Messengers of cell death: apoptotic signaling in health and disease

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Background and Objectives. Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis. Understanding the molecular basis of apoptosis signaling may reveal novel clues for lymphomagenesis.

Evidence and Information Sources.Pro-apoptotic signaling is mediated by specific ligands and surface death receptors (extrinsic pathway of apoptosis regulation), which are capable of delivering a death signal from the microenvironment and can activate the execution of apoptosis in the cell cytoplasm and organelles. Death receptors include tumor necrosis factor-receptor 1, Fas, death receptor (DR) 3, DR4, DR5 and DR6, whereas death ligands include tumor necrosis factor-α, lymphotoxin, Fas-Ligand, Apo3-Ligand and TRAIL (*T*NF-*R*elated *A*poptosis-*I*nducing *L*igand). Once activated, death receptors recruit adaptor proteins, which in turn recruit initiator caspases, giving rise to a pro-apoptotic complex termed the death-inducing signaling complex (DISC). Besides being triggered from microenvironmental signals, apoptosis can also be activated from inside the cell through specific cell sensors residing in the cell nucleus and cytoplasm (intrinsic pathway of apoptosis regulation). The intrinsic pathway of apoptosis leads to the formation of a pro-apoptotic complex termed an apoptosome. Both the extrinsic and the intrinsic pathways of apoptosis signaling converge into a common pathway causing the activation of the effector enzymes caspases. Consistent with the role of apoptosis as a main regulator of B-cell homeostasis in the germinal center, the pathogenesis of several germinal-center-derived lymphomas is characterized by deregulation of one or more steps of the apoptosis signaling pathways.

Perspectives. Tumor-specific alterations in the apoptotic machinery may represent new potential targets for molecular therapy of lymphoma.

Key words: apoptosis, lymphoma, TRAIL, FAS, death receptor.

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Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of tissue homeostasis and is morphologically characterized by cell shrinkage, membrane remodelling and blebbing, chromatin condensation and DNA and cellular fragmentation into apoptotic bodies. Pro-apoptotic signaling may be mediated by specific ligands and surface receptors (extrinsic pathway of apoptosis regulation), which are capable of delivering a death signal from the microenvironment and can activate the execution of apoptosis in the cell cytoplasm and organelles.[1] Apoptosis can also be activated from inside the cell through specific cell sensors residing in the cell nucleus and cytoplasm (intrinsic pathway of apoptosis regulation).[1] Both the extrinsic and the intrinsic pathways of apoptosis signaling converge into a common pathway causing the activation of effector enzymes termed caspases.[1]

Pathways of apoptotic signaling

Extrinsic pathway of apoptotic signaling

Death receptors are a family of membrane receptors, also named tumor necrosis factor receptor (TNF-R), that transduce pro-apoptotic signals from the extracellular space into the intracellular milieu (Figure 1A). The TNF-R family contains six members including TNF-R1, Fas, death receptor (DR) 3, DR4, DR5 and DR6 which have in common an extracellular cysteine-rich domain, required for ligand binding, and an intracellular death domain (DD) required for apoptotic signal transduction (Figure1A).[2,3] The ligands for these receptors form a family of related cytokines, collectively named as the TNF family, containing TNFα, lymphotoxin (LTα), Fas-Ligand (Fas-L), Apo3-Ligand (Apo-3-L) and TRAIL (*T*NF-*R*elated *A*poptosis-*I*nducing *L*igand).[2,3] These ligands function in an autocrine or paracrine manner and, upon binding, cause trimerization of their respective cell surface membrane receptors, which is an essential requirement for apoptotic signaling (Figure 1B).[3,4]

Once activated, death receptors recruit adaptor proteins through the homophilic interaction of their own DD to the DD of the adaptor proteins (Figure 1C). Beside carrying the DD, adaptor proteins also contain a death effector domain (DED), which is involved in the next step of apoptotic signaling along the extrinsic pathway (Figure 1C). In fact, the DED of the adaptor protein interacts in a homophilic manner with the DED of the apoptosis initiator enzyme, procaspase-8, which is thus recruited into the death inducing signaling complex

