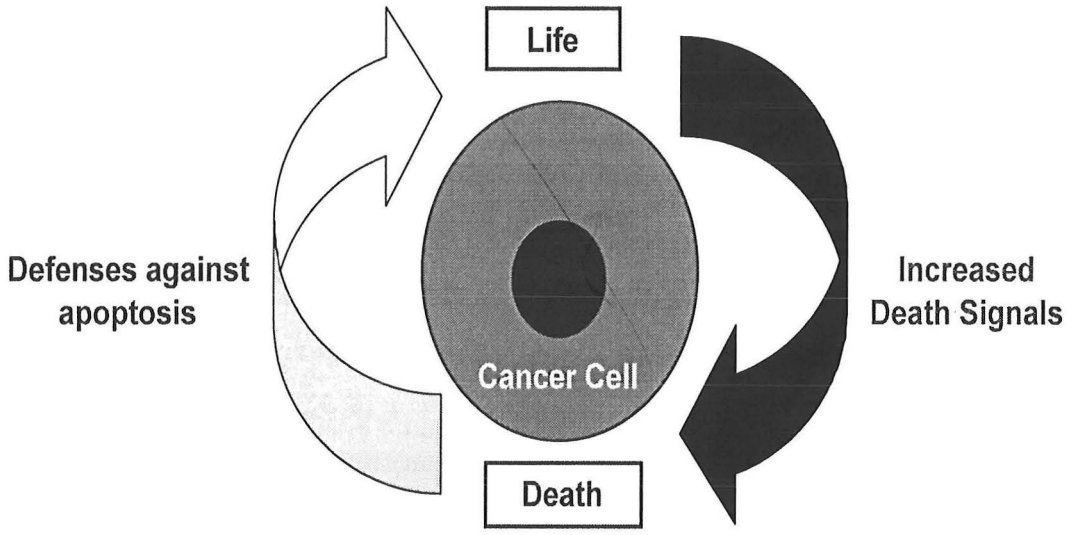
REGULATION OF APOPTOSIS: IMPLICATIONS FOR CANCER BIOLOGY AND THERAPY



INTERNAL MEDICINE GRAND ROUNDS

Preet M. Chaudhary, MD, PhD

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This is to acknowledge that Preet M. Chaudhary, MD, PhD, has not disclosed any financial interests or other relationships with the commercial concerns related directly or indirectly to this program. Dr. Chaudhary will be discussing off-label uses in his preparation.

Preet M. Chaudhary, MD, PhD, Assistant Professor of Medicine and Molecular Biology, Division of Hematology/Oncology, UT Southwestern Medical Center.

Research Interests: Our laboratory is interested in studying the signal transduction by the members of the Tumor Necrosis Factor Receptor Family and their role in pathogenesis of various human disease conditions. Current projects in the laboratory include regulation of signaling via the death receptors, EDAR, XEDAR and TAJ; role of HHV8-encoded vFLIP in the pathogenesis of AIDS-associated malignancies; and molecular biology of Ewing's sarcoma.

Introduction

Apoptosis is an evolutionarily conserved and genetically regulated form of cell suicide which plays an important role in development and maintenance of tissue homeostasis in multicellular organisms [1, 2]. Apoptosis plays an essential role in the elimination of mutated or transformed cells from the body and as such evasion of apoptosis is one of the hallmarks of cancer [3]. Morphologically, apoptotic cells exhibit several characteristic features, such as cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation [2]. Molecularly, most apoptotic pathways involve a sensor that detects an injurious signal, a signal transduction network which relays and amplifies the signal and an execution machinery which dismantles the cell. Although a comprehensive review of the molecular control of apoptosis is beyond the scope of this discussion, the following section provides a brief introduction to the major players involved in this process.

Molecular control of apoptosis

Caspases, the proteases for cell death

Apoptotic cell death is orchestrated by the activation of caspases, a family of cysteine proteases that cleave their substrates at aspartic acid residues [4-6]. To date, 14 mammalian caspases have been identified, a subset of which are involved in the regulation of apoptosis (Fig. 1). Caspases are normally expressed in the cells as inactive zymogen and are converted into their active form at the onset of apoptosis by a process involving proteolytic processing followed by assembly of the subunits into an active tetramer. Caspases can be broadly divided into initiator (upstream)

and effector (downstream) caspases [4-6]. Initiator caspases, such as caspase –8, -9 and –10, possess long N-terminal prodomains that function as protein recruitment modules by interacting with proteins which initiate caspase activation. These caspases are first to be activated following a proapoptotic stimulus and are responsible for activating the effector caspases. The effector caspases, primarily caspase-3, -6 and –7, contain short N-terminal prodomains and are dependent on the upstream caspases for their processing and activation. Once activated, these caspases carry out majority of the substrate proteolysis during apoptosis.

