Biology of Hodgkin's lymphoma

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A B S T R A C T

The Hodgkin and Reed/Sternberg (HRS) tumor cells in classical Hodgkin lymphoma (HL) are derived from mature B cells. However, they have largely lost the B cell-specific gene expression program and express a mixture of genes typical for various hematopoietic cell types. A grossly deregulated network of transcription factors contributes to this mixed lineage phenotype. The transforming events involved in the generation of the malignant HRS cells are only partly understood, but multiple genetic lesions in members of the NF-κB signaling pathway have been identified, including frequent mutations in the TNFAIP3 tumor suppressor gene. Recent studies revealed that the frequent genomic amplification of the chromosomal region 9p24 in HRS cells involves at least four pathogenetically relevant genes: JAK2, PD-1 ligands 1 and 2, and JMJD2C. There is currently a controversial discussion whether HRS cell clone members with cancer stem cell features exist, and if so, whether such cells are present among the typical HRS cell population or have a distinct phenotype.

Introduction

In the current WHO lymphoma classification, Hodgkin lymphoma (HL) is subdivided into a classical form and a nodular lymphocyte predominant form.[1] Classical HL, which accounts for about 95% of cases, is further subdivided into nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte depleted HL. This subclassification is largely based on differences in the morphology of the tumor cells and the histological picture.

HL is a very peculiar and hence fascinating malignancy, due to several specific features. First, the tumor cells, named Hodgkin and Reed/Sternberg (HRS) cells in classical HL and lymphocyte predominant (LP) cells in nodular lymphocyte predominant HL (NLPHL), are rare in the lymphoma tissue and usually account for only about 1% of the cells. The vast majority of other cells in the lymphoma tissue resembles an inflammatory infiltrate and is composed of T cells, B cells, plasma cells, neutrophils, eosinophils, histiocytes, mast cells, and others. Although the tumor cells are rare, HL is still a fatal disease if left untreated (with some exceptions for NLPHL).[2] Second, whereas in all other lymphomas, the tumor cells retain key immuno-phenotypic and gene expression similarities with their cells of origin, HRS cells in classical HL show a very "mixed" phenotype, which does not resemble any normal cell of the hematopoietic system.[3] Third, although deregulation of numerous signaling pathways is a hallmark of all leukemias and lymphomas, it appears that HRS cells are rather unique in the extent to which multiple signaling pathways show a deregulated and partly aberrant activation in these cells.

Deregulated transcription factor networks in HRS cells

The detection of rearranged and somatically mutated immunoglobulin (Ig) variable (V) region genes in isolated HRS and LP cells unequivocally established the mature B cell origin of these cells, as Ig gene rearrangements and somatic hypermutation are B cell specific-processes.[4–7] LP cells of NLPHL also show a mature B cell phenotype, with expression of key B cell transcription factors (e.g., Bcl-6, Pax-5, Oct-2) and differentiation markers (e.g., CD20).[8] However, HRS cells of classical HL express only few B cell markers and express multiple markers of other hematopoietic cell types, which was one reason why the origin of HRS cells has been enigmatic for a long time.[9–11] As such a dramatic "reprogramming" to a mixed-lineage phenotype is unique among lymphoid malignancies, this is likely a key factor for HL pathogenesis. Thus, there is much interest in understanding the mechanisms that cause this loss of the B cell phenotype and the upregulation of genes not normally expressed by B cells. Several factors contributing to the "reprogramming" have been identified in recent years. The B cell transcription factors Oct-2, BOB1, and Pu.1 are strongly downregulated in HRS cells, which explains why many of their target B cell genes are also not expressed.[10,12,13] The silencing of these and other factors is also influenced by epigenetic mechanisms, including DNA methylation in the promoter regions of these genes.[14] Another main B cell transcription factor, E2A, is still expressed, but its activity is blocked in HRS cells by high levels of the two E2A inhibitors ABF-1 and ID2.[15,16] Notably, ID2 is normally expressed by natural killer and dendritic cells, supporting their

