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The Molecular Basis for the Generation of Hodgkin and Reed-Sternberg Cells in Hodgkin's Lymphoma

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Abstract

Hodgkin's lymphoma (HL) is a lymphoid neoplasm with a low frequency of malignant tumor cells, known as Hodgkin and Reed-Sternberg (H-RS) cells, in a background of mixed cellular infiltrates. Despite extensive studies on H-RS cells, the molecular mechanisms of their growth and regulation have remained uncertain for a long period. Recently, constitutively activated nuclear factor–κB (NF-κB) was reported to be a unique and common characteristic of H-RS cells that prevents the cells from undergoing apoptosis. NF-κB triggers proliferation and provides a molecular basis for these cells' aberrant growth and cytokine gene expression. In HL pathogenesis associated with Epstein-Barr virus infection, the activation of NF-κB is induced by viral latent membrane protein 1 (LMP1). Coupled with recent insights into the molecular mechanisms of activation of NF-κB signaling in H-RS cells, this review discusses a linkage between LMP1 and HL via CD99, which has recently been reported to be down-regulated by LMP1 through the NF-κB signaling pathway. This down-regulation leads to the generation of cells with H-RS phenotypes related to the clinical and histologic characteristics of HL. *Int J Hematol.* 2003;77:330-335.
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1. Introduction

Hodgkin's lymphoma (HL) is morphologically characterized by a low occurrence of neoplastic mononuclear Hodgkin (H) and multinucleated Reed-Sternberg (RS) cells surrounded by abundant non-neoplastic infiltrates in the tumor tissue [1]. Since HL was first reported as a distinct clinical entity, it has become recognized as one of the most difficult lymphomas to study at the cellular as well as the molecular level because of the rarity of the malignant H-RS cells (less than 1% of the cells in an involved lymph node) and the presence of its histologically distinct subtypes [2]. In addition, because of the uncontrolled production of markers normally expressed in cells from different hematopoietic lineages, H-RS cells vary between B, T, and myeloid phenotypes. The Revised European American Lymphoma and World Health Organization classification recently distinguished lymphocyte predominance HL from classic HL that consists of 4 subtypes (lymphocyte-rich, nodular-sclerosing, mixed cellularity, and lymphocyte-depleted). This classification reflects the diversity in clinical presentation and behavior, morphology, phenotype, and molecular features of HL [3]. Despite apparent heterogeneity in their genotypes and phenotypes, individual H-RS cells show similar histopathologic features, such as the typical bizarre-looking morphology and the deregulation of various cytokines and growth factors, suggesting a common molecular mechanism in their generation. Recent advances in the comprehension of the etiology and pathogenesis of HL are noteworthy and have been made by studies of immunophenotyping, genotyping, and cytokine production of H-RS cells in tissue specimens or in cell lines derived from HL tissues. In particular, studies employing single-cell micromanipulation techniques with polymerase chain reaction amplification of RNA and genomic DNA have shed light on the identification of the cellular origin of H-RS cells, which has long been a matter of debate. A clonal rearrangement of the V, D, and J segments of the immunoglobulin heavy chain locus has been detected in the H-RS cells of most patients, and sequence

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analyses of the rearranged VDJ regions from these H-RS cells have demonstrated a high load of somatic mutations, indicating that these cells had originated from germinal center B-lymphocytes or from B-cells at a later stage of differentiation in few exceptional cases [4-7]. In addition, micromanipulation of single cells also identified the recurrence of constitutive nuclear factor–κB (NF-κB) activity in H-RS cells, which is currently considered the common molecular defect that forms the basis for the occurrence of HL.

2. Constitutive Activation of the NF-κB Signaling Pathway in H-RS Cells

The NF-κB/Rel family of transcription factors mediates inducible gene expression in response to numerous pathogens and cytokines and is also known to regulate a wide variety of genes whose products play fundamental roles in inflammatory and immune responses. NF-κB consists of homodimers and heterodimers in a variety of gene products related to the v-Rel oncoprotein [8]. In most cells, NF-κB factors are sequestered in the cytoplasm with NF-κB inhibitor (IκB). Various cellular stimuli trigger the phosphorylation of IκB, which leads to ubiquitination and the subsequent degradation of IκB and the resultant release of NF-κB subunits into the nucleus [9-13].

