Pathogenesis of Hodgkin’s lymphoma

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Abstract: In Hodgkin’s lymphoma (HL), the B cell origin of the tumour cells, the Hodgkin and Reed-Sternberg (HRS) cells, has been disclosed by molecular single cell analysis about 10 yr ago. This finding formed the basis for various studies aimed to better understand the pathogenesis of this peculiar malignancy and the pathophysiology of the HRS cells. Work of our groups in this regard was focussed recently on two main topics, namely the study of differential gene expression in HRS cells and the pathogenesis of composite lymphomas. Composite lymphomas are combinations of HL and B cell non-Hodgkin lymphomas, that turned out to be often clonally related. By molecular analysis of several composite lymphomas for potential transforming events, we identified examples of both shared as well as distinct transforming events. Comparing gene expression profiles of HL-derived cell lines with the corresponding profiles from other B cell lymphomas and normal B cell subsets revealed a global down-regulation of the B cell-specific gene expression signature in HRS cells. Moreover, we identified aberrant expression and activity of multiple receptor tyrosine kinases in HRS cells of classical and to a lesser extend lymphocyte predominant HL, which appears to be a unique feature of HL, and may offer novel strategies for treatment.

In Hodgkin’s lymphoma (HL), the Hodgkin and Reed-Sternberg (HRS) cells, which represent the tumour cells of this malignancy, usually account for less than 1% of cells in the tumour tissue. This has for a long time hampered their molecular analysis. However, through the establishment of microdissection techniques to isolate HRS cells and the analysis of single HRS cells by sensitive PCR methods, it became possible to study these cells at the molecular level. These studies revealed the clonality and B cell nature of the HRS cells and provided evidence that HRS cells are derived in most cases from a subset of germinal centre (GC) B cells (1–3). Only in rare cases, HRS cells derive from T cells (4, 5). Microdissection and single cell PCR methods were also instrumental to study HRS cells for potential transforming events, such as mutations in proto-oncogenes or tumour suppressor genes (6–8). Recently, we applied these methods to the analysis of composite lymphomas, which are rare combinations of a HL and a B cell non-Hodgkin lymphoma (NHL). The results of this analysis will be discussed below.

Due to the problems in the analysis of primary HRS cells, extensive efforts were undertaken to establish cell lines from HRS cells as in vitro models of these cells. This turned out to be extremely difficult, and in the last 30 yr, only about 10 cell lines were established that are still existing. Since the attempts to establish cell lines were so rarely successful, it is an important question whether the lines are derived from the HRS cells in the patient, and whether the few lines are indeed representative of HRS cells (notably, all cell lines were derived from blood, bone marrow or pleural effusion, but none from lymph node biopsies). Indeed, only for one of the lines, L1236, the derivation of the cell line from the HRS cells in the patient was proven (9). Nevertheless, several important new findings about the pathogenesis of HL were initially identified analysing HL cell lines and were then confirmed for primary HRS cells. For example, we performed gene expression profiling with several HL cell lines, and the main findings of that study were confirmed for HRS cells in lymph node biopsies, as discussed below (10–12).

Pathogenesis of composite lymphomas

In rare cases a combination of classical HL and B cell NHL occurs in the same patient, either concurrently or sequentially. Important insights
into the development of those composite lymphomas were provided by single cell PCR analysis for rearranged Ig V genes of the two lymphomas. Thus, clonal relationship between the two different tumours was demonstrated in the majority of the cases analysed (13–20). Interestingly, in many of the clonally related cases, the rearranged V genes of the two lymphomas showed both shared as well as distinct somatic mutations in the HRS and the NHL cells. This strongly indicates that in these cases the two lymphomas developed separately from a common (premalignant) precursor and that these cases do not represent a transformation of one of the lymphomas into the other. Moreover, since somatic mutations are introduced into rearranged V genes of B cells participating in germinal centre (GC) reactions (21), the V gene mutation pattern in these cases suggests that decisive steps in the malignant transformation process happened in distinct members of a proliferating GC B cell clone.