(DISC) (Figure 1D). Next, procaspase-8 is activated proteolytically into caspase-8 and further activates effector caspases along the common pathway of apoptosis (Figure 1E-F).[3,4]

Two specific examples of the extrinsic pathway of apoptotic signaling in lymphoid cells are represented by the FasL-Fas pathway and the TRAIL-DR4/DR5 pathway (Figure 1). FasL is a cytokine synthesized as a membrane protein and released in homotrimers from the cell surface by metalloproteinase-mediated proteolytic cleavage.[5,6] FasL belongs to the TNF family and is predominantly expressed on activated T-cells and natural killer cells.[5,6] The Fas receptor is a membrane protein belonging to the TNF-R family and is highly expressed in activated lymphocytes.[5,6] FasL homotrimers engage three Fas receptors that bind to the DD of the adaptor protein FADD (for *Fas Associated Death Domain*), which in turn recruits procaspase-8 into the DISC through DED-mediated homophilic interactions (Figure 1).[5,6] TRAIL, like FasL, is primarily expressed as a membrane protein although there is evidence that a soluble form of TRAIL may also exist.[7,8] TRAIL can interact with five distinct death receptors belonging to the TNF-R family.[7,8] Two of these receptors, DR4 and DR5, contain a cytoplasmic DD and can elicit apoptosis when stimulated by TRAIL (Figure 1).[7,8] Both TRAIL and the DR4 and DR5 receptors are widely expressed in human tissues. Stimulation of the DD containing TRAIL receptors DR4 and DR5 results in both cases in the formation of a DISC through the recruitment of FADD and subsequently of procaspase-8, which in turn is proteolytically activated and triggers the caspase cascade (Figure 1).[7,8]

Instrinsic pathway of apoptotic signaling

Mitochondria are induced to release cytocrome c into the cytosol in response to stress such as DNA damage induced by chemotherapeutic agents or irradiation or withdrawal of growth factors and survival stimuli.[9] Upon release into the cell cytoplasm, cytochrome c recruits the caspase adaptor molecule Apaf-1 and the apoptosis initiator enzyme termed procaspase-9.[9] Together, cytochrome c, Apaf-1 and caspase-9 form a holoenzyme complex called an apoptosome.[9] The apoptosome, through the enzymatic activity of caspase-9, activates the effector caspases along the common pathway of apoptosis.[9]

The extrinsic and the intrinsic apoptotic pathways are intimately connected (Figure 2). For example, caspase-8 generated by the extrinsic pathway activates, by proteolysis, the pro-apoptotic factor Bid, a Bcl-2 family member.[10] Upon cleavage, Bid translocates to mitochondria, induces cytochrome c release and thus leads to formation of the apoptosome.[10]

Common pathway of apoptotic signaling

Activation of caspases is central for the execution of apoptosis. Caspases are a family of intracellular cysteine proteases produced as inactive zymogens that cleave their substrates at aspartic acid residues.[11] Caspases may be hierarchically stratified into upstream initiator caspases and downstream effector caspases.[11] Initiator caspases, namely procaspases-8, -9, -10, have two main functions. First, the DED domain of initiator caspases allows their association with adaptor proteins of the DISC and of the apoptosome (Figures 1D and 2D). Second, the enzymatic domain of initiator caspases triggers the activation of the downstream effector caspases in a cascade fashion (Figures1F and 2E).[11] In contrast, the effector caspases, namely caspase-3, -6, -7, lack the DED domain and thus largely depend upon upstream caspases for their activation. In turn, the effector caspases are responsible for cell death by proteolysis of cellular substrates (Figures 1 and 2).[11]