The role of NF-κB in oncogenesis was first implicated in fatal lymphomas of birds induced by the v-*rel* gene and in human malignancies associated with rearranged and/or amplified genes encoding the NF-κB family factors [14]. The importance of NF-κB activation in HL was also demonstrated in cultured HL cell lines and primary H-RS cells, in which elevated NF-κB DNA-binding activities were consistently observed in the nucleus [15]. Immunohistochemical staining of biopsy samples of HL patients with an anti-RelA antibody confirmed abundant NF-κB–RelA activity in RS cells. Although c-Jun and JunB overexpression in tumor cells from all patients with classic HL was recently reported, the AP1 activities in these patients still showed synergy with NF-κB [16]. Furthermore, when a dominant negative IκBα that is not inducibly degraded was overexpressed, Hodgkin cell lines showed decreases in nuclear NF-κB activity and proliferation rates and demonstrated an enhanced apoptosis [17]. The antiapoptotic effects of NF-κB have been documented in many other cell types, including mouse B-cells [18]. The presence H-RS cells with nonfunctional immunoglobulin genes that should have been negatively selected implies the rescue of the cells from apoptosis by aberrant NF-κB activation. Thus, the roles of NF-κB as a direct requirement for human neoplastic disease suggest that the H-RS cells result from deregulated NF-κB expression.

The abnormal NF-κB activation in H-RS cells is associated with molecules closely linked to its signaling pathway, such as a mutated IκB, a constitutive activity of kinases upstream from IκB, or a modification of NF-κB, which renders NF-κB insensitive to inhibition by IκB. In fact, clonal deleterious somatic defects in the IκBα gene, such as a deletion or a point mutation, have been detected in some HL cell lines and H-RS cell cases and have resulted in the cells becoming functionally null for IκBα activity [19-21]. However, in other cases of H-RS cells that maintain wild-type IκBα alleles expressing IκBα protein, an activation of upstream factors leading to rapid degradation of IκBα, such as aberrant IκB kinase (IKK) or NF-κB inducing kinase (NIK) activities, appeared to be the reason for NF-κB activity in the nucleus [22].

3. Members of the Tumor Necrosis Factor Receptor Family and NF-κB Activation in H-RS Cells

Roles of some members of the tumor necrosis factor receptor (TNFR) family, such as CD30 and CD40, have long been suggested to be involved in the NF-κB activation of H-RS cells. Generally, the TNFRs transmit their signal through the direct recruitment of TNFR-associated factors (TRAFs) [23]. Although the molecular mechanisms by which TRAFs activate downstream effector proteins remain largely unknown, the current data suggest that TRAFs interact with different types of kinase, such as NIK, MAPK/extracellular response kinase, and transforming growth factor β–activated kinase, which are associated with IKKs leading to NF-κB activation [12,24,25]. Overexpression of CD30 in vitro has been demonstrated to activate NF-κB through self-aggregation and recruitment of TRAF2, TRAF5, and NIK in H-RS cells in a constitutive, ligand-independent way [26,27]. CD40-mediated signal transduction also activates NF-κB, and CD40-induced NF-κB activation has been demonstrated to be mediated by the proteolysis of TRAF3 in a Hodgkin cell line [28,29]. CD40 is expressed at significantly higher levels on HL cell lines than on other lymphoma cells, with a highly distinctive staining pattern irrespective of the antigenic phenotype or histologic subtype [30]. In both CD30 and CD40 cases, it was also suggested that their activation could be achieved by cross-linking their matching ligands on either the H-RS cell or on adjacent infiltrating lymphocytes [31,32].

Epstein-Barr virus (EBV) latent protein 1 (LMP1) is also a member of the TNFR superfamily, and constitutively activates NF-κB–mediated transcription, regardless of ligand stimulation of NF-κB. Because of its high frequency of occurrence (about 40%), LMP1 has also attracted considerable attention as a key molecule for activating NF-κB in H-RS cells from HL patients [33]. LMP1 is a viral pseudoreceptor, and the signaling regions of LMP1 have extensive functional homology to that of CD40, but LMP1 has no significant sequence homology with CD40. Although their signaling domains have been shown to be interchangeable, LMP1 and CD40 do not interact with exactly the same set of molecules, indicating that the signaling pathways may differ in some aspects.

