

## **The Molecular and Cellular Origins of Hodgkin's Disease**

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Hodgkin's disease is divulging its secrets at last. Despite the fact that Hodgkin's disease was the first lymphoma to be recognized as a distinct clinical entity (1), it has proved to be one of the most difficult lymphomas to approach molecularly. The clonal, malignant Hodgkin/Reed-Sternberg (H/RS) cells of Hodgkin's disease represent <1% of the cells in an involved lymph node and are characteristically surrounded by a mixture of granulocytes, plasma cells, and T cells. Although several cell lines have been derived from patients with Hodgkin's disease, it has been difficult to prove, in most cases, that the cell lines are clonally related to the H/RS cells of the patients. Therefore, molecular approaches to the etiology and pathogenesis of Hodgkin's disease have relied on single cell micromanipulation of H/RS cells coupled with PCR amplification of RNA and genomic DNA. These techniques have shed considerable light on the cellular origins of Hodgkin's disease through analysis of the antigen receptor genes of H/RS cells (2). These techniques have also led to the identification of the first recurrent molecular abnormality of H/RS cells: mutations in the I $\kappa$ B $\alpha$  gene (3, 4; and, in this issue, Jungnickel et al. [5]). This finding specifically implicates the nuclear factor  $\kappa$ B (NF- $\kappa$ B) transcription factor signaling pathway in the pathogenesis of Hodgkin's disease. Once revealed, this association seems quite natural given the intense inflammatory component of Hodgkin's lymphoma lesions and the central role that NF- $\kappa$ B plays in the normal regulation of inflammatory responses (6).

**Cellular Origins of Hodgkin's Disease.** The normal cellular counterpart of the H/RS cell has long been enigmatic due to the promiscuous expression in H/RS cells of markers that are normally expressed in distinct hematopoietic lineages. H/RS cells can variably express T cell markers (e.g., CD2, CD4, granzyme B), B cell markers (e.g., CD19, CD20), and myeloid markers (e.g., CD15, c-fms) (7, 8). Within the lymphoid lineages, the most lineage-specific molecular events involve rearrangement and mutation of the antigen receptor genes. Analysis of micromanipulated H/RS cells from nodular sclerosis Hodgkin's disease, the most common subtype, revealed that most patients had a clonal rearrangement of the V, D, and J segments of the IgH chain locus in the H/RS cells, indicating that these cells

descended from B lymphocytes (2). Sequence analysis of the rearranged VDJ regions from these H/RS cells demonstrated a high load of somatic mutations, often resulting in nonfunctional Ig V regions (9). Inasmuch as somatic hypermutation of Ig genes occurs characteristically in germinal centers of secondary lymphoid organs (10), these findings suggested that H/RS cells arise from germinal center B cells or from B cells at a later stage of differentiation.

Most recently, evidence has accumulated that the initial transforming event in Hodgkin's disease occurs within the germinal center B cell itself (11, 12). This view arose from analysis of rare patients with "composite lymphomas" in which Hodgkin's disease coexists with a non-Hodgkin's lymphoma (NHL). In these patients, the H/RS cells and the NHL cells had identical VDJ rearrangements at the IgH chain locus and also shared certain somatic mutations within the VDJ region. The H/RS and NHL cells both accumulated additional somatic mutations that were not shared by the other cell type. As previously observed in other NHL cases, the VDJ regions of the NHL cells of these patients showed a pattern of ongoing somatic mutation, suggesting that the initial transforming event(s) occurred within the germinal center microenvironment. Since the H/RS cells and the NHL cells were clonally related, the simplest explanation of these findings is that the initial transforming event occurred in a germinal center B cell that was the precursor of both the NHL cells and the H/RS cells. From this perspective, it is also clear that the H/RS cells and/or the NHL cells in these patients must have acquired secondary molecular lesions that account for the strikingly different phenotypes of these two cell types.

