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REVIEW ARTICLE

Apoptosis in cancer: Key molecular signaling pathways and therapy targets

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Abstract
Apoptosis is a physiological process vital for embryologic development and the maintenance of homeostasis in multicellular organisms, but it is also involved in a wide range of pathological processes, including cancer. In mammalian cells, apoptosis has been divided into two major pathways: the extrinsic pathway, activated by proapoptotic receptor signals at the cellular surface, and the intrinsic pathway, which involves the disruption of mitochondrial membrane integrity. Although many of the proteins vital for apoptosis have been identified, the molecular pathways of cellular death still remain to be elucidated. This review provides references concerning the apoptotic molecules, their interactions, the mechanisms involved in apoptosis resistance, and also the modulation of apoptosis for the treatment of cancer.

Programmed cell death (PCD) is a major component of normal development, the maintenance of homeostasis, and elimination of damaged cells in unicellular and multicellular organisms. Depending on morphological alterations and caspase involvement, cell death can be classified into different types: apoptosis, autophagy, necrosis, mitotic catastrophe, paraptosis, and slow cell death [1].

Apoptosis, a controlled and energy-dependent process, is the best-described form of programmed cell death. Its deregulation can lead to cancer, autoimmune and degenerative diseases, explaining the increasing interest in elucidating the apoptosis pathways for disease etiology and therapeutic modulation. Many of the morphological changes that cells undergo during apoptosis have been observed by light and electron microscopy, including cell shrinkage and chromatin condensation with pyknosis formation. The most important characteristic features of apoptosis include cytoplasmic membrane blebbing, formation of apoptotic bodies and the phagocytosis of apoptotic bodies by neighboring cells or macrophages.

For a long time necrosis has been considered a passive and uncontrolled process of cell death. However, evidence is accumulating that necrosis may be as well-regulated as apoptosis [2]. Necrosis often results in inflammation and adaptive immunity, whereas no inflammatory reaction is associated with apoptosis. One explanation for this difference in behavior is that apoptotic cells do not release their cellular constituents into the surrounding tissue and are quickly phagocytosed by macrophages and parenchymal cells without the production of inflammatory cytokines [3]. Whether a cell dies by necrosis or apoptosis partially depends on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu. More recently, a molecule called high mobility group box-1 protein (HMGB1), released by dying cells, has been shown to determine whether the immune system is activated or not [4]. Necrotic cells, but not apoptotic cells, release HMGB1, which stimulates the immune system to produce inflammatory cytokines.

The immunosuppressive effect observed during apoptosis is not only mediated by soluble factors released from the dying cells. A new concept has been developed regarding the suppression of the response of phagocytes and non-classical phagocytes (including

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epithelial, endothelial, and fibroblastic cells) following engulfment of the apoptotic corpse [5].

Autophagy, from the Greek words *auto* meaning self and *phagein* meaning eating ("self-eating"), is a lysosomal degradation pathway essential for homeostasis under normal conditions. In addition, autophagy can assume the cell-killing role when apoptosis is not an option [6]. Autophagy is induced at low levels under normal conditions, but it becomes exacerbated in response to genotoxic or ER stress stimuli during apoptosis-deficient conditions [7]. On the other hand, autophagy suppresses apoptosis in Myc-dependent lymphomas, inducing tumor growth and promoting the survival of cancer cells that express the apoptosis-inhibiting Bcl2 family under hypoxic conditions [8,9]. These data suggest a complex role for autophagy in tumor formation and that its inhibition might provide therapeutic benefit (Figure. 1).

Mechanisms of apoptosis

The two apoptosis pathways are the extrinsic and intrinsic pathways. The extrinsic pathway operates via death receptors on the cell surface, and the intrinsic pathway depending on mitochondria is activated by loss of growth factor signals or in response to lethal stimuli from inside the cell. Both pathways are interconnected with other signaling proteins, such as NK-κB and p53-MDM2, and converge at the level of the effectors proteolytic enzymes, called caspases.

The caspase cascade

The process of apoptosis is carefully controlled, involving an energy-dependent cascade of molecular events. The morphological changes of apoptosis are due to the action of well-preserved and efficient cysteinyl aspartate-specific proteases called caspases. Caspases are widely expressed in most cells in an inactive proenzyme form, and when activated by proteolytic processing they activate other procaspases in turn, thus amplifying the apoptotic signaling pathway and leading to cell death [10]. So far, 14 caspases have been identified, 11 of which are present in mammals. They are subdivided, depending on their activity, into proinflammatory (caspases 1, 4, 5, 11, 12, 13 and 14) and proapoptotic caspases. The latter are grouped as initiator caspases (2, 8, 9, 10) and effector caspases (3, 6, 7). Little is known about some proinflammatory caspases, such as caspase 11, which has been reported to regulate apoptosis and cytokine maturation during septic shock [11]; caspase-12, which mediates endoplasmic-specific apoptosis in mice and rats; caspase 13, which is found only in cattle; and caspase 14, which is highly expressed in embryonic tissues.

