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Review

B-cell Differentiation, Apoptosis and Proliferation in Diffuse Large B-cell Lymphomas

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Abstract. *Diffuse large B-cell lymphomas (DLBCL) represent the most common type of adult non-Hodgkin's lymphomas in Western countries and are characterized by heterogeneous clinical, histological, immunophenotypic and genetic features. Recent investigations using cDNA and oligonucleotide microarrays have identified molecularly distinct groups of DLBCL with respect to the B-cell differentiation gene expression profile: the germinal center (GC) B-cell-like DLBCL, the activated B-cell-like DLBCL and the type 3 DLBCL. The GC B-cell-like DLBCL were characterized by the expression of genes of the normal GC B-cells, the activated B-cell-like DLBCL were characterized by the expression of genes that are normally induced during in vitro activation of peripheral blood B-cells, while the type 3 DLBCL did not express either set of genes at a high level. Patients with GC B-cell-like DLBCL had more favorable clinical outcome than those with activated B-cell-like or type 3 DLBCL. Immunohistochemical studies have shown that the bcl6/CD10/MUM1/CD138 B-cell differentiation immunophenotypes are prognostically relevant and may predict the cDNA classification in a sizable fraction of DLBCL. In the last few years, there has been accumulating molecular and immunohistochemical evidence indicating links between B-cell differentiation gene expression profiles and expression of apoptosis and cell cycle-associated genes in DLBCL. The present review summarizes data with respect to the relationships between B-cell differentiation, apoptosis and proliferation in DLBCL.*

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Diffuse large B-cell lymphomas (DLBCL) account for approximately 40% of all non-Hodgkin's lymphomas of the Western world and are characterized by heterogeneous clinical, histological, immunophenotypic and genetic features (1-11). DLBCL are detected as primary or secondary forms both at the nodal and extranodal levels (5, 6). The significant variability in cell morphology, immunophenotype and clinical profile justifies the identification of variants and subtypes. Among the latter, the primary mediastinal subtype corresponds to a distinct clinicopathological entity (5, 6). Over recent years, there has been accumulating evidence that diverse mechanisms disrupting the molecular pathways that regulate B-cell differentiation, apoptosis and proliferation are involved in the pathogenesis of B-cell lymphoid malignancies, including DLBCL (12-87).

Recently, increased understanding of the pathobiology of DLBCL has been made possible by using cDNA and oligonucleotide microarrays for the analysis of the global gene expression profile of DLBCL (76-82). On the basis of the B-cell differentiation gene expression profiles, three molecularly distinct histogenetic groups of DLBCL have been identified: the germinal center (GC) B-cell-like DLBCL, the activated B-cell-like DLBCL and the type 3 DLBCL (79-81). The GC B-cell-like DLBCL were characterized by the expression of genes of the normal GC B-cells (*e.g.* bcl6, CD10, CD38), the activated B-cell-like DLBCL were characterized by the expression of genes that are normally induced during *in vitro* activation of peripheral blood B-cells, while the type 3 DLBCL did not express either set of genes at a high level (79-81). The DLBCL gene expression subgroups have distinct mechanisms of malignant transformation, which indicates that they are pathogenetically distinct diseases. Indeed, the translocation t (14;18), which involves the bcl2 gene, the amplification of the c-REL locus on the chromosome 2p and ongoing immunoglobulin somatic mutations were observed in GC B-

cell-like but not in activated B-cell-like DLBCL (80, 83). By contrast, activated B-cell-like DLBCL are characterized by activation of the NF-kB pathway and high expression levels of NF-kB target genes, including those that encode the interferon regulatory factor 4 (IRF4/MUM1), the cell adhesion molecule CD44, the anti-apoptotic genes c-FLIP, bcl2, bcl-xl, TRAF1, TRAF2, c-IAP1 and c-IAP2 and the cell cycle-associated gene cyclin D2 (78-80). These findings indicate links between B-cell differentiation, apoptosis and proliferation in DLBCL. With respect to the clinical relevance of the molecular classification of DLBCL, patients with GC B-cell-like DLBCL had more favorable clinical outcome than those with activated B-cell-like or type 3 DLBCL (79-81).

Since the cDNA microarrays technology is expensive and not generally available, many studies have successfully used immunohistochemical analysis for the histogenetic classification of DLBCL in routine histopathology specimens (3-6, 9, 10, 36-40, 57, 60-62, 84-87). Of particular importance, is the study of Hans *et al.*, who correlated cDNA microarrays and immunohistochemical results in order to examine the reliability of bcl6/CD10/MUM1 B-cell differentiation immunophenotyping for classifying DLBCL (57). They showed that the classification of DLBCL into GC and non-GC B-cell-like groups, based on the bcl6/CD10/MUM1 B-cell differentiation immunophenotypes, is prognostically relevant and predicts the cDNA classification in 71% of GC B-cell-like and 88% of activated B-cell-like or type 3 DLBCL (57). On the other hand, the B-cell differentiation proteins bcl6, CD10 and MUM1 have been related to the status of apoptosis and proliferation in cell lines and B-cell lymphoid malignancies (88-105) and have been implicated in the pathogenesis of various B-cell lymphoid malignancies (106-146).

In the present review, we summarize data with respect to the relationships between B-cell differentiation, apoptosis and proliferation in DLBCL, because these relationships seem to be important for the further understanding of the pathobiology and the clinical behavior of DLBCL (38, 43, 57, 60, 61, 76-82, 85, 87, 117, 123, 131-133, 145). We particularly focus on the B-cell differentiation proteins bcl6, CD10, MUM1 and CD138, which have been found to be useful for the histogenetic classification of DLBCL into GC and non-GC subgroups in routine histopathology specimens (57, 61, 62, 145).

Physiological role and expression patterns of the B-cell differentiation proteins bcl6, CD10, MUM1 and CD138 in normal B-cells and B-cell malignancies

The proteins bcl6, CD10, MUM1 and CD138 are involved in normal B-cell differentiation and and are considered to be implicated in the pathogenesis of various types of B-cell malignancies (106-146).