4. EBV LMP1 Expression and NF-κB Activation in H-RS Cells

EBV, a ubiquitous human herpesvirus, has been reported to be associated with many human malignancies, such as HL, endemic Burkitt lymphoma, nasopharyngeal carcinoma, and posttransplantation lymphoproliferative disease. The EBV infection of resting B-cells in vitro leads to the establishment of immortalized lymphoblastoid cell lines. Among the EBV antigens that cooperate in the transformation of normal B-lymphocytes, LMP1 plays a key role. Expression of LMP1 induces the oncogenic transformation of established fibroblast cell lines and suppresses the senescence of mouse pri-

mary embryonic fibroblasts [34]. In addition, transgenic mice with LMP1 linked to the immunoglobulin heavy chain promoter develop B-cell lymphomas at a high frequency, and LMP1 expression is likely to cause alterations in cell growth, demonstrating the tumorigenic potential of LMP1 in vivo [35-37]. As for LMP1's association with HL, among the first clues was the finding that LMP1 is highly expressed in H-RS cells in EBV-associated HL. In addition, overexpression of LMP1 favors the formation of multinucleated H-RS cells in L-428, an HL-derived cell line [38].

LMP1 is an integral membrane protein comprising 386 amino acids and consists of a short amino (N)-terminal cytoplasmic stretch, 6 transmembrane sequences, and a long carboxyl (C)-terminal cytoplasmic region with no significant extracellular domain. The C-terminal region mediates its pleiotropic activities through at least 3 independent signaling pathways: the NF-κB pathway through C-terminal activation region 1 (CTAR1) and CTAR2, the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway via activation of JAK3 through CTAR3, and the SAPK/ERK kinase/c-Jun N-terminal kinase (SEK/JNK) pathway through CTAR2.

NF-κB activation by LMP1 is mediated 25% through the direct interaction of TRAFs with CTAR1 and 75% through TNFR-associated death domain protein (TRADD) binding to CTAR2 [35,36,39]. The core of the TRAF-binding domain of CTAR1, amino acids 201 to 210, contains the TRAF2-binding consensus sequence PXQXXD [36,39,40-42]. Changing the proline residue at position 204 of the domain and the glutamine at position 209 to alanine residues abolishes the association of LMP1 with TRAFs and severely impairs LMP1-induced NF-κB activation via CTAR1 [38,39]. In addition, the overexpression of a dominant negative TRAF2 mutant was demonstrated to inhibit NF-κB activation via CTAR1 and CTAR2, a finding implying a positive role for TRAF2 in the NF-κB activation signal [43,44]. Furthermore, NF-κB activation by CTAR1 is enhanced by TRAF1 overexpression and inhibited by TRAF3 overexpression [39,45]. These findings indicate that TRAF1 and TRAF2 mediate NF-κB activation through CTAR1, whereas TRAF3 may have an inhibitory role in this process.

CTAR2, the second NF-κB–activating domain, spans amino acids 352 to 386. In contrast to CTAR1, CTAR2 does not interact directly with TRAF proteins but interacts with TRADD, which synergistically interacts with CTAR2 to activate NF-κB [35,46-48]. The 169 N-terminal amino acids of TRADD bind to TRAF2, and truncated TRADD proteins have been shown to inhibit LMP1-mediated NF-κB activation, presumably by acting as dominant negative mutants [49-51]. However, TRADD does not induce apoptosis on binding to LMP1, as TRADD does when it is recruited to TNFRI, indicating a fundamental difference between the 2 cell surface receptors [52]. Apoptosis would be due to the inefficient recruitment of downstream effectors of apoptosis such as the Fas-associated death domain and caspases by the conformational or posttranslational modification of TRADD in the resulting LMP1-TRADD complex. Alternatively, a cofactor critical for TRADD-mediated apoptosis may be absent in the LMP1-TRADD complex, or LMP1 may apply an inhibitor of the apoptotic pathway or induce a powerful antiapoptotic process.