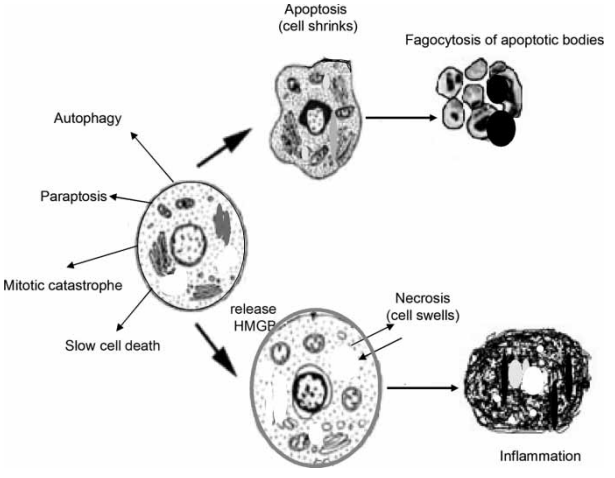

Figure 1. Various models of cell death, including apoptosis and necrosis, depend on HMGB protein release with the death stimulus. Other models have been proposed to define the caspase-independent pathway of cell death: paraptosis, autophagy, mitotic catastrophe and slow cell death.

The caspases are modulated by several endogenous cellular factors, including the inhibitor of apoptosis (IAP) proteins. These polypeptides have a common amino-terminal, 70-residue domain, called the baculovirus inhibitor repeat (BIR) domain, which appears to bind and inhibit active caspases. There are eight mammalian BIR-containing proteins (BIRPs) identified thus far, including the inhibitors of apoptosis 1 and 2 (IAP1 and IAP2), X-linked inhibitor of apoptosis (XIAP), survivin, neuronal inhibitor of apoptosis (NIAP), and BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) [12]. Livin, also known as melanoma-specific inhibitor of apoptosis protein (ML-IAP), has been identified as a new member of the IAP family. Recent studies suggest that ML-IPA is expressed in some tumor cells and several fetal tissues and that it might regulate apoptosis by sequestering Smac and preventing it from antagonizing the XIAP-mediated inhibition of caspases, rather than by directly inhibiting caspases [13].

Members of the IAP family contain one or more BIR domains, each of them having different functions. XIAP, NAIP, c-IAP1 and c-IAP2 each contain three BIR domains. In XIAP, the third BIR domain (BIR3) inhibits the activity of caspase 9, whereas the region between BIR1 and BIR2 specifically targets caspase 3 and caspase 7 [14]. Many IAPs also have another protein motif, the RING domain, which can recruit E2 ubiquitin-conjugating enzymes (UBCs) and catalyze the transfer of ubiquitin onto target proteins, leading to proteasomal degradation. Through their ubiquitin E3 ligase activities, IAPs appear to regulate NF-κB family transcriptional activators, which have also been associated with malignancy. c-IAP1 and cIAP2 promote the

degradation of NF-κB-inducing kinase, the central Ser/Thr kinase in the non-canonical NF-κB pathway [15]. cIAP1 and c-IAP2 contain a caspase recruitment domain (CARD) with an unknown role (Figure. 2).

The activity of IAP family members can be modulated by other endogenous cell factors released by mitochondria. For example, the inhibitory effects of XIAP can be overcome by the antagonizing functions of the mitochondrial proteins, Smac/DIABLO and Omi/HtrA2.

The ubiquitous c-FLIP polypeptide contains a prodomain similar to that of procaspase-8, but lack a caspase active site and may inhibits procaspases 8 and 10.

Extrinsic pathway or death receptors pathway

The extrinsic pathway is initiated by stimulation of the transmembrane death receptors by specific ligands released by other cells. Death receptors may belong to the tumor necrosis factor family (TNF), which is composed of many members. Members of the TNF receptor family have a cysteine-rich extracellular subdomain that allows them to specifically recognize their ligands and a cytoplasmic domain of about 80 amino acids called the "death domain" (DD), which plays a critical role in transmitting the death signal from the cell surface to the intercellular pathways.

Currently, six death receptors are known, including TNF receptor 1 (TNF-R1, also called DR1, p55, p60, or CD120a), Fas (also called DR2, CD95, or APO-1), DR3 (also known as Apo-3, LARD, TRAMP or WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, also known as DR4 or Apo-2), TRAIL-R2 (DR5, KILLER, or TRICK2), and DR6 [16]. The extrinsic pathway is initiated by binding of the transmembrane death receptors with their specific ligands (FasR/FasL, TNFR1/TNFα, DR-4/TRAIL, DR-5/TRAIL, DR3/Apo-3L/TWEAK). Once activated, the intracellular domains of these receptors (DD) bind to the adaptor protein Fas-associated death domain (FADD) or TRADD (TNFR1-associated death domain protein) to form the death inducing complex (DISC) with recruitment of pro-caspase 8. Next, procaspase 8 is proteolytically activated and serves as the 'initiator' caspase, further activating downstream effectors proteins such as caspase 3 and 7 to initiate cell degradation, causing inevitable apoptosis.