The molecular analysis of such clonally related composite lymphomas permits the unique opportunity to study the time point and the role of transforming events in the multistep process of lymphomagenesis, to gain insights into the development of two distinct malignancies from a common precursor and to better understand the pathogenesis of HL. Six clonally related composite lymphomas were analysed for transforming events that have been described to be present in cases of HL and/or B-NHL (manuscript in preparation) (Table 1).

Hallmarks of many B-NHL are chromosomal translocations, juxtaposing one of the immunoglobulin loci with one of several proto-oncogenes (22). However, in HL, Ig-associated chromosomal translocations have not been determined on a molecular level so far. Interestingly, in three cases of composite lymphomas combining HL with mantle cell lymphoma (MCL) and HL with follicular lymphoma (FL) identical hallmark translocations (Igh/bcl-1 and Igh/bcl-2) were obtained from the B-NHL and the corresponding HL (Table 1). Further sequence analysis of the bcl-2 and bcl-1 translocational breakpoints showed loss of nucleotides and addition of non-germline encoded nucleotides (typical for VDJ recombination) directly at the junctions of Igh and the translocated oncogenes in both NHL and HL. Hence, the common B cell precursor of the composite lymphomas most likely acquired these Ig-associated translocations during misguided VDJ recombination in an early developmental step.

Mutations in the tumour suppressor genes IκBα CD95 and ATM have been found in a fraction of cases of HL or NHL and presumably contribute to the pathogenesis of these malignancies (6, 23–27). However, in none of the composite lymphoma cases analysed mutations in these genes were detected in the HL or the NHL.

A remarkable finding of the analysis of transforming events was the restriction of two clonal replacement mutations in the p53 gene to the DLBCL cells of a composite lymphoma case combining HL and DLBCL (Table 1). Those two mutations were demonstrated to be located on different alleles and were found at positions that have been previously reported to be frequently mutated in aggressive NHL, suggesting a pathogenic role for these mutations (28, 29). The presence of the mutations exclusively in the DLBCL suggests a late transforming event, which happened most likely in the GC because of the occurrence of shared

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**Table 1. Characteristics of six clonally related composite lymphomas**

<table>
<thead>
<tr>
<th>Case</th>
<th>Composite lymphoma</th>
<th>HL and NHL present in the same tissue</th>
<th>Mutated V genes</th>
<th>Shared and distinct V gene diversity</th>
<th>Intraclonal V gene diversity</th>
<th>EBV</th>
<th>Igh-associated translocations</th>
<th>P53 gene mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HL</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Igh/bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>HL</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Igh/bcl-2</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>HL</td>
<td>Yesa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>–</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>SMZL</td>
<td>Noa</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>HL</td>
<td>Yesb</td>
<td>Only distinct</td>
<td>Two subclones</td>
<td>Yes (one subclone)</td>
<td>No</td>
<td>Igh/bcl-1</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>HL</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>–</td>
<td>Two clonal mutations on separate alleles</td>
</tr>
</tbody>
</table>

1The data on the rearranged Ig V genes and the EBV status are taken from Refs 14, 15, 17–19.
2For each of the lymphomas, the breakpoint in the HRS cells and B-NHL cells was identical.
3NHLs were first diagnosed 3–15 yr before diagnosis of the composite lymphomas.
4Indication of two copies of the same clonal rearrangement with distinct mutation patterns was obtained in the previous single cell analysis (from Ref. 17).
5–: not analysed; HL: Hodgkin’s lymphoma; FL: follicular lymphoma; B-CLL: B cell chronic lymphocytic leukemia; SMZL: splenic marginal zone lymphoma; MCL: mantle cell lymphoma; DLBCL: diffuse large cell lymphoma.
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and distinct somatic V gene mutations in HRS as well as DLBCL cells. This late separate transforming event might have prompted the evolution of the DLBCL from a common precursor in terms of separation of the two lymphoma clones.

In conclusion, the molecular analysis of several composite lymphomas supports the concept that the development of such lymphomas is characterised by both early shared transforming events and distinct events occurring at later stages that then determine the development of the different malignancies.