Bcl6 protein is a POZ/zinc finger sequence-specific transcriptional repressor, which is involved in lymphocyte activation, differentiation, proliferation, apoptosis and migration (108, 146). Bcl6 was found to be required for GC formation, antibody-affinity maturation and T-helper-2-mediated responses (106-108, 146). Mice deficient in bcl6 fail to form GC and mount reduced levels of T cell-dependent antibody responses (146). Bcl6 (-/-) mice develop a massive inflammatory response in many organs characterized by eosinophilic infiltration and hyper-IgE production, a typical Th2 hyperimmune response (146). This suggests a negative role of bcl6 in the Th2 pathway. By functioning as a potent transcriptional repressor of various target genes, bcl6 modulates IL-4, B-cell receptor and CD40L signals for normal B-cell development (108). Bcl6 inhibits lymphocyte activation by inhibiting the expression of CD69 and CD44 and inhibits differentiation of GC B-cells toward plasma cells by inhibiting the expression of the Blimp-1 gene, which is a transcriptional repressor with a key role in plasmacytic differentiation (108). Bcl6 represses a group of genes (cyclin D2 and p27) that are involved in cell cycle control (108). Studies of bcl6 (-/-) mice have revealed that bcl6 acts as a negative regulator of inflammation by repressing the expression of chemokines (108). Structural alterations of the bcl6 promoter region, including chromosome translocation and somatic hypermutation, represent the most frequent genetic lesions associated with non-Hodgkin's lymphoma, especially of DLBCL (3, 9, 108, 146). Chromosomal translocations involving the 5' noncoding domain of the bcl6 gene at band 3q27 are observed in about 40% of DLBCL and 10-15% of follicular B-cell lymphomas, juxtaposing the gene to promoters from a variety of partner chromosomes (most commonly in immunoglobulin heavy chain loci) (109-114). Mutations within the 5' noncoding domain of the bcl6 gene occur frequently in GC cells and GC lymphomas, including DLBCL, follicular B-cell lymphomas and Burkitt lymphomas (115-118). A subset of mutations specifically associated with DLBCL cause deregulated bcl6 transcription (118). These mutations affect two adjacent bcl6 binding sites located within the first noncoding exon and prevent bcl6 from binding its own promoter, thereby disrupting its negative autoregulatory circuit (118). Expression of the bcl6 protein occurs almost always in follicular B-cell lymphomas, in about 50-80% of DLBCL and in a sizable fraction of Burkitt lymphomas, but it appears that bcl6 protein expression is not associated with alterations of the bcl6 gene (120, 121, 131-137).

CD10 protein is a cell surface metalloproteinase that reduces cellular response to peptide hormones (5, 6, 122). Identified substrates are largely neural or humoral oligopeptides including growth and chemotactic factors and the enzyme functions to terminate signaling by degrading

the ligand (122). Among hematopoietic cells, CD10 is expressed by immature B- and T-cells, by the GC B-cells, by granulocytes and by cells of various lymphoid malignancies (122). Regarding B-cell lymphomas, CD10 protein is expressed in almost all Burkitt lymphomas, in most follicular B-cell lymphomas and in about 30-40% of DLBCL, whereas marginal zone/mucosa associated lymphoid tissue (MALT) lymphomas and mantle cell lymphomas are CD10-negative (5, 6, 120-124).

MUM1/IRF4 protein is encoded by the MUM1 gene, which has been identified as a myeloma-associated oncogene. This gene is activated at the transcriptional level as a result of t(6;14)(p25;q32) chromosomal translocation and by virtue of its juxtaposition to the 3' a enhancer locus of the immunoglobulin heavy chain gene (IgH) (9, 125). It belongs to the interferon regulatory factors (IRF), which is generally induced by interferon and plays a role in cell proliferation, cell survival and in resistance against viral infections. MUM1/IRF4 expression was found to be highest in plasma cells among the B-cell lineage and in activated T-cells among T lineage lymphocytes (3, 5, 6, 9, 125). There is evidence that MUM1 expression may denote the final step of intra-GC B-cell differentiation and subsequent steps of B-cell maturation toward plasma cells (3, 5, 6, 9, 125). MUM1 protein is expressed in about 50-75% of DLBCL, and in a part of marginal zone and small lymphocytic lymphoma, whereas follicular B-cell lymphomas and mantle cell lymphomas are MUM1-negative (3, 5, 6, 9, 125-128).

CD138 protein is an integral transmembrane proteoglycan belonging to the syndecan family, which mediates cell-to-extracellular matrix interactions (129). In normal lymphoid tissue, CD138 protein is expressed by plasma cells and by bone marrow-derived B-cell precursors (5, 6). Regarding B-cell lymphomas, CD138 protein is expressed on the surface of tumor cells of various origins including multiple myeloma, Hodgkin's lymphomas and certain HIV-associated lymphomas, whereas DLBCL are very rarely CD138-positive (5, 6, 129, 130). CD138 protein has important effects on myeloma cell growth, survival, adhesion and invasion (129).

The aforementioned proteins can be used as an immunohistochemical panel for the histogenetic classification of DLBCL since bcl6 and CD10 are expressed by GC B-cells, MUM1 by late GC and post-GC B-cells and CD138 by post-GC B-cells (5, 6). On the basis of the bcl6/CD10/MUM1 immunohistochemical patterns, two major immunophenotypic profiles were distinguished according to the pattern of differentiation: a) the GC B-cell-like differentiation immunophenotype (composed of the bcl6+/CD10+/MUM1, bcl6+/CD10-/MUM1- and bcl6-/CD10+/MUM1- patterns) and b) the non-GC B-cell-like differentiation immunophenotype (composed of the bcl6+/CD10-/MUM1+ and bcl6-/CD10-/MUM1+ patterns) (57, 61).

Apoptosis and proliferation in diffuse large B-cell lymphomas

Before we discuss the relationships between B-cell differentiation, apoptosis and proliferation in DLBCL, we briefly summarize the basic concepts regarding cell cycle and apoptosis in normal cells.