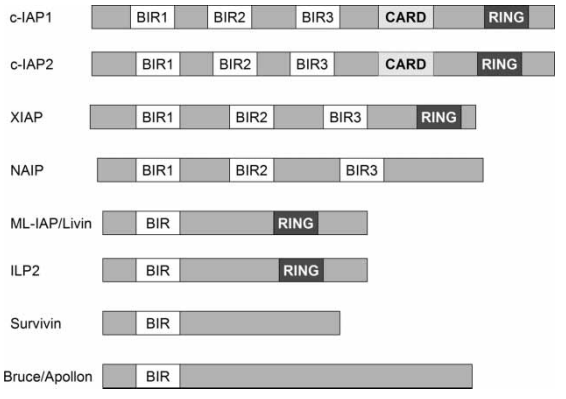

Figure 2. Proteins of the inhibitor of apoptosis family (IAP) include c-IAP1, c-IAP2, XIAP (X-linked IAP), ML-IAP (melanoma IAP/livin), NAIP (neuronal apoptosis inhibitory protein), and survivin. Some of the IAPs contain a protein motif, the RING domain, which can recruit E2 ubiquitin-conjugating enzymes. cIAP1 and c-IAP2 contain a caspase recruitment domain (CARD) with an unknown role.

Two types of intracellular signaling have been defined for the extrinsic apoptotic pathway that are characterized by the generation of high (type I) or low (type II) levels of DISC and caspase 8 activation upon receptor stimulation. In type I, caspase 8 stimulation is sufficient to activate effector caspases and to induce apoptotic death. In contrast, further amplification is needed in the type II cells, where active caspase 8 cleaves BID protein to tBID, which can bind to pro-apoptotic BAX and BAK, resulting in mitochondrial membrane permeabilization and the release of mitochondrial proteins cytochrome C and DIABLO [17].

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) can initiate apoptosis by direct caspase activation, as described above, or indirectly via the release of apoptogens from mitochondria. Recent studies suggest that TRAIL induced mitochondrial apoptotic pathway is potentiated by phospholipids scramblase-3 [18]. Phospholipids scramblase (PLS) are enzymes that play an important role in bidirectional movement of membrane lipids, which are critical for mediating apoptosis in many cell types. PLS3 is a phospholipid scramblase from mitochondria that facilitates changes in mitochondrial membrane lipids that promote the release of apoptogenic factors with activation of caspase 9 and effector caspase 3.

The death receptor pathway is regulated at different levels. First, expression of death receptors varies among different cell types at different stage of development. For example, DR4 and DR5 appear to be expressed in the normal adult thymus and in a variety of tumor cells, but not in other normal tissues. The DR3 and DR6 signaling pathways are less characterized. DR3 can co-stimulate T cells, but its role as an apoptosis inducing receptor is less clear [19]. Second, some cells express so-called "decoy receptors", cell surface or secreted proteins that bind death ligands with high affinity but are unable to transduce signals to cytoplasmic adaptor molecules [20]. Although several decoy receptors of the TNF

receptor family have been identified, the most important one involved in many diseases is DcR3. Also known as TR6 or M68, DcR3 is a member of the TNF receptor family and can bind to the Fas ligand to inhibit its ability to induce apoptosis. A third mechanism for inhibiting death receptor signaling involves the down-regulation of procaspase-8. For example, the ubiquitous c-FLIP polypeptide contains a prodomain similar to that of procaspase-8, but lack a caspase active site (Figure. 3).

In addition, the binding of TRAILR with its ligands can result in the activation of other signaling pathways, including the phosphoinositide 3-kinase (PI3K)–Akt, nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNKs) and p38) pathways. Activation of these additional pathways has a proliferative effect that regulates different physiological processes, such as hematopoiesis and T cell activation and survival, with TRAIL being an important molecule involved in the surveillance and elimination of developing tumors [21].

Intrinsic pathway or the mitochondrial pathway

The intrinsic pathway, involving the mitochondria, is usually activated by the loss of growth factor signals or in response to lethal stimuli from inside the cell, such as DNA damage, oxidative stress, hypoxia, or chemotherapeutic drugs.

