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Epidemiology and etiology of Hodgkin's lymphoma

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Introduction

Thomas Hodgkin published his description of seven patients suffering from massive enlargement of lymph nodes and spleen as a new disease entity as early as 1832. Nevertheless, some of the key questions regarding the etiology of Hodgkin's lymphoma (HL) have only been answered in the last few decades [1].

Systematic analyses of epidemiological data pointed towards an infectious agent as a potential cause for this disorder and the development of new sophisticated research tools in the field of molecular biology enabled the identification of the Epstein–Barr virus (EBV) as a putative transforming agent. Recently, single-cell technology made the Hodgkin and Reed–Sternberg (HRS) cells accessible to molecular analyses and provided evidence for their derivation from a preapoptotic germinal center B cell (GCB) in almost all cases.

Epidemiology

Incidence and age of onset

Hodgkin's lymphoma is an uncommon disorder with an annual incidence of 2–3 per 100 000 in Europe and the USA [2]. In industrialized countries the onset of HL shows a bimodal distribution with a first peak in the third decade and a second peak after the age of 50. Men are affected by HL slightly more often than women among all subtypes, except for the nodular-sclerosing subtype that occurs slightly more often in young females than in male patients. Among the group of young adults, the most common subtype is nodular-sclerosing (NS) HL occurring at a higher frequency than the mixed-cellularity (MC) subtype. The frequency of MC increases with age, while that of NS reaches a plateau in the group >30 years of age. The lymphocyte-predominant (LP) and the lymphocyte-depleted (LD) subtypes both occur at lower frequencies. Lymphocyte-depleted subtype is diagnosed at a frequency of <1% in western countries [3]. According to the WHO classification, NS, MC and LD are summarized in 'classical' (cHL), while LPHL is referred to as a distinct entity. Interestingly, there is a great difference in incidence between developing and industrialized countries. In developing countries, the disorder appears predominantly during childhood and its incidence decreases with age, while in industrialized countries, young children are only rarely affected by HL in contrast with young adults where incidence increase with age.

Epstein–Barr virus (EBV)

Epidemiological studies pointed towards a viral etiology of HL. In developed countries, there is an association between early birth order, low number of siblings and playmates, high maternal education, single family dwellings in childhood and occurrence of HL in younger patients [4, 5]. These characteristics had also been observed in the epidemiology of poliovirus infection and suggested that HL represented the rare consequence of infection with a common virus at a late age of onset [6]. An infectious pathogenetic agent is also in agreement with the clinical characteristics of HL, e.g. fever, night sweat, weight loss and the laboratory findings of elevated erythrocyte sedimentation rate (ESR) or interleukin 6 (IL-6) in the serum (for review see [7]).

Several studies suggest that EBV may be a transforming agent in HL. Patients with a history of EBV-related infectious mononucleosis are at a two- to three-fold higher risk for development of HL. Mueller et al. [8] analyzed EBV titers in pre-disease sera and found an enhanced level of EBV activation prior to onset of HL.

To substantiate the role of EBV in HL, a number of authors investigated the role of EBV in HRS cells using novel molecular techniques and found that EBV DNA is present in HL cases more frequently in developing countries than in developed countries. Interestingly, EBV-positivity in underprivileged patients and children from industrialized countries shares the frequency of that observed in developing countries [9, 10]. In western countries, about 50% of all cases of classical HL are EBV-positive, i.e. carry the virus within the tumor cells, with the NS subtype being positive in 15–30% of cases and the MC subtype harboring EBV DNA in up to 70% of cases [11]. By comparison, ≥90% EBV-positivity of HRS cells has been demonstrated in developing countries [10].

Hodgkin and Reed–Sternberg cells in EBV positive patients show an expression pattern of EBV-encoded genes, termed type 2 latency, which resembles that found in endemic nasopharyngeal carcinoma or in a subset of T-cell lymphomas. This pattern includes expression of the EBV latent genes *LMP1*, *LMP2a* and *EBNA1*. Since EBV is present in about 50% of cases in the western world, investigators were

prompted to search for other viruses involved in the pathogenesis of HL. However, until now, the role of other viruses in the pathogenesis of HL is uncertain.

Taken together, these data suggest that EBV is involved in the transformation process in EBV positive HL cases. In EBV negative cases, a 'hit and run' mechanism was hypothesized, but evidence of EBV as a transforming agent in negative cases is scarce [12, 13]. Therefore other transforming mechanisms must be taken into account in HL.

