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Review

Cell Cycle and Apoptosis Deregulation in Classical Hodgkin Lymphomas

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Abstract. *Classical Hodgkin lymphomas (cHL) have now been recognized as B-cell lymphomas with some exceptional cases of T-cell origin. In recent years, there has been accumulating evidence that Hodgkin and Reed-Sternberg (H/RS) cells, the presumed neoplastic-cell population in cHL, are characterized by a profound disturbance of the cell cycle and apoptosis regulation. The constitutive activation of the nuclear factor (NF)-kB pathway, which is considered to be involved in the proliferation and survival of H/RS cells. Moreover, substantial evidence that H/RS cells have defective cell cycle and apoptosis regulation has been provided by studies showing that these cells are characterized, in a large proportion of cases, by alterations of the p53, Rb and p27 tumor suppressor pathways, overexpression of cyclins involved in the G1/S and G2/M transition such as cyclins E, D2, D3, A and B1, overexpression of cyclin-dependent kinases such as CDK1, 2 and 6 and overexpression of anti-apoptotic proteins such as c-FLIP, bcl-xl, c-IAP2, X-linked IAP and survivin. Recent studies suggest that interleukin 13 (IL-13) is an important growth and survival factor in H/RS cells. Furthermore, the Epstein-Barr Virus (EBV), which is present in H/RS cells in about 30-50% of cHL, has been shown to affect the cell cycle and apoptosis regulation in cHL. The present review summarizes data with respect to the cell cycle and apoptosis deregulation in cHL.*

Hodgkin lymphoma, which accounts for approximately 30% of all lymphomas, is composed of two different entities: the rare lymphocyte predominant Hodgkin lymphoma and the more frequent classical Hodgkin lymphoma, representing approximately 95% of all Hodgkin lymphomas (1, 2). Hodgkin and Reed-Sternberg (H/RS) cells, the neoplastic-cell population in classical Hodgkin lymphoma (cHL), constitute only a minor component of the tumor, whereas the majority of the malignancy is composed of a mixed inflammatory infiltrate variably composed of lymphocytes, eosinophils, macrophages, plasma cells and fibroblasts (1, 2).

A central issue in cHL research was the cell(s) of origin of H/RS cells. Evidence has accumulated that H/RS cells harbor clonally rearranged and somatically mutated immunoglobulin genes, indicating their derivation, in most cases, from germinal center (GC) B-cells (3-6). Under normal conditions, GC B-cells, that lack a functional high-affinity antibody, undergo apoptosis in the germinal center. H/RS cells show a characteristically defective B-cell differentiation program, lose the capacity to express immunoglobulin and, therefore, should die (3-6). However, H/RS cells escape apoptosis and instead proliferate (3-6). These findings raised the questions of how H/RS cells resist apoptosis, acquire self-sufficiency in growth signals and proliferate. Therefore, many studies focused on the mechanisms regulating cell cycle and apoptosis in H/RS cells and provided evidence that these cells are characterized by a profound disturbance of the cell cycle and apoptosis regulation (7-35). In this respect, of particular importance is the constitutive activation of the nuclear factor (NF)-kB pathway in HL-cell lines and neoplastic tissues, which is considered to be involved in the proliferation and survival of H/RS cells (3-6, 12-16). Moreover, substantial evidence that H/RS cells have defective cell cycle and apoptosis regulation has been provided by studies showing that these cells are characterized by alterations of the p53, Rb and p27 tumor suppressor pathways (17-34), overexpression of cyclins involved in the G1/S and G2/M transition such as cyclins E, D2, D3, A and B1 (11, 26, 29, 33-35), overexpression of

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cyclin-dependent kinases such as CDK1, 2 and 6 (33) and overexpression of the anti-apoptotic proteins c-FLIP, bcl-xl, c-IAP2 and survivin (3-6, 23, 28, 33). Furthermore, recent evidence suggests that interleukin 13 (IL-13) is an important growth and survival factor in H/RS cells (36). Finally, the Epstein-Barr Virus (EBV), which is present in about 30-50% of cHL (1-6), has been shown to affect the cell cycle and apoptosis regulation in cHL (33). The present review summarizes data with respect to the cell cycle and apoptosis deregulation in cHL.

Cell cycle and apoptosis

The basic concepts regarding cell cycle and apoptosis regulation in normal and pathological lymphoid cells are reviewed in references 37-44.