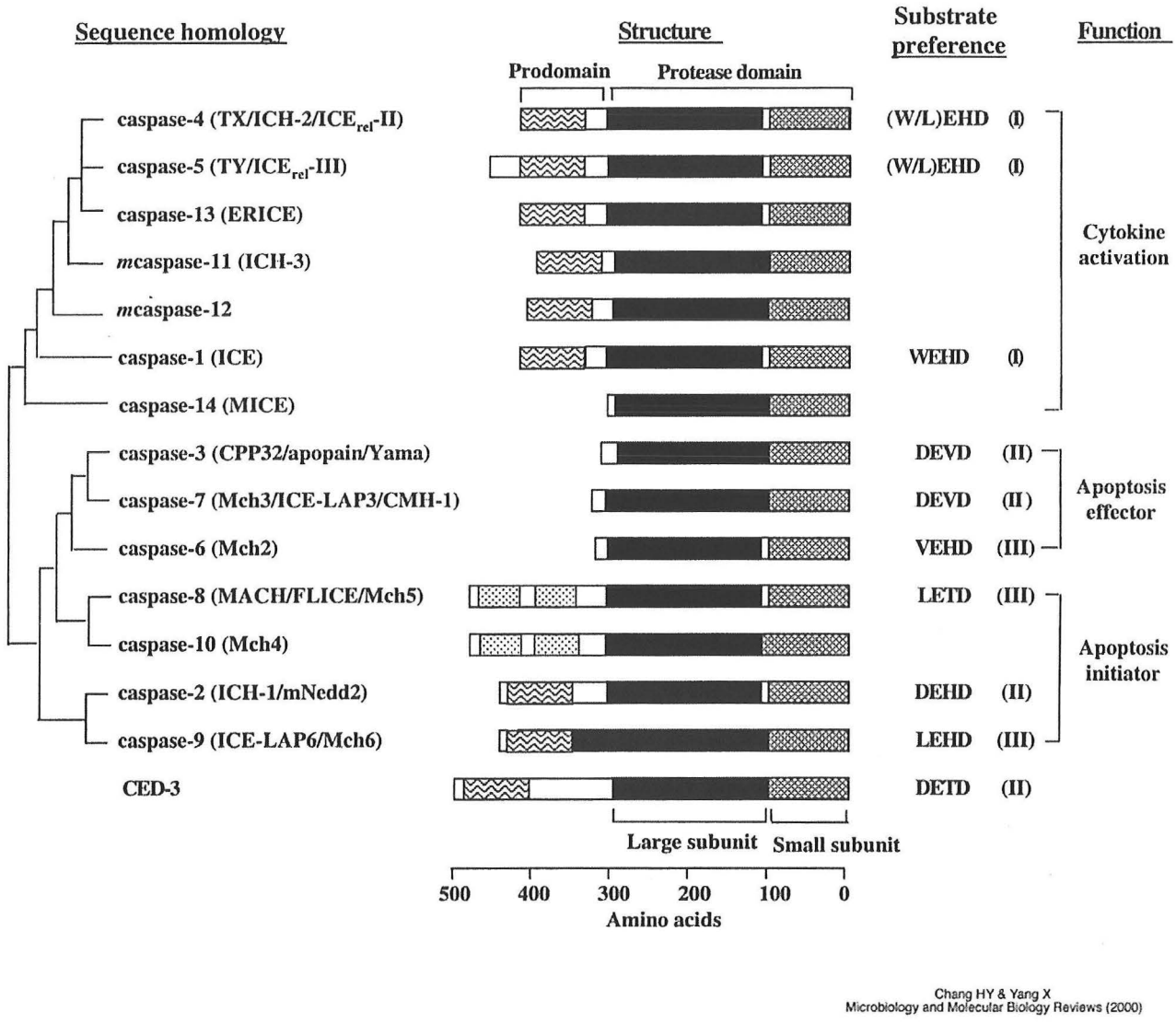


FIG. 1. Mammalian caspase family and C. elegans caspase CED-3. All mammalian caspases are of human origin except for murine caspase-11 and -12, for which no human counterparts have been identified yet. Phylogenetic relationships are based on sequence similarity among the protease domains. Alternative names are listed in parentheses after each caspase. Dotted box, DED domains; wavy boxe, CARD domain. Substrate preferences at the P1 to P4 positions are indicated. Based on the substrate specificity, caspases are divided into three groups (indicated in parentheses). Reproduced from Ref (4).

Pathways for caspase activation

To date, two major pathways for caspase activation have been described, which are headed by caspase-8 and –9, respectively [4-8]. The death receptors, such as Fas, TNFR1, Death Receptor-

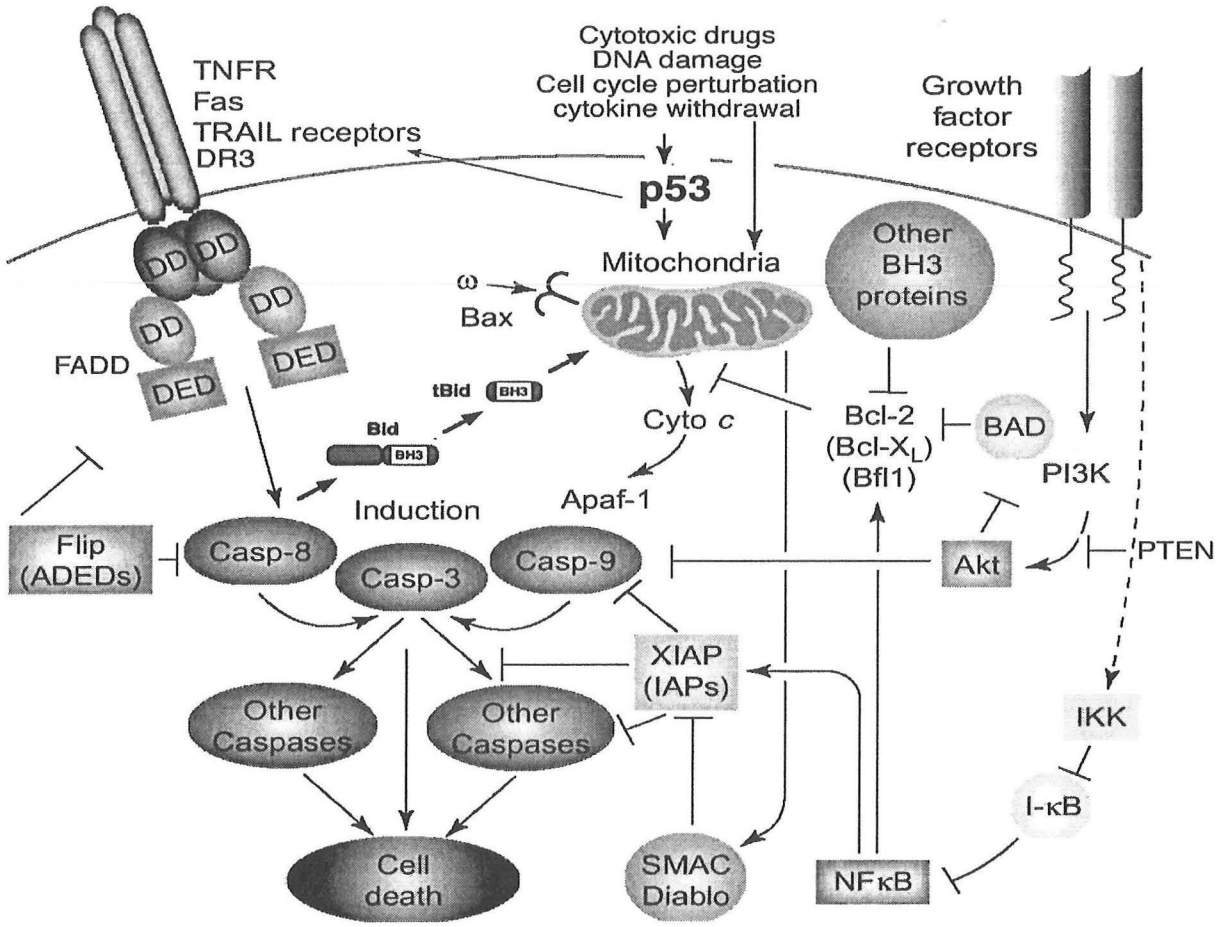


Fig. 2. Mechanisms of caspase activation and suppression. Two major pathways for caspase activation in mammalian cells are presented, the extrinsic (left) and intrinsic (right). The extrinsic pathway is triggered by members of the TNF-family of cytokine receptors such as TNFR1, Fas and TRAIL receptors. These proteins recruit adapter proteins including Fadd, to their cytosolic Death Domains (DDs), which then bind pro-caspases, particularly pro-caspase-8, that contain another protein interaction motif (the Death Effector Domain [DED] domain) that binds a complementary domain in Fadd. This pathway is under suppression by DED-containing antagonists of Fadd and pro-caspase-8, such as Flip (Anti-apoptotic DED [ADED] family proteins). The intrinsic pathway is triggered by release of cytochrome c from mitochondria, induced by various stimuli, including elevations in the levels of pore-forming pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol, cytochrome c binds and activates Apaf1, allowing it to bind and activate pro-caspase-9. Thispathway is suppressed by anti-apoptotic Bcl-2 family proteins, which prevent cytochrome c release. The anti-apoptotic Bcl-2 family proteins, in turn, are suppressed by BH3-only proteins such as BAD that heterodimerize with death-suppressors such as Bcl-2 and Bcl-XL. Other BH3-only killer proteins (not shown) are regulated by cell stimuli, including Bim (released from disrupted microtubules), Noxa (included by p53), Hrk (induced by NGF deprivation), BAD (dephosphorylated by Calcineurin during pathological Ca2+ influx). Active caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3, but may also activate other caspases. Caspase-3, in turn, cleaves and activates directly or indirectly other effector caspases such as caspases-6 and -7. Activate caspases, including caspases-3, -7, and -9 can be directly inhibited by some IAP-family proteins such as XIAP. IAPs, in turn, are suppressed by SMAC/Diablo, which is released from mitochondria. NF-B induces expression of apoptosis suppressors, including certain IAP-family genes and some anti-apoptotic Bcl-2 family genes. The kinase Akt can phosphorylate and inactivate BAD, as well as caspase-9. The schematic is an oversimplification of the events that occur in vivo. Reproduced from reference 7.