An additional complication is that the four histologically distinct subtypes of Hodgkin's disease (nodular sclerosis, nodular lymphocyte-predominant, mixed cellularity, and lymphocyte depletion) may well represent diseases with separate molecular and cellular etiologies. Nodular lymphocyte-predominant Hodgkin's disease is also derived from a B cell, as indicated by clonal VDJ rearrangements of the IgH chain locus in the malignant lymphocytic and histiocytic (L&H) cells of this disease (13-15). The VDJ regions of L&H cells show a pattern of somatic mutation that suggests that mutations continued to accumulate after the initial transforming event and were positively selected by antigen. L&H cells express BCL-6 (16), a transcriptional repressor protein characteristically expressed in germinal center B cells (17). Taken together, these data suggest that the L&H cells of nodular lymphocyte-predominant Hodgkin's dis-

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ease are derived from germinal center B cells and retain at least part of the germinal center B cell phenotype.

In contrast, the H/RS cells of nodular sclerosis Hodgkin's disease frequently lack BCL-6 expression (18) and do not show evidence of ongoing somatic hypermutation of their Ig genes (2). Further differences between germinal center B cells and H/RS cells were uncovered during high-throughput expressed sequence tag (EST) sequencing of cDNA libraries prepared from individual H/RS cells (19). Many established germinal center B cell markers, including CD10 (20), *a-myb* (21), and 8-oxoguanine-DNA glycosylase (OGG-1 [22]), were not observed during sequencing of the H/RS libraries but were repeatedly observed in a library derived from tonsillar germinal center and memory B cells (19). Thus, although the initial transforming event leading to an H/RS cell may occur in a germinal center B cell, much of the biology of this stage of B cell differentiation does not appear to be retained by this malignant cell.

With the emerging view that most H/RS cells derive from germinal center B cells, what accounts for the ectopic expression of T cell and myeloid markers in some cases? In this issue, Mischen et al. (23) examined three Hodgkin's disease cases in which the H/RS cell was found to express cytotoxic T cell markers (granzyme B, T cell intracellular antigen 1 [TIA-1], and/or perforin) by immunohistochemistry. In two cases, the IgH chain loci had VDJ rearrangements and the TCR- $\beta$  locus was in germline configuration. Surprisingly, the third case had clonal VDJ rearrangements of the TCR- $\beta$  locus and germline configuration of the IgH chain locus. This observation, together with the fact that some Hodgkin's cell lines also have TCR gene rearrangements (8), demonstrates that classical nodular sclerosis Hodgkin's disease can, in rare cases, be derived from a T cell. Therefore, in these cases, the expression of the cytotoxic T cell markers by the H/RS cells may not be ectopic after all.

Nonetheless, the aberrant expression of T lineage markers in the other two cases remains unexplained and suggests that global changes in gene expression patterns are a consequence of the transforming events leading to Hodgkin's disease. Promiscuous expression of lineage markers is a feature of uncommitted stem cells (24). Of note in this regard is the fascinating, stem cell-like behavior of pro-B cells from mice deficient in Pax5, a key differentiation factor in the B cell lineage (25). Pax5<sup>-/-</sup> pro-B cells fail to differentiate further along the B cell lineage and instead behave as uncommitted hematopoietic progenitor cells (25). Intriguingly, Pax5<sup>-/-</sup> pro-B cells aberrantly express genes from many hematopoietic lineages, including *c-fms* and perforin, two of the genes aberrantly expressed in H/RS cells. Thus, it may be worth investigating whether the transforming events of H/RS cells interfere with the expression and/or function of transcription factors that are involved with cell fate and commitment decisions in the B cell lineage.