The 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) (37, 38). Cyclins are expressed in a cell cycle-dependent manner and are divided into two main functional families. The G1/S 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 G2/M family includes cyclin A, which is involved in DNA synthesis, S-phase completion and preparation for mitosis and cyclins B1 and B2, which control the onset, sequence of events and completion of mitosis. Cyclins D complex with either CDK4 or CDK6 in the early G1-phase and they regulate the activity of the restriction point that controls the transition through the late G1-phase. 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 on ubiquitin-mediated degradation 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. Toward the end of the S-phase, cyclin A activates CDK1, thereby signaling 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 achieves its full biological activity by the nuclear translocation of the complex and is maintaned up to the metaphase–anaphase transition in mitosis. Cyclin-dependent kinase inhibitors (CDKI) negatively regulate the kinase activity of the complexes composed of cyclins and CDKs. There are two known families of CDKIs. The INK4 family includes four 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. Important roles in the control of the cell cycle progression are played by the p53, Rb and p27 tumor suppressor pathways (37, 43). 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 p53 protein is regulated *via* interactions with proteins such as Hdm2, which acts as ubiquitin ligase for p53, allowing targeting of p53 to the ubiquitin-mediated proteolytic network. The enzymatic activity of Hdm2 is controlled by p14; complexes of p14/ Hdm2 proteins are devoid of ubiquitin ligase activity and thus stabilize p53 protein. Central to the Rb (p16-cyclin D-CDK4/6-Rb) pathway is the regulation of phosphorylation of the Rb protein (pRb). Hypophosphorylated pRb binds and inactivates transcription factors, notably the E2F-DP, important for the transition from G1- to S- phase; phosphorylation of pRb by CDK 4/6 kinases results in release of the E2F-DP and subsequent initiation of gene transcription. One of the genes induces by the E2F-DP is cyclin E, which also phosphorylates pRb. 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. 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 (39-42). 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 including TNF-R1, Fas/CD95, Death Receptor (DR) 3, DR4, DR5 and DR6, which have in common an intracellular death domain (DD) required for apoptotic signal transduction; the specific ligands for the TNFR family belong to the TNF family including TNFa, Fas-ligand, lymphotoxin (LT)-a, Apo-3-Ligand and TNF Related Apoptosis Inducing Ligand (TRAIL) (39-42). Binding of a death ligand to a death receptor induces activation of the death receptor; once activated, death receptors recruit adaptor proteins [*e.g.* Fas associated death domain (FADD) for the case of Fas/CD95], which contain a DD and a death effector domain (DED).

The DED of the adaptor proteins interact 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 the death inducing signaling 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 (39-42). 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 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 complex called apoptosome. Procaspase 9 is activated by auto-proteolytic cleavage into caspase 9. The extrinsic and intrinsic pathways of apoptosis signaling are intimately connected and both pathways 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, translocates to the mitochondria, releases cytochrome c and leads to the formation of apoptosome. 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) (39-42). The FLIP proteins may regulate the extrinsic pathway by binding to the DISC, thereby inhibiting the activation of procaspase 8. The bcl2 family comprises both pro-apoptotic (*e.g.*, bax, bak, bok, bad, bik, bim, bid) and anti-apoptotic (*e.g.*, bcl2, bcl-xl and mcl1) members. The anti-apoptotic bcl2 family members may regulate the intrinsic pathway by preventing the mitochondrial release of cytochrome c. 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.

Cell cycle and apoptosis deregulation in classical Hodgkin lymphomas

Central issues in Hodgkin lymphoma research were the questions of how H/RS cells resist apoptosis and acquire self-sufficiency in growth signals. H/RS cells are considered to derive, in most cases, from GC B-cells (3-6). However, H/RS cells lose the capacity to express immunoglobulin and, therefore, should die; nevertheless, H/RS cells escape apoptosis and instead proliferate (3-6). Indeed, H/RS cells are characterized by a high growth fraction as shown by the immunohistochemical overexpression of Ki67 (MIB1), which stains cells in all cycle phases except G0 (21, 27, 33). This suggests that a large number of H/RS cells enter the cell cycle. However, the expression of proliferation-related antigens in H/RS cells is associated with absence of normal progression through mitosis (10). As a result, H/RS cells do not rapidly outnumber the reactive component, which represents the overwhelming majority of the tumor tissue. To explain this phenomenon, it was suggested that H/RS cells have defective cytokinesis (8-11). Indeed, a number of studies using flow cytometric, histological and immunohistochemical methods reported that H/RS cells display aberrant cell cycle features such as S-phase disorder, frequent aneuploidy and abortive mitoses, with arrest at the metaphase-ana/telophase transition leading to the formation of characteristic multinucleated cells and/or considerable cell deletion, often in the form of mummified cells (7-11).

Moreover, substantial evidence that H/RS cells have defective cell cycle regulation has been provided by studies showing that these cells are characterized by immunohistochemical overexpression of cyclin-dependent kinases (CDK) such as CDK1, 2 and 6 and cyclins such as cyclins D2, E, A and B1, whereas overexpression of cyclin D3 is less frequent and overexpression of cyclin D1 is rather uncommon in most studies (11, 26, 27, 29, 33-35).

Immunohistochemical overexpression of cyclin D1 in H/RS cells is uncommon in cHL (2-20%) (27, 29, 33, 35), suggesting that cyclin D1 may not support the proliferation of H/RS cells in most cHL.