Regulation of apoptosis signaling

Multiple antagonists are involved in the modulation of apoptosis at the level of the intrinsic, extrinsic and common pathways. For example, in the intrinsic pathway, the anti-apoptotic proteins of the Bcl-2 family prevent mitochondrial release of cytochrome c.[12] In the extrinsic pathway, regulation may occur both at the membrane and at the cytosol level. At the membrane level, the expression of inactive TNF-R such as DcR1 (for death decoy receptor 1) or DcR2, which are transmembrane receptors devoid of a DD, antagonizes TRAIL-mediated apoptosis,[3] while at the cytosol level the expression of anti-apoptotic DED-containing proteins, such as FLIP, may compete with the adaptor protein FADD for binding to procaspase-8.[13] Finally, caspase activation is directly regulated by a family of inhibitors of apoptosis proteins (IAP), which inhibit both effector and initiator caspases.[13]

Apoptotic signaling in normal and neoplastic germinal center

Apoptosis signaling in the normal germinal center

The transition from naïve B-cells to antigen-experienced B-cells takes place in a highly specialized microenvironment termed the germinal center (GC). Within the GC, the B-cell maturation program involves a series of genetic events that include somatic hypermutation of the IgV genes and Ig class switch, aimed at selecting functional, high affinity and antigen-specific B-cells. A stringent selection mechanism must be in place to ensure the survival of only immunocompetent B-cells and the death of aberrantly developed low affinity or autoreactive B-