*Molecular Origins of Hodgkin's Disease.* The first indication of a role for NF- $\kappa$ B in Hodgkin's disease came with the observation that several Hodgkin's disease cell lines and primary H/RS cells from one patient had constitutive NF- $\kappa$ B DNA binding activity in the nucleus (26). The NF- $\kappa$ B

family of transcription factors consists of homo- and heterodimers of a variety of gene products related to the *v-rel* oncoprotein (6). Most cells do not have nuclear NF- $\kappa$ B because one or more inhibitory proteins belonging to the inhibitor of NF- $\kappa$ B (I $\kappa$ B) family sequester the NF- $\kappa$ B factors in the cytoplasm. A wide variety of cellular stimuli leads to the phosphorylation, ubiquitination, and subsequent degradation of I $\kappa$ B, allowing NF- $\kappa$ B factors to travel to the nucleus. The situation is a bit more complex in mature B cells in that the NF- $\kappa$ B1/c-rel heterodimer can be constitutively present in the nucleus while the NF- $\kappa$ B1/relA heterodimer is inducible (27, 28). Hodgkin's cell lines resembled mature B cells in having nuclear NF- $\kappa$ B1/c-rel but differed from mature B cells by the constitutive presence of NF- $\kappa$ B1/relA in the nucleus (26).

A priori, the mechanisms underlying this phenomenon could include constitutive activity of kinases upstream of I $\kappa$ B, mutation or loss of I $\kappa$ B, or modification of NF- $\kappa$ B rendering it insensitive to inhibition by I $\kappa$ B. Several reports now show that mutation of the I $\kappa$ B $\alpha$  gene is the culprit in a subset of Hodgkin's cases (3–5). In two Hodgkin's disease cell lines, L428 and KM-H2, one allele of I $\kappa$ B $\alpha$  is deleted and the other allele has a deletion or point mutation which results in a truncated I $\kappa$ B $\alpha$  protein lacking the COOH terminus. No I $\kappa$ B $\alpha$  protein could be observed in KM-H2, suggesting that the severely shortened protein from the mutated allele is unstable. The truncated I $\kappa$ B $\alpha$  protein in L428 cells was stable but was unable to associate with NF- $\kappa$ B heterodimers. Thus, the I $\kappa$ B $\alpha$  mutations and deletions in these cell lines made the cells functionally null for I $\kappa$ B $\alpha$  activity.

Since these Hodgkin's disease cell lines were established from treated patients late in the natural history of the disease, it was important to determine whether I $\kappa$ B $\alpha$  alterations are clonal events that occur in the H/RS cells of untreated patients. Definitive proof that this is the case came from one untreated Hodgkin's case in which all H/RS cells that were micromanipulated from a lymph node biopsy contained two different mutant I $\kappa$ B $\alpha$  alleles, each resulting in a COOH-terminal truncation of the protein (5). In the 19 patients studied thus far, 5 had truncating mutations of I $\kappa$ B $\alpha$  on at least 1 allele (3–5).

However, most Hodgkin's disease cell lines and patients apparently have wild-type I $\kappa$ B $\alpha$  alleles and express I $\kappa$ B $\alpha$  protein. Nonetheless, the NF- $\kappa$ B pathway may also play a role in these cases. For example, some Hodgkin's disease cell lines have wild-type I $\kappa$ B $\alpha$  alleles yet have constitutive nuclear NF- $\kappa$ B1/relA DNA binding complexes (29). In such cases, constitutive activity of I $\kappa$ B kinases, leading to rapid degradation of I $\kappa$ B $\alpha$ , appears to account for the presence of NF- $\kappa$ B in the nucleus (29). Although the cytokines secreted by the Hodgkin's disease cell lines can induce nuclear NF- $\kappa$ B (29), the possibility of molecular defects in the components of the I $\kappa$ B kinase cascade should be investigated. Since the H/RS cells of 17–41% of Hodgkin's disease patients harbor EBV (30), the EBV-encoded protein latent membrane protein 1 (LMP-1) may activate NF- $\kappa$ B in these cases by promoting I $\kappa$ B $\alpha$  turnover (31). Finally,

three TNF family receptor–ligand pairs have the potential to activate NF- $\kappa$ B in Hodgkin's disease: CD40 present on the H/RS cell may be engaged by CD40 ligand on the surrounding T cells in the Hodgkin's lesions (32), TNF- $\alpha$  produced by the H/RS cell may act in an autocrine fashion via TNF- $\alpha$  receptors (33), and CD30 on the H/RS cells may be cross-linked by CD30 ligand on either the H/RS cell or on surrounding cells (34).