Another line of evidence showing a close association between oncogenesis and NF-κB activation by LMP1 is the presence of 2 functional domains tightly linked with the transforming potential of LMP1 and located in its C-terminal region; these domains are transformation effector site 1 (TES1) and TES2 [35,53,54]. TES1 is mapped at the 45 amino acids proximal to the membrane, and TES2 corresponds to the last 35 amino acids at the C-terminal end. Deletion of TES1 abolishes B-lymphocyte transformation by EBV in vitro, and TES2 enhances the long-term growth potential of EBV-transformed B-lymphocytes. TES1 and TES2 overlap with CTAR1 and CTAR2, respectively, a finding implicating the critical role of NF-κB and AP1 activities in the process of B-lymphocyte transformation by EBV.

The LMP1-induced NF-κB signaling pathway is associated with factors positioned upstream of IKKs but downstream of TRAFs, because dominant negative forms of NIK and IKK mutants have been shown to suppress LMP1- and TRAF2-induced IκBα phosphorylation and NF-κB transactivation [55]. In a recent study of primary biopsies from patients with HL, the serine/threonine kinase Tpl-2/Cot was identified as a component of LMP1 signaling downstream of TRAF2 [52].

Because all naturally occurring LMP1 deletion variants isolated from HL patients still fully stimulate NF-κB–mediated transcription, the integrity of LMP1-dependent NF-κB–mediated transcriptional activation seems very important for EBV-associated HL. Moreover, forced expression of LMP1 in an EBV-negative Hodgkin cell line induced an increased number of RS cells, supporting the hypothesis that identical signal transduction pathways are associated with the generation of RS cells of EBV-negative and EBV-positive HL. The result suggests that a deregulated cellular factor associated with the NF-κB signaling pathway may be also acting in LMP1-negative HL.

5. Functional Consequences of NF-κB Activation in H-RS Cells: Roles of CD99 in the Generation of H-RS Cells

Many of the genes known to be expressed in H-RS cells are NF-κB target genes [56,57]. LMP1 can induce most of the phenotypic changes associated with EBV transformation of resting human B-lymphocytes, including induction activation of NF-κB [58-60]. LMP1 expression in B-cell lines causes extensive phenotypic changes that are characterized by the induction of surface antigens (CD21, CD23, CD30, CD40, CD44, Fas), adhesion molecules (ICAM1, LFA1, LFA3), and antiapoptotic proteins (A20, Bcl2, Bfl1, Mcl1). The importance of LMP1-induced inhibition has recently been highlighted, and this inhibition includes down-regulation of the expression of surface antigens such as CD99, CD10, and CXCR4 and the inhibition of transforming growth factor β signaling and Smad-dependent activation of transcription [61-63].

CD99 is a strongly sialoglycosylated 32-kDa transmembrane protein that closely resembles typical signal-transducing sialomucin-type glycoproteins, such as CD34 and CD43 [64-66]. The CD99 protein is encoded by a pseudoautosomal gene *mic2*, is located in Xp22.32-pter and Yp11-pter, and

thus escapes X chromosome inactivation [67]. CD99 was initially described as a human thymus antigen and as a putative adhesion molecule (termed E2) involved in the spontaneous formation of rosettes of T-cells with erythrocytes. Although its ligand has not yet been identified, CD99 has been well established to function as a signal-transducing molecule in several cellular systems [68-71].