Inheritance

For family members of patients affected by HL, their own increased risk of developing HL ranges from three- to nine-fold that of expected values, which leads to the hypothesis that at least a proportion of cases are the consequence of an inherited disorder [14]. Mack et al. [15] found that out of 179 monozygotic twin pairs with HL, in 10 of these both twins developed HL, strongly supporting the idea of a genetic component in a subset of HL. A recent study that analyzed the Swedish Cancer Registry and compared the data with a matched healthy cohort found that HL was fourth in a list of cancers with high familial indices, just behind cancer affecting eye or testis (52 first degree relatives/8766 HL cases) [16]. However, familial HL is estimated to contribute to only a minority of cases. Additionally, no consistent mechanisms of inheritance have been identified so far and evidence for a genetic aberration present in all cases of familial HL is lacking.

Etiology

The cell of origin

The affected tissue in HL is characterized by a heterogenous infiltrate with typical mono- and multinucleated giant cells in an inflammatory background composed of stroma, lymphocytes, histiocytes, eosinophils and monocytes. In classical HL, the giant cells are termed Hodgkin and Reed–Sternberg (HRS) cells, while in LPHL, they are termed lymphocytic and histiocytic (L&H) cells. Typically, HRS cells represent only 0.1–1% of the affected tissue, and therefore systematic analysis of these cells has been a frustrating effort for a long period.

Immunophenotyping studies showing expression of different lineage markers left the question of the origin of HRS cells unanswered. For example, HRS cells lack most typical B-lineage markers such as CD20, sIg or CD79a. In contrast, markers typical for other cell types are expressed by HRS cells, including CD15 (granulocytes), CD30 (monocytes and T-cells) (reviewed in Stein and Hummel [17]), Perforin (T-cells), Syndecan (Memory B cells), Fascin and TARC (dendritic cells) [18–21]. L&H cells of LPHL differ from HRS cells in cHL in that they consistently express typical B-lineage markers such as CD20, sIg or the J-chain [22]. Consequently, L&H cells can be considered malignant B cells that lack expression of other entity-defining cell surface molecules.

As immunophenotyping did not lead to the identification of the cell of origin of the HRS cells, molecular approaches were applied to solve this question. Ralf Küppers was the first to show that HRS cells harbor somatically mutated clonal rearranged immunoglobulin (Ig) heavy chain genes by using single-cell polymerase chain reaction (PCR) on micromanipulated primary HRS cells [23].

From these results it was concluded that HRS cells are derived from germinal center B cells. Amplification of identical rearranged and mutated Ig genes from different HRS cells from the same patient also answered the question of dignity since clonality—the key criterion of malignancy—of HRS cells was hereby shown (reviewed in Kuppers and Rajewsky [24]). In addition, it could be shown that HRS cells do not only expand clonally within one affected lymph node, but also clonally disseminate in advanced stage disease and relapse even after clinical complete remission [25–29]. Recent publications demonstrate that a probably small subset of cHL exists with T-cell derivation of the respective HRS cells [30, 31]. Thus, HRS cells are derived from germinal center or post-germinal center B cells in the majority of cases. In contrast, L&H cells in LPHL harbor ongoing mutations in their rearranged Ig genes and, therefore, seem to be malignant B cells at a different maturation stage [32].

The B-cell paradox

Extended single-cell studies in the 1990s have illustrated the B-cell genotype of the HRS cell in most cases. These findings are in contrast with the immunophenotype of the HRS cell lacking a consistent pattern of B-cell specific surface markers. Despite harboring rearranged and mutated Ig genes, HRS cells do not express sIg. Several mechanisms may account for this observation. First, mutations in the VDJ regions of the rearranged Ig genes that lead to abrogation of the coding capacity ('crippling mutations') have been described in about 25% of primary cHL cases [33, 34]. Second, mutations in regulatory regions important for Ig transcription have been reported and may account for missing Ig expression in a subset of cHL cases [35–37]. Third, in almost all cases, loss of the transcription factors BOB1, Oct-2 and PU.1 represent consistent explanations for the lack of sIg in cHL [38–40]. So far, it is not understood which of these mechanisms primarily underlie the complete absence of sIg in cHL.

During the germinal center reaction, B cells express the transcription factors STAT6, NFκB, PU.1, BOB1 and Oct-2 (reviewed in Henderson and Calame [41]). Expression of these transcription factors reflects the specific developmental stage of B cells undergoing the process of affinity maturation. While HRS cells of cHL constitutively express STAT3, STAT6 and NFκB, they lack expression of genes PU.1, BOB1 and Oct-2 [42–44, 38–40]. Thus, HRS cells display a heterogeneous *phenotype*, a germinal center B-cell derived *genotype*, and probably the *transcriptome/proteome* of a defective B cell. In contrast, L&H cells are homogeneous in phenotype, genotype

and transcriptome/proteome, underlining the different biology of LPHL.