The cell cycle progression is regulated in response to many intracellular and extracellular signals. These signals are integrated into stop or go messages by a complex network of signal transduction pathways that channel information towards specific regulatory events. Current models suggest that cell cycle regulation is achieved through a family of serine/threonine kinase holoenzyme complexes consisting of regulatory cyclin subunits that bind to and activate catalytic cyclin–dependent kinases (CDK) (reviewed in 3, 10, 147-149). Cyclins are expressed in a cell cycle-dependent manner and are divided in to two main functional families. The G1 family includes the cyclins D1, D2, D3 and E, which are important for the passage of cells through the G1-phase and their entry into the S-phase. The other family includes the cyclins A, B1 and B2. Cyclin A is involved in DNA synthesis, S-phase completion and preparation for mitosis. Cyclins B1 and B2 control the onset, sequence of events and completion of mitosis. Cyclins D complex with either CDK4 or CDK6 in the early G1-phase of the cell cycle and they are thought to be involved in regulating the activity of the restriction point that controls the transition through the late G1-phase of the cell cycle. The cyclin E/CDK2 complex acts at the G1/S boundary. Accumulation of the cyclin E/CDK2 complex depends on the E2F transactivation of the cyclin E gene and by ubiquitin-mediated destruction of the protein. Once the cell enters the S-phase, cyclin E is degraded and the activation of CDK2 is taken over by cyclin A. The cyclin A/CDK2 complex is important for the initiation and the maintenance of DNA synthesis. Activation of CDK2 by cyclin A is necessary for the continuation of the S-phase, but toward the end of S-phase, cyclin A activates CDK1. This signals the completion of the S-phase and the initiation of the G2-phase. The G2/M transition is triggered by the cyclin B/ CDK1 complex (mitosis promoting factor) which regulates the onset, sequence of events and completion of mitosis. The full biological activity mitosis promoting factor is achieved by the nuclear translocation of the complex and is maintained up to the metaphase-anaphase transition in mitosis. The kinase activity of the complexes composed of cyclins and CDKs are negatively-regulated by cyclin–dependent kinase inhibitors (CDKI) (reviewed in 3, 10, 147-149). There are two known families of CDKIs. The INK4 family includes four closely related ankyrin repeat containing genes (p16/INK4A, p15/INK4B, p18/INK4C and p19 (p14)/INK4D), which bind to CDK4 and 6 and prevent

D-type cyclin binding and activation. The CIP/KIP family includes three genes (p21/CIP1, p27/KIP1 and p57/KIP2), which target CDK 2, 4 and 6. The cell cycle progression is controlled by the p53, Rb and p27 tumor suppressor pathways (reviewed in 3, 10, 147-149).

The p53 (p14-Hdm2-p53-p21) pathway regulates cell cycle arrest in G1- and G2-phases. P53-dependent G1/S arrest can be mediated through p53-mediated induction of p21 and p53-dependent G2/M arrest can be mediated by repression of the promoters of cyclin B1 and CDK1. The activity and the stability of the p53 protein is regulated *via* interactions with proteins such as mdm2, which allows targeting of p53 to the ubiquitin-mediated proteolytic network. The Rb (p16-cyclin D-CDK4-Rb) pathway regulates the restriction point by inhibiting transcription of genes necessary for the transition from G1- to S-phase. Central to the Rb pathway is the regulation of phosphorylation of the Rb protein (pRb). pRb is a member of the 'pocket-protein' gene family, which also include p130/pRb2 and p107. Hypophosphorylated pRb binds and inactivates transcription factors, notably the E2F1, important for the transition from G1- to S-phase. When pRb is phosphorylated the E2F1 transcription factor is released. Phosphorylation of pRb is stimulated by cyclin D-CDK4/6 complexes and inhibited by the p16 and other CDKIs including p27. The two pathways (p53 and Rb) are linked through the 9p21 locus in which reside two CDKI genes, the CDKN2A and CDKN2B genes. The CDKN2A gene encodes p16 protein which is involved in the RB1 pathway, whereas the p14/ARF protein binds to Hdm2 and promotes Hdm2 degradation, thereby abrogating the Hdm2-mediated degradation of p53. Central to the p27 (p27-cyclin E-CDK2) pathway is the CDKI p27, which may act as a mediator of G1 arrest. P27 is phosphorylated by cyclin E-CDK2 and this modification signals the proteolytic degradation of p27 protein *via* ubiquitination-proteasomal degradation; in this process, SKP2 mediates degradation of p27 by acting as ubiquitin ligase for p27 protein.

Apoptosis is morphologically defined by alterations including cell shrinkage, nuclear fragmentation and chromatin condensation. The apoptotic process is genetically controlled through a network of positive and negative elements (reviewed in 150-152). Apoptosis can be initiated by two alternative convergent pathways, the extrinsic pathway, which is mediated by cell surface death receptors and the intrinsic pathway, which is mediated by mitochondria. In both pathways, cysteine aspartyl-specific proteases (caspases) are activated that cleave cellular substrates resulting in the characteristic morphological and biochemical alterations of apoptosis.

The extrinsic pathway involves cell surface death receptors belonging to the Tumor Necrosis Factor-Receptor (TNF-R) family, which includes TNF-R1, Fas/CD95, Death Receptor (DR) 3, DR4, DR5 and DR6. These receptors are characterized by an extracellular cysteine-rich domain and an intracellular death domain (DD) crucial for transduction of the apoptotic signal. The specific ligands for the TNF-R family belong to the TNF family, which includes TNFa, Fas-ligand, lymphotoxin (LT) a, apo-3-ligand and TNF related apoptosis inducing ligand (TRAIL). Binding of a death ligand to a death receptor induces activation of the death receptor by homotrimerization. Once activated, death receptors recruit adaptor proteins [*e.g.* Fas associated death domain (FADD) for the case of Fas/CD95] through interaction between the DD of the death receptors with the DD of the adaptor proteins. The adaptor proteins contain the death effector domain (DED) which interacts with the DED of the apoptosis initiator enzyme procaspase 8. The resulting complex, consisting of trimerized death receptor (*e.g.* Fas /CD95), adaptor protein (*e.g.* FADD) and procaspase 8, is called death inducing signalling complex (DISC). Procaspase 8, after recruitment in the DISC, is activated by auto-proteolytic cleavage into caspase 8.