3, -4 and -5 (DR3, DR4 and DR5) trigger apoptosis via the recruitment of adaptor protein FADD, which subsequently helps in the recruitment and activation of procaspase 8. The interaction between FADD and procaspase 8 is mediated by the prodomain of procaspase 8 that contains two homologous death effector domains (DED). Activated caspase 8 leads to activation of downstream effector caspases either directly (Type I cells) or via an amplification loop involving truncated Bid (tBid) mediated release of cytochrome c from the mitochondria (Type II cells) [9-11]. On the other hand, drug-induced apoptosis is generally believed to be mediated by caspase-9. This pathway is triggered by the release of cytochrome c from the mitochondria, which forms a multi-protein apoptosome complex with Apaf-1 and procaspase 9 in the presence of dATP [8, 12, 13]. Procaspase 9 is activated upon recruitment to this complex and in turn activates the effector caspases. However, cytochrome c-mediated caspase activation may not be sufficient to lead to cell death. This is due to the presence of a family of proteins, called the IAPs (inhibitor of apoptosis protein), which can bind and inhibit the active caspases in the apoptosome. This inhibition is relieved by the release of another mitochondrial protein, called Smac/Diablo, which binds to the IAPs and release active caspases from their inhibitory influence [14, 15]. Apoptosis-inducing factor (AIF) and endonuclease G are two additional proteins which are released from the mitochondria and are involved in chromatin condensation and DNA fragmentation associated with apoptosis [16, 17]. The death receptor and mitochondrial pathways for caspase activation are also referred to as the "extrinsic" and "intrinsic" apoptosis pathways, respectively.

Bcl-2 family members are regulators of mitochondrial apoptotic signals

At least 15 Bcl-2 family members have been identified in mammalian cells and are characterized by the presence of at least one of four conserved motifs known as the Bcl-2 homology domains (BH1 to BH4) [8, 18]. These proteins can be divided into three major groups based on their function and sequence homology. Most pro-survival members contain at least the BH1 and BH2 domains. The pro-apoptotic members of this family can be further subdivided into two subfamilies based on their structure. While Bax, Bak and Bok contain BH1, BH2 and BH3 domains and resemble Bcl-2 in structure, seven other pro-apoptotic members contain only the central BH3 domain and otherwise are unrelated to any known protein [8, 18]. These BH3-only family members are usually located in other cellular compartments and translocate to the

mitochondria in response to an apoptotic stimulus where they presumably interact with Bax and Bak [8, 18]. This interaction is believed to induce a conformation change in the latter proteins leading to their oligomerization. The oligomerized Bax and Bak are believed to form a pore in the mitochondrial outer membrane from which the apoptogenic proteins, such as cytochrome c and Smac, can leak into the cytosol [8, 18]. Alternatively, these proteins may destabilize the mitochondrial outer membrane through an as yet unknown mechanism. The pro-survival Bcl-2 family members, such as Bcl-2 and Bcl-xL, are believed to block the apoptotic program by preventing the oligomerization of Bax and Bak.

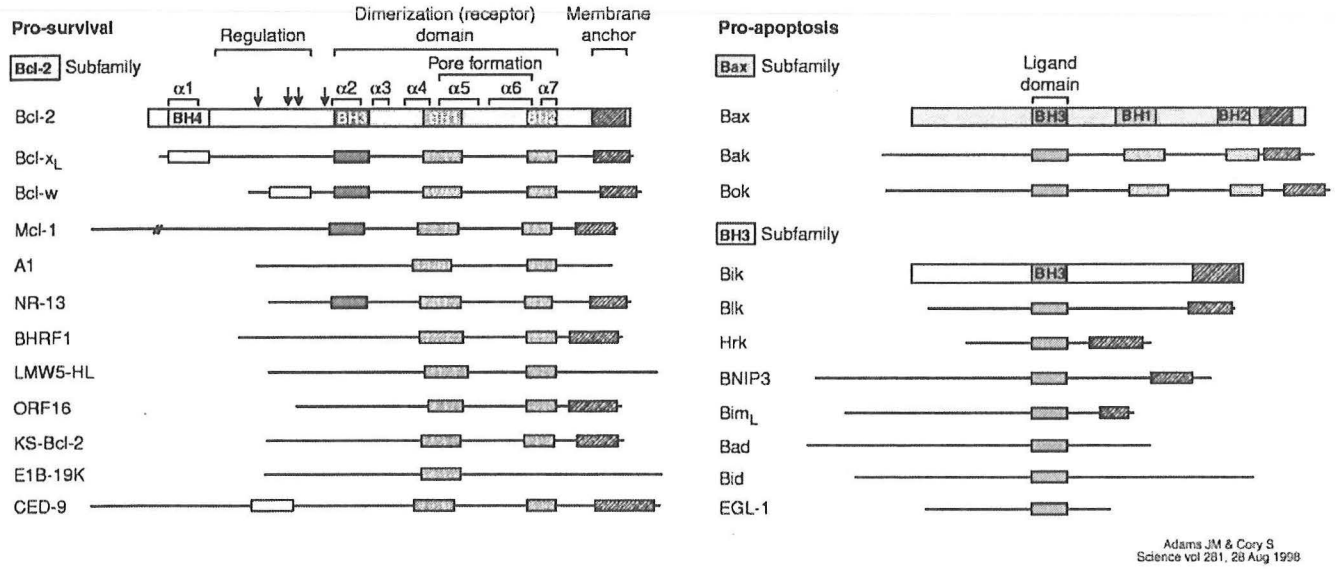


Fig. 3. The wider Bcl-2 family. Three subfamilies are indicated: The Bcl-2 cohort promotes cell survival, whereas the Bax and BH3 cohorts facilitate apoptosis. BH1 to BH4 are conserved sequence motifs. The functional domains of Bcl-2 are described in the text. The Bax subfamily resembles the Bcl-2 subfamily but lacks a functional BH4 domain. Except for the BH3 domain, the BH3 subfamily is unrelated to Bcl-2. All proteins compared are mammalian (usually human), except for NR-13 (chicken), CED-9, and EGL-1 (C. elegans), and the viral proteins BHRF1, LMW5-HL, ORF16, KS-Bcl-2, and E1B-19K. Reproduced from ref 18.

p53: The Death Star

The p53 protein is the most commonly mutated gene in human cancer and lies at the heart of a stress response pathway that prevent the growth and survival of potentially malignant cells [19]. A wide variety of cellular stress events can activate p53, such as DNA damage, oncogene activation, telomere attrition, hypoxia and loss of normal growth and survival signals. Although activation of p53 is known to induce several responses in the cells, the two best understood are its ability to induce growth arrest and apoptosis. Either of these two responses prevents

replication of cells harboring oncogenic mutations and so serves as a safeguard against tumor development [20].

Numerous p53-dependent target genes have been identified which are involved in the different downstream functions of p53. In general, each p53 response is mediated by the activation of