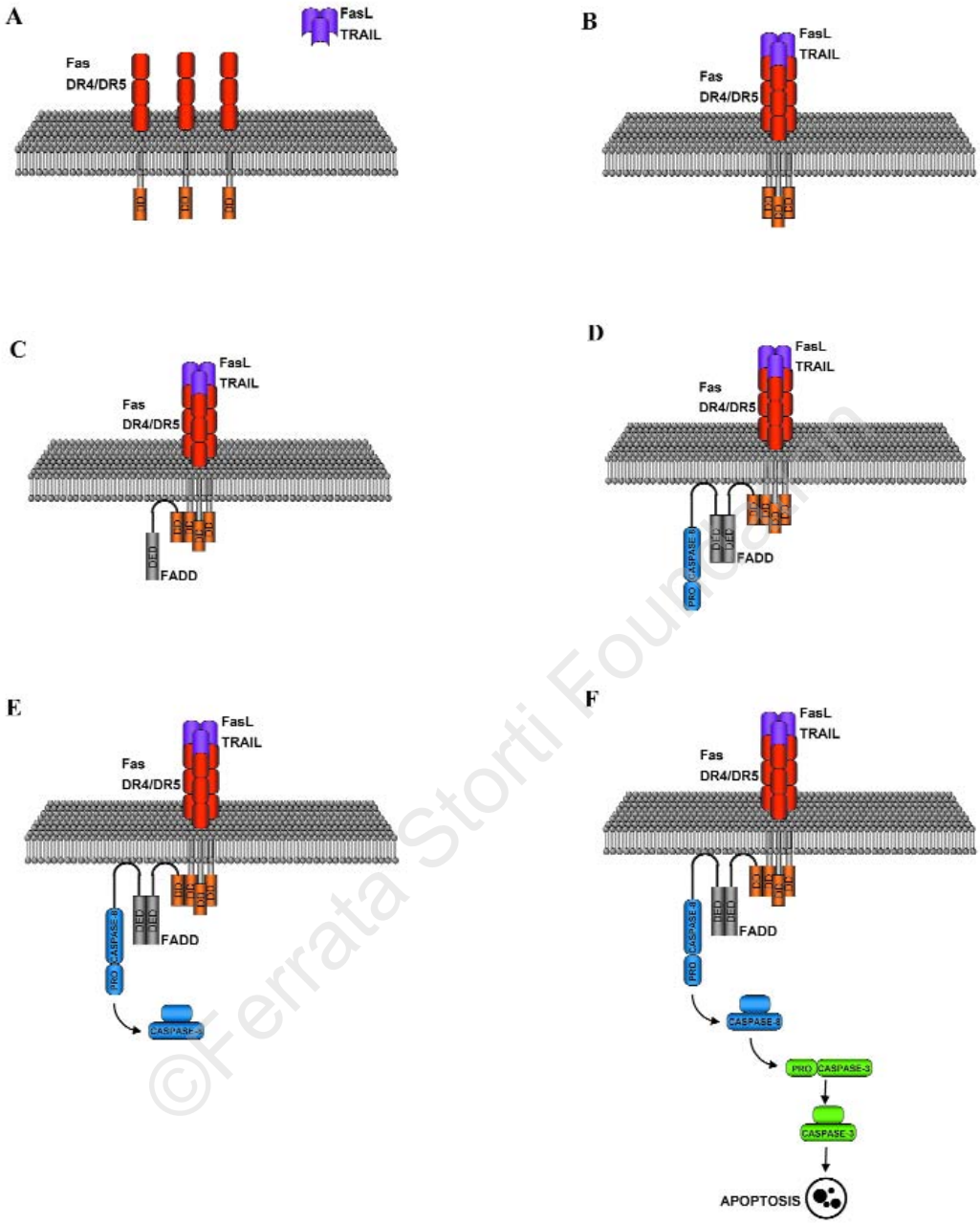

Figure 1. Extrinsic pathway of apoptotic signaling. Panel A. Fas, DR4 and DR5 are transmembrane receptors belonging to the family of death receptors. In general terms, a death receptor is composed of an extracellular domain containing the ligand binding site, a transmembrane domain and an intracellular domain, termed the death domain (DD), involved in apoptotic signal transduction. In the absence of its own death ligand, such as FasL for Fas and TRAIL for DR4 and DR5, the death receptor is in an inactive form. Panel B. Engagement by a death ligand induces death receptor activation by homotrimerization. Panel C. Once activated, death receptors recruit adaptor proteins through the homophilic interaction of their own DD to the DD of the adaptor proteins such as FADD, in the case of Fas, DR4 and DR5. Characteristically, the adaptor protein contains an additional domain, the death effector domain (DED), necessary for procaspase recruitment. Panel D. In the next step, the DED domain of the adaptor protein interacts in a homophilic manner with the DED of an apoptosis initiator enzyme termed procaspase-8. The formed complex is called DISC. Panel E. Procaspase-8 is synthesized as an inactive precursor that is activated by auto-proteolytic cleavage after recruitment in the DISC. Panel F. Activation of caspase-8 triggers the activation of downstream effector caspases such as procaspase-3 in a cascade fashion. Activated caspase-3 is responsible for cell death by proteolysis of cellular substrates.