The transformation process of HRS cells is not yet fully understood

Despite progress in the understanding of the cellular origin of HRS cells, there is little evidence on the initial transformation process. EBV has been identified as a virus with potential involvement in the transformation of a portion of cHL cases. Additionally, constitutive activation of NFκB seems to be a central mechanism that leads the way out of the hostile environment of the germinal center and contributes to proliferation and apoptosis resistance.

EBV and NFκB

EBV represents a good candidate for an initial transforming agent in HL, since it is found in HRS cells of 50% of cases [11]. Furthermore, infected HRS cells display expression of EBV encoded latent genes *LMP1*, *LMP2* and *EBNA1*. Both latent genes have transforming capacity. LMP1, for instance, can transform primary B cells, possibly by mimicking the function of constitutively active CD40, a transmembrane receptor molecule of the TNF receptor family [45]. Physiologically, CD40 ligation results in activation of a signaling cascade terminating in activation of the transcription factor NFκB. NFκB itself initiates transcription of proinflammatory and antiapoptotic genes such as *bcl-2*. Constitutively activated NFκB has recently been demonstrated to be a characteristic feature of HRS cells [44]. Additionally, abrogation of constitutive NFκB activation results in massive spontaneous apoptosis of HRS cells by downregulation of an antiapoptotic signaling network, thereby providing evidence for its central role in transformation and acquisition of the apoptosis-resistant phenotype [46]. Thus, *LMP1* as the EBV encoded gene with the highest transforming potential may induce the activated phenotype seen in HRS cells with constitutively activated NFκB.

Recent publications describe mutations in the *IκB*a gene in some cases of primary and cultured HRS cells [47–49]. These mutations might underlie activation of NFκB in a minority of cases since wild-type IκBα keeps NFκB bound in the cytoplasm, thereby preventing its translocation to the nucleus and subsequent transcriptional activity. Several other mechanisms might also be involved in NFκB up-regulation including RANK expression in HRS cells, or autocrine IL13 signaling [50, 51].

Apoptosis resistance

B cells acquire a high load of somatic mutations in their Ig genes during the process of hypermutation in the germinal center reaction. This process gives rise to a big repertoire of diversified Ig genes and may result in B cell receptors (BCR) with high affinity for the corresponding antigen. B cells are selected for expression of high-affinity surface Ig (sIg) in the germinal center and leave it as memory B cells or plasma cells, a process that has been termed affinity-maturation (for review see Rajewsky [52]). GCBs that do not fulfil this criterion are effectively eliminated by Fas-receptor mediated apoptosis [53, 54]. One characteristic feature of HRS cells is the complete lack of sIg expression. One would expect such a cell to be negatively selected in the germinal center. The HRS cell instead escapes apoptosis and leads to systemic disease as it expands clonally. Consequently, Fas-resistance of HRS-derived cell lines has recently been experimentally demonstrated [55].

Physiologically, cross-linking of the Fas-receptor leads to clustering of a membrane-bound intracellular signaling platform, the death-inducing signaling complex (DISC). Autoproteolytical cleavage of the cysteine protease caspase-8 at the DISC leads to activation of the caspase cascade resulting in the cleavage of DNA through effector-caspases and, finally, cell death [56–58]. Consequently, defects may be located at different levels of apoptosis signaling. First, mutations in the *Fas* gene have been described that result in disturbed lymphocyte homeostasis and development of a lymphoproliferative disorder. Second, clustering of the DISC may be interrupted due to overexpression of the antiapoptotic protein c-FLIP. Third, imbalances between expression levels of mitochondrial players of apoptosis, such as the Bcl-2 family of proteins, are well known causes of apoptosis resistance and enhanced response to proliferative signals. Finally, effector caspases at the end of the signaling cascade may be nonfunctional or lacking, thus preventing DNA fragmentation and membrane blebbing.

In cHL, the acquisition of a Fas-resistant phenotype is probably closely related to the transformation process because of the necessity to survive the negative selection process of the germinal center. *Fas* gene mutations have been reported in only a minority of primary cases of cHL and direct evidence for imbalances between pro- and antiapoptotic bcl-2 family members is lacking [59]. Additionally, lack of effector caspases does not seem to be a prominent feature of HRS cells in cHL [60]. We have recently analyzed expression of c-FLIP in cultured and primary HRS cells of cHL and found constitutive expression in almost all analyzed cases. These data support the hypothesis that a constitutively expressed antiapoptotic c-*FLIP* gene in cHL may be a central mechanism for apoptosis resistance in HRS cells [61].

Genetic alterations

Four types of genetic instability may be distinguished in human cancer [62]:

- subtle DNA sequence changes (point mutations including microsatellite instability);
- chromosomal instability;
- translocations and
- gene amplifications and deletions.

