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REGULATION OF SURVIVAL PATHWAYS BY IL-3 AND INDUCTION OF APOPTOSIS FOLLOWING IL-3 WITHDRAWAL

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TABLE OF CONTENTS

1. ABSTRACT

The production of hematopoietic cells is regulated by the availability of key cytokines such as interleukin-3 (IL-3). IL-3 promotes the survival and proliferation of bone marrow-derived hematopoietic cells. Recent studies using IL-3-dependent cell lines have begun to shed light on the regulation of apoptosis in cytokine-dependent cells. These studies indicate that IL-3 inhibits apoptosis by stimulating the activation of cellular kinases, including phophatidylinositol (PI) 3-kinase and Akt kinase. On the other hand, withdrawal of IL-3 leads to the activation of caspase proteases and a commitment to cell death. This review will discuss the current state of knowledge regarding the molecular mechanisms of: a) suppression of apoptosis by IL-3, and b) activation of apoptosis following IL-3 withdrawal.

2. INTRODUCTION

IL-3, along with GM-CSF, is produced and secreted by activated T cells (1-3). Once released, these cytokines promote the survival, proliferation, and maturation of bone marrow-derived multipotential and lineage-committed hematopoietic progenitors (1-7). The fact that IL-3 is produced by activated T cells has led to the hypothesis that IL-3 plays an important role in the expansion of hematopoietic populations during inflammation (2). Moreover, attenuation of an inflammatory response may require depletion of IL-3 and resultant apoptotic death of the expanded IL-3-dependent cells. Although recent gene knockout experiments have raised questions about the precise role of IL-3 (8-11), studies using IL-3-dependent cells have provided an excellent working model for understanding the development and death of cytokine-dependent cells.

In addition to providing insight into the process of normal hematopoiesis, an understanding of apoptosis regulation in cytokine-dependent cells also may provide clues regarding the origin and progression of certain leukemias and lymphomas. The observation during the late 1980's that greater than 85% of patients with follicular B cell lymphoma overexpress the antiapoptotic protein Bcl-2 (12-14), raised the possibility that abrogation of apoptotic pathways may represent a common mechanism of malignant progression. More specifically, abnormal expression or function of apoptosis regulatory molecules could play a role in converting normal cytokine-dependent cells into cancerous cells. For this reason, it is first important to identify and understand the normal mechanisms of apoptosis regulation in cytokine-dependent cells. In the following sections, we will describe what is known about survival pathways that are activated in IL-3 stimulated cells and apoptotic pathways that are activated in IL-3-deprived cells.

3. SIGNALS MEDIATED BY THE IL-3 RECEPTOR

The effects of IL-3 are mediated by a high-affinity cell surface receptor (15-17). The receptor for IL-3, like the receptors for GM-CSF and IL-5, consists of two subunits, the alpha subunit (or IL-3alpha) and the beta subunit (or $beta_c$). Both the IL-3alpha and the $beta_c$ subunit span the membrane once and are oriented with their amino termini outside the cell. Neither subunit contains intrinsic kinase activity in its cytoplasmic domain. The IL-3alpha subunit is a 378 amino acid protein (human) which contains a single cytokine receptor module (CRM) in its extracellular domain and a short 53 amino acid cytoplasmic domain (18). The $beta_c$ subunit is an 881 amino acid protein (human) with two CRMs in its extracellular domain and a cytoplasmic domain of 432 amino acids (19). Strikingly, the $beta_c$ subunit of the IL-3 receptor is also shared by the receptors for GM-CSF and IL-5 (18, 20). On the other hand, the IL-3, GM-CSF, and IL-5 receptors each contain distinct alpha subunits (18, 21-23). Thus, the ligand binding specificities of these receptors are determined by their unique alpha subunits.

The binding of IL-3 to its cognate receptor results in dimerization of the IL-3alpha and $beta_c$ subunits, followed by tyrosine phosphorylation of $beta_c$ (24-28). Extensive mutational analyses have determined that while the cytoplasmic domains of both subunits are important for normal receptor activation, it is the cytoplasmic domain of the $beta_c$ subunit which is primarily responsible for signaling (25, 29-32). Since $beta_c$ is common to the IL-3, GM-CSF, and IL-5 receptors, studies of $beta_c$-mediated signaling have interchangably used IL-3, GM-CSF, or IL-5 as stimulating ligands.