The intrinsic (or mitochondrial) pathway is induced in response to stress stimuli such as DNA damage caused by chemotherapeutic agents, UV- or γ-irradiation or withdrawal of survival signals such as growth factors, cytokines or hormones. The intrinsic pathway is triggered by stimulation of the mitochondrial membrane (*e.g.*, by translocation into mitochondria of the bcl2 family of proteins resulting in alterations in the mitochondrial membrane permeability) and the consequent release of cytochrome c and other apoptogenic factors from the intramembraneous space of mitochondria. Cytochrome c recruits the caspase adaptor molecule called APAF1 (apoptotic protease-activating factor-1) and the apoptosis initiator enzyme procaspase 9. Together, cytochrome c, APAF1, procaspase 9 and ATP form a holoenzyme complex called apoptosome. Procaspase 9 is activated by auto-proteolytic cleavage into caspase 9. The extrinsic and intrinsic pathways are intimately connected and both pathways of apoptosis signaling converge into a common pathway causing the activation of effector or executioner caspases 3, 6 and 7. Interestingly, in some cells (type I cells) the amounts of active caspase 8 are sufficient to induce apoptosis by the death-receptor pathway, but in other cells (type II cells) these amounts are not sufficient and mitochondria are used as amplifiers. In type II cells, activation of the intrinsic (or mitochondrial) pathway is mediated by the bcl2 family member bid protein, which is cleaved by active caspase 8 and translocates to the mitochondria.

The apoptotic machinery is tightly regulated and various proteins control the apoptotic process at different levels. Important roles in the regulation of apoptosis are played by the FLIP proteins (FADD-like interleukin-1 β-converting

enzyme-like protease [FLICE/caspase 8]-inhibitory proteins), the bcl2 family of proteins and the IAP proteins (inhibitor of apoptosis proteins). The FLIP proteins interfere with the death receptor pathway by binding to the DISC, thereby inhibiting the activation of procaspase 8. The bcl2 family comprises both pro-apoptotic and anti-apoptotic members that are classified by sequence homology into four α-helical segments called BH1-BH4. The highly conserved anti-apoptotic members (*e.g.*, bcl2, bcl-xl and mcl1) contain all four BH3 domains. The more conserved multidomain pro-apoptotic members (*e.g.*, bax, bak, bok) contain the BH1, BH2 and BH3 domains. In contrast, the BH3-only members (*e.g.*, bad, bik, bim, bid) contain only the BH3 minimal death domain. The functional balance of anti-apoptotic *versus* pro-apoptotic influences determines a cell's susceptibility to apoptosis. The mechanisms of action of some of these proteins, principally homodimerization and heterodimerization, have been extensively investigated (reviewed in 152). For example, bax homodimers promote apoptosis, while bcl2 homodimers and bcl2/bax heterodimers inhibit apoptosis; similarly, bcl-xl inhibits apoptosis, while bcl-xs and bak inhibit the anti-apoptotic function of bcl-xl. The IAP family of proteins (*e.g.*, XIAP, c-IAP1, c-IAP2, NAIP, ML-IAP, ILP2, survivin) may suppress apoptosis by binding to and inhibiting caspases or may act as E3-ubiquitin ligases, promoting the degradation of the caspases that they bind.

Over recent years there has been increasing evidence that diverse mechanisms resulting in the deregulation of cell cycle and apoptotic pathways are involved in the pathogenesis of DLBCL (1-3, 8-10, 19-24, 32-44, 52-54, 59-62, 78-83, 152-154). Importantly, structural alterations of the bcl6 gene, which is involved in the regulation of cell cycle, apoptotic and B-cell differentiation pathways, represent the most frequent genetic lesions in DLBCL (3, 8, 9, 108-118, 146). Disruption of the p53 pathway (*e.g.*, p53 inactivation by p53 gene mutations), Rb pathway (*e.g.*, p16 inactivation by promoter hypermethylation, deletion or mutation or hyperactivation of CDK/cyclin-D complexes by overexpression of cyclin D3) and p27 pathway (*e.g.*, p27 inactivation by increased protein degradation or by sequestration in CDK4/cyclin D3 complexes) may result in cell cycle deregulation in DLBCL (3, 5, 6, 8-10). Apoptosis deregulation may result from impairment of the pathways regulated by the bcl2 family members (*e.g.*, the translocation t (14;18), which involves bcl2 gene) (3, 5, 6, 8-10).

B-cell differentiation, apoptosis and proliferation in diffuse large B-cell lymphomas

An important aim of studies analyzing the B-cell differentiation gene expression profile of B-cell lymphomas is the identification of the cell of origin of a given B-cell lymphoma (8). Thus, the cell of origin of a B-cell lymphoma refers to the relationship between the B-cell differentiation gene expression profile of the tumor on clinical presentation and a normal stage of B-cell differentiation (8). This relationship has usually been assessed by a combination of histological appearance, immunophenotype and some genetic features (4, 5, 8). Recently, the analysis of the global gene expression profile using the methods of cDNA and oligonucleotide microarrays has provided important information a) for the relationships between B-cell differentiation gene expression profile of DLBCL and normal stages of B-cell differentiation and b) for the relationships between B-cell differentiation gene expression profile and expression status of apoptosis and proliferation-associated genes in DLBCL (1, 2, 76-82). The methods of cDNA and oligonucleotide microarrays quantitate, in parallel, the mRNA levels of tens of thousands of genes (76). For cDNA microarrays, the polymerase chain reaction (PCR) products of cDNA clones are spotted on filters or glass slides and, for oligonucleotide microarrays, oligonucleotide probes are deposited or synthesized directly on the surface of a silicon wafer (76-82). The evaluation of the gene expression profiling data can be performed by unsupervised or supervised learning methods (76-82). Unsupervised learning methods aggregate samples from tumors into groups based on their gene expression profiles without *a priori* knowledge of specific relations, whereas supervised learning methods aggregate samples from tumors based on known differences (*i.e.*, cured *versus* fatal disease) and produce transcriptional profiles of the defined groups.

The method of cDNA microarrays was used by Alizadeh *et al.* (79) and Rosenwald *et al.* (80). Alizadeh *et al.* (79) showed that there is a diversity in gene expression profile of DLBCL, apparently reflecting the variation in tumor cell proliferation, host response and B-cell differentiation status of the tumor. Based on the B-cell differentiation gene expression and using unsupervised learning methods (hierarhical clustering), Alizadeh *et al.* (79) identified two molecularly distinct groups of DLBCL, the germinal center (GC) B-cell-like DLBCL and the activated B-cell-like DLBCL. The GC B-cell-like DLBCL were characterized by the expression of genes of the normal GC B-cells (*e.g.* bcl6, CD10, CD38) and the activated B-cell-like DLBCL were characterized by the expression of genes that are normally induced during *in vitro* activation of peripheral blood B-cells. The gene expression signature of the GC B-like DLBCL included a host of new genes (*e.g.* BCL-7A, LMO2) (79). BCL-7A was cloned as part of a complex chromosomal translocation in a Burkitt lymphoma cell line and was found to be rearranged in a mediastinal large B-cell lymphoma cell line (79). LMO2 is translocated and overexpressed in a subset of T-cell acute lymphoblastic leukemia and may have a role in inhibiting B-cell differentiation (79). The gene expression signature of the activated B-cell-like DLBCL included IRF4/MUM1, and anti-apoptotic genes such as c-FLIP and bcl2 (79). c-FLIP is a dominant-negative mimic of

caspase 8, which can block apoptosis mediated by Fas and other death receptors (79). The patients with GC B-cell-like DLBCL had more favorable clinical outcome than those with activated B-cell-like or type 3 DLBCL (79).