What are the functional consequences of constitutive nuclear NF- $\kappa$ B in H/RS cells? Many of the genes known to be expressed in H/RS cells are also NF- $\kappa$ B target genes. I $\kappa$ B $\alpha$  itself is a well-documented NF- $\kappa$ B target gene that is transcriptionally activated by NF- $\kappa$ B, resulting in a negative feedback loop that attenuates NF- $\kappa$ B signaling (35, 36). In the majority of Hodgkin's disease cases, 10–90% of the H/RS cells expressed high levels of I $\kappa$ B $\alpha$  as judged by immunohistochemistry (3). Other known NF- $\kappa$ B target genes expressed in H/RS cells include intercellular adhesion molecule 1 (ICAM-1), GM-CSF, IL-6, and TNF- $\alpha$ . Bargou et al. (37) directly manipulated the NF- $\kappa$ B system in several Hodgkin's cell lines by overexpressing a dominant negative version of I $\kappa$ B $\alpha$  which cannot be inducibly degraded. As a consequence, these cell lines had decreased nuclear NF- $\kappa$ B, decreased proliferation rates, an enhanced apoptotic response to removal of serum from the culture medium, and decreased ability to form tumors in immunodeficient mice (37). A role for NF- $\kappa$ B in cellular transformation of B cells was previously demonstrated by the translocation of NF- $\kappa$ B2 (38) and the amplification of c-rel (39) in NHLs.

The antiapoptotic effects of NF- $\kappa$ B have been documented in many cell types (6), including mouse B cells (40). The observation that H/RS cells contain nonfunctional Ig genes suggests that they are derived from germinal center B cells that should have been negatively selected (2), but were rescued from apoptosis by aberrant NF- $\kappa$ B activation. Indeed, these results raise the possibility that normal positive selection of germinal center B cells may involve transient activation of NF- $\kappa$ B after interaction of the B cell with antigen-bearing follicular dendritic cells. Most importantly, though, these studies suggest that pharmacological manipulation of the NF- $\kappa$ B system might have therapeutic potential in Hodgkin's disease. However, given the variety of ways in which NF- $\kappa$ B can be activated in Hodgkin's disease, any therapeutic intervention will have to be tailored to the particular molecular lesion in each patient.

*Insights into Hodgkin's Disease from Genomics.* Given the likelihood that NF- $\kappa$ B activation is only one step in a multistep transformation process, how can we get clues to the other pathways that may be aberrant in Hodgkin's disease? Increasingly, the answer to this question will come from the emerging field of functional genomics. In particular, genomic-scale gene expression profiling using DNA microarrays has the potential to transform our understanding of human cancer (41, 42). This technology can simultaneously quantitate the expression of tens of thousands of genes in parallel, giving biologists a bird's eye view of the molecular pathways that are engaged in a cancer cell. The

similarity of a cancer cell to a particular stage of normal differentiation can be defined by the expression of scores of lineage-restricted genes instead of the handful of markers currently in use. Databases of genes that are responsive to various cytokines, signal transduction pathways, or transcription factors will soon help biologists deconvolute the gene expression profiles of cancer cells. Eventually, genomic-scale gene expression analysis may be used to guide cancer patients to the therapies that are most appropriate to the signaling pathways that are engaged in their particular tumors.

DNA microarrays of 950 genes were used to profile gene expression in 2 Hodgkin's cell lines, L428 and KM-H2 (43). The gene expression in each of these cell lines was measured relative to the gene expression in a lymphoblastoid B cell line transformed with EBV. Since the LMP1 protein of EBV would be expected to activate NF- $\kappa$ B target genes in the lymphoblastoid cell line, the expression of some NF- $\kappa$ B target genes in the Hodgkin's cell lines may have been missed by this analysis. Nevertheless, these microarray experiments revealed the expression of several genes in the Hodgkin's cell lines that could play important and previously unrecognized roles in the pathophysiology of this disease.