In addition to tyrosine phosphorylation of $beta_c$, ligand binding induces a number of other cellular responses,

including: A) tyrosine phosphorylation of Jak2 kinase (33), Stat5 (34, 35), phosphatidylinositol (PI) 3-kinase (36), Vav (37), Shc (38-40), and PTP1D phosphatase (41), B) activation of Jak2 kinase (33), Stat5 (34, 35), PI 3-kinase (36), Ras (42, 43), Raf-1 kinase (44), and MAP kinase (45, 46), C) transcription of c-*myc*, c-*fos*, and c-*jun* (47, 36), D) cellular proliferation, and E) suppression of apoptosis. This diversity of responses may seem somewhat remarkable since $beta_c$ itself does not exhibit intrinsic kinase activity. However, numerous studies have supported a model wherein ligand-induced heterodimerization of IL-3alpha and $beta_c$ causes a conformational change resulting in the activation of Jak2 kinase that is preassociated with the receptor (33). The activation of Jak2 then leads to phosphorylation of $beta_c$ on several tyrosine residues in its cytoplasmic domain. These phosphorylated tyrosine residues serve as docking sites for SH2-containing adaptor or signaling molecules such as Shc (38-40) and hematopoietic cell phosphatase (48). When brought into close proximity with activated Jak2 kinase, the SH2-containing molecules themselves can become targets for phosphorylation and activation.

Recent experiments have demonstrated that distinct biochemical pathways are responsible for IL-3-induced DNA synthesis and IL-3-mediated suppression of apoptosis. In IL-3-dependent pro-B (Ba/F3) or pro-myeloid (32D) cells, treatment with genistein during IL-3 stimulation was found to block induction of DNA synthesis, but not suppression of apoptosis (49). This distinction between survival and proliferation pathways has been defined in even greater detail through studies using $beta_c$ receptor mutants (36, 49). Such studies have identified three distinct functional domains in the human $beta_c$ cytoplasmic region: a membrane proximal domain (amino acids 455-517), a membrane distal domain (a.a. 544-763), and a C-terminal domain (a.a. 763-881). Deletion of the C-terminal domain by truncating $beta_c$ at amino acid 763 increases ligand-dependent PI 3-kinase activity and tyrosine phosphorylation of $beta_c$ and Shc, indicating that the C-terminal region may be important in negative regulation. The C-terminal region is not essential for induction of DNA synthesis or suppression of apoptosis. Removal of the membrane distal domain by truncation of $beta_c$ at amino acids 517 or 544 results in receptors which retain the ability to promote c-*myc* transcription and DNA synthesis, but fail to mediate activation of Ras and the Raf-1/MAP kinase pathway, induction of c-*fos* and c-*jun*, or suppression apoptosis. Removal of the membrane proximal domain by truncation at amino acid 455 results in a receptor which is unable to bind Jak2 kinase, and unable to mediate transcription of c-*myc* and induction of DNA synthesis (33, 36). Thus, in summary, the membrane proximal domain is important for ligand-dependent activation of Jak2, transcription of c-*myc*, and stimulation of DNA synthesis, while the membrane distal domain is required for activation of Ras/Raf-1/MAP kinase, induction of c-*fos*/c-*jun*, and suppression of apoptosis (33, 36, 49).

The mutational studies described above have determined that the membrane distal domain of $beta_c$ is critically important for mediating survival signals from the IL-3 receptor. Since this region of $beta_c$ also is important for activation of Ras and the Raf-1/MAP kinase pathways, attention has focused on these molecules as potential mediators of survival. Kinoshita *et al*. (50) have shown that expression of mutant activated Ras restores Raf-1 activation and cell survival in cells expressing the $beta_c$ mutant lacking the membrane distal domain. The ability of activated Ras to promote survival may be mediated in part by Raf-1 kinase, since overexpression of constitutively activated Raf-1 can suppress apoptosis in IL-3-deprived cells (50). At the same time, Raf-1-independent pathways also appear to be involved, since expression of an activated Ras mutant which is unable to activate Raf-1 can also support survival (50). The Raf-1-independent pathways are sensitive to wortmannin, a specific inhibitor of PI 3-kinase, suggesting the involvement of this enzyme in $beta_c$- and Ras-mediated survival pathways (50, 51). The potential role of PI 3-kinase will be discussed in greater detail in the next section.

Finally, it should be noted that in the mouse, two closely related IL-3 receptor beta subunits, $beta_c$ and $beta_{IL-3}$ (52), have been identified (as opposed to only one beta subunit in all other species studied). The $beta_c$ subunit, as described above, serves as a common beta subunit for the IL-3, GM-CSF, and IL-5 receptors. By contrast, $beta_{IL-3}$ is specific to the murine IL-3 receptor, associating only with the murine IL-3alpha receptor subunit. The role of $beta_{IL-3}$ in IL-3 receptor-mediated signaling in the murine system is largely undefined.