The findings of Alizadeh *et al.* (79) were confirmed by Rosenwald *et al.* (80), who constructed a molecular predictor of risk using genes with expression patterns that were associated with survival. Three gene expression subgroups were identified: GC B-cell-like, activated B-cell-like and type 3 DLBCL. Patients with GC B-cell-like DLBCL had the highest five-year survival rate. GC B-cell-like DLBCL were characterized by two common oncogenic events, bcl-2 translocation and c-REL amplification (80). The c-REL (human c-rel) proto-oncogene (on 2p12-16) encodes for the transcription factor NF-kB, which consists of dimers of subunits belonging to the family of REL/NF-kB proteins (c-REL, p65/RELA, RELB, p50/p105, and p52/p100) (153-155). These dimers bind to a common sequence motif known as the NF-kB site. NF-kB transciptional activity is regulated by members of the inhibitor of the kB (IkB) family of proteins, which binds to NF-kB dimers and retains them in the cytoplasm (153, 154). Exposure to various extracellular stimuli (*e.g.*, proinflammatory cytokines) activates the IkB kinase (IKK) complex which phosphorylates NF-kB bound IkB. This targets IkB for ubiquitin-dependent degradation and allows the liberated NF-kB dimers to translocate to the nucleus. Depending on the stimulus and the cellular context, NF-kB can activate pro-apoptotic (*e.g.*, CD95, CD95L, TRAIL receptors), anti-apoptotic (c-FLIP, bcl2, bcl-xl, c-IAP1, c-IAP2) and cell cycle (cyclin D1, cyclin D2, c-myc) genes (151, 153-155). REL/NF-kB proteins have been involved in normal B-cell development, proliferation and survival. Activation of the Rel/NF-kB signal transduction pathway has been associated with a variety of malignancies as well as in inflammatory and immune responses (153, 154). In addition to the three major molecular subgroups of DLBCL, Rosenwald *et al.* (80) also identified individual genes with expression patterns that correlated with survival. Most of these genes were characteristic of GC B-cells, proliferating cells, reactive stromal and immune cells in the lymph node, or major-histocompatibility-complex class II.

As an alternative approach, Shipp *et al.* (82) used oligonucleotide microarrays for analyzing the gene expression profile of DLBCL. They applied a supervised learning prediction method to identify cured *versus* fatal or refractory disease. The algorithm classified two categories of patients with very different five-year overall survival rates (70% *versus* 12%). The model also effectively delineated patients within specific International Prognostic Index (IPI) risk categories who were likely to be cured or to die of their disease. Genes implicated in the DLBCL outcome included some that regulate responses to B-cell-receptor signaling, critical serine/threonine phosphorylation pathways and apoptosis (82).

A novel statistical classification method (statistical predictor), that focuses on those genes that discriminate the GC B-cell-like and activated B-cell-like DLBCL subgroups with highest significance, was developed by Wright *et al.* (81). This method assigns a tumor to a DLBCL subgroup and estimates the probability of membership in one of two DLBCL subgroups. This method classified two categories of patients with different five-year overall survival rates (62% for GC *versus* 26% activated B-cell-like DLBCL). Interestingly, this method can classify DLBCL into biologically and clinically distinct subgroups irrespective of the technique used to measure gene expression (cDNA or oligonucleotide microarrays). Furthermore, Wright *et al.* (81) showed that activated B-cell-like DLBCL express a subset of genes that are characteristic of plasma cells, particularly those encoding endoplasmic reticulum and Golgi proteins involved in secretion (for example, XBP-1 transcription factor that regulates the unfolded protein response in the endoplasmic reticulum).

Of particular interest are the relationships between the activation of the REL/NF-kB signal transduction pathway and the B-cell differentiation gene expression profile of DLBCL (78, 79). Davis *et al.* (78) showed that activated B-cell-like DLBCL are characterized by constitutive activation of the NF-kB pathway and high expression levels of NF-kB target genes, including those that encode the MUM1/ IRF4, the cell adhesion molecule CD44, the cell cycle-associated gene cyclin D2 and the anti-apoptotic genes c-FLIP, bcl2, bcl-xl, TRAF1, TRAF2, IAP1 and IAP2 (78). The tumor necrosis factor receptor-associated factors (TRAFs) are involved in the signal transduction of several members of the TNFR superfamily (*e.g.*, TNFR2, LT-βR, CD40, CD30, LMP1). These members of the TNFR family, like TNFR1 and Fas/CD95, are known to transduce signals regulating apoptosis and proliferation, but lack the intracellular death domain present in TNFR1 and Fas/CD95 (3, 10). It has been shown that TNFR2, LT-βR, CD40, CD30 and LMP1 exert their function by interacting with TRAF1, 2, 3, 5 or 6, whereas the Fas/CD95 and TNFR1 receptors bind to another group of molecules, including FADD, RIP and TRADD by their death domain (3, 10). In the study of Davis *et al.* (78), the two cell lines of activated DLBCL were found to have high nuclear NF-k B DNA binding activity, constitutive IkB kinase (IKK) activity and rapid IkB alpha degradation. These features were not seen in cell lines of GC B-like DLBCL. Retroviral transduction of a super-repressor form of IkB alpha or dominant negative forms of IKK beta was toxic to activated B- cell-like DLBCL cells but not GC-B DLBCL cells (78). DNA content analysis showed that NF-kB inhibition caused both cell death and G1-phase growth arrest (78). Further information on the involvement of the NF-kB pathway in DLBCL was provided by Houldsworth *et al.* (138), who analyzed the relationship between REL

amplification and REL function and correlated the results with the GC and activated B-cell-like gene expression profiles. They showed that amplification of the REL locus (2p12-16) is not associated with accumulation of the nuclear active form of REL, as evaluated by immunofluorescence analysis. REL amplification was detected in all subgroups of DLBCL, while high levels of nuclear-located REL were more frequently detected in activated B-cell-like DLBCL. The results of Houldsworth *et al.* (138) indicated that the 2p12-16 amplification does not lead to abnormal REL activation, suggesting that REL may not be the functional target of the amplification event.