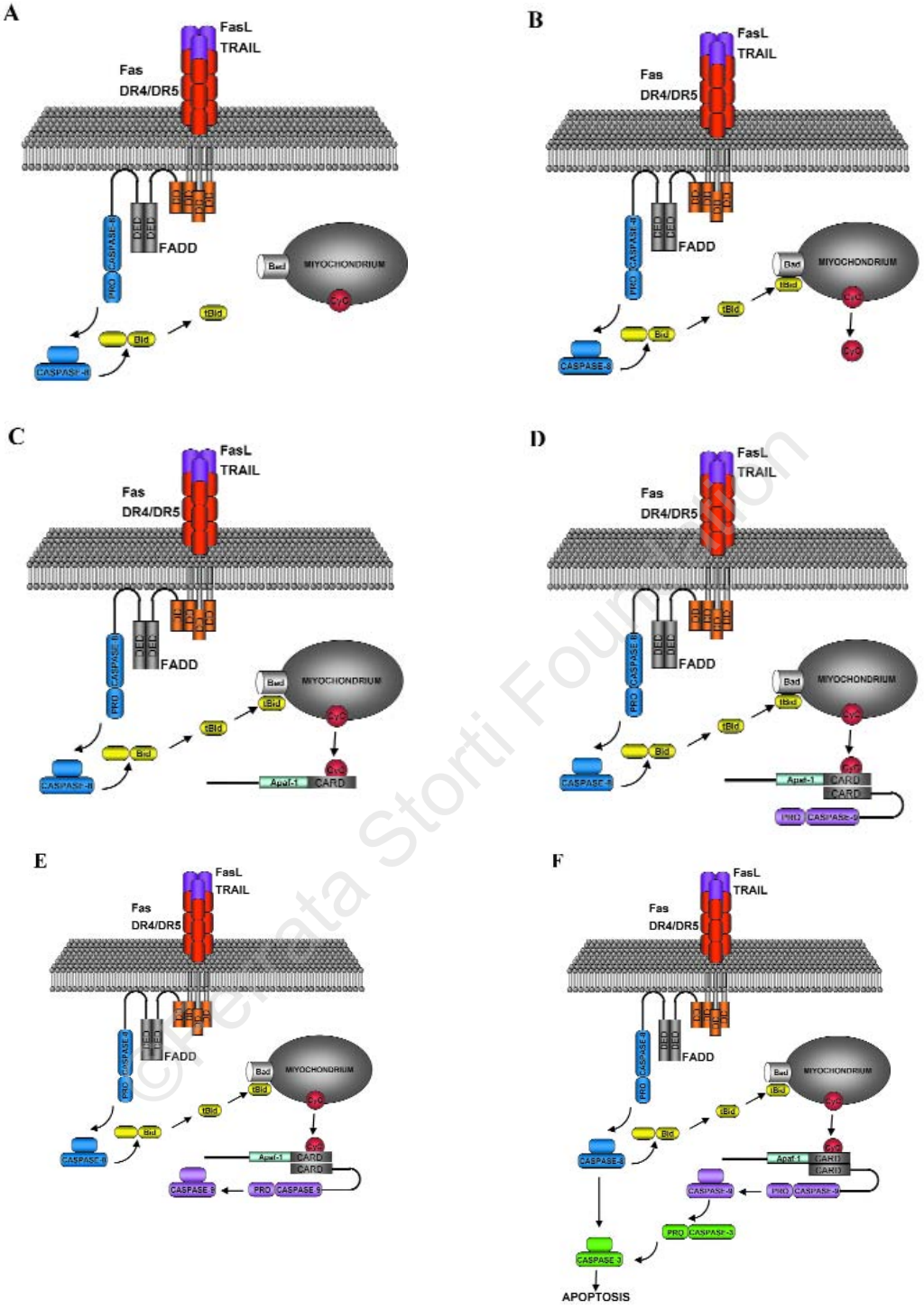

Figure 2. Interconnections between extrinsic and intrinsic apoptosis signaling pathways. Panel A. The extrinsic and the intrinsic pathways are interconnected. Activated caspase-8 triggers the cleavage of the cytosolic pro-apoptotic protein Bid, which thus becomes activated. Panel B. Activated Bid translocates to the mitochondrial membrane and activates Bad, another pro-apoptotic protein belonging to the family of Bcl-2 proteins. Activated Bad disrupts the mitochondrial membrane causing the release of cytochrome C (CyC) into the cytosol. Panel C. Cytochrome C in turns recruits the caspase adaptor molecule Apaf-1 leading to exposure of the caspase recruitment domain (CARD). Panel D. Apaf-1 recruits the initiator caspase procaspase-9, through homophilic interaction of its own CARD to the CARD of procaspase-9. Together, Apaf-1 and procaspase-9 form the apoptosome enzymatic complex. Panel E. The apoptosome, through the enzymatic activity of activated caspase-9, triggers the activation of the downstream effector caspase-3, inducing cell death.

cells, and to ensure a relatively constant B-cell number despite periodic expansions during immune responses. In this view, lymphocytes are subjected to cell death checkpoints at many steps during their lifespan in the GC.[14-16]

A first checkpoint involves the extrinsic pathway of apoptosis. B-cells residing within the GC exhibit a default phenotype that is characteristic of apoptosis-sensitive cells. In fact, GC B-cells become sensitive to Fas-mediated apoptosis after Fas upregulation caused by the interaction through CD40 on B-cell membranes, and CD154 on surrounding T-cells. On this basis, the FasL-Fas pathway is a major player in the negative selection of autoreactive B-cells generated upon the somatic hypermutation process.[14-16]