4. Bcl-2, BAD, AND AKT KINASE

In the previous section we began by discussing signaling events that originated with the cell surface IL-3 receptor, then followed these pathways into the interior of the cell. In this section we will focus on molecules and events in the interior of the cell and then trace our way back towards the receptor. This will serve to link known intracellular regulators of apoptosis with receptor-mediated survival signals. We will begin with a discussion of the Bcl-2 oncoprotein.

The *bcl-2* protooncogene was initially identified as a cellular gene located at the site of a frequent chromosomal translocation in follicular B cell lymphomas (12, 13). Follicular B cell lymphomas represent one of the most common cancers of the human hematopoietic system. Greater than 85% of these lymphomas, as well as 20% of diffuse B cell lymphomas, exhibit a t(14;18) chromosomal translocation, which results in elevated expression of wild-type Bcl-2 protein (52, 12, 14). A considerable body of experimental evidence indicates that Bcl-2 contributes to the progression of these diseases by blocking apoptosis in cells that are normally destined to die. For example, targeted overexpression of Bcl-2 in the B lymphocytes of transgenic mice results in extended B cell survival and an expanded B cell compartment (54).

The deleterious effects of Bcl-2 overexpression may not be limited to cancers of hematopoietic cells. Deregulated expression of Bcl-2 has also been documented in neuroblastoma (55), androgen-independent prostate cancer (56), breast cancer (57, 58), melanoma (59, 60), and gastrointestinal cancer (61). Thus, the disruption of apoptotic pathways by Bcl-2 or Bcl-2-like molecules may be a common step during tumor development.

The expression of Bcl-2 also appears to play an important role in the normal development and maintenance of the hematopoietic system. In the adult, Bcl-2 is expressed in bone marrow progenitor cells representing all lineages, suggesting a role in the survival of these immature cells (62). Further clues about the role of Bcl-2 have come from *bcl-2*$^{-/-}$ gene knockout mice (63, 64). These mice are born normal, and initially demonstrate apparently normal hematopoiesis. However, shortly after birth the knockout mice exhibit massive lymphocyte apoptosis. Furthermore, thymocytes from these mice undergo accelerated apoptosis in response to dexamethasone or radiation. Eventually, most *bcl-2*$^{-/-}$

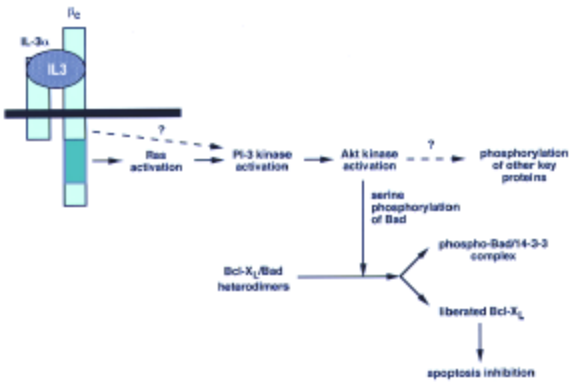

Figure 1. Potential pathway for suppression of apoptosis by IL-3.

knockout mice die prematurely from polycystic kidney disease.

In cell culture sytems, Bcl-2 has been shown to block apoptosis in response to a variety of apoptotic stimuli, including cytokine withdrawal (65, 66). A great deal of effort has been invested to determine the molecular mechanism of Bcl-2 action. Bcl-2 is a 25 kDa integral membrane protein that is localized primarily to membranes of the mitochondria, endoplasmic reticulum, and nucleus (67-69, 106, 107). Although the primary amino acid sequence of Bcl-2 is not homologous to any known enzymatic proteins, a number of Bcl-2 related proteins have been isolated and cloned. Together these proteins constitute the Bcl-2 protein family (70). Functional studies have determined that the different members of the Bcl-2 protein family act either to suppress (Bcl-2, Bcl-X_L, Mcl-1, A1) or promote (Bax, Bcl-X_S, Bad, Bak, Bik, Bid) apoptosis (70-72). Members of the Bcl-2 protein family can homodimerize or heterodimerize with other members of the family. Importantly, the functional properties of these proteins are altered by heterodimerization interactions (73, 74).