differentiation and inhibiting a B cell development of lymphoid precursors. HRS cells also express the important myeloid colony stimulating factor 1 receptor (CSF1R).[17] The mechanism for its aberrant expression in the HRS cells involves the reactivation of an endogenous retrovirus located upstream of the CSF1R gene.[17] The lost suppression of this retroviral element is at least partly caused by downregulation of the corepressor CBFA2T3.[17] This downregulation appears to affect the expression of many other endogenous retroviral sequences, so that potentially many genes may be aberrantly expressed in HRS cells through the activity of reactivated retroviral elements. HRS cells also express the T cell transcription factors, Notch1 and GATA3.[18–20] Notch1 is a main T cell lineage differentiation factor, and in early lymphocyte development, suppresses B cell genes and thus a B cell development, and promotes T cell differentiation. GATA3 is typically expressed by T helper type 2 cells. We recently showed that the high activities of Notch1 and NF-κB in HRS cells contribute to aberrant GATA3 expression in these cells and that GATA3 activity plays a role in the cytokine expression and signaling pattern of these cells.[21] Further factors that have been implicated in the downregulation of B cell genes in HRS cells include the polycomb group gene BMI-1, active STAT5, and in the Epstein–Barr virus (EBV)-positive cases, the EBV-encoded genes latent membrane protein 1 (LMP1) and latent membrane protein 2a (LMP2a) (reviewed in ref.[3]).

Thus, multiple factors contribute to the grossly deregulated gene expression program in HRS cells. However, it is still not clear how this "reprogramming" of the HRS cells is initiated, and whether so far undetected oncogenic events are involved in this process.

Mechanisms causing constitutive NF-κB activity

The transcription factor family NF-κB consists of five members – Rel, RelA (p65), RelB, p50, and p52 – which function as homo- or heterodimers.[22] A canonical and a non-canonical NF-κB signaling pathway is distinguished. In the canonical pathway, NF-κB is kept inactive by binding to IκBα or other members of the IκB family in the unstimulated stage, which retains the NF-κB dimers in the cytoplasm. Upon activation of the NF-κB signaling pathway, IKK kinases induce the degradation of the IκB factors, so that the NF-κB dimers can translocate to the nucleus and activate the transcription of their target genes.[22] In the non-canonical pathway, inactive precursor proteins are expressed in the absence of stimulatory signals. Upon stimulation of this pathway, the NIK kinase processes the p100 precursor of p100/RelB heterodimers into the active p52 form, which then translocate as p52/RelB dimers into the nucleus. NF-κB activates multiple genes involved in inflammation, survival, and proliferation, including IL6, IL13, CCL5, BclXL, and FLICE inhibitory protein (FLIP). In normal B cells, NF-κB is only transiently activated. However, several types of B cell lymphomas, including HL, show a constitutive activation of NF-κB. The pathogenetic role of this activation is evident from the observation that inhibition of NF-κB in HL cell lines causes the apoptotic death of the cells.[23]

Multiple mechanisms likely contribute to the constitutive NF-κB activation in HRS cells. First, HRS cells express several surface receptors known to activate NF-κB, including CD30, CD40, and RANK.[8] HRS cells are often in direct contact with CD40L expressing T cells, and CD30L-positive eosinophils, and mast cells are regularly seen in the HL microenvironment, suggesting ligand-mediated activation of the CD30 and CD40 receptors.[24–26] Signaling through Notch1, TACI, and BCMA presumably also contributes to NF-κB activation.[8] Second, in about 30–40% of cases of classical HL, the HRS cells are infected by EBV, and in these cases, LMP1 is expressed, which is known to mimic an activated CD40 receptor and activate NF-κB.[27] Third, genetic lesions in HRS cells play an important role in the deregulated NF-κB activity. These lesions include genomic gains of the NF-κB factor Rel and of the NF-κB activating kinase NIK, and inactivating mutations in the genes NFKBIA (encoding IκBα) and NFKBIE (encoding IκBε). Gains of Rel and NIK are found in about 40% and 20% of cases, respectively,[28–30] whereas NFKBIA and NFKBIE mutations have been detected in approximately 10% of cases.[31–35] We and others recently identified mutations in the TNFAIP3 gene, encoding the NF-κB inhibitor A20, as a frequent genetic lesion in HRS cells: 40% of HL cases showed such mutations.[36,37] TNFAIP3 mutations are also frequent in classical HL cell lines, as four out of six lines in the initial analysis showed inactivating TNFAIP3 mutations.[37] In the SUP-HD1 classical HL cell line, we also recently detected an inactivating mutation in exon 2 (Figure 1). Interestingly, most mutated HL cases were EBV-negative, and TNFAIP3 inactivation was seen in 70% of EBV- cases of classical HL.[37] These findings also prompted the analysis of further regulators of the NF-κB pathway for mutations, but mutations in the NF-κB inhibitors CYLD and TRAF3 were rare. CYLD inactivation was found in one of eight HL cell lines analyzed, but in none of ten primary cases of classical HL.[38] Similarly, destructive TRAF3 mutations were identified in one of six classical HL cell lines studied, but not in isolated HRS cells from seven cases of classical HL (own unpublished data).