Immunohistochemical overexpression of cyclin D2 in H/RS cells is a prominent feature of most cHL (70%-80%) (27, 34). This expression pattern differs strongly from the low levels of cyclin D2 expression in reactive lymph nodes and most B-cell malignancies (except B-cell chronic lymphocytic leukemias and lymphoplasmacytic lymphomas) (34, 43, 45, 46). Up-regulation of cyclin D2 expression in H/RS cell lines has been shown to result from constitutive activation of NF-kB and activator protein (AP)-1 in H/RS cells (13, 15, 16). On the basis of the immunohistochemical and the *in vitro* findings, it was suggested that overexpression of cyclin D2 may support the proliferation of H/RS cells (13, 15, 16, 27).

Immunohistochemical overexpression of cyclin D3 in H/RS cells was observed in 30%-58% of cHL (27, 33, 35). This range of expression shows similarities to that observed in diffuse large B-cell lymphomas (47-49). Overexpression of cyclin D3 in H/RS cells might reflect their increased proliferative activity, since cyclin D3 is the principal D-type cyclin mediating G1 progression in human B-cells (43) and cyclin D3 expression in H/RS cells was positively correlated with the expression of cyclin A, cyclin B1, cyclin D2, MIB1 (Ki67), CDK1 and CDK6 (27, 33).

Immunohistochemical overexpression of cyclin E in H/RS cells is a striking feature of most cHL (82%-90%) (27, 33, 35). This expression pattern differs strongly from the low levels of cyclin E expression in reactive lymphoid tissue and other lymphomas (47, 50). Overexpression of cyclin E in H/RS cells may, at least partially, explain cell cycle aberrations in cHL in view of the findings that high levels of cyclin E expression impair S-phase progression, increase chromosomal instability and polyploidy and maintain CDK2 activity whose down-regulation is required for exit from mitosis (51, 52). Increased expression of cyclin E in H/RS cells may reflect their increased proliferative activity, since cyclin E overexpression in H/RS cells was positively correlated with the expression of CDK2, MIB1 (Ki67), cyclin A and cyclin B1 (33).

Immunohistochemical overexpression of cyclins A and B1 in H/RS cells is a common feature of most cHL (90-95%) (26, 27, 33). This range of expression shows similarities to that observed in diffuse large B-cell lymphomas (53). Overexpression of cyclins A and B1 may, at least partially, explain the abortive mitoses of H/RS cells (8), since cyclin A can delay chromosome alignment and anaphase (54) and the cyclin B1/ CDK1 complex (mitosis promoting factor) is involved in chromosome condensation, nuclear membrane breakdown and mitotic spindle formation (55). In this respect, the cyclin B1/CDK1 complex (mitosis promoting factor) was profoundly deranged in H/RS cells, as evidenced by: a) the markedly low fraction of large atypical cells expressing cyclin B1 simultaneously in the cytoplasm and the nucleus, and b) the absence of correlation of the fraction of large atypical cells expressing cyclin B1 simultaneously in the cytoplasm and the nucleus with the anaphase/telophase indices (56).

Immunohistochemical overexpression of cyclin T1 in H/RS cells is common in cHL (57). The expression of the cyclin T1/CDK9 complex was found to be related to the differentiation program and the cell cycle status in normal and malignant B-cells. Indeed, cyclinT1 and CDK9 proteins are highly expressed in normal and malignant precursor and germinal center B-cells, which are characterized by increased proliferation, whereas these proteins were undetectable in normal mantle cells and plasma cells, as well as in mantle cell and marginal cell lymphomas (57).

Immunohistochemical overexpression of the cyclin-dependent kinases (CDK) 1, 2, 6 and 9 in H/RS cells is common and may reflect their increased proliferative activity, since the expression of CDK1, CDK2 and CDK6 in H/RS cells was positively correlated with the expression of cyclin D3, cyclin E and Ki67 (MIB1) (33, 57). Moreover, since the activation of CDK6 may induce the transcription of p18 through E2F1 activation (58), the concomitant CDK6 high expression and p18 low expression in H/RS cells suggests impairment of this autoregulatory network in cHL (32). Interestingly, there is a link between cell cycle arrest and terminal B-cell differentiation (59). The p18 requirement in terminal B-cell differentiation is specific since other CDKIs such as p19, p21 and p27 cannot compensate the p18 deficiency (59). Therefore, the link between cell cycle arrest and terminal B-cell differentiation in H/RS cells may, at least in part, be related to the defective B-cell differentiation status of these cells (60-62).

Some studies sought to ascertain whether groups with distinct cellular kinetic properties could be delineated in cHL. Spina *et al*. (10) revealed the existence, independently of histological subtype, of two distinct large groups of HL with different kinetic event index (kinetic event index =mitotic index + DNA fragmentation index). Bai *et al*. (27) revealed the existence of 2 cluster solution for the proliferation profile (combined expression values counted for the proliferation-associated proteins Ki67, cyclin A and cyclin B1), thereby permitting clear separation of cHL into distinct groups with low and high proliferative activity. The identification of distinct clusters with respect to the proliferation profile indicates that groups with distinct cellular kinetic properties can be delineated in cHL.