Mitochondria, very specialized organelles, have an outer membrane (OM) separated from an inner membrane (IM) by an intermembrane space (IMS). The IMS contains many proteins involved in cell death induction, such as cytochrome c (cyt C), apoptosis-inducing factors (AIF), Omi/HtrA2, EndoG and Smac/DIABLO. All of the stimuli that cause changes to the inner mitochondrial membrane produce the opening of the mitochondrial permeability transition pore (MPT), loss of the mitochondrial transmembrane potential and release of sequestered pro-apoptotic proteins from the intermembrane space into the cytosol. After release into the cytoplasm, cytochrome C stimulates apoptosome formation (a complex including apoptotic protease-activating factor [Apaf-1], dATP, cytochrome c and caspase 9) followed by activation of caspase 9. The 'initiator' caspase 9 causes the activation of the 'executioner' caspases (3, 6, 7), which cleave vital substrates, resulting in cellular death. The catalytic activity of cytochrome C is modulated by members of the inhibitor of apoptosis protein family (IAP), which are in turn controlled by two other mitochondrial proteins, Smac/DIABLO and OMI/HtrA2 [22].

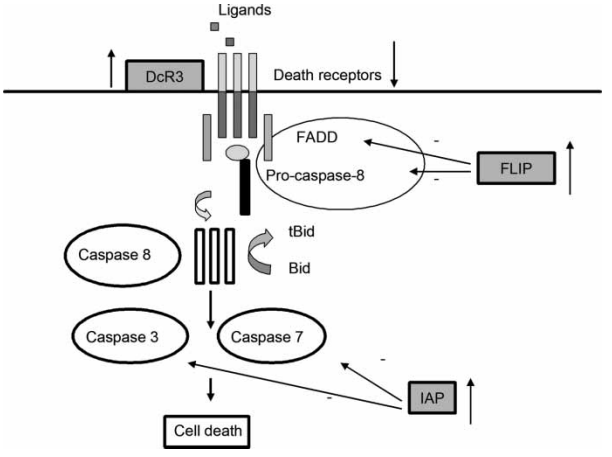

Figure 3. The extrinsic pathway of apoptosis and its regulation. Death receptor signaling can be inhibited at a different levels, including: overexpression of decoy receptors, diminished expression of death receptors, overexpression of IAP molecules that can inhibit caspase 3 and 7 activity, or overexpression of cellular or viral FLIP molecules that can bind to the death effectors domain of FADD and prevent procaspase 8 recruitment.

Apoptosis inducing factor (AIF) is a mitochondrial oxidoreductase associated to the inner mitochondrial membrane. During apoptosis AIF can be released by proteolysis and translocate to the nucleus where it participates in chromatin condensation and DNA fragmentation. A newly discovered member of the AIF family is AMID (apoptosis-inducing factor-like mitochondrion-associated inducer of death). Despite its name, its precise cellular localization and its role during apoptosis are unclear. Recent studies in human leukemia Jurkat T-cells suggest increased expression and plasma membrane association of AMID after apoptosis induction [23].

The intrinsic pathway is controlled by interactions between proapoptotic and antiapoptotic members of the Bcl-2 protein family. There are at least 20 proteins in the Bcl-2 family, which are divided into 3 groups. Group I members are anti-apoptotic, whereas groups II and III are pro-apoptotic. The members of the Bcl-2 family can be defined by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). Each of these BH domains has a different function, and family members contain one or more of them. Whereas most antiapoptotic proteins (including Bcl-2, Mcl-1, Bcl-w, Bcl-xL, and A1) contain all four Bcl-2 homology domains and protect cells exposed to diverse cytotoxic conditions, proapoptotic proteins may lack one or several of the BH domains and can be divided into two subgroups. Members of the first group, BH3-only domain proteins (including Bid, Bim, Bik, Bad, Bmf, Noxa, Puma, and Hrk), act as damage sensors and direct antagonists of Bcl-2 and the other pro-survival proteins. The other proapoptotic group contains the BH 1-3 domains (Bax, Bak, Bok and Bcl-x_s) and

directly activates other proapoptotic family members. Antiapoptotic Bcl-2 proteins exert their activity by binding the proapoptotic members Bax and Bak, preventing mitochondrial damage [24]. Recently, a novel Bcl-2 protein, named Bcl-x_{AK}, has been identified that contains BH4 and BH2 domains but lacks BH3 and BH1 [25]. The overexpression of Bcl-x_{AK} triggers apoptosis in human melanoma cells. This is the first Bcl-2 protein that induces apoptosis without a functional BH3 domain (Figure. 4).