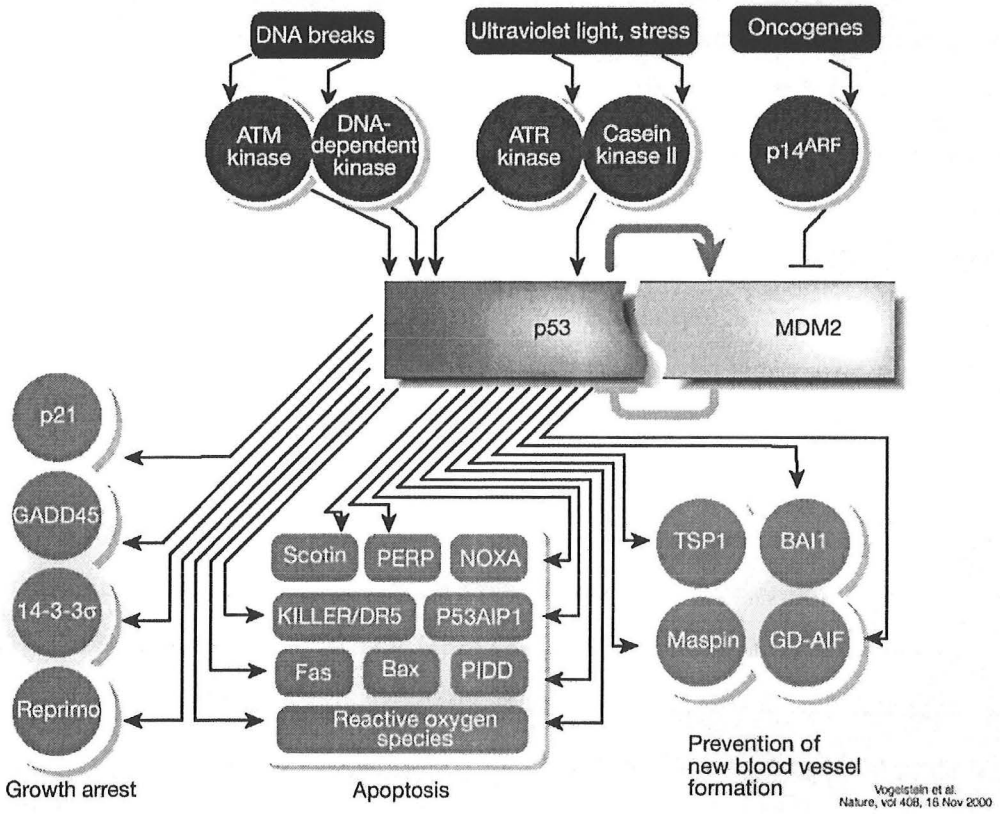


Figure 4 The p53 network. Activation of the network (by stresses such as DNA damage, ultraviolet light and oncogenes) stimulates enzymatic activities that modify p53 and its negative regulator, MDM2. This results in increased levels of activated p53 protein. The expression of several target genes is then activated by binding of the activated p53 to their regulatory regions. These genes are involved in processes that slow down the development of tumours. For example, some genes inhibit cell-cycle progression or the development of blood vessels to feed a growing tumour; others increase cell death (apoptosis). A negative feedback loop between MDM2 and p53 restrains this network. Many other components of this network, not shown here, have been identified. Similarly, p53 activation results in a variety of other effects, including the maintenance of genetic stability, induction of cellular differentiation, and production of extracellular matrix, cytoskeleton and secreted proteins. The components of the network,and its inputs and outputs, vary according to cell type. p53 is a highly connected 'node' in this network. It is therefore unsurprising that the loss of p53 function is so damaging, and that such loss occurs in nearly all human cancers. Reproduced from 21.

several genes. In the case of apoptosis, p53 is believed to induce the expression of genes involved in both the intrinsic and extrinsic apoptotic pathways [21-28]. p53-controlled genes that

can trigger cytochrome c release from the mitochondria include the proapoptotic Bcl-2 family members Bax and Bak, and inhibitors of anti-apoptotic Bcl2 family members Noxa, p53AIP1 and PUMA [25-27]. Interestingly, a recent study suggested that Apaf1 is a downstream target of p53 as well [29]. Among the genes belonging to the extrinsic pathway, p53 induces the expression of the death receptors Fas/CD95 and DR5/TRAIL-R2/Killer [30, 31]. A p53-induced increase in the expression of the death receptors may be a potential mechanism by which transformed cells are more susceptible to killing by the immune system [31].

Survival pathways

Somatic cells in multicellular organisms are totally dependent for their survival upon the continuous availability of trophic factors [32]. Despite a wide array of trophic factors and receptors, many of these receptors use common intracellular signaling pathways to promote cell survival. Four pathways that have been extensively characterized for their anti-apoptotic role are the phosphatidylinositol 3-kinase (PI3K)/Akt, the Nuclear factor kappa B (NF-κB), the Ras/mitogen-activated protein kinase, and the Jak/signal transducers and activators of transcription (STAT) pathways [32]. The anti-apoptotic effect of the PI3K/Akt pathway is believed to be mediated via the phosphorylation of its target proteins, such as BAD, procaspase 9, Forkhead transcription factor and Mdm2 [32, 33]. Several recent studies have documented the protective effect of the NF-κB pathway against apoptosis induced by the death receptors, chemotherapeutic drugs and irradiation [34-39]. These effects are mediated via the transcriptional upregulation of genes such as TRAF1 and 2, cIAP1 and 2, Bcl-2 homolog A1/Bfl-1, IEX-1 and XIAP [40-42].

Role of apoptosis in prevention of cancer

Rarity of cancer

Cancers are diseases of uncontrolled proliferation of somatic cells which kill by invading, eroding and destroying the surrounding normal tissue repair [20, 43]. A common defect fueling this deregulated cellular proliferation involves mutations in genes which normally control various aspects of growth in multicellular organisms. In large, long-lived multicellular organisms, such as man, there exists a substantial and continuous need for cellular proliferation for development as well as maintenance and repair [20, 43]. This life-long need for cellular

proliferation has to be balanced against the constant threat of cancer arising as a result of deregulated growth caused by mutations in the same genes which control normal cellular proliferation repair [20, 43]. Thus, the organism must find a way to allow cellular proliferation only when needed while effectively suppressing this activity at other times. The rarity of cancer highlights the remarkable efficiency with which this goal is normally achieved. With an estimated mutation rate of 1in 2 X 10^7 per gene cell division, some 10^{14} target cells in the average human and the large number of genes controlling various aspects of cellular growth, it is remarkable that cancer arise on average less than once in every three lifetimes repair [20, 43]. This is even more striking when one considers that oncogenic mutations foster the clonal expansion of the affected cell, thereby increasing the probability of further oncogenic events.

Cell proliferation and apoptosis are coupled [20, 43]

One of the main mechanism by which organisms protect themselves against the development of cancer from deregulated activity of oncogenes is by coupling cellular proliferation to apoptosis repair [20, 43]. Increased tendency to apoptosis in cells transformed by oncogenes was noticed in many early studies and was initially attributed to the fact these proteins force cells into "unprepared" cell cycle by overriding cell cycle checkpoints and inducing "mitotic catastrophe" [20, 43]. However, more recent studies, based on the genetic and molecular analysis of the processes of cell division and apoptosis, favor the hypothesis that cell proliferation and apoptosis are coupled — the tendency of cells to undergo apoptosis is a normal consequence of engaging the cell's proliferative machinery [20, 43].

One of the best examples of an oncogene with pro-apoptotic potential is *c-myc*. Deregulated expression of *myc* genes is frequently seen in many forms of human cancer and the growth promoting ability of Myc protein is well documented. However, Myc is also a powerful inducer of apoptosis, especially under conditions of stress, genotoxic damage or depleted survival factors, which has led to the hypothesis that the innate apoptotic potential of Myc serves as an in-built foil to its oncogenic capacity [20, 44-46]. A remarkable proof of this hypothesis was provided by the ability of Bcl-2 to accelerate formation of B cell lymphomas in Myc transgenic mice by enhancing lymphocyte survival, not by further stimulating their Myc-induced proliferation [47, 48]. Myc-induced apoptosis in fibroblasts grown in low serum is also

suppressed by a survival signaling pathway triggered by IGF-1, which routes through Ras and PI3K/Akt, and leads to phosphorylation of Bad as well as by disruption of Fas signaling [49].

Another example of a growth promoting oncoprotein with potent pro-apoptotic ability is the adenoviral E1A protein [20]. During normal adenovirus infection, E1A causes accumulation of p53 which would cause apoptosis and abrogate viral replication were it not for the anti-apoptotic products of the E1B genes [20, 50]. Both the growth promoting and pro-apoptotic functions of E1A have been mapped to its NH2-terminal region, further supporting the argument that these activities are linked [51].