H-RS cells from the lymph nodes of HL patients consistently lack CD99 expression on their surfaces, and induced LMP1 expression has been demonstrated to directly down-regulate CD99 in B-cell lines at the transcriptional level via the NF-κB pathway. This finding possibly indicates a role for LMP1-induced down-regulation of CD99 in the generation of H-RS–like cells [72,73]. Lymphoblastoid cell lines lacking CD99 display most of the typical characteristics of H-RS cells and are identical to those found in lymph nodes of HL patients, such as abundant cytoplasm and bilobate or multilobate nuclei with prominent nucleoli, the expression of intracellular CD15, the decreased expression of CD45RB, slower kinetics of cell proliferation, and in chromosome number abnormalities [74]. Because the regulation of cell surface expression of major histocompatibility complex (MHC) class I antigens is one of the important mechanisms by which H-RS cells of HL escape from host immune surveillance, the lack of CD99 also causes a low level of surface MHC class I antigen expression by delaying the trafficking of newly synthesized MHC class I molecules from the Golgi apparatus to the plasma membrane [75]. In addition, CD99 deficiency not only causes somatic mutations that take place in the variable region of the immunoglobulin genes of H-RS cells but also increases intercellular aggregation due to the atypical expression of surface molecules, which is similar to the cell rosetting around H-RS cells frequently found in HL lesions [76,77]. Examination of CD99 expression in H-RS cells from HL patients and HL-derived cell lines such as L428 and KMH2 have confirmed the marked down-regulation of CD99 surface molecules. The facts that spontaneously occurring CD99-deficient cells also show phenotypes similar to H-RS cells and that all these H-RS phenotypes shown in CD99-deficient B-cells are completely rescued by the forced expression of CD99, strongly imply that loss of CD99 recapitulates the phenotypic changes seen in H-RS cells.

Because of complexity of the disease, a genetic locus with a defect that could explain all the cellular changes accompanying the pathogenesis of HL has not, to date, been pinned down. The analysis of chromosomal aberrations in H-RS cells has not resulted in the description of a specific HL gene. However, HL is sometimes familial, and the genetically identical siblings of affected monozygous twins carry a 100-fold increased risk of the disease [78]. Cases of families coinheriting both HL and Leri-Weill dyschondrosteosis, the gene for which is located on the short-arm pseudoautosomal region of the X and Y chromosomes, have been reported [79], and genetic study of the 2 loci by linkage analysis has suggested the localization of a putative gene for HL in the pseudoautosomal region [80]. Because the *mic2* gene encoding CD99 maps to the pseudoautosomal region, these investigators' data provide indirect evidence of a linkage between CD99 and HL.

The accumulated data described above therefore suggest close interrelationships of 3 factors, the presence of LMP1, the loss of CD99, and the enhanced NF-κB activity, in the generation of H-RS cells. As a transmembrane molecule that constitutively activates NF-κB activity in a ligand-independent manner [81], LMP1 seems to regulate the transcription of CD99 via NF-κB mediating the suppression signal from the cytoplasm into the nucleus, because inhibition of the NF-κB activity of LMP1 can restore CD99 expression.

However, the loss of CD99 may not be the exclusive cause for the pathogenesis of LMP1-associated HL, because the increased generation of multinucleated H-RS cells has been demonstrated by LMP1 overexpression in EBV-negative L428 cells originally lacking CD99 [82]. The observation suggests that although the loss of CD99 plays an important role in the pathogenic process leading to the formation of H-RS cells, LMP1 has additional functions for accelerating the process independently of CD99 down-regulation, which suggest the cooperation of both CD99-dependent and CD99-independent LMP1-induced pathways to generate H-RS cells in a synergistic way.

6. Perspectives

Because of the number of molecular tools used in immunophenotypic and karyotypic studies, there recently has been a significant advance in the understanding of the common molecular defects that form the basis for the occurrence of HL, and activation of the NF-κB pathway has been suggested as a common denominator in cases of HL. On the basis of observations that LMP1 activation of NF-κB markedly reduces CD99 expression at the transcriptional level and that the subsequent loss of CD99 expression induces typical H-RS phenotypes in vitro in a B-cell line, the loss of CD99 expression via the NF-κB pathway is likely to play a critical role in the pathogenic sequences that lead to the formation of H-RS cells, at least in part if not totally. The idea suggests the possibility of manipulating NF-κB–mediated CD99 expression in HL cases for therapeutic purposes. Future study should define downstream factors associated with the CD99 signaling pathway that transduce extracellular signals from CD99 into the cytoplasmic compartment. Such findings will clearly explain the pleiotropic roles for the loss of CD99 function in the generation of H-RS cells.

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