of the Cullin family of E3 ubiquitin ligases, as a functional inhibitor of Myc-induced apoptosis. We have shown that CUL7 can cooperate with Myc to drive colony formation in soft agar. CUL7 appears to exert its anti-apoptotic function by binding directly to p53 to promote polyubiquitination, and siRNA-mediated knock-down in human neuroblastoma cells led to the increase in p53 protein levels and p53-dependent apoptosis [127].

The ATM and ATR kinase pathways have also been shown to cooperate with deregulated Myc in driving p53-mediated apoptosis. The kinases have been shown to act independently and in concert with one another following various types of DNA damage. Both kinases phosphorylate p53, stabilising the protein by blocking interaction with Mdm2 [128]. p53 accumulation in response to deregulated Myc was abrogated by treatment of fibroblasts with caffeine, which inhibits ATM and ATR [129], a finding that was strengthened with the in vivo observation that ATM deletion abrogated Myc-induced p53-dependent apoptosis [130]. The authors also suggest that DNA damage is a result of deregulated Myc expression, and ATM kinase mediates the p53 response [130]. Although ATM/R can phosphorylate and activate p53 upon deregulated Myc expression in the absence of ARF, the presence of both ARF and ATM/R can activate p53 more strongly than when only either ARF or ATM/R is present [131]. This finding suggests that both ARF and ATM/R kinases cooperate in activating p53 in response to Myc.

Myc was linked to the extrinsic pathway of apoptosis when it was observed that serum-starved Myc-expressing cells did not die when antagonists to CD95/Fas were present. The authors also showed that Rat1 cells, normally refractory to CD95-induced death, could be made sensitive with ectopic expression of Myc [132]. A similar cooperation was observed with N-Myc in neuroblastoma cells [133]. As with the intrinsic pathway, much of the data indicated that Myc was sensitising the cells to apoptosis by amplifying the death signal at the mitochondria [134]. A more direct role in death receptor-mediated apoptosis was discovered when the observation was made that Myc up-regulates expression of the TRAIL receptor DR5 at the cell surface and increases caspase-8 processing [135], and siRNA to GSK3β and FBW7 mimic this effect [136]. GSK3 and FBW7 have been recently shown to act in the same pathway to target Myc for degradation at T58 [137,138]. Similarly, N-Myc over-expression sensitised SHEP neuroblastoma cells to TRAIL-induced killing by up-regulating DR5 receptors [139]. In helping to promote the same pathway, Myc has also been shown to bind the promoter region of the TRAIL-signalling inhibitory protein c-FLIP by chromatin immunoprecipitation in U2OS cells, leading to down-regulation of c-FLIP, and increased apoptosis as determined by caspase-3 activation and cell cycle analysis [140].

Myc-induced apoptosis has also been linked to the protein translation machinery. Myc has been shown to bind to the promoter of translation initiation factor eIF4E, and to activate the promoter in reporter assays [141]. Constitutive expression of eIF4E is sufficient to block Myc-induced apoptosis in rat embryo fibroblasts and cause an increase in transformation [142,143]. The signal from the activated PI3K pathway flows through PKB/AKT and mTOR such that when PKB and mTOR are active, eIF4E is also active [144]. Data indicates that eIF4E at least partially blocks cytochrome c release from the mitochondria, and that Myc repression of BCL-XL is abrogated in REFs, possibly by increased translation of BCL-XL mRNA [145]. eIF4E-binding protein 1 (4EBP1) binds and inactivates eIF4E, and an activated form of 4EBP1 was shown to modestly increase apoptosis caused by Myc [146]. Finally, a recent report in which Eμ-Myc mice were crossed to eIF4E transgenic mice had a reduced apoptotic index and clearly demonstrated cooperation in vivo [147]. The cooperation was also tested in the adoptive stem cell lymphoma model, where it was confirmed that over-expression of PKB/AKT or eIF4E could cooperate with Myc to reduce the latency of lymphoma onset [148]. These authors also demonstrated that rapamycin, which inhibits mTOR, markedly sensitised cells to undergo Myc-induced apoptosis when also treated with doxorubicin, an observation also evident with serum withdrawal-induced apoptosis of murine fibroblasts expressing deregulated Myc [149]. Interestingly, although eIF4E-dependent protein translation is shut down during apoptosis, translation of Myc continues through use of an internal ribosome entry segment [150].

Many other over-expressed or inactivated proteins have been shown to cooperate with Myc, affect Myc-induced apoptosis and drive transformation. These include, but are not limited to, Met [151], the guanidine-nucleotide exchange factor Tiam1 [152], transcription factors TEL2 [153] and Runx2 [154], macrophage migration inhibitory factor [155], and Bin1 [156–159]. Early work using retroviral insertion in the Eμ-Myc mouse lead to the identification of the cooperating oncogenes Pim-1 and -2, and Emi-1 and Bmi-1 [160]. The cooperation between Bmi-1 and
Myc has been further characterised, and it appears that Bmi-1 is responsible for repressing the INK4a locus [161,162], a similar function perhaps to Twist. Clearly, it will be important to definitively determine what role these and other co-operators play in human neoplastic disease, their cell- and tumour-type specificities, and how this knowledge can be exploited to develop novel therapeutic strategies to target Myc-deregulated tumours.