The mechanisms which could underlie the aberrant cell cycle features of H/RS cells have been investigated by studies using immunohistochemical and molecular biology methods. Evidence has been provided that cell cycle deregulation in H/RS cells may be due, at least in part, to alterations in the p53 (p14-Hdm2-p53-p21), Rb (p16-cyclin D-CDK4/6-Rb) and p27 (p27-cyclin E-CDK2) tumor suppressor pathways (17-33).

Inactivation of the p53 pathway in H/RS cells has been suggested because of the very frequent immuno-histochemical overexpression of p53, Hdm2 and p21 proteins, which differs strongly from the low levels of expression of these proteins in reactive lymphoid tissue (18-23, 26-30, 33). This inactivation does not appear to result from p53 gene mutations (17, 22), but rather from Hdm2 protein overexpression in H/RS cells, which has been associated with the presence of alternative transcripts of Hdm2 lacking the adhesion to p14 (ARF), its inhibitory protein (30). Indeed, immunohistochemical studies showed absence of Hdm2/p14 nucleolar complexes and revealed different localization of Hdm2 (nucleoplasm) and p14

(nucleoli) in H/RS cells (30). In some cases of cHL, overexpressed Hdm2 protein was related to Hdm2 gene amplification (20). The absence of Hdm2/p14 nucleolar complexes and the binding of overexpressed Hdm2 protein to overexpressed p53 protein in H/RS cells may be sufficient to inactivate the p53 pathway and may explain the very low frequency of p53 gene mutations in cHL (30). High p53 expression in H/RS cells was associated with high MIB1, cyclin A, cyclin B1, cyclin E, CDK6 and p21 expression, suggesting that overexpressed p53 protein in H/RS cells is unable to induce cell cycle arrest (18, 21, 27, 28, 33). This is likely to reflect the p53 inactivation in H/RS cells, which impairs the induction of p53-transactivated genes involved in the G1/S and G2/M checkpoints (36, 37, 56, 63, 64). With respect to the G1/S checkpoint, p53 induces cell cycle arrest by inducing the expression of p21 (36, 37). However, since H/RS cells in most cHL had the p53+/p21+ phenotype and p53 is inactivated, it is possible that p21 up-regulation is p53-independent in cHL (37, 38, 41). With respect to the G2/M checkpoint, p53 down-regulates the expression of cyclin B1 and CDK1 by repression of their promoters and up-regulates the expression of 14-3-3σ, which modulates the subcellular localization of cyclin B1/CDK1 complexes, as the binding of 14-3-3σ to CDK1 results in retention of the kinase in the cytoplasm (56, 63, 64). Thus, p53 inactivation may, at least partially, explain the cyclin B1/ CDK1 overexpression and the disturbed nuclear localization of Mitosis Promoting Factor components (cyclin B1 and CDK1), which are both features of H/RS cells (33, 57).

Inactivation of the Rb pathway in H/RS cells has been suggested because of the frequent immunohistochemical underexpression of p16 and p18 proteins, while immunohistochemical underexpression of Rb protein was less frequent (21, 24, 27, 28, 31-33). This inactivation could result from p15/INK4b, p16/INK4a and p18/INK4c promoter region hypermethylation (p16 promoter region homozygous deletion and mutations are rare), cyclin D [1, 2, 3] overexpression and/or CDK6 overexpression in H/RS cells (21, 24, 27, 28, 31-35).

Inactivation of the p27 pathway in H/RS cells has been suggested because of the frequent immunohistochemical underexpression of p27 protein and the overexpression of cyclin E and CDK2 proteins (27, 29, 33). This inactivation could result from increased p27 protein degradation mediated by SKP2, which acts as ubiquitin ligase for p27 (33). Indeed, since SKP2 is overexpressed in 84% of cHL and is inversely related to p27, high expression of SKP2 may mediate degradation of p27 protein in H/RS cells (33). High p27 expression in H/RS cells was associated with high cyclin A, cyclin E, CDK2 and CDK6 expression (27, 33), suggesting aberrant p27 expression since normal cycling lymphoid cells have very low levels of p27 protein (43, 65-68). This might be due to inactivation of overexpressed p27 protein because of binding to D-type cyclins, in view of the findings that p27-cyclin D3 nuclear colocalization was detected in a subset of aggressive B-cell lymphomas showing concomitant high p27/cyclin D3 expression and high growth fraction (65). In this context, p27 might be protected from CDK2-mediated degradation because of p27 sequestration in cyclin D3/CDK4 complexes (65).