In addition to deregulation of dominant oncogenes such as Myc and E1A, cancers also arise due to functional inactivation of growth suppressive regulatory pathways. A good example of such a growth suppressive pathway which is frequently disrupted in human cancer is one involving the retinoblastoma (Rb) protein [52]. The Rb protein is a key regulator of the cell cycle by virtue of its ability to bind and inactivate the E2F protein, a transcription factor involved in the activation of several genes necessary for progression through G1 to S phase. Cells lacking Rb or those with mutations in E2F that prevents its interaction with Rb demonstrate accelerated S phase entry which is however accompanied by increased apoptosis [53-56]. Consistent with the above in vitro results, transgenic mice with functional inactivation of Rb gene in the choroid plexus develop slowly growing microscopic tumors with high apoptotic rates. However, crossing of these animals with p53 -/- null mice led to rapidly growing tumors with low apoptotic rates [3, 57, 58].

Implications for cancer biology and therapy [20, 43]

Although the exact mechanism of Myc-induced cell death is unclear, it is believed to sensitize cells to a wide range of mechanistically different triggers of apoptosis including DNA damage, nutrient deprivation, interferon, hypoxia, and death ligands such as TNF, FasL and TRAIL [46]. As many of these agents exert no direct effect on cellular proliferation, these results argue against the hypothesis that oncogene-induced apoptosis is the result of an intracellular growth conflict [20, 43]. Furthermore, as many of the above stimuli are also encountered by the incipient tumor cells, these results support to the hypothesis that growth-deregulating mutations sensitize

cells to a wide variety of apoptotic triggers, which unless counterbalanced by appropriate survival signals automatically remove the affected cells [20, 43] (Fig. 5). Because such survival signals are usually provided by the neighboring cells, this ensures that somatic cells remain mutually interdependent for survival, thereby limiting the proliferative autonomy of any single cell [20, 43]. Moreover, since such signals are usually available only within discrete somatic environments, this effectively traps the somatic cells within specialized trophic microenvironments in the body and thus acts as a major defense against metastatic spread [20, 43].

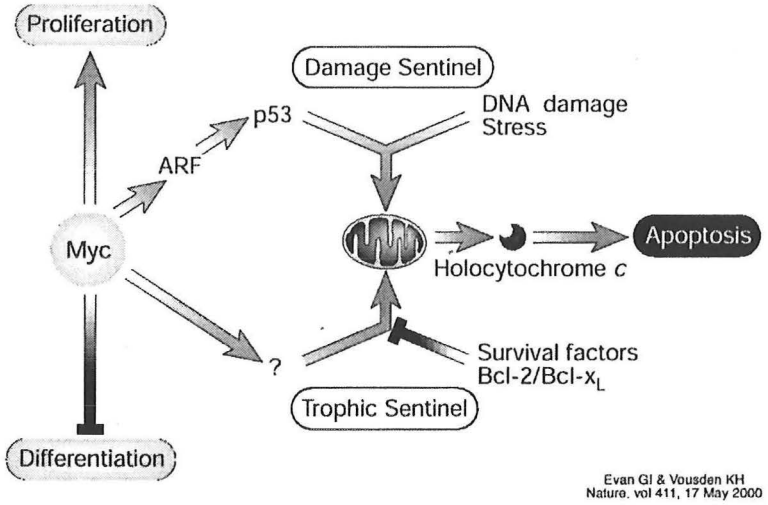


Figure 5 Activation of growth-deregulating lesions triggers 'sentinel' functions that guard the cell against acquiring mutations or propagating into an inappropriate somatic compartment. The more powerful and persistent the growth signal, the more potent and persistent the sentinel function. In this example, the oncoprotein Myc is shown activating a p53 damage sentinel through the ARF/MDM-2 pathway, thereby sensitizing the cell to any DNA damage. Myc also promotes release of holocytochrome c from the mitochondrion into the cytosol where it triggers apoptosis. Release of holocytochrome c is inhibited by paracrine 'survival' signals that are typically restricted both in supply and location. Clonal outgrowth driven by relentless Myc expression outstrips survival factor availability, triggering the 'trophic sentinel' to kill the cell. Reproduced from 43.

The sensitization to apoptosis induced by oncogenes remains the most significant Achilles' heel of the cancer cells and explains the remarkable sensitivity of most primary cancer cells to cancer chemotherapy drugs as compared to their normal counterparts, a fact exploited by most classical cancer therapeutics [20, 43]. Unfortunately, the processes that govern the malignant transformation and tumor progression are evolutionary in nature. As such, this therapeutic window is eventually eroded by further mutations in genes critical to the cell's apoptotic

response, a phenomenon further hastened by the underlying genomic instability inherent to cancer cells [20, 43].

Evading Apoptosis: a hallmark of cancer

In recent years, genetic alterations in a number of genes involved in the regulation of apoptosis have been identified in different disease conditions including various forms of human cancers and have led to the realization that acquired resistance towards apoptosis is a hallmark of most and perhaps all types of cancers [3, 66]. While a comprehensive review of all mutations in apoptosis related genes is beyond the scope of this discussion, the following examples will illustrate the importance of this pathway to the natural history of cancer.

Genetic alteration leading to the disruption of the extrinsic (death receptor) pathway

Neuroblastoma (NB) is a childhood tumor that accounts for approximately 10% of pediatric cancers. The best known genetic abnormalities in NB are the amplification of *MYC*N (33%) and loss of heterozygosity (LOH) and translocations involving several chromosome regions, such as 1p36 (26%), 2q33 (30%), 11q (24%), 14q (22%) and 18q (31%) [59, 60]. Although NB is usually responsive to chemotherapy at diagnosis, recurrent disease is often chemoresistant and is associated with *MYC*N amplification [59]. Amplification of MYCN is also frequent in aggressive, high stage and invasive NB tumors in older children (N-type) which are usually chemoresistant and is usually absent in low-stage, non-invasive tumors in younger children (S-type) that frequently undergo spontaneous remission [59, 61, 62]. Since caspase-8 gene is located at 2q33, a region of frequent LOH in neuroblastoma, recent studies have examined the expression of this protein in NB [59, 61, 62]. Interestingly, loss of caspase 8 message and protein was frequently seen in NB cell lines (13 out of 18) and was strongly correlated (13 out of 13) with MYCN amplification. Furthermore, 67% of patient tumor samples with MYCN amplification also demonstrated loss of caspase 8 expression. Methylation of caspase 8 promoter region was the primary mechanism by which its expression was silenced in both the cell lines and tumor samples [59, 60].

Recently, in collaboration with the laboratory of Dr. Adi Gazdar, we have examined the status of caspase 8 in lung cancer cell lines and tumor samples and discovered that its expression is lost in most (27 of 34, 79%) of small cell lung cancer (SCLC) cell lines but was retained in 22 non-

SCLC (NSCLC) cell lines [63]. In addition, based on the methylation of caspase 8 promoter, we estimated that approximately 58% SCLC, 30% bronchial carcinoids and 0% NSCLC lack caspase 8 expression [63]. However, alternative mechanism of apoptosis evasions may be operative in NSCLC as well. For example, a high fraction of lung and colon cancer cell lines were found to upregulate the expression of a decoy receptor for the Fas ligand, which could block apoptosis by competing with the Fas death receptor for binding to the ligand [64].

Genetic alterations leading to the disruption of the intrinsic (mitochondrial) pathway

A typical example of a genetic alteration in cancer leading to the disruption of the intrinsic death pathway is one involving the Bcl-2 gene [65, 66]. Most follicular lymphomas are characterized by a t(14:18) translocation involving the bcl-2 locus on chromosome 18 and the heavy chain immunoglobulin (Ig) segments on chromosome 14. In addition, in approximately 3-10% of chronic lymphocytic leukemias, the 5' flanking region of Bcl-2 gene is rearranged and linked to the Ig light chain genes in chromosome 2 or 22. In both cases, the chromosomal re-arrangements result in the deregulation and over-expression of Bcl-2 protein, which protects cells from apoptosis. However, the presence of Bcl-2 translocation is not sufficient for the development of lymphoma, which requires additional genetic alterations [65].