The p53 gene is a well-known tumor suppressor gene that encodes a nuclear protein with a critical role in the regulation of cell death. In response to different cellular stresses, p53 stops cell-cycle progression through expression of its target gene, p21. When the cell cannot repair the cellular damage, p53 promotes apoptosis. It seems that p53 uses multiple pathways to induce cell death [26]. It is well established that p53 transactivates a variety of apoptotic factors, such as Bax, Puma, Noxa, and p53-regulated-inducing protein 1 (p53AIP1) and also can translocate to the mitochondria where it binds to the antiapoptotic proteins Bcl-2 and Bcl-xL. In addition, p53 can stimulate the production of reactive oxygen species and Fas/CD95 to redistribute to the cell surface. Therefore, when p53 is inhibited, cell proliferation is accelerated. Indeed, p53 function is compromised in the majority of human cancers.

In normal cells, MDM2 is an essential regulator of p53. MDM2 and p53 regulate each other through an autoregulatory feedback loop. Upon activation, p53 transcribes the MDM2 gene and, in turn, the MDM2 protein inhibits p53 activity. Many models that explain p53 inhibition by MDM2 have been observed. First, MDM2 binds directly to the N-terminal p53 transactivation domain and inhibits its transcriptional activity [27]. Second, MDM2 functions as an E3 ubiquitin ligase, which leads to both the export of p53 to the cytoplasm and its proteasomal degradation. Overexpression of MDM2 due to the amplification of the MDM2 gene has been observed in several human cancers. Targeting MDM2 with small molecules to reactivate p53 has emerged as a promising new cancer therapeutic strategy [26] (Figure. 5).

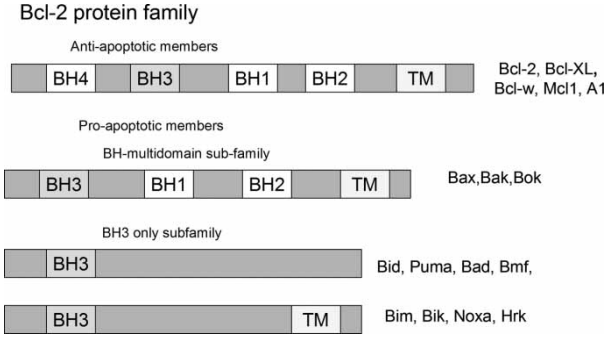

Figure 4. The extended Bcl-2 family. This family comprises pro-survival proteins, which share the Bcl-2 homology (BH) domains, and pro-apoptotic proteins that contain BH1-3 domains or only the BH3 domain.

Cancer: Mechanisms of apoptosis resistance

Cancer is a genetic disease in which a succession of genetic mutations is observed. It is now believed that some cancers are caused by the lack of cell death, rather than an increased rate of proliferation.

Transformed cells are recognized by the immune system based on the expression of abnormal molecules on the cell surface and the abnormal behavior of preneoplastic cells that respond to oncogenic stress. The immune system distinguishes between normal programmed cell death (PCD), which appears in development and homeostasis, and pathogen-induced PCD. This is possible using specific receptors expressed on the surface or within the cytoplasm of innate immune effectors that recognize so-called pathogen-associated molecular patterns (PAMPs). In addition to PAMPs, the so-called "danger-associated molecular patterns" can trigger the immune response. Efficient recognition of transformed cells and their elimination is called "immunosurveillance" [28]. Transformed cells often escape from immunosurveillance due to their capacity to

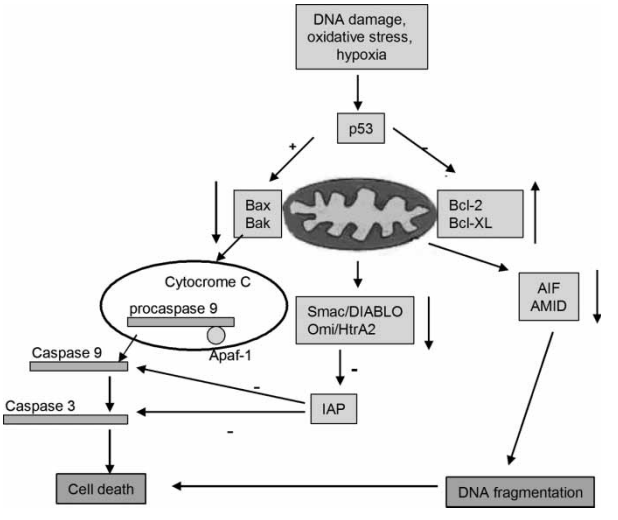

Figure 5. The intrinsic pathway and its regulation. The mitochondrial pathway can be inhibited at different stages, including: elevated expression of anti-apoptotic Bcl-2 family members, diminished expression of proapoptotic Bcl-2 family members, overexpression of IAP family members that can inhibit caspase 3, caspase 9 and caspase 7 activities, diminished expression of AIF family members or certain molecules expressed in the mitochondria (Smac/Diablo, Omi/HtrA2) that regulate the expression of IAP family members.

hide the abnormal molecules present on their surface by targeting specific pro-survival strategies or by inhibiting apoptosis pathways [29]. In tumorigenesis, disturbance of both extrinsic and intrinsic pathways of apoptosis is involved.