Besides cell cycle arrest, p53 and other cell cycle regulators are also involved in the regulation of apoptosis (37, 38, 41, 43, 44). DNA damage induces p53 protein, which mediates cell cycle arrest through p53-mediated induction of p21; in the case of ineffective repair of the DNA damage, p53 may induce apoptosis through up-regulation of the pro-apoptotic bax protein and down-regulation of the anti-apoptotic bcl2 protein (37-39, 41). Thus, it is possible that in cHL the inability of the overexpressed wt p53 protein to induce cell cycle arrest may, alternatively, lead to activation of the p53-induced apoptotic program. However, no immunohistochemical correlation was found between p53, bax and bcl2 protein expression status in cHL (23, 28, 33). Interestingly, high p21 and p27 protein expression in H/RS cells was associated with low apoptotic index, suggesting that these CDKIs have a protective role against apoptosis (29).

Deregulated expression of various apoptosis-associated molecules is common in cHL and, in recent years, evidence has accumulated that the constitutive activation of the NF-kB pathway in H/RS cells is of particular importance for explaining the apoptosis deregulation in cHL (3-6, 69-84). NF-kB consists of dimers of subunits belonging to the family of REL/NF-kB proteins (c-REL, p65/RELA, RELB, p50/p105 and p52/p100) (69, 70). 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 bind to NF-kB dimers and retain them in the cytoplasm (69, 70). Exposure to various extracellular stimuli (*e.g.*, pro-inflammatory 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 (69, 70). Constitutive activation of NFkB has a central role in the pathogenesis of cHL by up-regulating an anti-apoptotic (*e.g.*, c-FLIP, bcl-xl, c-IAP2, TRAF1, Bfl1/A1, IEX-1) and proproliferative (*e.g.*, cyclin D2) gene expression program in H/RS cells (3-6, 13, 15, 16, 27, 33, 85-89). In addition, NFkB up-regulates the expression of IL13 and CD40, which play a critical role in B-cell proliferation and are involved in the pathogenesis of cHL (3-6, 13, 15, 36). The introduction of a dominant–negative mutant of IkBa (which irreversibly keeps NFkB bound in the cytoplasm) into H/RS cells resulted in their massive apoptosis (12, 15). Immunohistochemical studies have shown nuclear localization of NFkB in H/RS

cells, thereby confirming its constitutive activation (14, 33, 71, 72). Several studies have provided explanations for the constitutive nuclear activity of NFkB in H/RS cells (73-85). Briefly, constitutive activation of NF-kB in H/RS cell precursors can be achieved: a) in a ligand-dependent fashion through various TNFR family members (through autocrine secretion of the cytokines TNF-a and LT-a, both of which bind TNFR, activation of CD30 by surrounding CD30L-positive eosinophils and mast cells, activation of CD40 by surrounding CD40L- positive T-cells or activation of RANK by RANKL on H/RS cells); and b) in a ligand-independent fashion through autonomously active CD30, CD40, RANK (Receptor activator of NF-kB) and Notch 1 signaling pathways, through the action of Epstein Barr Virus (EBV)-encoded LMP-1 protein, by mutations of the IKBA gene leading to inactivation of IkB-a protein, or by amplification of the NFkB/REL locus (3-6, 73-84).

Activation of NF-kB through various TNFR family members is mediated by the Tumor Necrosis Factor Receptor-associated factor (TRAF) molecules (90, 91). TRAFs are adapter proteins that bind to the cytoplasmic region of TNFR family members and recruit other proteins to form an active signaling complex that ultimately triggers the activation of the IkB kinase complex (IKK) (91). To date, six distinct TRAF molecules have been identified, termed TRAF1 through TRAF6 (91). TRAF 1, 2, 5 and 6 with appropriate TNFRs induce NF-kB activation (91). TRAF 1, 2 and 5 bind TNFRII, CD30, CD40, RANK and LMP1; TRAF6 binds to CD40 and RANK (91). It should be noted that the function of TNFRI and CD95/Fas is not primarily mediated by TRAF binding, but through association of their death domain with molecules such as FADD, TRADD and RIP (39, 40). With respect to cHL, TRAF1, 2, 5 and 6 are expressed in H/RS cell lines and TRAF1 and 2 are commonly expressed in H/RS cells in cHL tissues, as shown by immunohistochemistry (92-96).

The identification of c-IAP2 as a NF-kB-dependent apoptosis inhibitor in H/RS cells is of interest since these cells express simultaneously high levels of active caspase 3 (97-99). In addition, H/RS cells express the apoptosis inhibitors survivin and X-linked IAP (XIAP), which also belong to the IAP family (33, 100). Thus, it is possible that the c-IAP2, survivin and XIAP expression in H/RS cells may protect these cells from caspase 3-mediated apoptosis. Most of the NF-kB-dependent regulators of apoptosis identified in H/RS have anti-apoptotic functions, except Fas/CD95 (15). In addition, inactivating CD95 gene mutations were found in only 10% of cHL (101, 102). Furthermore, CD95 and CD95 L immunohistochemical expression is strong in H/RS cells and surrounding lymphocytes, respectively, in most cHL (98, 103). Thus, CD95 activation in H/RS cells should have promoted apoptosis. However, the anti-apoptotic c-FLIP (FADD-like IL-1b converting enzyme inhibitory proteins) is overexpressed in cultured H/RS cells and H/RS cells in cHL tissues (97, 98). Thus c-FLIP may be one of the molecules inhibiting the effects of Fas/CD95 activation in H/RS cells.