Recently the crystal structure of the Bcl-2 family member, Bcl-X_L, was elucidated, revealing striking similarities between Bcl-X_L and the pore-forming proteins diptheria toxin and the colicins (75). Based on this observation, subsequent experiments have determined that Bcl-X_L (76), Bcl-2 (77, 103), and Bax (74, 103), all have ion channel-forming ability when studied in artificial lipid membranes. The prominent localization of Bcl-2 family members to the mitochondria suggests that the mechanism of action of these molecules may be to regulate the permeability of the mitochondria to ions or potentially to other larger molecules (The role of mitochondria will be discussed in greater detail in the next section). Interestingly, pores that are formed by the proapoptotic molecule Bax exhibit properties that are different from those formed by the antiapoptotic molecule Bcl-2 (74, 103). Moreover, the unique properties of the Bax channel can be blocked by heterodimerization with Bcl-2 (74).

In addition to serving as a channel-forming protein, Bcl-2 can also act as a binding or docking site for other proapoptotic molecules, such as Bad (78). Although Bad is a member of the Bcl-2 protein family, it is not anchored to a membrane and exists as a cytoplasmic protein. Overexpression of Bad can inhibit the ability of Bcl-2 to prevent apoptosis in IL-3-deprived cells (78). However, in the presence of IL-3, Bad becomes phosphorylated on serine 112 and serine 136, and Bad-promoted apoptosis is suppressed (79). Zha *et al.* (79) have found that unphosphorylated Bad binds to Bcl-X_L (and possibly Bcl-2), while phosphorylated Bad is bound up by the cytoplasmic protein 14-3-3 (Figure 1). Thus, IL-3-mediated phosphorylation of Bad likely serves to free up antiapoptotic Bcl-2 family members to perform their function of inhibiting apoptosis.

Since the ability of IL-3 to promote phosphorylation of Bad appears to be important in the suppression of apoptosis, efforts have been made to identify the kinase that is responsible for Bad phosphorylation. Recently, Datta *et al.* (80) discovered that Akt kinase can phosphorylate serine 136 of Bad, both *in vitro* and *in vivo*. Furthermore, Akt-mediated phosphorylation of serine 136 appears to be sufficient for inhibition of apoptosis in cultured cells. These findings are consistent with previous studies which have shown that activated Akt can inhibit apoptosis under a number of different circumstances, including cytokine withdrawal (81, 51, 82-85). Importantly, the discovery that Akt can phosphorylate Bad provides an important link to IL-3 receptor-initiated signaling. This is because Akt kinase is known to be activated by PI 3-kinase (86-89), and PI 3-kinase is activated soon after IL-3 binds to its receptor (36). PI 3-kinase, like Akt, has been shown in a number of different studies to suppress apoptosis (90-92, 51, 82, 85). Activation of PI 3-kinase may occur via Ras-dependent pathways (93, 94), but also may involve Ras-independent mechanisms. Once activated, PI 3-kinase phosphorylates inositol phospholipids at the D-3 position. Akt activation results, at least in part, by the binding of PI 3-kinase-generated phosphorylated lipids to the pleckstrin homology domain of the Akt molecule (95, 96).

Figure 1 presents a summary of a potential mechanism for IL-3-mediated suppression of apoptosis. Briefly, the binding of IL-3 to its receptor results in Ras activation, through sequences represented by the membrane distal domain of the $beta_c$ receptor subunit. Activation of Ras may lead to the activation of PI 3-kinase. Alternatively, PI 3-kinase may be activated through a Ras-independent pathway. Activated PI 3-kinase phosphorylates inositol phospholipids, which then bind to and activate Akt kinase. Akt then phosphorylates Bad, and potentially other apoptosis-regulatory molecules, resulting in the association of Bad with 14-3-3. The sequestration of Bad by 14-3-3 frees up antiapoptotic proteins such as Bcl-X_L or Bcl-2, allowing them to bind to proapoptotic molecules such as Bax and inhibit apoptosis.

5. MITOCHONDRIAL CHANGES DURING APOPTOSIS

The discovery that several Bcl-2 family members are localized to the outer mitochondrial membrane has raised speculation that mitochondria play an important role in the regulation of apoptosis. In support of this, recent work has shown that a number of mitochondrial events occur during early apoptotic cell death. These events include: A) release of cytochrome c from the mitochondrial intermembranous space into the cytoplasm (97, 98), B) mitochondrial swelling and outer membrane rupture (99), and C) loss of mitochondrial membrane potential (100). Each of these mitochondria-associated events has been demonstrated in IL-3-deprived FL5.12 pro-B cells (99), as well as other cell lines stimulated with a variety of different apoptotic stimuli.