The death receptor pathway is regulated, as specified above, at three different levels. All of the levels can be disturbed in cancer cells. Concerning the death receptors, decreased expression of the Fas receptor has been observed in hepatomas as compared to normal hepatocytes, and its downregulation might contribute to evasion of the immune system during liver carcinogenesis [30]. In addition, DR4 and DR5 appear to be expressed in a variety of tumor cells, but not in normal tissues. Second, several decoy receptors of the TNF receptor family have been identified, the most important one being DcR3. DcR3 is over expressed in a variety of human cancers, such as colon, lung, and stomach cancer. Some studies suggest that DcR3 can be used to predict lymph node invasion in patients with gastric cancer [31]. A third mechanism for inhibiting death receptor signaling involves the downregulation of procaspase 8 by specific molecules such as c-FLIP. Overexpression of c-FLIP is observed in many carcinomas, with interruption of the death signal by its binding to FADD and competitive inhibition of the recruitment of procaspases 8 and 10 [32].

The intrinsic pathway is controlled by interactions between proapoptotic and antiapoptotic members of the Bcl-2 protein family. Alteration in the expression of either type occurs frequently in many human cancers. Bcl-2 is overexpressed in a variety of cancers and was the first apoptosis-related gene recognized to play a role in tumorigenesis. Elevations in Bcl-2 protein levels are commonly found in many hematopoietic malignancies including multiple myeloma (MM), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL) [33]. Furthermore, elevations in Mcl-1 have been reported in acute leukemia after relapse from chemotherapy [34]. Mutated or downregulated Bax and Bak are also observed in certain cancers [35].

Inhibitors of apoptosis proteins (IAPs) are overexpressed in many human cancers, and their level of expression has been associated with treatment resistance. For example, the overexpression of c-IAP1, c-IAP2, NAIP, XIAP and survivin has been detected in breast cancer [36]. In addition c-IAP2 is overexpressed in low and high grade pancreatic intraepithelial neoplastic lesions and pancreatic ductal adenocarcinomas. Thus, it is considered an early event in the progression of pancreatic cancer [37]. XIAP is overexpressed in esophageal cancer tissues compared with normal tissues and might confer resistance to apoptosis induction by caspase 3 activation and promote tumorigenesis [38].

Half of all human cancers have mutations in the tumor-suppressor gene p53, considered to be the "guardian of the genome". In human cancer, the function of p53 is inhibited by its primary cellular inhibitor, MDM2. Overexpression of MDM2 due to amplification of the MDM2 gene has been observed in many human cancers. Since p53 plays a central role in apoptosis induction, alteration of the p53 pathway influences the sensitivity to apoptosis and modifies the body homeostasis.

The nuclear factor-kappa B (NF-κB) pathway is an important signaling pathway in tumorigenesis which regulates tumor cell proliferation, controls apoptosis, promotes angiogenesis, and stimulates invasion and metastasis. Due to its multiple roles in tumorigenesis, inhibition of (NF-κB) alone or in combination with chemotherapy may lead to growth inhibition or tumor cell death [39].

Cancer: Target therapy involving the apoptotic pathway

The greatest problem limiting effective cancer treatment may be drug resistance which can involve multiple mechanisms including influx or efflux of drugs, resistance to death stimuli, or activation of proliferation pathways. Strategies to overcome tumor resistance to either extrinsic or intrinsic apoptotic pathways are under investigation. These include activation of the extrinsic pathway through proapoptotic receptors, restoration of p53 activity, inhibition of the Bcl-2 family of proteins, BH3-only mimic proteins, caspase modulation, IAP inhibition, and proteasome inhibition.

Targeting pro-apoptotic receptors in cancer

The potential targeting the extrinsic apoptotic signaling pathway as a therapeutic strategy was revealed more than 15 years ago when Trauth and colleagues demonstrated that a single intravenous injection of an APO-1 antibody induced regression of a xenotransplanted human B-cell tumor in nude mice within a few days [40]. The TRAIL pathway is an attractive therapeutic target for cancer treatment because tumor cells are more sensitive to the TRAIL-dependent apoptosis pathway than are normal cells. While the pro-apoptotic ligand FasL has cytotoxic activity against many tumor cells, hepatic toxicity limits the use of Fas-targeted therapy [41]. Two classes of pro-apoptotic receptors antagonists (PARAs) that target DR4 or DR5 have been developed. Recombinant human (rh) Apo2L/TRAIL activates both receptors while monoclonal