Since the cDNA microarrays technology is expensive and not generally available, many studies have used immunohistochemical analysis for the histogenetic classification of DLBCL in routine histopathology specimens (36, 37, 57, 60-62, 84-87, 133-137). Of particular importance is the study of Hans *et al.* (57), who correlated cDNA microarrays and immunohistochemical results and showed that the classification of DLBCL into GC and non-GC B-cell-like groups based on the bcl6/CD10/MUM1 differentiation immunophenotypes predicts the cDNA classification in 71% of GC B-cell-like and 88% of activated B-cell-like or type 3 DLBCL (57). In addition, recent immunohistochemical studies indicated relationships between the B-cell differentiation, apoptosis and proliferation in DLBCL (60, 61, 86, 117). Indeed, in DLBCL a) the GC B-cell-like bcl6/CD10/MUM1 differentiation immunophenotype was associated with high apoptotic index, high expression of the pro-apoptotic proteins bax, bak and bid and low expression of the anti-apoptotic protein bcl-xl and b) the expression of the GC B-cell related bcl6 and CD10 proteins was positively correlated with the apoptotic index and the expression of Ki67, cyclin A, bax, bak and bid proteins and negatively correlated with the expression of bcl-xl (60, 61, 86, 117). It was, therefore, suggested that DLBCL with GC B-cell-like immunophenotype are associated with increased apoptosis and that the expression of the GC B-cell-related bcl6 and CD10 proteins is associated with increased apoptosis and proliferation (60, 61).

The above immunohistochemical data can be related to *in vitro* data indicating a dual role of bcl6 in the apoptosis and cell cycle regulation and to *in vitro* and *in vivo* data indicating an association between CD10, apoptosis and proliferation (86, 88-105).

With respect to the relationship between bcl6 and apoptosis, the data in the literature indicate that the role of bcl6 as a promotor or inhibitor of apoptosis may depend on the cellular context and the experimental approach (88-98). Some studies reported that bcl6 may protect cells from apoptosis (95-98). Kumagai *et al.* (95) used the differentiating mouse myogenic cell line C2C12 and showed that adenovirus-mediated overexpression of bcl6 is related to terminal differentiation and to enhanced viability of the differentiating myocytes by preventing apoptosis. They found that apoptosis was induced by high expression levels of bcl6 antisense mRNA, whereas apoptosis was prevented by adenovirus-mediated expression of bcl6 sense mRNA. They suggested that bcl6 may protect mouse myocytes fom specific stressors *e.g.*, serum starvation (95). In keeping with this notion, Kojima *et al.* (96) examined spermatogenesis in bcl6-deficient (-/-), bcl6 heterozygous (+/-) and bcl6 (+/+) mice and showed that bcl6 may play a role as a stabilizer in protecting spermatocytes from apoptosis induced by stressors such as heat shock. Baron *et al.* (97) used the Epstein-Barr virus-negative Burkitt lymphoma BJAB cell line expressing high levels of bcl6 and found that bcl6 represses the human programmed cell death-2 (PDCD2) gene which had been associated with apoptosis in immature thymocytes. To support these findings, Baron *et al.* (97) showed that the immunohistochemical localization of PDCD2 protein expression is inversely related to that of bcl6 protein in germinal center and follicular mantle cells of human tonsils. They suggested that bcl6 may down-regulate apoptosis by means of its repressive effects on the PDCD2 gene (97). Korusu *et al.* (98) used Daudi and Raji B-cell lymphoma cell lines that overexpress bcl6 or its mutant bcl6-Ala333/343. They provided evidence that bcl6 overexpression did not have any significant effect on cell proliferation, but prevented increase in reactive oxygen species and inhibited apoptosis induced by chemotherapeutic reagents such as etoposide. However, some other studies reported that high expression of bcl6 may induce apoptosis (88-91). Zhang *et al.* (88) found that overexpression of bcl6 induces apoptosis in murine fibroblast NIH3T3 cells and showed that a 17 aminoacid sequence in the middle portion of bcl6 is responsible for inducibility of apoptosis in these cells. Albagli *et al.* (89) used a tetracyclin-regulated human osteosarcoma cell line U2OS stably transfected with bcl6 that could be induced in very high expression levels after removal of tetracyclin. They found that bcl6 induces dose-dependent growth suppression which was correlated with delayed S-phase progression and triggering of apoptosis, whereas a truncated bcl6 derivative used as control failed to induce apoptosis (89). Yamochi *et al.* (90) used a recombinant adenovirus to express bcl6 in CV1 and HeLa cells. They found that the viability of these cells was markedly reduced secondary to apoptosis. In their study, bcl6-overexpressing cells accumulated at the subG1- and G2/M-phase and bcl6-induced apoptosis was preceded by down-regulation of bcl2 and bcl-xl. Based on their findings, Yamochi *et al.* (90) suggested that bcl6 might regulate the expression of the apoptosis repressors bcl2 and bcl-xl. In keeping with the findings of Yamochi *et al.* (90), Tang *et al.* (91) described a novel pro-apoptotic repression program that is activated by nuclear localization of the forkhead transcription factor AFX

and involves bcl6 and the anti-apoptotic gene bcl-xl. They used transfectants of HeLa cells and showed that an AFX-induced transcriptional program induces bcl6 which directly binds to and suppresses the promoter of the bcl-xl gene (91). On the basis of these latter *in vitro* findings, it can be suggested that the significantly lower expression of the anti-apoptotic protein bcl-xl that was found in DLBCL with GC B-cell-like immunophenotype (61) may be due, at least in part, to down-regulation of bcl-xl expression induced by bcl6 overexpression.