Metastatic melanoma presents another example of a cancer with defects in the mitochondrial death pathway. These highly lethal cancers are highly drug resistant despite the presence of wild-type p53 gene. A recent study suggests that Apaf-1 expression is frequently lost in metastatic melanoma cell lines and correlates with their resistance to anticancer drugs-induced apoptosis [67].

Genetic alterations leading to loss of p53

Although resistance to apoptosis can be acquired by cancer cells through a variety of means, one of the most common mechanism is via mutations involving the p53 tumor suppressor gene [68, 69]. p53 mutations are the most common genetic abnormality found in human cancers and the p53 pathway is involved in the vast majority of tumors without mutations in p53, which attests to its pivotal role as a bulwark against the expansion of transformed cells [68, 69].

Mechanism of inactivating p53	Typical tumours	Effect of inactivation
Amino-acid-changing mutation in the DNA-binding domain	Colon, breast, lung, bladder, brain, pancreas, stomach, oesophagus and many others	Prevents p53 from binding to specific DNA sequences and activating the adjacent genes
Deletion of the carboxy-terminal domain	Occasional tumours at many different sites	Prevents the formation of tetramers of p53
Multiplication of the MDM2 gene in the genome	Sarcomas, brain	Extra MDM2 stimulates the degradation of p53
Viral infection	Cervix, liver, lymphomas	Products of viral oncogenes bind to and inactivate p53 in the cell, in some cases stimulating p53 degradation
Deletion of the $p14^{ARF}$ gene	Breast, brain, lung and others, expecially when p53 itself is not mutated	Failure to inhibit MDM2 and keep p53 degradation under control
Mislocalization of p53 to the cytoplasm, outside the nucleus	Breast, neuroblastomas	Lack of p53 function (p53 functions only in the nucleus)

Vogelstein et al
Nature, vol 408, 16 Nov 2000

There are several reasons why human cancers show such a high incidence of p53 functional inactivation. First, in addition to apoptosis, p53 is a strong transducer of growth arrest, which after certain types of DNA damage is irreversible; although alive, such cells are genetically dead [20]. Second, as discussed above, p53 is activated in response to a large number of stresses encountered during carcinogenic progression ranging from oncogenic deregulation at the early stage to hypoxia when the tumor reaches macroscopic size [43]. Thus, there is a strong pressure for the tumor cells to loose p53 function. Third, p53 is a major determinant of sensitivity of cells to chemo- and radiotherapy. For example, tumors that frequently contain p53 mutations (melanoma, lung cancers, colorectal tumors, bladder and prostate cancers) often respond poorly to radiation and chemotherapy while those with wild-type p53 (childhood ALL and germ cell tumors) respond well [66]. However, when the latter tumors relapse, they frequently demonstrate p53 mutations. Finally, p53 mutations are acquired during transformation to a more aggressive disease state, e.g. from chronic phase CML to blast crisis and from indolent follicular lymphoma to diffuse aggressive form [66].

Genetic alterations leading to activation of survival pathways in cancer

An alternative mechanism by which tumors escape apoptosis is via the activation of the survival pathways. Genetic alterations leading to the activation of the PI3-K/Akt pathway are a common mechanism of apoptosis evasion during tumorigenesis as examplified by activating mutations in

both the upstream and downstream components of this pathway in a wide variety of human cancers [70]. For example, amplification of members of the receptor tyrosine kinase family capable of activating PI3-K such as platelet-derived growth factor receptor (*PDGFR*) and epidermal growth factor receptor (*EGFR*) genes have been demonstrated in glioblastoma [71, 72]. Similarly, overexpression of Akt/PKB has been demonstrated in a proportion of ovarian and breast cancers. However, the most frequent cause of activation of the PI3-K/Akt pathway in human cancers is via the loss of tumor suppressor PTEN, which is mutated in a high proportion of high grade glioblastomas, prostate, thyroid, breast and endometrial cancers [73]. In addition to the above sporadic tumors, germline mutations of PTEN are associated with three autosomal dominant familial cancer syndromes with increased susceptibility for benign hamartomas throughout the body early in life, as well as increased incidence of cancers of the breast, thyroid and brain [73].

The NF-κB pathway represents another survival pathway that is frequently activated during the development of cancer [42, 74]. Nuclear factor-kappa B (NF-κB) is a transcription factor composed of heterodimers of at least five sub-units. Interestingly, chromosomal rearrangements and amplificatons have been detected in the regions of the genome containing most of the NF-κB subunits in various lymphoid malignancies.

Gene	Alteration	Type of cancer
Hematopoietic tumors		
v-rel	Overexpression	Avian & muiine ymphoma, myeloma
c-rel	Amplification	Diffuse & follicular large cell lymphoma
	Rearrangement/ overexpression	Primary mediastinal B-cell lymphoma
	Rearrangement/ overexpression	Diffuse large cell lymphoma
	overexpression	Avian B- & T-cell lymphoma
rela	Rearrangement,	non Hodgkin's lymphoma, myeloma
	Amplification	Diffuse large cell lymphoma
	Constitutive NF-κB activity	Hodgkin's disease
nfkb1	Rearrangement	Acute lymphoblastic leukemia
nfkb2	Rearrangement/ overexpression	Cutaneous T-cell lymphoma
	Rearrangement	non Hodgkin's lymphoma, myeloma
	Rearrangement	B-cell chronic lymphocytic leukemia
Solid tumors		
c-rel	Overexpression	Non-small cell lung carcinoma
rela	Amplification	Various solid tumors
	Splicing variant	Non-small cell lung carcinoma
nfkb1	Overexpression	Non-small cell lung carcinoma
nfkb2	Overexpression	Breast & colon carcinoma

Table 2: Genetic alterations of NF-κB subunits in human cancers (References 42, 74)

Similarly, mutations in the gene encoding IκBα, a protein which binds and retains NF-κB in an inactive form in the cytoplasm, have been detected in Hodgkin's lymphoma and are suggested to contribute to constitutive active NF-κB seen in this disease [75]. Constitutive NF-κB activation has been also linked to cellular transformation by several oncogenic viruses. For example, Tax protein of human T-cell leukemia virus (HTLV-1) and the latent membrane protein (LMP) of the Epstein-Barr virus (EBV) are believed to induce cellular transformation via the activation of this pathway [42, 74]. We have recently demonstrated that orf-K13, a protein encoded by the Human herpes virus 8, is a strong inducer of NF-κB and may be responsible for the constitutive NF-κB activation seen in primary effusion lymphoma cells [76]. Finally, increased NF-κB activity has been implicated in the pathogenesis of several solid tumors, such as breast, colon, pancreatic, thyroid and bladder cancers [77].

Therapeutic Agents targeting the apoptosis pathway

Agents targeting the death receptor pathway

TRAIL: Although several ligands of the TNF family, such as TNFα, FasL, LTα, can induce apoptosis in transformed cells, the potential utility of these agents in the treatment of cancer is limited by their acute systemic toxicity. TRAIL (TNF-Related Apoptosis Inducing Ligand, also called Apo2L) is a relatively new member of the TNF family and induces apoptosis via binding to two distinct receptors, designated Death Receptors 4 and 5 (DR4 and DR5), respectively. TRAIL has been shown to induce cytotoxic or cytostatic effects *in vitro* in a number of transformed cell lines of (32 out of 39) while having no toxicity on a majority of non-transformed cells [78, 79]. Treatment of athymic mice bearing solid tumors with TRAIL/Apo2L induced tumor cell apoptosis, reduced progression and improved survival. Of particular interest was the lack of detectable toxicity after repeated intravenous injections of Apo2L in non-human primates [78]. Finally, TRAIL/Apo2L had a synergistic antitumor effect when combined with the chemotherapeutic drugs 5-fluorouracil and CPT-11 [78]. Further pre-clinical studies of TRAIL/Apo2L are in progress to determine its safety when administered over longer time periods and to define the doses suitable for clinical studies.