Considering the various types of genetic lesions in components of the NF-κB pathway in HRS cells, the question arises whether these are cooperating or mutually exclusive events. Indeed, for many cancers, the concept has been proposed that there is usually only one genetic lesion per oncogenic pathway,[39] and the observation that TNFAIP3 mutations are largely restricted to EBV-negative cases of classical HL (see above) shows that these NF-κB activating events are largely mutually exclusive. For the other lesions, this issue cannot be answered with primary cases, as for all the genes analyzed by us and others, independent collections of cases were studied. However, we have a clearer picture for the HL cell lines (Table 1). Notably, several line show genetic lesions in more than one of the oncogenes and tumor suppressor genes of the NF-κB pathway.[32–34,37,38] Therefore, it appears that we have the unusual situation that multiple genetic lesions cause the deregulation of one transcription factor. As some mutations affect the canonical and others the non-canonical NF-κB pathway, it appears to be a selective advantage for the HRS cells to deregulate both arms of this pathway, which likely

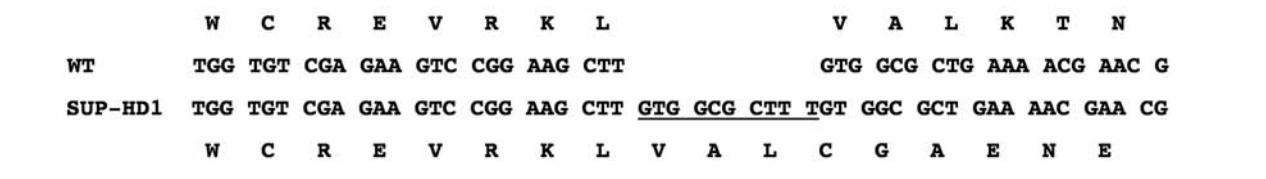

Figure 1. TNFAIP3 mutation in HL cell line SUP-HD1. PCR amplification and sequence analysis of the coding exons of TNFAIP3 in the classical HL cell line SUP-HD1 according to the protocol published in ref.[37] revealed that SUP-HD1 harbors a frameshift-causing insertion (which is largely a duplication of the sequence further 3') in exon 2. The 10 bp insertion is underlined. As only the mutated sequence was obtained, this is either a homozygous mutation (perhaps caused by an uniparental disomy event), or the other allele of TNFAIP3 is deleted. In any case, no functional A20 can be generated. The corresponding aminoacid sequences are given above the wild type and below the SUP-HD1 DNA sequences. The 3' part of exon 2 is shown. The SUP-HD1 exon 2 sequence has been submitted to the EMBL database under accession number FR775799.

Table 1. Multiple genetic lesions in regulators of NF-κB activity in HL cell lines.

HL cell line	NFKBIA	NFKBIE	TNFAIP3	CYLD	TRAF3	REL
L428	+	+	-	-	-	+
L591	-	n.a	-	-	n.a.	n.a.
L1236	-	n.a.	+	-	-	+
KMH2	+	n.a.	+	+	-	+
HDLM2	-	n.a.	+	-	-	n.a.
UHO-1	n.a.	n.a.	+	-	+	n.a.
SUP-HD1	n.a.	n.a.	+	-	-	n.a.