antibodies have been developed that act as agonists of either DR4 (mapatumumab) or DR5 (lexatumumab, Apomab, AMG655, Cs-1008, LBY-135) [42–46]. Stimulation of the proapoptotic receptors DR4 and DR5 shows promising safety and efficacy in several diverse preclinical cancer models. These agents have shown minimal cytotoxicity of normal cells and have been demonstrated to kill cells that are resistant to standard chemotherapy or restore the sensitivity of tumor cells to chemotherapy when combined with standard treatment. The most common adverse events that have been reported include fever, fatigue, thrombocytopenia, and manageable digestive toxicities. Moreover, several of these agents have successfully met the safety criteria of phase I clinical trials. Phase II trials are being initiated to assess the safety and efficacy of specific PARAs both in combination with standard cytotoxic chemotherapy or with targeted agents in a variety of tumors types. Because cancer cells are not universally sensitive to treatment, there has been extensive research to identify and characterize potential biomarkers for sensitivity to Apo2/TRAIL. Recent studies have identified the *myc* oncogene and O-glycosylation enzymes as potential markers for Apo2/TRAIL sensitivity in cancer cells [47,48].

Targeting intracellular caspase inhibitors

Members of the IAP family have been investigated as therapeutic targets for the treatment of multiple diseases including cancer due to the overexpression of its members in many tumors. There are currently several preclinical studies involving the administration of small molecule drugs that mimic the activity of Smac by binding to IAPs and preventing their interaction with caspases or use antisense-based inhibitors of IAPs [49,50]. Another possibility to deplete IAP expression is a molecular method named RNA interference (RNAi), which consists of synthesizing and transferring small interfering RNA (siRNA) into cancer cells to block the overexpression of IAPs or other molecules. Treatment with XIAP siRNA in combination with chemotherapy can efficiently decrease XIAP expression and induce cellular apoptosis [38]. On the other hand, normal cells are less dependent on IAPs, thus providing an important advantage for these novel agents. FLIP [FLICE (FADD-like ICE)-inhibitory protein] is an endogenous inhibitor of extrinsic apoptosis signaling. Recently, two compounds that reduce the expression of c-FLIP and sensitize cells to Apo2L/TRAIL-induced apoptosis have been described [51].

Targeting the p53-MDM2 interaction

Small-molecule inhibitors of the MDM2-p53 interaction have been developed. The first ones, called the nutlins, were reported in 2004, but in the last four years several new classes of small molecule MDM2 inhibitors have been discovered, including Nutlin-3, MI-219, and MI-63 [52,53]. The nutlins have been shown to inhibit tumor growth by inducing cell cycle arrest and cell death. In several xenograft models of human cancer nutlin-3 and MI-219 have shown strong antitumor activity in the presence of wild-type p53, but lack activity against tumors deficient in wild-type p53 [53]. The wild-type status of p53 appears to be the major determinant of the antitumor activity of MDM2 inhibitors. In addition, it has recently been shown that nutlin 3 can downregulate TNFα-induced activation of the NF-?B reporter genes in lung cancer cells [54]. Importantly, during treatment with these small molecules, no visible signs of toxicity in the animals were observed as assessed by necropsy studies and antibody weight. MDM2 inhibitors are promising therapeutic compounds with several small molecules now in preclinical development. Because the activity of MDM-2 is p53 dependent and 50% of tumors contain p53 mutations, it is reasonable to identify a biomarker of p53 activity. A member of the transforming growth factor β superfamily called macrophage inhibitory cytokine-1 has been proposed as a biomarker for p53 activity [55].

Targeting Bcl-2 family proteins

Bcl-2 is overexpressed in a variety of cancers and was the first apoptosis-related gene recognized to play a role in tumorigenesis. Treatment of tumors with Bcl-2 anti-sense oligonucleotides can reduce tumor growth and clinical trials have shown promising preliminary results including improvements in the response rate and progression free survival in patients with chronic lymphocytic leukemia and advanced, relapsed melanoma [56,57]. RNAi may also be a useful method by which to downregulate the expression of anti-apoptotic genes; several small molecule antagonists of Bcl-2 or Bcl-XL are in early phases of development [58]. Promising results have been reported in lung cancer, where in combination with chemotherapy, it was possible to sensitize resistant cell lines and to induce apoptosis [59]. Another strategy to enhance the response of apoptotic stimuli would be to stimulate the expression of pro-apoptotic molecules. This approach is under investigation [60].

There is now great interest in developing drugs that mimic the action of the BH3 domain by binding to one or more of the Bcl-2 like proteins and triggering apoptosis. Selective interactions of the

BH3-only proteins with pro-survival molecules are now well documented [61]. Several short peptides that could be used either as tools to understand the roles of the Bcl-2 family of proteins or to develop a new generation of anticancer drugs have been developed. One of these is GX15-070 which binds to Bcl-2, Bcl-XL, Bcl-w and Mcl1 and has activity in multiple cancer cell lines tested [62]. AT-101 has cytotoxic potency similar to that of GX15-070, and was effective in a phase I open-label trial in patients with previously untreated chronic lymphocytic leukemia. This compound had manageable gastrointestinal toxicities and has been tested in phase II studies in combination with rituximab with promising results [63]. The other two drugs: ABT-737 and ABT-263 bind to Bcl-2, Bcl-xL and Bcl-w, but not with Mcl-1 or A1, and induced stable regression of both human lung cancer and hematological disease in mouse xenograft models with minimal effects on platelet counts [64,65]. In addition, ABT-737 can enhance TRAIL-mediated cytotoxicity by unsequestering Bim and Bak in human pancreatic cancer cell lines [66].