Besides NF-kB, another family of transcription factors, found to be constitutively active in cHL and which may influence the cell cycle status of H/RS cells, is the signal transducer and activator of transcription (STAT) family (104, 105). STATs are activated by cytokine signaling through the activation of Janus kinase (Jak) family members, which phosphorylate the STAT family (104, 105). STATs are located in the cytoplasm and their tyrosine phosphorylation leads to STAT dimerization and translocation of the activated transcription factor to the nucleus (104, 105). Seven members of the STAT family have been so far identified; STAT1, 2, 3, 4, 5 and 6, and each is activated by a distinct set of cytokines (104, 105). STAT3, 5a and 6 are constitutively activated in H/RS cells and the expression of STAT1 and 3 is associated with high proliferative index and high expression of CDK1, 2 and 6 in H/RS cells (15, 33, 106, 107). STAT 3 is activated by several cytokines such as IL-6, 10, 2, 7, 9 and 15 (108). STAT3 phosphorylation in cHL was independent from signaling through the IL-6 receptor and subsequent activation of Jak, indicating disruption of normal regulatory networks (106). This finding may be explained by the recurrent amplification of the Jak2 genomic locus in cHL (76). Nuclear phosphorylated-STAT3 immunohistochemical expression, indicating constitutive STAT3 activation, was found in H/RS cells in 85% of cHL tissues, but it was not specific for cHL since such expression was also found in most NHL and reactive cells within cHL (107). STAT 5 is activated by several cytokines such as IL-2, 4, 7, 9, 15, 3 and 5 (108). Nuclear phosphorylated-STAT 5 immunohistochemical expression, indicating constitutive STAT5 activation, was found in H/RS cells in 25% of cHL tissues; this suggests that STAT 5-dependent cytokines are not involved as autocrine growth factors in most cHL (107). STAT 6 is activated by the cytokines IL-4 and 13 (108). Nuclear phosphorylated-STAT6 immunohistochemical expression, indicating constitutive STAT6 activation, was found in H/RS cells in 78% of cHL tissues, but rarely in reactive cells and other NHL (107). Expression of phosphorylated STAT6 was shown to depend on IL-13 signaling in HL cell lines (107). In addition, IL-13 and IL-13 receptor have been detected in H/RS cells in cHL by *in situ* hybridization and immunohistochemistry and have been found to be important for proliferation of H/RS cell lines (109-111). The aforementioned findings indicate that IL-13 is an autocrine growth factor for H/RS cells and suggest that constitutive activation of STAT6 through IL13 may contribute to the H/RS cell proliferation (107-111). Furthermore, IL-13 may be involved in the pathogenesis of cHL in cooperation with

CD40, since IL-13 and CD40 activation promote B-cell survival through induction of the anti-apoptotic molecule bcl-xl (112) that is commonly expressed by H/RS cells in cHL tissues (33, 86, 87).

Besides NF-kB and STATs, another transcription factor, found to be constitutively active in cHL and which may influence the cell cycle status of H/RS cells, is the transcription factor AP1 (16). AP1 is composed of homo or heterodimers formed by related Jun (c-Jun, Jun B, Jun D), Fos (c-Fos, Fos B, Fra1, Fra 2) and ATF (activating transcription factor) family proteins (113, 114). Transcription of AP1 family members is stimulated by extracellular signals which trigger activation of the mitogen activated protein kinase (MAPK) families; these include the c-Jun/NH2-terminal kinase (JNK) family, the extracellular signal regulated kinase (ERK) family and the p38 MAP kinase family (113, 114). Under physiologic conditions, activation of the MAPK cascades by surface receptor/ligand interactions is involved in regulating proliferation, apoptosis and differentiation (113, 114). In H/RS cells, a MAPK-independent constitutive activation of AP1 with strong c-Jun and Jun B immunohistochemical expression was revealed (16). Jun B expression was under the control of NFkB, whereas c-Jun was up-regulated in an autoregulatory process in H/RS cells (16). AP1 target genes in H/RS cells comprise cyclin D2 and the proto-oncogene c-MET, which are both strongly expressed in H/RS cells (16). Interestingly, the c-MET/Hepatocyte growth factor pathway has been suggested as important for the survival of H/RS cells (115). Furthermore, the MEK/ERK pathway was aberrantly active in HL cell lines (116). This pathway is shared by CD30, CD40 and RANK and activation of the respective receptors increases ERK phoshorylation and promotes the survival of HL cell lines (116). Inhibition of this pathway was found to induce G2/M arrest or apoptosis and was associated with modulation of the expression of the apoptosis regulators bcl2, mcl1 and c-FLIP in HL cell lines (116).