However, TRAIL/Apo2L may be already playing a significant role in the anti-cancer activity of agents currently in clinical use. A recent study suggested that ATRA (all-*trans* retinoic acid)

induces apoptosis of acute promyelocytic leukemia blasts via the induced expression and paracrine action of TRAIL [80]. Exogenous addition of TRAIL was able to induce apoptosis in APL cells as well as an ATRA-resistant cell line, suggesting that TRAIL may have a role in the treatment of both ATRA-sensitive and -resistant APL. In addition to inducing the expression of TRAIL, ATRA is also known to induce the expression of caspase 8, which might facilitate TRAIL-mediated apoptosis of leukemic cells. Since cancer chemotherapeutic drugs are known to induce the expression of the TRAIL receptor DR5, the above results may provide a molecular explanation for the improved outcome of APL patients treated with ATRA plus chemotherapy. In addition to ATRA, TRAIL is also induced by interferons and is believed to play a major role in the anti-tumor activity of these cytokines against a number of human cancers [81-83].

Caspase 8 activators: MRIT/cFLIP is a catalytically inactive homolog of caspase 8, which competes with caspase 8 for binding to the DISC. High level expression of MRIT/cFLIP has been shown to protect cells against death receptors-induced apoptosis. CDDO (2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid) is a synthetic analogue of the naturally occurring triterpenoids present in herbal remedies and has potent antiproliferative and antiinflammatory activities [84]. Several studies suggest that CDDO induces apoptosis via the activation of caspase 8 and sensitizes cells to TRAIL-induced apoptosis , which have been recently linked to the downregulation of MRIT/cFLIP expression [85-87].

Agents targeting the mitochondrial pathway

Agents targeting Bcl-2: Bcl-2 is an attractive therapeutic target because of its overexpression in a wide variety of human cancers and its association with resistance to chemo- and radio-therapy. One strategy to block the expression of Bcl-2 is through the use of anti-sense oligonucleotides. Antisense oligonucleotides targeting *Bcl-2* mRNA have been shown to induce apoptosis in leukemia cell lines and to eradicate tumor in a severe combined immune deficient mouse model of human Non-Hodgkin's lymphoma. Based on these encouraging results, clinical trials of a Bcl-2 antisense oligonucleotide (G3130; Genta Inc) have been started [88-92]. In 21 heavily pretreated patients with Bcl-2 positive relapsed NHL, a 14 day subcutaneous infusion of G3139 resulted in one complete response, 2 partial responses and 8 patients with stable disease. Bcl-2 protein level was reduced in seven out of 16 assessable patients [91]. The only notable toxicity

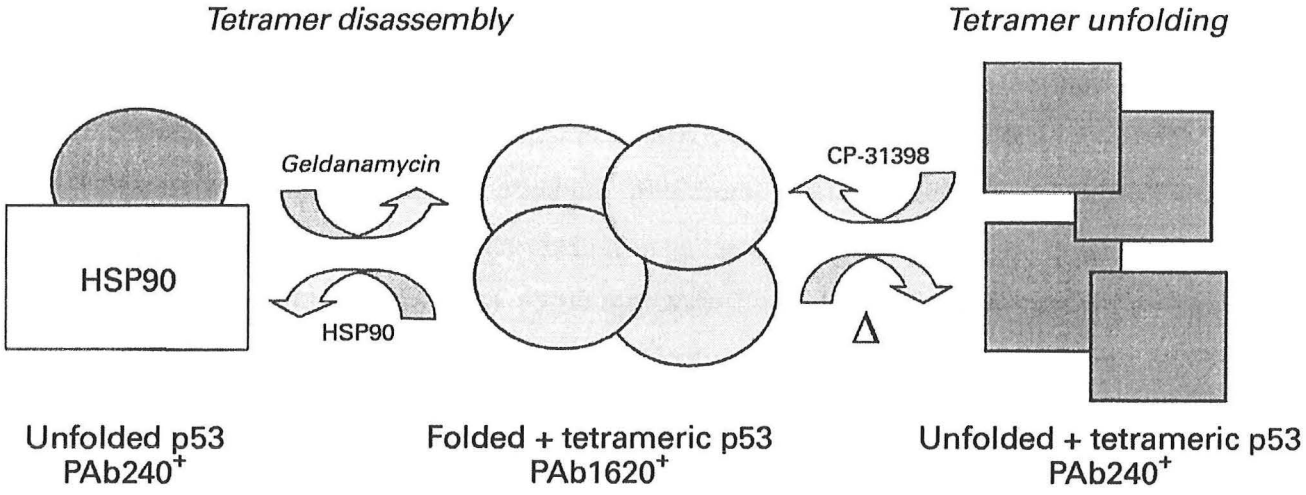
was thrombocytopenia. Clinical trials are currently underway to test the efficacy of G3139 in combination with chemotherapy in several malignancies besides NHL [89, 90, 92]. Alternative approaches to inhibiting the function of Bcl2, via the use of synthetic compounds which can directly bind to Bcl-2 protein or those that mimic BH3-only class of death agonists, have been described as well [93, 94].

Agents targeting Smac/Diablo: Smac/Diablo may provide another attractive target for drug development [8, 95]. Mutational analysis has revealed that the N-terminal four aminoacids (Ala-Val-Pro-Ile) of the mature Smac/Diablo protein are required for binding to XIAP and activating the apoptosome [95]. These four aminoacid residues pack into a small surface groove of 892Å, which suggests that a small molecule could be designed to mimic Smac activity and activate caspases selectively in cells with elevated levels of IAPs [95]. Recent studies demonstrating the ability of a seven aminoacids peptide containing the above four residues (NH2-Ala-Val-Pro-Ile-Ala-Gln-Lys-COOH) to activate caspase activity indicate that Smac/Diablo may turn out to be an attractive target for cancer drug discovery [8, 95].

Agents targeting the p53 pathway

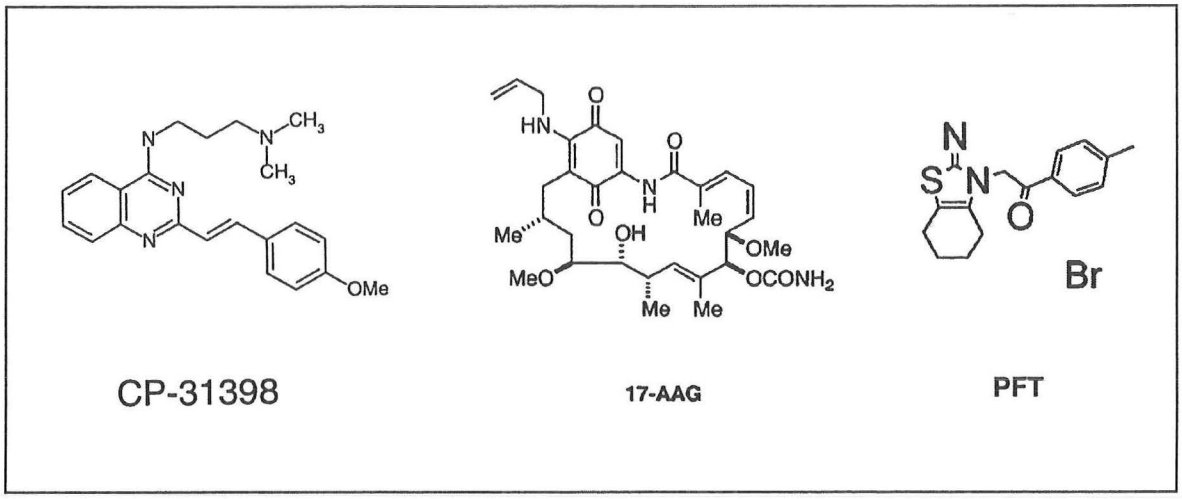
The tumor suppressive activity of p53 is absolutely dependent on its ability to act as a sequence specific transcription factor, which, in turn, is dependent on the ability of this protein to assemble into rigid three-dimensional tetramers. Most p53 mutations found in human cancer are present in its central DNA binding domain (DBD). These mutations reduce the thermodynamic stability of the protein, resulting in the production of a transcriptionally inactive unfolded protein (108) (Fig. 6). Screening for agents which could stabilize the active conformation of p53 has led to the isolation of a class of compounds which could restore p53 function in cells with mutated p53 [96]. The lead compound of this class, CP-31398, not only turned on the transcription of p53-responsive genes in cells with mutant p53 but also inhibited the growth of tumor xenografts in mice without significant toxicity [96]. Studies are in progress to generate derivatives of the above compounds with improved specificity and potency. If successful, this class of compounds may have great potential in the treatment cancers with mutated p53 either alone or in combination with classical chemotherapeutic agents or TRAIL.