Targeting protein degradation: E1 inhibitors

Protein degradation plays a vital role in controlling the activity of key molecules involved in cell cycle progression and apoptosis, including the p53 and NK-κB pathways. Recently, great advances have been made in our understanding of the fundamental importance of the ubiquitin-proteasome pathway in diverse biological processes. Ubiquitin-activating enzymes, also known as E1 enzymes, catalyze the first step in the ubiquitination process that targets a protein for degradation via the proteasome. Alterations in ubiquitination are observed in a wide range of pathological conditions, including cancer. Several classes of proteasome inhibitors that block degradation of ubiquitinylated proteins have been developed [67]. Bortezomid, approved by the FDA for the treatment of relapsed and refractory multiple myeloma, has promising anticancer activity in multiple other tumor types by effecting cellular proliferation, survival, apoptosis, and angiogenesis [68]. Early phase I and II trials have suggested activity for bortezomid in lung cancer [69].

PYR-41 is another proteasome inhibitor that increases the level and activity of p53, as well as the levels of MDM2 and p21, another p53 target gene. PYR-41 can increase apoptosis in cells with wild-type p53 and works by inhibiting several key steps of the NK-κB pathway. PYR-41 inhibits both proteasome-dependent and proteasome-independent ubiquitinylation, but its molecular mechanism needs to be more fully elucidated [67].

Single agent therapy may be more likely to result in the development of resistant tumors, a finding thought to be less common with the use of combinations of drugs. The ligation of TRAILRs in cells that cannot initiate an apoptotic signal results in activation of the NK-κB and PI3K-Akt pathways with stimulation of cell proliferation, survival, and cell migration. This provides a scientific rationale to combine agents that target the TRAIL pathway with additive effects on apoptosis (Table 1)[70–77].

The examples mentioned represent some of the current research in the apoptosis field and provide

Table 1. Effect of various agents on Apo2/TRAIL in preclinical studies.

Agent	Tumor type	Combination	Effects	Ref
Conventional chemotherapy	**Colon** COLO205, HCT116, COLO201	**CAM or 5-FU+rhApo2L/TRAIL**	Synergic effect with tumor regression or complete tumor ablation in mouse xenografts	94
	Prostate PC3	**DOX+Apo2L/TRAIL**	Synergic effect increased apoptosis and decreased tumor growth	95
Irradiation	**Prostate** In mice (PC3)	**Irradiation+rhApo2L/TRAIL**	Synergic effect by upregulation of DR5, Bax, Bak	96
Bcl-2 inhibitors	**Prostate** Cell lines (C42)	**BH3I-22 (Bcl-X_L inhibitor)+rhApo2L/TRAIL**	Synergic effect by activation of caspase 8 and Bid	97
Proteasome inhibitors	**Colon** Cell lines (COLO205, HCT15, KM12, SW620)	**BOR+rhApo2L/TRAIL**	Sensitization of KM12, and SW620 but not colo 205 or HCT15 sensitization	98
IAP antagonist	**Leukemia** Cell lines (Jurkat)	**Smac-7 or Smac-4+Apo2L/TRAIL**	Synergic effect by activation of caspase 3 and downregulation of XIAP, cIAP1 and survivin	99
	Neuroblastoma, melanoma, pancreas	**Synthetic Smac peptides+Apo2L/TRAIL**	Sensitization to Apo2L/TRAIL-induced apoptosis	100
MDM2 inhibitors	**Leukemia**	**Nulin3+rhApo2L/TRAIL**	Synergic effect	101

examples of the use of specific target therapy in improving the outcome of anti-cancer therapy.

Conclusion

Identifying the key proteins involved in apoptosis represents an attractive way to prevent the development of many diseases including cancer. Understanding how these proteins affect the apoptotic pathways may lead to more effective cancer treatments. Research efforts over the past decade have contributed to our knowledge regarding the characterization of the molecules involved in apoptosis, the relationships between these molecules, and the key molecules that can be used in targeted therapy.

The discovery of apoptosis pathways and the development of specific molecules that induce apoptosis of tumor cells suggest that cell death can be targeted therapeutically. However, the success of pro-apoptotic therapies has been limited, perhaps because of our lack of understanding the complexity of cell death regulation.

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