6. Hints at the sequel

Despite the frustration sometimes felt by researchers who have been scrutinising Myc and its role in cancer for 25 years, it is now, more than ever, an exciting time to be studying Myc. Recent advances in experimental model systems as well as genomics and proteomics provide new opportunities that should allow researchers to discover the unexpected and answer questions they previously could only pose. For example, all of the studies mentioned so far have demonstrated Myc driving apoptosis in a cell-autonomous manner. Using developing Drosophila wing imaginal discs engineered to contain clones expressing different levels of dmyc, two groups clearly demonstrated that cells expressing higher levels of Myc were capable of inducing cell death in neighbouring cells [163,164], although further work is required to resolve the mechanism of signal transmission. The results could have far reaching implications on just how a high Myc-expressing tumour might act towards its tissue environment [165].

Several new technologies have already begun to impact our understanding of Myc biology. For instance, the study of novel cooperators in vivo has exploded recently, with the adoptive stem cell transfer technique used extensively by the Lowe lab and collaborators [166]. This technique was initially used to study cooperation of over-expressed oncogenes in the haematopoietic compartment, but has since been modified to allow for the concomitant study of an over-expressed oncogene and knock-down of a tumour suppressor by stable integration of siRNAs [167]. This will allow many of the cooperating mutations studied in vitro to be verified in vivo without the requirement of a viable knock-out animal. The true power of siRNA knock-down technology is also finally being realised with vastly improved siRNA libraries which are already yielding results through library screens and synthetic lethal relationships [135,136,168].

One of the most exciting areas of research today is focused on microRNAs (miRNAs), non-coding RNAs previously thought to have no real function, but that have recently been shown to act as regulatory units for mRNA expression [169,170]. A trio of reports in the summer of 2005 showed that miRNAs could act as potential oncogenes and tumour suppressors. He and colleagues demonstrated the tumour-specific up-regulation of a cluster of miRNAs, known as the mir-17-92 polycistron. When this cluster of miRNAs was used in the adoptive stem cell transfer system into Eμ-Myc mice, a marked decrease in latency of pre-B cell disease was found. The tumours showed much-reduced TUNEL staining when compared to the Eμ-Myc tumours [171]. It was also determined that two miRNAs induced by Myc, one of which is encoded by the above cluster, negatively regulate E2F1 expression. The mechanism suggests a dampening of the proliferative feedback loop generated by the induction of E2F1 by Myc and vice versa [172]. The third paper in the set could hold clues to why cDNA expression array analysis might be confounded in its ability to identify Myc-regulated genes involved in transformation and apoptosis. This large study revealed miRNA expression patterns were better able to classify tumours than conventional mRNA expression patterns, and that miRNA alterations in cancer are at least as important as changes in mRNA when considered from a prognosis perspective [173]. It seems that it will be very important in the future to consider cancer and apoptosis from the point of view of miRNAs. Since miRNA expression levels do not always accurately represent protein levels, quantitative proteomic approaches are improving and could reveal which proteins are actually up- and down-regulated [174,175].

Finally, the true holy grail of Myc research is on the horizon. It will soon be possible to cut through the myriad of genes regulated by Myc to determine those genes critically important in cellular transformation and apoptosis. Chromatin immunoprecipitation has become the gold standard to definitively assess endogenous transcription factor binding to DNA. By combining this powerful method with the technology of DNA promoter-region arrays we can rapidly screen through many thousands of genes [176,177], including eventually non-coding regulatory RNAs. Determining the genetic programme by which Myc drives apoptosis, we can combine the functional and biochemical data to develop tangible strategies to attack the latent death programme abrogated in tumour cells. This so-called “ChIP-on-chip” technology will also help answer several key questions challenging the Myc field. Is Myc binding to a gene regulatory region sufficient for function? Does the cohort of genes bound by Myc change under deregulated conditions, or are the bound genes merely regulated inappropriately? What signals and co-factors are involved in changing the genetic programme? By combining ChIP-on-chip with expression array analysis, we can begin to address these questions.

7. Denouement

Deregulated Myc is currently thought of as a poor prognostic indicator for cancers, yet this hallmark may provide novel therapeutic options in the future. Researchers hope to exploit this tumour-specific feature to drive the death of cancer cells. By learning which molecules effect and abrogate Myc-induced apoptosis, the goal is to develop novel therapeutic strategies that will overcome these anti-apoptotic mechanisms. By identifying cooperating pathways, therapies targeting each can be combined to eradicate tumour cells. More precise knowledge of the differences in the genetic programmes driven by normal and deregulated Myc will lead to more specific therapies, reducing off-target effects. If these strategies prove successful, a significant advance will be realised against tumours harbouring deregulated Myc. We wish our fellow researchers, past, present and future, a happy anniversary.
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