+ denotes presence of a genetic lesion, - a wildtype sequence. For NFKBIA, NFKBIE, TNFAIP3, CYLD, and TRAF3, inactivating point mutations and deletions are considered; for REL chromosomal gains. L591 is the only EBV-positive HL cell line. n.a.: not analyzed

leads to deregulation of more NF-κB target genes than through activation of only one of the pathways. Moreover, one may speculate that because of the multilevel regulation of NF-κB activity, a mutation in only one component would not be sufficient to cause the very strong constitutive NF-κB activity in HRS cells.

A recent gene expression profiling study of isolated LP cells of NLPHL revealed that also these lymphoma cells show constitutive NF-κB activity.[40] However, the mechanisms for this activity appear to be quite distinct from those identified in HRS cells of classical HL. LP cells do not express CD30, they are not surrounded by CD40L expressing T cells, they are virtually never EBV-infected, there is no indication for Rel gains, and inactivating mutations in NFKBIA or TNFAIP3 were not found in a molecular analysis of ten cases of NLPHL and the LP cell-derived cell line DEV.[41]

The pathogenetic role of the 9p24.1 amplification in HRS cells

In 2000, Joos and colleagues reported that gains or amplifications of the chromosomal region 9p23-p24 are frequent in HRS cells and can be found in about a third of cases.[42] Later, they showed that such gains were also present in three of four HL cell lines analyzed.[43] 9p24 gains are also frequent in primary mediastinal B cell lymphoma (PMBL), a B cell lymphoma with numerous similarities to classical HL.[8,44] The amplified region harbors the JAK2 gene, an important factor of the JAK/STAT cytokine signaling pathway. This pointed to a potential role of the JAK/STAT pathway in HL pathogenesis. Constitutive activation of this pathway was indeed validated, as active forms of STAT3, STAT5, and STAT6 were found in HRS cells.[45–48] Moreover, inhibition of STAT activity in HL cell lines impaired cell proliferation.[46,49,50] A role of JAK/STAT deregulation in HL pathogenesis was further supported by the finding that HRS cells in about 40% of HL carry somatic mutations in the SOCS1 gene, a main negative regulator of STAT activity.[51] Activation of the JAK/STAT pathway in HRS cells also involves signaling through cytokines, in particular IL13 and IL21.[47,52,53] Thus, there is strong evidence that activation of the JAK/STAT signaling pathway through cytokine signaling and genetic lesions is a major factor in HL pathogenesis.

Two recent publications now indicate that the 9p24 amplifications have additional pathogenetic consequences. Green *et al.* revealed that the programmed death-1 (PD-1) ligand genes 1 and 2 (PD-L1 and PD-L2, respectively), which are also located in the amplicon, show an increased expression in the HRS cells of those HL cases with gains of the 9p24 region.[54] PD-1 is an inhibitory receptor expressed on T cells, and there is evidence that HL-infiltrating T cells are functionally impaired through PD-1 ligand/PD-1 signaling.[55,56] Thus, increased PD-L1 and PD-L2 expression by HRS cells likely contributes to the immunosuppressive microenvironment in classical HL. Enforced PD-1 ligand expression in HRS cells with 9p24 gains is not only due to increased gene dosage of the PD-L1 and PD-L2 genes, but also through further transcriptional upregulation of these genes by JAK2.[54]

Based on an RNA interference screen of genes located in the 9p24 amplicon region, Rui and colleagues identified a further gene in this region with pathogenetic relevance.[57] Downregulation of expression of the histone demethylase JMJD2C was toxic for a HL cell line and cell lines of PMBL, which harbored the 9p24 amplification. Importantly, not only JMJD2C modulates histones, but also JAK2 has been reported to modify histones, by phosphorylating histone H3. Indeed, further experiments showed that both proteins modulate the epigenetic state in HL and PMBL, and that they cooperate in this regard to promote proliferation and survival of HRS and PMBL cells.[57]

Taking these findings together, the pathogenetic role of the amplification of 9p24 in HRS cells (and PMBL) involves at least four genes, and the tumor-promoting

mechanisms include activation of the JAK/STAT cytokine signaling pathway (JAK2 gains), suppression of tumor-infiltrating T cells (PD-L1 and PD-L2 gains), and epigenetic remodeling (JAK2 and JMJD2C gains).