Epstein-Barr Virus (EBV) and cell cycle/apoptosis deregulation in classical Hodgkin lymphoma

EBV has been associated with the pathogenesis of cHL in about 30-50% of cases (117-149). In normal host epithelial and lymphoid cells, EBV infection induces two types of infection, *i.e.* lytic infection with production of infective virions and latent infection, in which no infective virions are produced and only a limited number of EBV genes are expressed (118-121). On the basis of *in vitro* and *in vivo* data, three patterns of EBV latency have been described: *type I latency*, expressing only Epstein-Barr nuclear antigen-1 (EBNA-1) gene (Burkitt lymphoma); *type II latency*, expressing EBNA-1, latent membrane protein-1, 2A and 2B (LMP-1, 2A and 2B) genes (cHL, NK/T-cell lymphomas, undifferentiated nasopharyngeal carcinomas); and *type III latency*, in which all EBNA (EBNA-1, 2, 3A, 3B, 3C, LP) and LMP (LMP-1, 2A, 2B) genes are expressed (lymphoblastoid cell lines [LCL]) (118-122).

Using molecular histology methods, EBV has been detected by DNA and/or RNA *in situ* hybridization in H/RS cells in about 30-50% of cases in Western countries; moreover, the EBV-encoded LMP1 and LMP2A proteins have been revealed by immunohistochemistry in H/RS cells in 30-50% of cHL cases (125-145). Using microarrays, the gene expression profile of cHL cell lines was similar to that of EBV-transformed B-cells (4).

Of particular importance for the pathogenesis of cHL is the detection of the EBV-latency proteins LMP1 and LMP-2A in H/RS cells, since these proteins are associated with EBV-mediated activation, transformation and deregulation of the cell cycle and apoptosis machineries of EBV-infected B-cells (118-124). LMP1 mimics a constitutively active CD40 receptor, a signaling pathway leading to the activation of the NF-kB through the TRAF pathway (118-124). Besides NF-kB, LMP1 also mediates activation of other signaling pathways such as AP1 activation through the JNK/c-Jun pathway, ATF2 (activating transcription factor 2) activation through the p38/MAPK pathway and Jak-STAT pathway activation through binding and activation of Jak3 (119-121). LMP1 may also mediate down-regulation of CD99, which leads to the generation of H/RS cells and induction of the cytokines IL-6 and IL-10, which might be involved in the EBV-induced growth activities (108, 119-126). LMP2A has effects on signal transduction by obstructing pathways that are triggered by ligation of the B cell antigen receptor complex (BCR) (119-122). Recently, important evidence supporting the involvement of LMP2A in the pathogenesis of cHL has been provided by the findings that LMP2A expression increases the expression of genes associated with cell cycle induction and inhibition of apoptosis (Ki67, cyclin A, PCNA, bcl-xl, survivin) and decreases the expression of B-cell specific factors (CD19, CD20, CD22, CD79a, Blk, Bnlk, Pax5, PU.1) (122). Since many of the LMP2A-induced alterations in gene expression were similar to those described in H/RS cells, it was suggested that LMP2A expression in EBV-infected B-cells may lead to the induction and the maintenance of an activated, proliferated state that ultimately result in cHL (122).

The cell cycle/apoptosis protein expression profiles in EBV-positive and EBV-negative cases of cHL have been extensively analyzed by immunohistochemistry resulting in conflicting results (9, 19, 21, 23, 25, 26, 28, 33, 35, 89, 92, 132, 141, 143, 144, 148, 149). Moreover, no correlation was found between the EBV status and apoptotic index as detected by the TUNEL method (9, 29, 33). Interestingly, a recent study of 288 cases of HL showed that EBV-positive

cases of cHL presented a different cell cycle/apoptosis immunohistochemical profile consisting of increased expression of STAT1 and STAT3 and decreased expression of p53, Hdm2, p27, cyclin E, CDK6 and bcl-xl proteins (33). This study demonstrated, in a large series of cHL, that EBV infection induces profound alterations in the cell cycle/apoptosis profile of H/RS cells (33).

Important mediators of the EBV-induced growth activity in cHL may be the EBV-induced cytokines (108). EBV induces the synthesis of various cytokines in B cells and, among them, IL-6 and IL-10 are important for the growth of EBV transformed cells (108, 150-154). Both IL-6 and IL-10 may be regulated by LMP1 *via* NF-kB and the p38 MAP kinase pathways (155-158). There is evidence that pathways activated by IL-6 and IL-10 (for example the Jak family of tyrosine kinases) are involved in lymphocyte growth and transformation (104, 105). The STATs, which are one substrate of the Jak family, are active in EBV immortalized cells and LMP1 can activate a STAT reporter which binds at least STAT1, STAT3 and STAT5 (159, 160). In addition, EBV-positive cHL are associated with immunohistochemical overexpression of STAT1 and STAT3 proteins (33). Therefore, STAT1, STAT3 and STAT5 may be involved in EBV-induced proliferation.

