Clonality and germinal centre B-cell derivation of Hodgkin/Reed–Sternberg cells in Hodgkin’s disease

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Summary

Molecular single-cell studies of Hodgkin and Reed–Sternberg (HRS) cells in Hodgkin’s disease (HD) have revealed the clonal nature of these peculiar tumour cells. HRS cells in classical HD as well as lymphocyte predominant (LP) HD originate from germinal center (GC) B cells in most cases, if not all. Whereas HRS cells in LP HD represent transformed antigen-selected GC B cells with evidence of ongoing immunoglobulin (Ig) V gene mutation, HRS cells in classical HD appear to often or always derive from GC B cells that have lost the capacity to express a functional antigen receptor. Using Ig gene rearrangements amplified from HRS cells as clonal markers for the tumour cells, it could be shown that the same HRS cell clone can disseminate in the patient and persist throughout the course of the disease. A common derivation of the tumour cells was recently demonstrated in two cases representing combinations of HD and non-Hodgkin’s lymphoma. Finally, V-gene analysis showed that viable cells enriched by magnetic cell sorting from HD patients as HRS cells indeed represent the HRS-cell population of the patient.

Key words: Hodgkin’s disease, Ig gene rearrangements, micromanipulation, Reed–Sternberg cell, single-cell PCR

Introduction

Clonality and lineage derivation of Hodgkin and Reed–Sternberg (HRS) cells in Hodgkin’s disease (HD) have long been subject of debate. Reasons for this are mainly due to the low frequency of the HRS cells in the tissue (usually less than 1% of the cells), the lack of a reliable purification procedure of the cells, and the establishment of only a low number of HD cell lines, for most of which the derivation from HRS cells is uncertain [1, 2]. Moreover, HRS cells in classical HD (i.e., the nodular sclerosis, mixed cellularity and lymphocyte depletion subtypes) show an inconsistent immunophenotype which is not directly related to any of the known hematopoietic lineages [3]. Only in the lymphocyte predominant (LP) subtype of HD has the regular expression of B-cell markers indicated a B lineage derivation of the HRS cells [4]. Indications for a clonal nature of the HRS cells have come from cytogenetic studies as well as from studies of the Epstein–Barr virus (EBV), which can be found in HRS cells in about half of the cases of classical HD [reviewed in 2]. However, these investigations did not address the question of lineage derivation of HRS cells.

Clonality of HRS cells

When single-cell polymerase chain reaction (PCR)-based methods became feasible, several groups established approaches to analyse single HRS cells for the presence of immunoglobulin (Ig) or T-cell receptor (TCR) gene rearrangements [5–11]. Since such gene rearrangements are highly diverse and specific for B and T cells, respectively, their detection in HRS cells would not only reveal the derivation of the cells from either the B or T lineage, but would also answer the question of clonality of the cells. The first single-cell studies of Ig gene rearrangements in HRS cells produced contradictory results, reporting either the detection of monoclonal [6] or mostly polyclonal [7, 8, 11] Ig gene rearrangements in HRS cells, or the lack of such rearrangements [9].

In our own analysis, we isolated single HRS cells from immunostained frozen tissue sections, using an approach that we had recently established [5, 6]. Clonal Ig gene rearrangements were detected in 16 of a total of 17 cases of classical HD [1, 6, 12–14]. One case was negative in the analysis, presumably due to insufficient DNA quality, because we also failed to amplify other genes from cells in this case (unpublished). Furthermore, clonal Ig gene rearrangements were amplified from each of six cases of LP HD [6, 15, 16].

In two of the cases of classical HD that we have recently analysed, CD30-positive cells were enriched from fresh lymph nodes using a magnetic-activated cell sorting based approach established by Radbruch et al. [14]. To determine whether the enriched large CD30-positive cells represent the HRS cells in the patient, single CD30+ cells with the morphology of HRS cells were isolated from cytospin preparations of the enriched cells, and HRS cells were micromanipulated from tissue sections of frozen samples of the same lymph nodes [14].
In both cases we amplified the same clonal Ig gene rearrangements (and no additional ones) from both sources of cells [14]. This shows that viable HRS cells were successfully enriched and demonstrates that HRS cells represent clonal populations throughout the affected lymph nodes.