With respect to the relationship between bcl6 and proliferation, the data in the literature indicate that the role of bcl6 as promotor or inhibitor of cell cycle progression and proliferation may depend on the cellular context and the experimental approach (89, 92-94, 99). Some *in vitro* studies reported that bcl6 expression was associated with impaired cell cycle progression and decreased proliferation (89, 93). Albagli *et al.* (89) showed that bcl6 mediates growth suppression associated with impaired S-phase progression in human U2OS osteosarcoma cells. Hosokawa *et al.* (93) established Ba/F3 pro-B cells carrying a human bcl6 transgene and they showed that induced bcl6 protein down-regulates the expression of the cell cycle protein cyclin A2 and inhibits cell proliferation. However, other *in vitro* studies reported that bcl6 may act as a promotor of cell cycle progression and proliferation (92, 94, 99). Allman *et al.* (99) showed that bcl6 protein expression was 34-fold higher in the rapidly proliferating GC B-cells than in the resting B-cells. Shaffer *et al.* (92) showed that bcl6 may promote cell cycle progression and maintain proliferation by repressing the expression of CDKI p27 and by blocking blimp-1 expression which represses c-myc expression. Shvarts *et al.* (94) have identified bcl6 as an inhibitor of antiproliferative p19 (ARF)-p53 signaling by using a senescence rescue screen for identifying genes that bypass the senescence response. Using primary murine embryo fibroblasts and primary B-lymphocytes, Shvarts *et al.* (94) showed that bcl6 overrides the senescence response downstream of p53 through a process that requires cyclin D1 expression, as cyclin D1 knockout fibroblasts are specifically resistant to bcl6 immortalization. In addition, bcl6 expression significantly extends the replicative lifespan of primary human B cells in culture (94). It was, therefore, suggested that bcl6 may act as an immortalizing oncogene by rendering cells unresponsive to antiproliferative signals emanating from the p19 (ARF)-p53 pathway during the senescence response (94). Taking into consideration the *in vitro* findings of Shaffer *et al.* (92) and Shvarts *et al.* (94), Bai *et al.* (60) hypothesized that the association between increased bcl6 expression and increased proliferation in DLBCL might be due, at least in part, to the possibility that bcl6 confers resistance to antiproliferative signals from the p19 (ARF)-p53 pathway and down-regulates the expression of the CDKI p27. In this context, the occurrence of aberrations in the p27 and/or p19 (ARF)-p53 and/or Rb-p16-cyclin D growth-inhibitory pathways (3), which are frequent events in DLBCL (3, 23, 24, 58, 59), might further enhance the proliferative activity of tumor cells. Relevant to this assumption may be the findings showing that altered p27/p53/Rb/p16 expression status is associated with enhanced tumor cell proliferation in DLBCL (58).

With respect to the relationship between CD10, apoptosis and proliferation, there are several lines of evidence indicating a positive correlation of CD10 expression with proliferation and apoptosis in both normal and malignant B-cells (86, 100-105). Indeed, a) GC cells which display high proliferation and have the propensity to undergo apoptosis, up-regulate CD10 protein expression on apoptotic induction, whereas CD10 protein expression is undetectable on other subsets of mature B-cells that are not characterized by high apoptosis (100, 104, 105); b) Burkitt lymphoma cells, which exhibit high proliferation and apoptosis, almost constantly express CD10 protein (4); c) CD10-positive B-acute lymphoblastic leukemia (B-ALL) cells were cycling cells with propensity to apoptosis whereas CD10-negative B-ALL cells had lower cycling capacities and were resistant to apoptosis (102); and d) B-chronic lymphocytic leukemia cells undergoing apoptosis *in vivo* and *in vitro* display expression of CD10 (103). The above findings in normal and malignant B-cells may be corroborated by the findings of Cutrona *et al.* (101), who reported that human postthymic and thymic T-cells express CD10 when undergoing apoptosis. The relationships between CD10 and apoptosis might be explained by the findings that CD10 might degrade cytokines that reach the cell when apoptosis has already started (101). Because a variety of cytokines may play a protective role in B- and T-cell apoptosis, CD10 expression may potentiate the apoptotic ability of B- and T-cells, by inhibiting the protective cytokine signals. This could be consistent with the capacity of CD10 to hydrolyze a variety of active peptides, including growth and chemotactic factors (101, 122). It is possible that CD10 participates in the process of selection in the germinal center and the thymus by increasing the threshold of cytokines required to prevent B- and T-cell apoptosis, respectively.

In contrast to the GC B-cell-like DLBCL, which are associated with increased apoptosis, the non-GC B-cell-like bcl6/CD10/MUM1 differentiation immunophenotype in DLBCL was associated with low apoptotic index, low expression of the pro-apoptotic proteins bax, bak and bid and high expression of the anti-apoptotic protein bcl-xl (60, 61). This association may be related to the findings that activated (non-GC) DLBCL are characterized by constitutive NF-Kappa B activity, which may up-regulate many anti-apoptotic NF-kB target genes such as bcl2, bcl-

xl, A1, TRAF1, TRAF2, c-IAP1 and c-IAP2 (78). Therefore, the higher expression of bcl-xl protein in DLBCL with non-GC B-cell like differentiation immunophenotypes may be due, at least in part, to up-regulation of bcl-xl expression induced by the constitutive NF-kB activity (61). In addition, the expression of the MUM1 protein, which characterizes the non-GC B-cell-like differentiation immunophenotypes, was negatively correlated with bax and bid expression and positively correlated with bcl-xl expression in DLBCL (60, 61). This latter association might be explained by the finding that the MUM1/IRF4 gene is also a NF-kB target (128).

Of particular interest are the relationships between the apoptosis-associated bcl2 gene and the B-cell differentiation gene expression profile in DLBCL. Indeed, increased expression of the bcl2 gene was associated with activated DLBCL by using cDNA microarrays for gene expression profiling (79). However, conflicting results were reported when the bcl2 expression was studied by immuno-histochemistry in relation to the bcl6/CD10/MUM1 B-cell differentiation immunophenotypes. Indeed, Hans *et al.* (57) found bcl2 protein expression in 59% of GC DLBCL and 43% of non-GC DLBCL. In addition, Colomo *et al.* (62) found bcl2 protein expression in 67% of bcl6 (+)/CD10 (+) GC, 50% of bcl6 (+)/CD10 (-) GC and 62% of post-GC DLBCL. In contrast, Bai *et al.* (60) found that high expression of bcl6 was associated with null/low bcl2 expression status. Moreover, Larocca *et al.* (119) showed that, in most cases of primary central nervous system DLBCL with increased bcl6 expression, the bcl2 protein was undetectable. These discrepancies might be due to different case selection, to different cut-off points for the evaluation of the immunohistochemical positivity and to decreased sensitivity of the immunohistochemical histogenetic classification in comparison to the cDNA classification (57).