An alternative approach to manipulating the p53 function involves targeting HSP90, a component of the molecular chaperone holoenzyme complex involved in protein folding and repair or degradation of damaged polypeptides (Fig. 6). Recent studies suggest that a HSP90-dependent pathway is involved in the unfolding of mutant p53. Moreover, the HSP90-mutant p53 complex has increased stability, which results in the accumulation of the mutant protein. Geldanamycin is an antitumor antibiotic which is an inhibitor of HSP90 [97]. Geldanamycin was shown to not only reduce the level of mutant p53 but also promote its refolding into the native tetrameric conformation. 17-AAG (17-allylamino geldanamycin), an analog of geldanamycin, is currently under Phase I trials at NCI and Memorial Sloan Kettering Cancer Center [97]. Finally, several gene-therapy based strategies to kill p53-deficient cells have been described as well [43, 108].



Hupp TR et al.
Biochem J. (2000) 352, 1-17

Figure 6 Pharmacological manipulation of p53 protein conformation in cancer cells
P53 protein can be assembled into native active tetramers, which bind to the conformationally sensitive monoclonal antibody PAb1620+, whereas unfolding and inactivation of the p53 tetramer can be quantified using the monoclonal antibody specific for unfolded p53 protein [monomer (ellipse) or tetramer (squares)], PAb240+. Two distinct classes of organic compounds have been shown to preserve the native PAb1620+ conformation of p53 in vitro and in vivo. One class of compounds, comprising the benzoquinone ansamycin class of antitumour fungal antibiotics (geldanamycin), inhibits the HSP90-dependent chaperone holoenzyme complex unfolding of p53 protein and forms a precedent for developing therapeutically relevant agents that modulate chaperone-dependent anti-apoptotic pathways. A second class of compounds (prototype being CP-31,398) presumably binds directly to native p53 protein and prevents its unfolding and inactivation by stabilizing the intrinsic thermoinstability (Δ) of the tetramer. Reproduced from ref 108.



In addition to its useful role in inducing apoptosis in tumor cells, p53 also plays a critical role in the induction of apoptosis in normal tissues in response to cancer chemotherapy and radiation therapy. As such, selective inhibition of p53 function in tissues, which are sensitive to chemo- and radiation-therapy, may be an attractive approach to limiting the side-effects of cancer therapies, particularly during the treatment of p53-deficient tumors. Komarov et al recently isolated a synthetic inhibitor of p53, designated pifithrin-α (PFT), which could reversibly block p53-induced transcriptional activation and apoptosis [98, 99]. PFT was shown to protect mice from lethal genotoxic stress associated with anticancer treatment while demonstrating no protective effect against p53-deficient tumors [98]. Studies are currently in progress to test whether local application of PFT can protect against chemotherapy-induced hair loss and gastrointestinal toxicity [99]. Finally, PFT may have utility in protecting against tissue damaged caused by other forms of stresses in which p53 has been implicated, such as hypoxia (cardiac and brain ischemia) and hyperthermia (burns and fever) [99].

Agents targeting the survival pathways

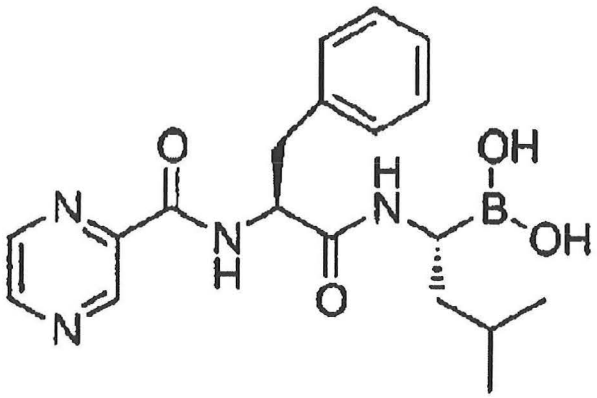
PI3-k/Akt pathway inhibitors

Since deregulated expression or activity of the growth factor receptors belonging to the protein tyrosine kinase family is a frequent cause of PI3-k/Akt over-activity in human cancers, agents inhibiting the activity of these receptors represent an effective approach to blocking the PI3-k/Akt pathway. HER2/ERB-2 is a member of the epidermal growth factor receptor family that is frequently overexpressed in breast (30%) and is associated with shortened time to relapse and poor overall survival. Herceptin® (Trastuzumab) is a humanized anti-HER2 antibody which has

shown clinical activity alone or in combination with chemotherapy in HER2 positive metastatic breast cancer [100]. Although the mechanisms behind the biological effects of Herceptin® are incompletely understood, downregulation of PI3-k/Akt pathway is believed to play a major role in its activity [101]. Interestingly, the PI3-k/Akt pathway has been known to protect cells against TRAIL-induced apoptosis, and combination of Herceptin® have been shown to enhance TRAIL-mediated apoptosis in breast and ovarian cancer cell lines that overexpress HER2 [102]. Other examples of agents in pre-clinical development or in active clinical use with major activity against the PI3-k/Akt pathway include ZD1839 (Iressa), which inhibits the Epidermal Growth Factor Receptor (EGFR or Erb1) [103], STI571 (Gleevec), which inhibits the Bcr/Abl tyrosine kinase and the various farnesyltransferase I inhibitors, which target the Ras family members [104].

NF-κB pathway inhhibitors

Under normal conditions, NF-κB is sequestered in the cytoplasm due to its association with the inhibitors of kappaB (IκB) proteins. The stimulators of NF-κB lead to phosphorylation of IκB proteins, which mark them for ubiquitination and subsequent degradation. The degradation of IκB proteins is carried out by the 26S proteasome, a multi-catalytic protease, responsible for the majority of protein turnover in the cells. PSI-341 is a selective proteasome inhibitor, which has



PS-341

potent anti-proliferative and pro-apoptotic activities against a large number of cancer cell lines *in vitro* and in tumor xenograft models *in vivo [97]*. PSI-341 has been shown to inhibit NF-κB activation induced by exposure to cancer chemotherapeutic drugs, leading to synergistic cancer killing [97]. In early clinical studies, PSI-341 has been well tolerated and has shown promising

biological activity in both hematological and solid tumor malignancies [97]. Inhibition of the NF-κB pathway may also contribute to the anti-cancer activities of thalidomide and arsenic trioxide, respectively [105-107].

Conclusions

The past decade has been a period of intense and exciting research in the field of apoptosis and has witnessed rapid advances in our understanding of the molecular biology and biochemistry of this process. This research has also led to a new appreciation of apoptosis not only as a major defense against the development of cancer but also as a key determinant of response to cancer therapy. However, the number of apoptosis related genes is still on the increase and it is likely that genetic alterations in several of them will be linked to different human cancers. A number of drugs targeting various molecules of the cellular apoptosis machinery are in various stages of clinical development and this number is also likely to increase in the future. The challenge ahead will be to tailor these molecular-targeted agents to specific genetic alterations in cancer, so as to take maximum advantage of the inherent sensitivity of cancer cells to apoptosis.

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