The cytokine profiles in EBV-positive and EBV-negative cases of cHL have been analyzed by immunohistochemistry and *in situ* hybridization (150, 151, 161). Two of these studies have shown a significant correlation between higher numbers of IL-10- and IL-6- expressing H/RS cells and positive EBV status in cHL (150, 151). In contrast, no difference in the percentage of IL-2-, IL-4- and IFN-γ-expressing H/RS cells was observed between EBV-positive and EBV-negative cases of cHL (151). Another immunohistochemical study reported higher numbers of IL-12-expressing reactive cells in EBV-positive cHL (161).

Relationships between B-cell differentiation program and apoptosis in classical Hodgkin lymphoma

Recent evidence suggests links between the relationships between the B-cell differentiation program and apoptosis in cHL. Indeed, the occurrence of somatic deleterious (crippling) mutations in the immunoglobulin genes of H/RS cells in about 25% of the B-cell cHL cases has led to the hypothesis that these cells are derived from pre-apoptotic GC B-cells (3, 162). More recent studies showed that the gene expression profile of cHL cell lines is characterized by the overexpression of genes, which are components of the activated DLBCL gene expression signature (cyclin D2, CD44, IRF4/MUM1, IkBa, c-FLIP, CCR7 and TNFa) (4, 13, 15, 16, 163, 164). In addition, a part of the cHL phenotype is based on NFkB-regulated genes (cyclin D2, IRF4/MUM1, IkBa, c-FLIP and CCR7) and both cHL and activated DLBCL show NFkB constitutive activation, which induces an anti-apoptotic and proproliferative gene expression program in these tumors (3, 4, 163, 164). Furthermore, cHL do not display features of GC-DLBCL, as shown by the low or null expression of typical GC B-cell differentiation proteins (*e.g.*, bcl6, CD10), the expression of the post-GC protein CD138 and the lack of ongoing mutations of the immunoglobulin genes in H/RS cells (3, 4, 60, 165-167). Thus, although cHL in most cases are derived from GC B-cells, they display a B-cell differentiation phenotype more similar to that of activated DLBCL (4). The lack of the GC B-cell differentiation phenotype in H/RS cells may be part of a general down-regulation of the B-cell lineage phenotype (3, 4, 60-62, 168-170). Thus, there is an open question as to whether the loss of the GC B-cell phenotype is related to the suggested pre-apoptotic origin of H/RS cells in which down-regulation of the GC B-cell markers occurs during neoplastic transformation or reflects a normal stage of the B-cell differentiation program (3, 4). In view of recent findings that the activated B-cell differentiation immunohistochemical immunophenotype is associated with decreased apoptosis profile in DLBCL (171-174), further studies on the expression of apoptosis regulators are required to clarify this relation in cHL.

Clinical relevance of the expression of cell cycle and apoptosis regulators in classical Hodgkin lymphoma

Many studies have analyzed the clinical relevance of the expression of cell cycle and apoptosis regulators in cHL using immunohistochemistry or gene expression profiling (21, 23, 33, 143, 144, 149, 175-178). In addition, a few studies analyzed the clinical relevance of the apoptotic index as detected by the TUNEL method (33, 144, 179). Shorter survival was significantly associated with high proliferation index (Ki67), high expression of the proliferating cell nuclear antigen (PCNA), high expression of bcl2, bcl-xl, bax and p53, low expression of Rb and caspase 3 and high apoptotic index (21, 23, 33, 143, 144, 175-177). By gene expression profiling, the good outcome cHL were characterized by up-regulation of genes involved in apoptosis induction (APAF, bax, bid, caspase 8, p53, TRAIL) and cell signaling, including cytokines and transduction molecules (IL-10, IL-18, STAT3), while the bad outcome cHL were characterized by up-regulation of genes involved in cell proliferation (Ki67) and by down-regulation of tumor suppressor genes PTEN (Phospatase and Tensin homolog deleted on chromosome 10) and DCC (Deleted in Colorectal Cancer) (178). In several studies, LMP1 expression was shown to have a favorable influence on the outcome of patients with cHL (21, 143, 145, 149), whereas in other studies no correlation was found between EBV status and prognosis of cHL (23, 33).

Conclusion

There is a strong body of evidence that H/RS cells of cHL are characterized by a profound disturbance of the cell cycle and apoptosis regulation. In this respect, of particular importance are the constitutive activation of the NF-kB pathway, alterations implicating various components of the p53, Rb and p27 tumor suppressor pathways and the activity of the IL-13/IL-13R autocrine growth loop. Furthermore, the Epstein-Barr Virus (EBV), which is present in H/RS cells in about 30-50% of cHL, has been shown to affect the cell cycle and apoptosis regulation in cHL.

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