Subtle DNA sequence changes are rare events affecting genes that are indispensable for the control of proliferation or apoptosis and, in the case of mismatch repair, involve genes that are important for DNA repair. In cHL, many oncogenes and tumor-suppressor genes have been studied, but until now, no consistent mutation pattern has been identified. For example, mutations affecting the *p53* or *Fas* genes are rare events in cHL [59, 63, 64]. Mutations in the *IκB*a tumor-suppressor gene have repeatedly been reported, but seem to affect only a small subset of cHL cases [47–49]. Finally, microsatellite instability was recently ruled out as a major mechanism governing transformation of HRS cells [65].

Analyses of chromosomal instability in cHL revealed a heterogeneous pattern with abnormal metaphases in 13–92% of cases, as determined by classical cytogenetics, and numerical aberrations were detected in 100% of HRS cells, as determined by FICTION, a modified fluorescence *in situ* hybridization (FISH) technique [66, 67].

Translocations that are typical for other lymphomas, like t(14;18) in follicular lymphoma or t(2;5) in anaplastic large cell lymphoma, do not seem to play a role in cHL [68, 69]. Other translocations are common but lack a consistent pattern, although a number of non-random breakpoints have been reported in cHL [66]. More recently, a novel translocation [t(2;14)(p13;q32.3)] involving the *BCL11a* gene in cHL was reported [70].

In contrast to these data, investigation of gene amplification and deletion is an increasing field. For example, Joos et al. [71] found a predominance of gains of chromosomal regions over losses in 12 primary cases of cHL by using comparative genomic hybridization (CGH). Additionally, by using the elaborate approach of single-cell PCR, another group analyzed primary HL cases and found a recurrent loss of heterozygosity (LOH) on 4q26 in 4/5 informative cases analyzed [72]. Although these data are preliminary (four markers were analyzed in seven cases), they point in the direction of a consistent pattern of genetic imbalance. By extending the number of patients studied to 16 and by applying the genome-wide GeneScan approach in combination with a highly sensitive PCR technique after laser-assisted micromanipulation of primary HRS cells, recurrent imbalances were recently detected in most cases on chromosome 6q25 (Re D, Starostik P, Massoudi N et al. in preparation).

In conclusion, while subtle DNA sequence changes and microsatellite instability do not seem to play a major role in cHL, chromosomal alterations in number and structure including translocations are frequent but lack a consistent pattern. In contrast, clonal alterations detected on the molecular level, such as whole gene amplifications or deletions comprising chromosomal regions as detected by LOH analyses, reflect a distinct and consistent pattern of genetic instability in cHL.

T-cells in Hodgkin's lymphoma

A characteristic feature of the affected tissue in cHL is the reactive infiltrate of numerous cell types surrounding a few malignant HRS cells. In contrast to other malignancies, tumor-infiltrating T-cells that represent the major cell population in the reactive infiltrate, are mostly $CD4^+$ Th2 cells [73]. Both the inability of bystander T-cells to secrete IL-2 and the predominant Th2 response with a lack of tumor-infiltrating cytotoxic T-cells (CTLs) may contribute to the failure of the immune system to mount an effective response against the HRS cells.

Several publications report on the secretion of immunosuppressive cytokines by HRS cells, such as TGF-β or IL-10, that may prevent killing by CTLs [74, 75]. Recently, it was demonstrated that HRS cells in cHL express the chemokine TARC at high levels. Additionally, the presence of the respective receptor CCR4 on the surrounding T-cells was shown [21]. TARC is a strong T-cell attractant, predominantly of the Th2 isotype. These data suggest that HRS cells participate in the prevention of an effective immune response by secreting immunosuppressive cytokines like TGF-β or IL-10 and by modulating the T-cell response towards a Th2 predominant isotype.

Summary

Although scientists have learned much about the derivation of HRS cells, little is known about the basic mechanisms that underlie malignant transformation of their precursors. The HRS cells in cHL have been shown to be derived from pre-apoptotic germinal center B cells in the majority of cases, while in some cases they seem to be of T-cell origin. The expression of EBV latent genes in EBV positive cases (50%) may be involved in transformation by up-regulation of the transcription factor NFκB. The transformation process in the EBV negative cases, however, is still not understood. Several studies have focused on the apoptosis resistant phenotype of HRS cells and recent data suggest that constitutively expressed c-FLIP may contribute to apoptosis resistance in cHL. Genetic instability is a typical feature of HRS cells and recent studies point to distinct genetic imbalances rather than subtle genetic alterations such as point mutations or microsatellite instability. Finally, the discovery that the HRS cells themselves contribute to the ineffective immune response by expressing immunosuppressive cytokines or by expressing chemokines that predominantly attract Th2 cells that are unable to kill, has widened our understanding of the environmental crosstalk of these peculiar cells.

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