The initial report of polyclonality of HRS cells in the collection of the cases analysed by H. Stein et al. was readdressed by reanalysing four of the cases originally classified as polyclonal. This reanalysis revealed the presence of only clonal Ig gene rearrangements in HRS cells in two of the cases [17, 18]. Moreover, three cases, which in the original publication were classified as 'mixed' because of the amplification of clonal as well as unique rearrangements, were later interpreted to represent clonal cases, presumably assuming that the unique sequences were due to cellular or other contamination [19]. In a recent analysis of 11 cases of LP HD, the same group found clonal Ig gene rearrangements in all cases [20].

Chan et al., who originally detected polyclonal Ig gene rearrangements in HRS cells when they analysed cell suspensions of paraffin-embedded material, used a different approach in later studies, and presently micro-manipulate HRS cells from tissue sections of paraffin-embedded biopsies. In those studies, HRS cells were found to represent clonal populations of tumour cells in all five cases of LP and the one case of classical HD analysed (from some of the cases, some unique rearrangements were amplified in addition to the clonal ones) [21, 22].

Taking these studies together, there is now ample evidence that HRS cells in classical as well as LP HD in most cases, if not all, represent clonal populations of tumour cells. It is likely that the initial reports of polyclonality of HRS cells or the lack of Ig gene rearrangements in those cases was due to technical matters. In particular, if clonal Ig gene rearrangements present in HRS cells are not amplified (e.g., because of somatic mutations at primer binding sites), then a low level of cellular contamination by normal B cells in the cell isolation procedure might mistakenly be interpreted as evidence for a polyclonality of the HRS cells.

**Germinal centre B-cell derivation of HRS cells**

In the studies of LP HD, intrACLonal diversity of V-gene sequences was observed in a large fraction of cases [6, 15, 16, 20, 22]. Since the process of somatic hypermutation is thought to be specific for and restricted to GC B cells [16], this finding indicates that the tumour cells in LP HD represent transformed GC B cells. This interpretation is in line with other histological and immunohistochemical features of LP HD [2, 4]. The pattern of somatic mutations in the productive V-gene rearrangements of HRS cells indicates that the tumour cells or their precursors have been selected for expression of functional antigen receptor [15]. In classical HD, ongoing mutation was not detected [1, 6, 12-14]. However, a GC B-cell derivation of the HRS cells is indicated by the V-gene sequence analysis in this type of HD also. All informative V-gene rearrangements were found to carry somatically mutated V genes [1, 6, 12-14]. Moreover, among the cases of classical HD in our study, mutations generating stop codons in otherwise productive V-gene rearrangements were found in four cases [6, 12]. Usually, GC B cells that acquire such 'crippling' mutations are efficiently eliminated within the GC by apoptosis and are not allowed to leave this microenvironment. Thus, the finding of tumour cells with such crippled V region genes indicates that malignant transformation happened within the GC allowing those cells to survive. Although only some of the cases carried stop codons within originally productive rearrangements, we speculate that HRS cells in classical HD may be derived from crippled GC B cells as a rule, because many types of crippling mutations (like those disturbing proper heavy or light-chain folding or leading to loss of antigen-binding) can not be easily identified. In line with this view, in a cell line established by V. Diehl et al. from the HRS cells of one of the cases of classical HD [1, 23], a mutation was found in the conserved octamer motif of the heavy-chain promoter, which likely explains the very low level of heavy-chain transcripts detected in the cell line [24]. Thus, in this case a crippling mutation was found outside the region initially analysed.

**Dissemination and persistence of HRS cells**

Most of the V-gene studies of single HRS cells were restricted to a single biopsy specimen and consequently could not address the question whether the clonality of HRS cells extends to all tissues involved in the disease in a given patient. Moreover, it is an interesting question whether relapses of the disease usually derive from the original tumour clone or represent independent (therapy-related?) malignancies.

In Cologne, three cases of classical HD were studied for the persistence and dissemination of HRS cells [13, 23, 25, 26]. From a relapsed case of mixed cellularity HD, a cell line (LI236) was established from the peripheral blood of the patient in 1994 [23]. Molecular analysis of LI236 cells and HRS cells micromanipulated from a HD-involved bone marrow specimen showed that the cell line stemmed from the HRS cells in the patient, thus demonstrating dissemination of the tumour cells into the peripheral blood [1]. Furthermore, using tumour-clone specific oligonucleotides in a PCR-based approach, the clonal V_{H1} gene rearrangement of the HRS cells was detected in a biopsy taken at primary diagnosis in 1991 and at a relapse of the disease in 1993, showing the persistence of the HRS cell clone throughout the course of the disease [25, 26]. In another case, two lymph node biopsies taken a year apart harboured the same HRS cell clone, and in the third case, the same HRS cell clone was detected in a lymph node and the spleen biopsied at
the same time and another lymph node biopsied one year later [13]. Persistence and dissemination of HRS cells in LP HD as well is indicated by two cases, one reported by Marafioti et al., the other by Ohno et al. [20, 22].