The role of Epstein–Barr virus in HL pathogenesis

Epstein–Barr virus (EBV), a member of the γ-herpes virus family, infects more than 90% of the human population worldwide. The reservoir of the virus are B cells, in which a latent infection is established. The EBV normally is a harmless passenger, but in rare cases, EBV is also involved in the pathogenesis of malignancies, mainly B cell lymphomas. One of these is HL, as EBV is found in the HRS cells in about 40% of cases of classical HL in the Western world (30). In EBV-harbouring HRS cells, three EBV-encoded latent proteins are expressed, namely the EBV nuclear antigen 1 (EBNA1) and the latent membrane proteins 1 and 2a (LMP1 and LMP2a) (30). The EBNA1 is required for the replication of the circular viral genome in proliferating cells. The LMP1 mimics an activated CD40 receptor, which plays a major role in the interaction of B and T cells and the survival of GC B cells. Indeed, LMP1 is a viral oncogene, because LMP1 transgenic mice develop B cell lymphomas (31). The cytoplasmic domain of LMP2a contains an ITAM (immunoreceptor tyrosine-based activation motif) that is also found in the coreceptors of the B cell receptor (BCR) (32). In the BCR, this motif mediates signal transduction of cross-linked BCR by binding of kinases, which are subsequently activated. Moreover, normal B cells strictly depend on BCR expression for survival, and the ITAM motif is essential also for this tonic survival signal (33, 34). There is evidence that LMP2a is capable of mimicking the presence of a functional BCR, because in LMP2a transgenic mouse models, B cells expressing LMP2a but lacking a functional BCR are generated in the bone marrow and are able to survive in the periphery (35, 36).

Based on the features of LMP1 and LMP2a discussed above, it has been proposed that these viral proteins could have a decisive role in the development of EBV-positive HL: by mimicking the main survival signals of GC B cells – CD40 signalling and Ig-crosslinking – LMP1 and LMP2a could allow the survival of GC B cells that would otherwise undergo apoptosis (GC B cells die as a default program by apoptosis, if they are not rescued from cell death by survival signals such as the ones mentioned above) (37). While this hypothesis may well explain the role EBV plays in the pathogenesis of EBV-positive HL, it is less clear whether LMP2a can have the same role also in the established HRS cell clone. As is discussed in more detail below, gene expression profiling studies showed that HRS cells have lost expression of many B cell-specific genes (12). These molecules include the Syk tyrosine kinase and the intracellular adaptor protein SLP-65 (BLNK), two important factors for BCR signalling. Since these molecules appear to be essential for the function of LMP2a as a BCR surrogate (38, 39), it is questionable whether LMP2a can replace the BCR survival signal in the established HRS cell clone via the conventional BCR signalling pathway.

Another issue regarding the role of EBV in the pathogenesis of HL relates to the question at which step in the pathogenesis of HL HRS cell precursors become infected by EBV. It has been proposed that in healthy virus carriers, EBV predominantly infects naive B cells and that (some of) these cells undergo GC reactions to gain access to the memory B cell compartment, the long-term reservoir of EBV (40). Thus, according to this scenario, EBV would be present already in a B cell that is driven into a GC reaction and that after acquisition of additional transforming events gives rise to a malignant HRS cell clone. However, a study of the viral strategies during infectious mononucleosis, the acute primary EBV infection, suggested that also GC and/or memory B cells can be directly infected (41, 42). While it is normally not possible to identify the differentiation stage at which an HRS cell precursor was infected by EBV, we recently encountered a composite mantle cell and classical HL in which only a fraction of HRS cells was EBV-infected (Table 1) (19). Interestingly, the EBV-positive and -negative HRS cells carried not only shared somatic mutations in the rearranged Ig V genes, but also a few mutations that were found only in the EBV + subclone. This suggests that the EBV infection happened in a member of a mutating GC B cell clone and that this cell as well as a non-infected other member of the clone later gave rise to the HRS tumour clone (presumably, the common precursor of the two HRS subclones already carried a shared transforming event). The alternative scenario, namely that EBV was lost from an originally homogenously EBV-infected HRS cell clone is unlikely due to the fact that the EBV− HRS cell subclone carried less somatic mutations than the EBV + subclone. Thus, this case is the first example for a HL case with indication that EBV infection happened in a GC B cell, suggesting that at least in a
fraction of cases of EBV-positive HL the viral infection may happen in GC and not in naive B cells.