The search for HRS stem cells

In several types of cancers, there is convincing evidence that not all tumor cells have the same proliferative capability and that a small subset of cancer stem cells is mainly responsible for sustaining the tumor clone.[58] As cancer stem cells show differences in their gene expression to the bulk of the tumor clone and often appear to be more chemotherapy resistant than their descendents, the identification and characterization of cancer stem cells is also of high clinical relevance.

In HL, years before the issues of cancer stem cells became an important topic, there was a discussion whether the rare morphologically visible HRS cells indeed account for the whole tumor clone, or whether other tumor clone members might exist among the many other cells in the lymphoma microenvironment. It was also debated what the relationship between the mononuclear Hodgkin and the multinucleated Reed/Sternberg cells is.

The detection of rearranged immunoglobulin heavy and light chain V region genes firmly established the derivation of HRS cells from mature B cells.[5,6] Moreover, as most rearrangements carry a high load of somatic mutations, and as the process of somatic hypermutation, which generates such mutations, is linked to antigen-activated B cells proliferating in germinal centers,[59] HRS cells derive from germinal center B cells or their descendents, with the pattern of mutations suggesting a derivation from germinal center B cells that normally would have undergone apoptosis.[5,60] Importantly, as all HRS cells of a clone carry the same Ig V gene rearrangements and (with very few exceptions) the identical somatic mutation pattern, putative HRS stem cells – if they exist – must carry the same Ig V gene rearrangements and mutations and hence must also derive from mature B cells.

Regarding the relationship between the mononucleated Hodgkin and the multinucleated Reed/Sternberg cells, there is now firm evidence from studies with HL cell lines that Hodgkin cells give rise to Reed/Sternberg cells through endomitosis.[61–63] Cell fusion is not involved in the generation of Reed/Sternberg cells from Hodgkin cells, or the generation of the HRS cell clone as such.[64,65] Reed/Sternberg cells had little proliferative capacity in *in vitro* studies,[61–63] and it has been suggested that nuclear disorganization and telomere loss in Reed/Sternberg cells causes their failure to undergo further cell division.[66] Thus, the mononucleated Hodgkin cells represent or harbor the proliferative pool of tumor cells that give rise to more Hodgkin cells and to Reed/Sternberg cells.

The question whether the HRS tumor clone consists of more cells than the typical CD30+ HRS cells was addressed in several studies. First, in two HL cases in which the HRS cells showed numerical chromosomal abnormalities, it was analyzed whether cells with such abnormalities were also present among CD30- cells in the HL microenvironment. A few CD30- cells with trisomies as seen in the respective HRS cells were reported, arguing for the existence of clone members among the CD30-negative cells.[67] However, numerical abnormalities are not a stringent clonal marker, and increased frequencies of normal B cells with chromosomal abnormalities have actually been reported for HL.[68] Second, it was argued that in EBV+ cases of HL, in which the HRS clone shows a monoclonal viral infection pattern, putative HRS clone members not visible as CD30+ HRS cells must also be EBV-infected. However, in a detailed study of microdissected HRS cells and CD30-EBV+ cells, few, if any of the small EBV-infected cells belonged to the HRS clone, arguing against the existence of HRS clone members among CD30- cells in the HL tissue.[69] In a third study, it was reported that clonotypic B cells can be found in the peripheral blood of HL patients. These cells had a B cell phenotype (CD19+ and surface Ig+) and expressed the putative stem cell marker ALDH (aldehyde dehydrogenase).[70] However, this study was criticized as none of the data presented unequivocally demonstrated a clonal relationship between the ALDH+CD19+ B cells in the peripheral blood and HRS cells in the tissue.[71] In another study that searched for HRS clone members in the peripheral blood of HL patients, using a highly sensitive PCR with HRS clone specific Ig V gene primers, no HRS cell-specific amplificates were obtained, arguing that HRS clone members are very infrequent or absent in the peripheral blood.[72] It is also important to consider that B cell clones generated in a germinal center can be very large.[73] Thus, one may potentially find with highly sensitive assays other memory B cell descendents from a germinal center B cell clone that gave also rise to an HRS cell clone. These cells, although clonally related to the HRS cells, may be non-malignant B cells, or pre-malignant cells that share some transforming events with the HRS cells. As these clone members will likely differ in the Ig V gene somatic mutation pattern from the HRS cells, a detailed study of the Ig gene rearrangements is needed to distinguish between putative HRS stem cells and pre-malignant clone members (Figure 2).