The release of cytochrome c from mitochondria is particularly intriguing. Apo-cytochrome c is encoded by a nuclear gene and is transported, by an unknown

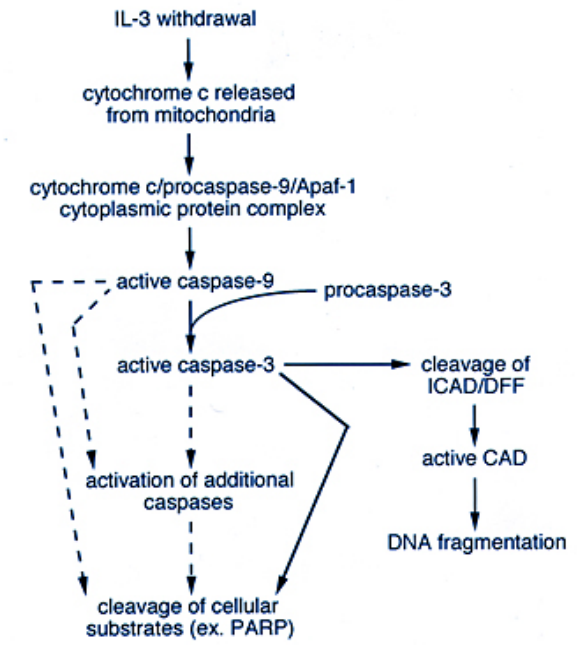

Figure 2. Potential pathway outlining caspase activation and cellular destruction following IL-3 withdrawal. Current experimental evidence has demonstrated that IL-3 withdrawal leads to cytochrome c release from the mitochondria, and eventual caspase-mediated cleavage of cellular proteins and fragmentation of genomic DNA. However, the mechanism of caspase activation in IL-3-deprived cells remains unknown. The figure shows a pathway for caspase activation that has been elucidated in cytokine-independent cells (97, 101, 102, 145-147).

mechanism, into the space between the inner and outer mitochondrial membranes. Once transported, apo-cytochrome c binds with heme and assumes its important role in oxidative phosphorylation. The release of cytochrome c from mitochondria during an early stage of apoptosis was initially discovered by the laboratory of Xiaodong Wang (97). This group was searching for cytoplasmic proteins that contributed to the activation of a class of apoptotic effectors, caspase proteases (caspases are discussed at length in the next section). One of the proteins they purified and determined to be important for activation of caspases was cytochrome c. As depicted in figure 2, subsequent studies (101, 102) have shown that cytochrome c that is released from the mitochondria forms a complex with two other proteins, procaspase-9 and Apaf-1 (a mammalian homolog of the *C. elegans* death protein CED-4). Formation of this complex in the presence of dATP results in the processing of inactive procaspase-9 to active caspase-9 (102). Active caspase-9 then promotes the processing and activation of caspase-3, which may lead to a cascade of other caspases being activated. Currently, it is unclear how cytochrome c contributes to the cytochrome c/Apaf-1/procaspase-9 activation complex, and how the redox potential of the cytochrome c heme group might be involved. Also, with respect to IL-3 withdrawal-induced apoptosis, it remains to be determined whether caspase-9 is the initial caspase that is activated.

Another unsolved problem concerns the mechanism of cytochrome c release from the mitochondria. One possible mechanism for this release would involve pores in the outer mitochondrial membrane. Since the proapoptotic molecule Bax has demonstrated pore-forming ability (74, 103) it has been intriguing to speculate that Bax may form the pore through which cytochrome c passes. The observation that Bcl-2, via heterodimerization, alters the properties of Bax pores (74), and also blocks the release of cytochrome c (104, 105), supports this idea. However, current experimental evidence suggests that the pores formed by Bax are more likely to be limited to the passage of ions or much smaller molecules (74, 103). Nonetheless, it remains a possibility that Bax, or some as yet unknown pore, may be responsible for transport of cytochrome c to the cytoplasm.

Another possible mechanism for the release of cytochrome c would involve rupture of the outer mitochondrial membrane. Vander Heiden *et al*. (99) have reported that apoptosis caused by IL-3 withdrawal (FL5.12) or stimulation of Fas antigen (Jurkat T leukemic cells) is marked by early swelling of the mitochondria and rupture of the outer membrane. These events, which can be blocked by expression of Bcl-X_L, may account for the release of cytochrome c. However, it is unclear whether mitochondrial swelling and outer membrane rupture occur universally in apoptotic cells, and whether membrane rupture actually precedes the earliest release of cytochrome c.