Within the scope of these findings, it appears that HRS cells often or perhaps always disseminate and persist in the patient, demonstrating the malignant nature of these cells.

The relationship between concurrent or consecutive HD and B NHL

In rare cases, a B-cell non-Hodgkin’s lymphoma (NHL) and HD occur in the same patient, either sequentially or concurrently [27, 28]. The most frequent combination is that of LP HD followed by a diffuse large-cell lymphoma [27, 28]. In light of the B-cell nature of HRS cells in HD, it is an interesting question whether the two malignancies stem from the same precursor or represent clonally independent diseases. Several cases of LP HD with concurrent or subsequent B NHL were investigated, and in most instances V-gene rearrangements amplified from the B NHL biopsies could not be detected in the LP HD specimens [29–33]. This indicates that the B NHL often represents a tumour clone distinct from the HRS cells of the LP HD. Recently, one case of B NHL followed by classical HD was studied by single-cell PCR [21]. Two different VH gene rearrangements were amplified from the lymphomas, demonstrating that the lymphomas were clonally independent. Since the patient received chemotherapy as well as radiotherapy after the diagnosis of HD, the B NHL might have been therapy-related.

We analysed two cases representing combinations of classical HD with B NHL: one composite lymphoma consisting of a follicular lymphoma and HD in the same lymph node, and one case in which a T-cell rich B-cell lymphoma was followed by HD three years later. A V-gene analysis of single, micromanipulated HRS and B NHL cells revealed that in both patients the two lymphomas harboured clonally related V-gene rearrangements (manuscript in preparation). The presence of somatic mutations shared between both lymphomas as well as mutations found in only either one of the entities indicates that the HRS and B NHL tumour cells derived from two members of a GC B-cell clone. These findings exemplify the close relationship between HD and some types of B NHL and provides compelling evidence for a GC B-cell derivation of the HRS cells in classical HD.

Analysis of T-cell receptor gene rearrangements in HD

Although it is now clear that the vast majority of cases of HD harbour Ig gene rearrangements and are thus B lineage derived, it can not be excluded that a small fraction of cases is derived from other lineages, such as T cells. A potential T-cell derivation in some cases of HD is indicated by the finding that in several cases of the disease, T-cell markers such as CD3 or granzyme B are expressed by the HRS cells [3, 34]. However, in two single-cell studies of a total of 22 cases of HD, not a single case was positive for TCR y gene rearrangements [10, 11].

We recently established an approach for amplifying TCR β gene rearrangements from single cells and applied this method to the study of T cells rosetting around HRS cells [35]. The aim of this study was to find out whether the phenomenon that HRS cells in HD are usually intimately associated with CD4+ T cells reflects a specific antigen-driven interaction. In that case, the T cells might be composed of an oligoclonal population and carry a restricted TCR β gene repertoire. However, the study of two cases of classical and one case of LP HD has revealed that the rosetting T cells represent a polyclonal population of cells [35]. This suggests that the HRS cells attract CD4+ T cells nonspecifically.

Conclusions

Molecular single-cell studies performed during recent years have convincingly shown that HRS cells in HD represent clonal populations of tumour cells. In classical as well as LP HD, the HRS cells likely derive from GC B cells: mutating, antigen-selected B cells in the case of LP HD, and crippled GC B cells that have lost the capacity to express antigen receptor in the case of classical HD. The usage of V-gene sequence information for designing tumour clone-specific probes has already proven useful in the study of dissemination and persistence of the tumour cells. In the future, this approach may become valuable for clinical applications such as monitoring disease progression and the detection of minimal residual disease or tumour cell contamination in stem cell harvest [36]. Finally, approaches for isolating highly enriched populations of viable HRS cells are now available, and this is expected to have a major impact on studies of gene expression of these peculiar cells. Such investigations will hopefully permit the solution of other central issues in the pathogenesis of HD, such as the identification of genes involved in the cellular interaction of the HRS cells with neighbouring cells, and the way in which the HRS cells in classical HD escape apoptosis.

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