A second checkpoint involves the intrinsic pathway of apoptosis. Positive selection and survival of antigen-specific B-lymphocytes simultaneously requires activation of B-cell receptor, stimulation by inflammatory cytokines and co-stimulation by the CD19/CD21 and CD40/CD154 complexes. All together, these stimuli are potent inducers of the Bcl-2 family of anti-apoptotic proteins, thus stabilizing the mitochondrial membrane and preventing cell death. In contrast, when these stimuli are absent or insufficient, as in the case of low affinity or autoreactive B-cells or after antigens and inflammatory cytokines have been cleared at the end of an immune response, B-cells undergo apoptosis through the intrinsic pathway triggered by the activation of the pro-apoptotic proteins Bim, Bax and Bak.[14-16]

Apoptosis signaling in neoplastic germinal center

Given the central role of apoptosis in the regulation of GC homeostasis, it is not surprising that lymphomas derived from GC B-cells carry alterations of genes involved in both the extrinsic and intrinsic pathways of apoptosis. Examples of deregulation of apoptotic signaling in lymphoma are represented by translocations of BCL-2, API2/MLT, BCL-10 as well as mutations of BAX, FAS, and caspase-10.[17,18]

BCL-2. Chromosomal translocations involving the BCL-2 gene are the molecular hallmark of follicular lymphoma and are also detected in a fraction of diffuse large B-cell lymphomas (DLBCL).[18] The translocation causes BCL-2 deregulated expression by placing BCL-2 under the control of the IgHμ enhancer, resulting in constitutively high levels of Bcl-2 protein.[18] Because of the somatic hypermutation mechanism associated with the IgH locus, the translocated BCL-2 can accumulate somatic point mutations.[18] These mutations may either contribute to deregulation of BCL-2 gene expression or alter the biochemical function of the Bcl-2 protein with enhanced activity.[18] Aside from chromosomal translocations, BCL-2 deregulation by gene amplification has also been described in 10-20% of DLBCL.[18]

API2/MLT and BCL-10. The translocation t(11;18)(q21;q21) is the most common structural abnormality in MALT-lymphoma, occurring in approximately 50% of cases.[19,20] The t(11;18) involves the API2 gene, mapping at 11q21, and the MLT gene, mapping at 18q21, and results in the API2/MLT fusion protein.

API2 is an inhibitor of apoptosis that interferes with the effector caspases-3, -7 and -9. MLT is a caspase-like protease containing a death domain (DD) and involved in the activation of the NF-κB transcription factor pathway.[19,20] Physiologically, MLT activates NF-κB only through the interaction with the BCL-10 protein.[21-23] In contrast, MLT in the context of the API2/MLT fusion protein undergoes self-activation, leading to induction of NF-κB and, consequently, NF-κB-mediated inhibition of apoptosis.[19,20]

The translocation t(1;14)(p22;q32) is an uncommon recurrent chromosomal aberration associated with MALT-lymphoma; it affects the BCL-10 gene.[19,24] Physiologically, BCL-10 and *MLT* form a tight complex that activates the NF-κB pathway.[19,24] The translocation results in deregulated expression of BCL-10 and constitutive activation of NF-κB.[19,24] Thus, the activation of NF-κB by either overexpressed BCL-10-MLT complexes or by *API2/MLT* fusion protein may represent the major pathogenetic mechanism involved in MALT-lymphoma.

BAX. Mutations inactivating the BAX gene have been identified in a fraction of Burkitt lymphoma (BL) cell lines which carry a phenotype characterized by resistance to Fas-mediated apoptosis.[25] However, several subsequent studies on primary tumors demonstrated that BAX mutations are rare in lymphomas of both immunocompetent and immunocompromised hosts.[26-28] Independently of BAX gene alterations, reduced expression of Bax protein has been identified in several lymphoid malignancies.[29,30]