In particular, the cytokine IL-13 was found to be expressed in each of the Hodgkin's disease cell lines studied, and IL-13 expression was subsequently found in H/RS cells of Hodgkin's disease by *in situ* hybridization (43). This important cytokine promotes T helper cell differentiation to the Th2 phenotype (44) and can influence the survival and/or proliferation of B cells (45, 46). The receptors for IL-13 and IL-4 have two signaling chains in common and thus can activate overlapping target genes (47). Interestingly, the Hodgkin's disease cell lines were also found to express the E4BP4/NF-IL3, a gene that was previously found to be an IL-4-regulated gene by DNA microarray gene expression analysis and by subtractive hybridization (41, 48). Thus, the expression of E4BP4/NF-IL3 may indicate autocrine signaling by IL-13 in the Hodgkin's disease cell lines. E4BP4/NF-IL3 encodes a transcription factor that prevents IL-3 withdrawal apoptosis (49) and thus could conceivably enhance the survival of H/RS cells. Another known IL-13 target gene, macrophage-derived chemokine (MDC [50]), was repeatedly encountered during the high-throughput sequencing of H/RS cell cDNA libraries (19), further supporting the possibility of IL-13 autocrine signaling in H/RS cells. MDC specifically attracts Th2 cells (51) and thus may contribute to the inflammatory milieu surrounding H/RS cells.

Intriguingly, neutralizing antibodies to IL-13 blocked the proliferation of the HDLM-2 Hodgkin's disease cell line (43), again supporting an autocrine signaling model and suggesting that IL-13 signaling could be attacked pharmacologically to the benefit of Hodgkin's disease patients. Although speculative, there could be a relationship between the antiproliferative effects resulting from IL-13 neutralization and from overexpression of dominant negative I $\kappa$ B $\alpha$ . The IL-13-stimulated transcription factor, signal transducer and activator of transcription 6 (Stat6), interacts physically

with NF- $\kappa$ B, and these two transcription factors synergistically activate some target genes (52–54). Although IL-13 normally counteracts NF- $\kappa$ B by activating I $\kappa$ B $\alpha$  transcription (55, 56), this effect may be mitigated in H/RS cells by mutations in the I $\kappa$ B $\alpha$  gene or by constitutive degradation of I $\kappa$ B $\alpha$ .

As with most DNA microarray gene expression studies, these experiments identified a host of other differentially expressed genes that suggest intriguing hypotheses requiring further work. The host of cytokines that H/RS cells are capable of secreting (8), exemplified by IL-13, IL-5, and GM-CSF in this DNA microarray analysis, most likely initiate the intense inflammatory reaction surrounding H/RS cells. It remains to be determined whether the NF- $\kappa$ B activation in H/RS cells is responsible for this cytokine expression and whether other signaling pathways are required in addition. Another gene expressed in both Hodgkin's disease cell lines, IFN regulatory factor 1 (IRF-1), encodes a transcription factor that can cooperate with NF- $\kappa$ B in activating certain genes (e.g., IFN- $\beta$  [57]) and thus may act synergistically with the constitutive NF- $\kappa$ B in H/RS cells. Two other overexpressed genes in Hodgkin's disease cell lines play important roles in other cancers: insulin-like growth factor II (IGF-II) is an antiapoptotic factor that is overexpressed during mouse pancreatic tumor progression (58), and urokinase plasminogen activator is an important prognostic marker in a variety of solid tumors that is likely to regulate cancer cell metastasis (59). In the future, the application of DNA microarray gene expression analysis to micromanipulated H/RS cells will present a considerable technical challenge, but will be well worth the effort. Current first-line treatments of Hodgkin's disease can cure roughly 80% of patients with early disease and 60% of patients with advanced disease (60). If DNA microarray gene expression analysis of H/RS cells could identify those patients who are likely to relapse, they could be offered early high-dose therapy coupled with hematopoietic transplantation (60), or other therapies suggested by our growing knowledge of the molecular lesions in Hodgkin's disease.

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