Another approach to search for a subpopulation of cells among the HRS cells with specific features in terms of proliferation and chemotherapy resistance involves a flow-cytometric strategy. The increased chemoresistance of some cancer stem cells appears to be closely related to their expression of drug transporters of the ABC family, which expel chemotherapeutical drugs from the cells. As ABC transporters also extrude the Hoechst dye 33342, negativity for Hoechst dye staining has been used to identify ABC+ cells, which are called 'side population cells', in flow cytometry studies.[74,75] Although cancer stem cells and side population cells are defined through different features of tumor cell subpopulations, these populations appear to overlap in several instances.[74,75] Two recent studies addressed the issue whether side population cells exist in HL cell lines. Side population cells were indeed found, accounting for less than 1% of cell line cells. These cells were small (i.e., Hodgkin cells), chemoresistant, and could repopulate a mixed population of Hodgkin cells and Reed/Sternberg cells.[76,77] Thus, these cells fulfill key criteria of tumor stem cells.[78] However, not all HL cell lines harbored side population cells,[77] arguing against a general role of these

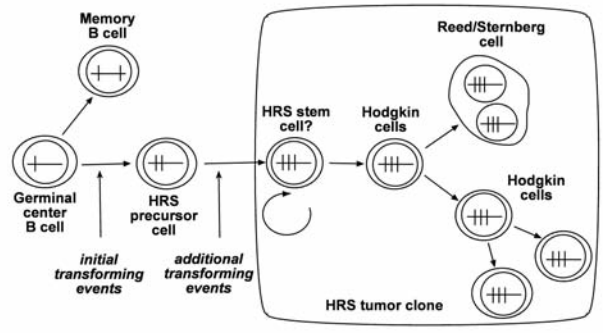

Figure 2. HRS cells and their precursors. The HRS tumor clone is most likely derived from germinal center B cells. As lymphoma development is a multi-step process, one can postulate that HRS precursor cells exist that carry some, but not all of the transforming events of the HRS clone. These cells may also persist in the patient. Among the tumor cells, the mononucleated Hodgkin cells are the proliferative compartment that give rise to more Hodgkin cells and through endomitosis to multinucleated Reed/Sternberg cells. These latter cells have little if any proliferative capacity. It is currently debated whether HRS stem cells exist that feed the HRS tumor clone. As HRS cells of a given clone carry identical Ig V gene rearrangements and an identical somatic mutation pattern of these rearrangements, also putative HRS stem cells must carry the same V region gene sequences. The pre-malignant HRS precursor cells carry the same Ig V genes and may have identical or partly different mutation patterns. As germinal center B cells clones can give rise to very large populations of long-lived memory B cells, cells with the same Ig V gene rearrangement as the HRS cells (but presumably different mutation patterns) may also be found among normal memory B cells. The horizonal lines in the cells indicate an Ig V gene rearrangement and the vertical lines exemplify somatic point mutations.

cells for the maintenance of the HRS tumor clone, and it remains to be shown whether side population cells clonally related to the HRS cells also exist *in vivo*.

Taken together, although the identification of side population cells in some HL cell lines and some other observations might indicate that HRS stem cells exist, it still remains unclear whether such cells exist *in vivo* in the patient, and if so, what their role is in the establishment and perpetuation of the HRS tumor clone. Moreover, it will be important to clarify whether side population cells are responsible for treatment failure in some patients.

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