In addition to cytochrome c, a protein called AIF (apoptosis-inducing factor) has been reported to be released from mitochondria during apoptosis, and appears to be involved in caspase activation (108). AIF is a 50 kDa protein which awaits purification and cloning. It will be interesting to see whether other critically important proteins are released from the mitochondria.

The loss of mitochondrial membrane potential (also referred to as mitochondrial permeability transition) has also been observed in a variety of different cell types in response to a variety of different apoptotic stimuli (100, 109). A poorly defined pore, capable of large conductance and located in the inner mitochondrial membrane, is responsible for mitochondrial permeability transition (98). Several studies indicate that the opening of this pore is preceded by mitochondrial swelling, outer membrane rupture, and cytochrome c release (104, 105, 99). Thus, the importance of mitochondrial permeability transition in the propagation of apoptosis signaling events remains unknown. However, as with other mitochondrial events, mitochondrial permeability transition is efficiently inhibited by expression of antiapoptotic Bcl-2 family members (109).

6. ACTIVATION OF CASPASE PROTEASES FOLLOWING IL-3 WITHDRAWAL

In a variety of model systems, the execution stage of apoptosis is known to be associated with the activation of a family of cellular proteases called caspases (110-112). Caspases are cysteine proteases which cleave substrate proteins after aspartate residues found in specific sequence contexts. To date, ten distinct members of the caspase protease family (caspase-1 through -10) have been identified and cloned (113). During apoptosis the activation of caspases often proceeds in a cascade-like fashion, with one member of the family serving to activate another (see figure 2). In this fashion the proteolytic signal may be amplified. Ultimately, caspase activation leads to the destruction of the cell, presumably due to the cleavage of multiple intracellular substrate proteins. The activation of caspases has been shown to occur during apoptosis caused by a variety of different stimuli, including treatment with chemotherapy or radiation (114-119), stimulation of cell surface Fas or TNF receptor (120-122), detachment

from extracellular matrix (123), or withdrawal of essential neurotrophic factor (124, 125). As discussed below, more recent data indicates that withdrawal of cytokines, such as IL-3, results in caspase activation in cytokine-dependent hematopoietic cells.

Detailed studies of caspase proteases have required convenient methods for assessing caspase activation. Many of these studies have made use of the fact that caspases are initially synthesized as inactive precursors. Activation involves proteolytic processing of the inactive procaspase form to two smaller subunits which associate into a heterotetrameric active enzyme form (110-112). Thus, the activation of a specific caspase can be assessed via Western blotting, by measuring conversion of the high molecular weight precursor form to smaller active subunits. In the same way, the cleavage of known caspase substrate proteins can be used as a measure of caspase activation. A number of important caspase substrate proteins have been identified, including poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair (126-131). PARP is known to be cleaved by several different members of the caspase family, and cleavage of PARP has commonly been used as a hallmark indicator of caspase activation. Finally, caspase activation can also be assessed by measuring the cleavage of fluorogenic peptides that are based on peptide cleavage sites in caspase substrate proteins. The most commonly used peptides contain the core sequence YVAD (recognized specifically by caspase-1), VAD (recognized by multiple caspases), or DEVD (recognized at least by caspase-3).

The role of caspase proteases in various apoptotic processes has been studied using two types of caspase inhibitors: 1) peptides based on cleavage sites in substrate proteins (132, 133), and 2) virally-encoded caspase inhibitors, including cowpox virus CrmA (134-136) and baculovirus p35 (137, 138) proteins. Experiments using the CrmA inhibitor have shown that caspases are important for apoptotic execution following neurotrophic factor withdrawal (124), Fas or TNFR stimulation (120-122), detachment from extracellular matrix (123), and treatment with chemotherapy (118).

Although relatively little work has been done to investigate the mechanism of IL-3 withdrawal-induced apoptosis, recent work has begun to document an important role for caspases in this process. Early studies by Kumar (139) demonstrated that a number of IL-3-dependent cell lines expressed mRNA for caspase-2. Moreover, expression of antisense caspase-2 in IL-3-dependent FDCP-1 cells (murine myeloid progenitor cell line) resulted in moderately enhanced survival following IL-3 withdrawal (139). Subsequent Western blotting experiments using extracts of Mo7e cells (IL-3-dependent human megakaryoblastic cell line) have revealed rapid processing/activation of caspase-2 and caspase-7 following IL-3 withdrawal (140). At later timepoints, caspase-3 was also activated, indicating that a cascade of caspases may be initiated in IL-3-deprived Mo7e cells.