B-cell differentiation in the primary mediastinal subtype of diffuse large B-cell lymphomas

Primary mediastinal large B-cell lymphoma (MLBCL) is a recently identified subtype of DLBCL, characteristically presenting as localized tumor in young female patients (5, 6, 139-145). With respect to the histogenesis, Pileri *et al.* (142) suggested that a sizable fraction of MLBCL are from activated GC or post-GC B-cells. This was based on the bcl6(+/-)/MUM1(+/-)/CD10(-/+)/CD138(-)/bcl2(+)/CD30(+) immunophenotype exhibited by MLBCL. In addition, more than half of the cases displayed bcl6 gene mutations (142). For a more precise molecular diagnosis and histogenetic classification of primary MLBCL, Rosenwald *et al.* (143) and Savage *et al.* (144) used gene expression profiling and showed a strong relationship between MLBCL and Hodgkin's lymphoma. Indeed, over one-third of the genes that were more highly expressed in MLBCL than in other DLBCL were also characteristically expressed in Hodgkin's lymphoma cells (143). PDI2, which encodes a regulator of T cell activation, was the gene that best discriminated MLBCL from other DLBCLs and was also highly expressed in Hodgkin's lymphoma cells (143). The genomic loci for PDI2 and several neighboring genes were amplified in over half of the MLBCL and in Hodgkin's lymphoma cell lines (143). MLBCL had low expression levels of multiple components of the B-cell receptor signaling cascade, a profile resembling that of Reed-Sternberg cells of cHL (144). Like cHL, MLBCL also had high levels of expression of the interleukin-13 (IL-13) receptor and downstream effectors of IL-13 signaling (Janus kinase-2 [JAK2] and signal transducer and activator of transcription-1 [STAT1]), TNF family members and TRAF1 (144). By immuno-histochemistry, MLBCL displayed increased expression of STAT1 and TRAF1 and c-REL protein was localized to the nucleus, consistent with activation of the NF-kappa B pathway (144).

Clinical relevance of the B-cell differentiation immunophenotypes

The clinical behavior of DLBCL has been extensively analyzed (11, 22-24, 32, 38-44, 52-54, 57, 62, 68, 71, 79-87, 117, 123, 132). Of particular interest are the findings showing that increased proliferation and apoptosis is associated with aggressive behavior in DLBCL (68-71).

Recently, the bcl6/CD10/MUM1 B-cell differentiation immunophenotypes were related to the clinical behavior of DLBCL. Indeed, a) the survival of DLBCL with GC B-cell-like immunophenotype was better than that of DLBCL with non-GC B-cell-like immunophenotype (38, 57, 85, 133, 145); and b) the immunohistochemical expression of bcl6 or CD10 proteins was associated with better overall survival (OS) whereas expression of MUM1 protein was associated with worse OS in DLBCL (57, 79, 87, 117, 123, 131, 132). Interestingly, the relationships between bcl6/CD10/MUM1 immunophenotypes and apoptosis may be helpful for the understanding of the clinical behavior of DLBCL (61). Indeed, high expression of the pro-apoptotic protein bax, which had been related to better five-year OS in DLBCL (43), was associated with the GC B-cell-like immuno-phenotype in these lymphomas (61). On this basis, it was hypothesized that GC B-cell-like DLBCL may be more susceptible to apoptosis and, as a consequence, may be more sensitive to treatment (61).

Besides bcl6, CD10, MUM1 and CD138, the immunohistochemical expressions of other proteins involved in B-cell differentiation, such as CD5, CD23, CD38, CD40

and CD44, were also analyzed with respect to their clinical relevance in DLBCL (85, 87, 133, 137, 156-163). CD5 is expressed in only about 10% of DLBCL and CD5-positive DLBCL were reported to be associated with shorter survival than CD5-negative DLBCL (156-158). CD44 is a family of cell surface adhesion glycoproteins that act as receptors for hyaluronate and exist in a variety of alternatively spliced isoforms and was reported to be a NF-kB target gene (78, 163). Normal lymphocytes express the CD44s and DLBCL may express CD44s and CD44v (especially CD44v6). In DLBCL expression of CD44s was associated with advanced-stage disease and shortened survival and expression of CD44v6 was found to be an independent predictor of poor survival (159-163). Interestingly, Tzankov *et al.* (85) analyzed the prognostic significance of CD44 expression in relation to the B-cell differentiation profile of DLBCL (activated *vs* GC B-cell-like subtypes). CD44v6 was expressed predominantly in activated DLBCL. Expression of CD44v6 correlated with disease stage and might contribute to lymphoma dissemination. CD44s-negative cases were separated into CD44v6-negative (OS, 82% at 70 months) and CD44v6-positive (OS, 58% at 70 months) (85). The prognostic significance of the B-cell differentiation proteins CD23 (marker of pre/early GC origin) and CD40 (marker for a GC phenotype) was analyzed by Linderoth *et al.* (137). CD40 was positive in 76% of the DLBCL cases and CD23 was positive in 16% of the cases (all CD5-negative and all CD40-positive). They showed that CD40 and CD23 positivity were associated with better OS in DLBCL (137).

Conclusion

In the last few years, DLBCL has become a good model for the study of lymphomas because of the accumulation of data revealing the occurence of molecularly distinct subtypes and showing alterations in specific key genes involved in the B-cell differentiation, cell cycle and apoptotic pathways. Studies using cDNA microarrays have identified three molecularly distinct subtypes of DLBCL: the GC B-cell-like DLBCL, the activated B-cell-like DLBCL and the type 3 DLBCL. Immunohistochemical studies have shown that the bcl6/CD10/MUM1 B-cell differentiation immunophenotypes may predict the cDNA classification in most DLBCL, suggesting that these immunophenotypes may be useful for the histogenetic classification of DLBCL in routine histopathology specimens. There is increasing evidence indicating links between B-cell differentiation gene expression profiles and expression of apoptosis and cell cycle-associated genes in DLBCL. These links may be important for the further understanding of the pathobiology and the clinical behavior of DLBCL.

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