Death associated protein (DAP)-kinase. DAP-kinase is a cytoplasmic serine/threonine kinase involved in the transduction of the apoptotic signal triggered by γ interferon. Inactivation of DAP-kinase gene through promoter hypermethylation is a common event in indolent B-cell lymphomas and functionally causes loss of γ interferon-mediated apoptosis.[31,32]

FAS. Germline mutations of FAS have been discovered as the underlying basis for the human and mouse *A*utoimmune *L*ym*P*hoproliferative *S*yndrome (ALPS), a disorder of lymphocyte homeostasis characterized by a high predisposition to the development of B-cell non-Hodgkin's lymphoma (B-NHL).[33] A putative role of FAS mutation in lymphomagenesis is additionally suggested by the fact that approximately 20% of GC-related B-NHL carry somatic mutations of FAS targeting both the DD as well as other domains of the protein.[34-39] Mutations of FAS

inhibit Fas-mediated apoptosis. In fact, mutations affecting the DD cause loss of FADD binding, a decrease in DISC formation and thus a concomitant decrease in apoptosis induction. Since most B-NHL are resistant to Fas-mediated apoptosis, other mechanisms beside Fas somatic mutations may be involved in generating the Fas-resistant phenotype, including deregulated membrane expression of Fas protein or alterations in downstream effector molecules.[34-40]

CASP-10. Somatic mutations of CASP10 have been identified in 15% of B-NHL. A subset of mutations inactivate the DED, which is necessary for caspase-10/FADD interaction in the DISC.[41,42]

DR4-DR5. Somatic mutations of TRAIL receptors DR4 and DR5 are rare events among B-NHL. Most of these mutations target the DD, resulting in malfunction of apoptotic signal transduction.[43] Malfunctioning of the TRAIL pathway in lymphomas devoid of TRAIL mutations may account for resistance to TRAIL-induced apoptosis. In this issue of Haematologica, Hussain *et al*. have tried to define the sensitivity and possible mechanisms of resistance to TRAIL in BL.[44] The authors identified a BL cell line that is sensitive to TRAIL-mediated apoptosis, but resistant to Fas. Since Fas-mediated apoptosis in BL cells is strictly correlated to the expression of Bax, Bak and Bcl-Xs, it is presumable that loss of expression of these pro-apoptotic proteins does not compromise sensitivity to TRAIL, and thus that TRAIL may induce apoptosis independently of the intrinsic pathway. These results underscore the utility of TRAIL-based therapeutic strategies for B-NHL.[44]

Interference with apoptosis signaling: a perspective for molecular therapy of lymphomas

Emerging knowledge about the proteins that constitute the apoptotic machinery and their alterations in lymphoid malignancies has revealed new potential targets for molecular therapy. With respect to the intrinsic pathway of apoptosis, for example, nuclease-resistant antisense oligonucleotides directed against BCL-2 mRNA are currently in phase II and III trials for patients with lymphoid malignancies.[45]

With respect to the extrinsic pathway of apoptosis, death receptors involved in apoptosis signaling have been considered as potential therapeutic targets due to their ability to kill cancer cells. This option was first tested using TNFα and FasL, but the results were disappointing because the impressive therapeutic effect was hampered by acute and severe toxicity observed in preclinical studies.[4,7]

More recently, TRAIL has appeared to be selectively cytotoxic for tumor cells, but not or only minimally toxic for normal tissues.[4,7] The tumor specific killing by TRAIL may be related to the expression of decoy receptors DcR1 and DcR2 on normal tissues but not on tumor cells. In preclinical studies, TRAIL effectively killed multiple myeloma, chronic lymphocytic leukemia and BL cells.[44,46-50] Moreover, TRAIL and chemotherapy may exert a synergistic cytotoxic effect. In fact, chemotherapeutic agents sensitize myeloma cells to TRAIL apoptosis by inducing overexpression of its death receptors.[4,7]

However, serious concerns have been raised about the use of TRAIL in cancer therapy, since its safety has been challenged by the potential hepatotoxicity of this compound.[4,7] Indeed, further preclinical trials are required to elucidate the spectrum of TRAIL toxicity before clinical trials.

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