**The lost B cell identity of HRS cells**

For the identification of genes differentially expressed between HRS cells and normal B cells we performed two different approaches. In one experiment, global gene expression of the HRS cell line L1236 was compared to the expression profile of flow cytometrically isolated tonsillar GC centroblasts using the serial analysis of gene expression (SAGE) method (11). In a second experiment, gene expression profiles of four HL-derived cell lines (L428, L1236, KMH2 and HDLM2) were compared to the profiles of four normal B cell subsets, namely naive B cells, memory B cells, centroblasts (CD77-positive proliferating GC B cells) and centrocytes (CD77-negative resting GC B cells), using Affymetrix microarrays (10). One of the observations made consistently in both types of experiments was the detection of a global down-regulation of most B cell-typical genes (Fig. 1) (12). For a number of these genes, it was already known that HRS cells usually lack expression of these molecules, such as CD19, CD20, CD79, immunoglobulin and the transcription factors Oct-2, Bob-1 and Pu-1 (43–46). However, the gene expression profiling studies identified many more genes that are normally expressed by B cells and that are down-regulated in HL cell lines, e.g. the kinases Syk, Lyn and Blk, the adaptor molecule SLP-65, and the transcription factors Pu-B and A-myb (12). For several of the molecules, we confirmed by immunohistochemistry that their down-regulation is not only a feature of the HL lines, but is also characteristic for HRS cells in HL biopsy specimens (12). Interestingly, HRS cells retain expression of key B cell-specific molecules important for B cell/T cell interaction (CD80, CD86, MHC class II) and surprisingly also of the B cell lineage commitment and maintenance factor Pax-5 (Fig. 1) (47, 48).

What could be the cause for this global down-regulation of B-lineage-specific genes? It has been discussed that the HRS cell phenotype could reflect a plasma cell differentiation, because plasma cells down-regulate many B cell-lineage molecules, such as CD19, CD20 and Blk, and HRS cells often express molecules that are upregulated in plasma cells (CD138, Mum-1) (49). However, the retained expression of MHC class II and Pax-5, and in particular the down-regulation of immunoglobulin expression argue against this hypothesis. Based on the puzzling observation that HRS cells usually express the B lineage maintenance factor Pax-5, but lack expression of several target genes of this transcription factor, we speculated that Pax-5 may be inactive because of somatic mutations. However, no mutations were found in three HL lines that were analysed for mutations in the Pax-5 gene (12).

Another possibility could be that the B cell phenotype of the HRS cells is suppressed by a dominant factor that is aberrantly expressed by HRS cells. Indeed, it has been reported that HRS cells express Notch-1, which plays an important role in early lymphocyte development by suppressing a B cell differentiation and supporting the generation of T cells from lymphoid precursors (50). Perhaps, the aberrant expression of Notch-1 by HRS cells contributes to the down-regulation of B cell markers in these cells.

*Fig. 1.* The peculiar phenotype of HRS cells. The HRS cells in classical HL have a phenotype that does not resemble any normal hematopoietic cell, and is very much different from the phenotype of its normal counterpart, i.e. GC B cells. Shown are examples for B-lineage-specific genes down-regulated by HRS cells, plasma cell markers expressed by HRS cells, markers of other lineages aberrantly expressed by HRS cells (TARC: dendritic cells, GATA-3: T cells, Notch-1: T cells, fascin: dendritic and myeloid cells; CD15: myeloid cells) and B lineage genes that show retained expression in HRS cells. The down-regulation of B cell markers is indicated by ‘x’ through the molecules.
Independent of the mechanism that causes the global loss of the B cell identity of HRS cells, it is an intriguing question whether this dramatic phenotypic change could be of advantage for the tumour cells and may hence be selected for during the pathogenesis of HL. On the background of the proposed origin of HRS cells in classical HL from pre-apoptotic GC B cells which failed to express a high-affinity BCR due to accumulation of disadvantageous V gene mutations, the loss of the B cell identity could indeed represent a strategy of the cells to escape from the stringent selection for expression of an appropriate BCR (a high affinity BCR in the case of GC B cells), that governs the life of a B cell, as discussed above.