A number of studies, using Ba/F3, Mo7e, 32D, or FDCP-1 cells, have observed cleavage of PARP protein following IL-3 withdrawal (140-143; see Figure 2). In general, PARP cleavage immediately preceded fragmentation of genomic DNA to oligonucleosomal-length fragments. The IL-3 withdrawal-induced PARP cleavage was inhibited both *in vivo* and *in vitro* by overexpression of Bcl-2, or by incubation with the inhibitory peptides z-VAD-fluoromethyl ketone (z-VAD-FMK) or z-DEVD-fluoromethyl ketone (z-DEVD-FMK) (141-143). These inhibitors also delayed DNA fragmentation and loss of cell viability, indicating an important role for caspases in apoptotic execution following IL-3 withdrawal (141, 142). On the other hand, YVAD peptide, an inhibitor of caspase-1, had no effect on PARP cleavage or apoptosis, suggesting that caspase-1 is not involved (142). In addition, PARP cleavage, DNA fragmentation, and loss of cell viability were not inhibited by expression of CrmA protein (143, 144), a potent inhibitor of apoptosis caused by neurotrophic factor withdrawal (124) or Fas stimulation (120-122). This suggests that the repertoire of caspases activated by IL-3 withdrawal is different from the repertoires activated by other apoptotic stimuli.

In still further experiments, extracts from IL-3-deprived cells have been shown to cleave the fluorogenic peptides DEVD-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) (140) and DEVD-4-methylcoumaryl-7-amide (DEVD-MCA) (142). The cleavage of DEVD-AFC and DEVD-MCA by these extracts has been interpreted to indicate that caspase-3 is an important mediator of IL-3 withdrawal-induced apoptosis. However, it is possible that other members of the caspase family also can cleave this peptide (133). Peptides thought to be specific for caspase-1 were not cleaved by extracts from IL-3-deprived cells (140, 142), consistent with experiments described above.

On a technical note, it should be pointed out that *in vivo* inhibition of PARP cleavage or apoptosis in the IL-3-dependent cells that have been studied, typically requires much higher concentrations of inhibitory peptide than is needed in other cell types (ex. Fas-stimulated Jurkat T leukemic cells). Perhaps the inhibitory peptides are poorly taken up, or are rapidly metabolized by IL-3-dependent cell lines.

Ultimately IL-3 withdrawal leads to fragmentation of genomic DNA and loss of cell viability. In related systems, protein purification has led to the discovery of a caspase-activated deoxyribonuclease (CAD) that mediates DNA fragmentation in apoptotic cells (145, 146). Normally, CAD exists as an inactive enzyme, complexed with an inhibitor called ICAD/DFF (145-147; see Figure 2). However, in the presence of an apoptotic stimulus, activated caspase-3 cleaves and inactivates the ICAD/DFF inhibitor (145-147). This enables CAD to perform its function of cleaving genomic DNA. It will be interesting to see whether ICAD/DFF and CAD are involved in DNA fragmentation during IL-3 withdrawal-induced apoptosis.

In summary, although it is now clear that IL-3 withdrawal leads to caspase activation and cleavage of caspases substrates, a number of questions remain unanswered: Which members of the caspase protease family are activated by IL-3 withdrawal, and in what order? Are different caspases activated in different hematopoietic lineages? What is the mechanism of caspase activation following IL-3 withdrawal? Which events commit cytokine-deprived cells to a pathway of apoptotic execution? Is CAD activated in IL-3-deprived cells? Further experiments will be needed to address these and other important questions.

7. THE IMPORTANCE OF p53

The ability of p53 to promote apoptosis has been well documented in a variety of systems. In the case of IL-3 withdrawal-induced apoptosis, p53 appears to be critically important (148-150). Studies using 32D cells have shown that overexpression of wild-type p53 results in accelerated apoptosis following IL-3 withdrawal (149). By