Expression of receptor tyrosine kinases in HRS cells

Receptor tyrosine kinases (RTK) are frequently involved in cellular transformation (51). Nevertheless, analysis of RTK expression in HRS cells has so far been limited to a few studies of individual RTKs, namely KIT, MET, HER2 and PDGFRA (52–58). The reported expression of HER2 and KIT in HRS cells could, however, not be confirmed by other groups and thus remains controversial (52, 55). The MET can be detected in HRS cells and also normal GC B cells with immunohistochemistry (57), implying that expression in HRS cells is not aberrant but likely due to the GC B cell origin of the HRS cells.

For a more comprehensive analysis of RTK expression we screened our global RNA expression data of four HL cell lines (10), where 50 of 57 known RTKs were represented by probes, for aberrant HRS cell-specific RTK expression. Eight RTKs, which were aberrantly expressed in at least two of the HRS cell lines were identified, and for most of these RTKs we confirmed the RNA expression data in Western blot analysis (59).

The expression of six of these RTKs was then analysed in primary cases of classical HL and also other B cell lymphomas. Immunohistochemistry revealed that the RTKs PDGFRA, DDR2, EPHB1, RON, TRKB and TRKA, were each expressed in HRS cells of 30–75% of cases (Fig. 2A). None of these RTKs was expressed in normal B cells. In B-NHL (MCL, FL, chronic lymphocytic leukemia, Burkitt lymphoma and diffuse large B cell lymphoma) expression was only occasionally observed with the most frequently expressed RTK found only in 7% of cases. The HRS cell-specific expression was further stressed by analysis of two composite FL/HL cases, where

![Fig. 2. Expression and activation of the PDGFRA receptor tyrosine kinase in HRS cells. Immunohistochemistry using alkaline phosphatase with Fast Red as substrate for PDGFRA (A) revealed that PDGFRA is expressed in the HRS cells of most classical HL cases. A large fraction of these cases coexpressed the PDGFRA high affinity ligand PDGFA (B), and activated, phosphorylated PDGFRA (C) could be demonstrated in a fraction of cases. Immunohistochemistry with a pan-phospho-tyrosine specific antibody using horseradish peroxidase with DAB as substrate revealed aberrantly high phospho-tyrosine contents in HRS cells of a large fraction of HL cases (D). The pan-phospho-tyrosine stainings were comparable to tumours, where activated receptor tyrosine kinases have important roles in cellular transformation, like ALK-positive anaplastic large cell lymphoma.](image-url)
PDGFRA expression was restricted to the HRS cells.

In the vast majority of cases at least one and in most cases several RTKs were coexpressed. Averagely RTK expression was more pronounced in nodular sclerosis subtype. Here on average, nearly four RTKs per case were found to be expressed, while in mixed cellularity subtype an average below two RTKs per case was observed. In lymphocyte-predominant HL on average only one RTK was expressed per case and the most frequently expressed RTK was found in only 30% of cases.

Activated RTKs are phosphorylated on several intracellular tyrosine residues and therefore we used antibodies specific for phosphorylated RTKs to analyse if they were not only aberrantly expressed but also activated. For PDGFRA and TRKA/B we could exemplarily show activation in considerable fractions of cases (Fig. 2C). In addition, antibodies detecting phospho-tyrosine in several proteins revealed that the HRS cells in many cases had elevated cellular phospho-tyrosine contents, comparable to other tumours, where activated RTKs play important roles in pathogenesis (Fig. 2D).

Constitutive TK activities in tumours are frequently due to mutations in the TKs. However, upon sequencing of the cDNAs of the RTKs under investigation from the HL cell lines, we found no activating mutations. As RTK activation could also be due to presence of the ligands in the HL infiltrate, we performed immunohistochemistry for the four RTK ligands for which suitable antibodies were available. We indeed found that the DDR2 ligand collagen type I and the TRKA ligand, NGF were present in several cases analysed; collagen type I in the sclerotic bands branching in the tumour infiltrate and NGF in granulocytes, respectively. This likely means, that these two RTKs can be activated in paracrine fashions in a considerable fraction of cases. For the PDGFRA and EPHB1 ligands PDGFA and EphrinB1, respectively, we observed expression in HRS cells in a number of cases (Fig. 2B). In most of the cases expressing PDGFA or EphrinB1 in the HRS cells, the HRS cells expressed also the corresponding RTK, suggesting autocrine activation in these cases.

Taken together, our analysis revealed aberrant expression and activation of several different RTKs in HRS cells of classical HL, most pronounced in nodular sclerosis HL, and at least for four RTKs activation seems to occur predominantly by their ligands in paracrine or autocrine fashions. Although no genetic changes causing deregulated RTK activity were identified, the unprecedented coexpression of multiple RTKs in a tumour together with the observed aberrant expression in the vast majority of cases indicates that aberrant RTK activities contribute to HL pathogenesis. These findings indicate that TK inhibitors, e.g. the PDGFRA inhibitor Imatinib, which is already in clinical use, may be potential therapeutics for classical HL, especially for treatment of nodular sclerosis HL.

**Expression profiling of microdissected HRS cells**

Since HL cell lines most likely do not retain the features of primary HRS cells in all important aspects, and since the rare HL lines may not be representative of typical primary HRS cells, as discussed above, we plan to study differential gene expression of microdissected HRS cells in comparison with various B-NHL, the main normal B cell populations and HL cell lines using gene expression profiling. Because of the rareness of the HRS cells in the tumour tissue there was the need to establish a reproducible and reliable amplification protocol for RNA from lasermicrodissected HRS cells. Different RNA isolation methods including cell lysis by detergent, two guanidine isothiocyanate-based methods and a salt precipitation method were tested for RNA yield and quality. Best results were obtained with a kit based on salt precipitation (Genta, Minneapolis, MN, USA). The T7 RNA polymerase-based RiboAmpTM RNA Amplification Kit (Arcturus, Mountain View, CA, USA) was used with slight modifications to amplify the RNA of 500–1000 lasermicrodissected or FACS-sorted cells by in vitro transcription. The in vitro transcribed RNA was then converted into cDNA and the double-stranded cDNA was subjected to the BioArray High Yield RNA Transcription Labelling Kit (ENZO, Farmingdale, NY, USA) in a modified manner. To test the reproducibility and reliability of the procedure total RNA of three independent replicates of 10^6 cells, 5 ng of diluted RNA and 500 FACS-sorted cells, all from the HL cell line L428, were amplified, labelled and hybridised to Affymetrix genechip microarrays. Array data were analysed according to the Affymetrix manual. All comparisons showed high reproducibility in terms of signal correlation, discrepant calls and other quality criteria. The protocols were then applied to lasermicrodissected cells of primary HL, other B cell lymphomas and FACS-sorted normal B cells subsets and HL cell lines. A first preliminary analysis of the data by unsupervised hierarchical clustering shows that the clustering is independent from the cell isolation method. The two main branches of the dendrogram subdivide the samples into normal B cells and lymphoma cells. Among the normal B cell subsets, there are three main subbranches separating plasma cells, resting peripheral blood cells (naive and memory B cells) and GC B cells (centro-
bifurcates and centocytes). In the branch of the lymphoma cells, the HL cell lines build a cluster of their own, separate from the primary HL cases. The preliminary data of the few lymphomas analysed so far indicate that, as expected, there are apparently significant differences between the HL cell lines and primary HRS cells. Interestingly, HRS cells of classical and lymphocyte predominant HL cluster close to each other.

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References