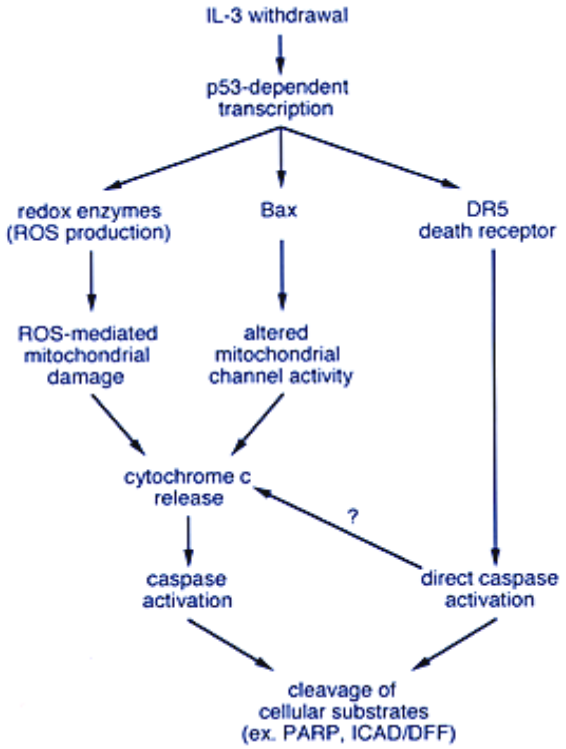

Figure 3. Potential pathways of p53-mediated apoptosis. The p53 protein plays an important role in IL-3 withdrawal-induced apoptosis (see text). In other systems, p53 has been shown to induce transcription of Bax, DR5 death receptor, and enzymes involved in the production and regulation of cellular reactive oxygen species (ROS). The figure depicts potential pathways of p53-mediated caspase activation.

contrast, disruption of p53 function by overexpression of dominant-negative p53 serves to protect IL-3-deprived 32D or DA1 cells from apoptosis (148, 149). Thus, p53 function seems to be required for IL-3 withdrawal-induced apoptosis of hematopoietic cells.

The precise mechanism whereby p53 promotes apoptosis in IL-3-deprived cells remains unclear. However, it seems likely that activation of caspase proteases may be a downstream effect of p53 action. In M1 myeloid leukemic cells, overexpression of wild-type p53 has been shown to elicit caspase activation (151). Incubation of these cells with IL-6 cytokine can inhibit the p53-mediated caspase activation.

Recent studies have provided clues regarding p53-mediated events that are more proximal than caspase activation, and suggest that early biochemical events may depend on the ability of p53 to act as a transcription factor (figure 3). These studies have shown that overexpression of p53 induces transcription of proapoptotic Bax protein (152, 153), as well as DR5 (154), a cell surface receptor that is directly linked to activation of caspases. In addition, Polyak *et al*. (155) have demonstrated that overexpression of p53 in a colorectal cancer cell line leads to the induction of a limited number of p53-inducible-genes (PIGS). Of the 14 PIGs they identified, several turned out to encode enzymes involved in the production or regulation of intracellular reactive oxygen species (ROS). Polyak *et al.* (155) proposed that p53-mediated induction of these enzymes could lead to the generation of ROS, oxidative damage to the mitochondria, release of proteins from the damaged mitochondria, and activation of caspases by the released mitochondrial proteins (figure 3). Future experiments will help to determine the role of p53-inducible genes and ROS in IL-3 withdrawal-induced apoptosis.

8. PERSPECTIVES

Athough much has been learned in recent years concerning the regulation and execution of apoptosis, many questions are left to be answered. One area of research that promises to be particularly exciting will be the characterization of channels formed by Bcl-2 family members. Studies of these channels should shed light on the regulation of mitochondrial function and the mechanism of cytochrome c release. In addition, these studies will help to determine how proteins such as Bad, through their interactions with antiapoptotic membrane channels, serve to promote apoptosis.

With respect to IL-3 withdrawal-induced apoptosis, it now seems clear that caspases play an important role in the execution process. However, questions remain regarding which caspases are activated, and in what order, following IL-3 withdrawal. Furthermore, it is not known whether the individual members of the caspase family are developmentally regulated or lineage-restricted during hematopoiesis. A clearer understanding of caspase expression patterns will be useful when asking whether caspase expression or function is altered in leukemias or lymphomas.

Finally, it remains to be determined, from a mechanistic perspective, how caspases become activated following IL-3 withdrawal. Evidence from other systems has delineated two distinct models of caspase activation. In one model, ligand-induced activation of a cell surface receptor (ex. Fas, DR5) results in direct activation of a caspase (ex. caspase-8) that is part of a signaling complex associated with the receptor cytoplasmic domain (156, 157). In the second model, depicted in figure 2, caspases become activated in response to the release of cytochrome c from the mitochondria. The release of cytochrome c has been observed following IL-3 withdrawal (99), suggesting that the activation of caspases in IL-3-deprived cells may occur via this second model. However, it remains possible that caspases are activated at the cell surface, or by some other unique mechanism, following IL-3 withdrawal. A greater understanding of caspase activation pathways will assist the development of therapies aimed at restoring apoptotic potential in apoptosis-resistant leukemias and lymphomas.

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