Hodgkin's disease ; Past, Present and Future.

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In the past decades, significant progresses have been made in the field of research on Hodgkin's disease[1,2]. Results obtained by the single cell analysis of Reed-Sternberg's cells on tissue sections, radically changed our understanding of this ever-debated disease[3]. Discovery of disturbed functions in some transcription regulatory factors also much clarified the pathophysiology of this disease. In this paper, a brief history and the molecular mechanisms of Hodgkin's disease so far understood will be reviewed.
Key words Hodgkin's disease, B cell origin, NF κ B, EB virus, follicular center cell

1. Efforts to elucidate origin of Reed-Sternberg's cells

Origin of Reed-Sternberg's cells (RS cells) remained unclear for long time, and whenever new techniques were established, they were utilized for the purpose of solving this problem. Thus, in early 1950, electron microscopic studies[3] were performed, only to fail on disclosing the presence of viral particles or other specific changes of the microorganellas. Raffled cytoplasmic border with variable cytoplasmic projections was considered to be reminiscent of histiocytes[4]. The next effort was done with an enzyme- histochemical approach[5]. RS cells were found to have lysosomal enzymes such as acid phosphatase, β-glucuronidase or glucosaminidase. These findings favored the origin of RS cells to be in the monocyte-macrophage system[6,7], although neuraminidase was always negative and phagocytic activity was seldom observed in the tissue sections.

Meanwhile, our knowledge on the transformation in response to antigens or mitogens, of small lymphocytes into large cells with a large cytoplasm and lysosomal activities, drastically changed our idea about histiocytic lymphomas or reticulum cell sarcomas, and also affected the understanding of RS cells as a histiocytic descendant.

In 1974, Paul Nakane developed an indirect immunoperoxidase method[8] and in 1975, a monoclonal antibody was introduced by Milstein[9]. Immunohistochemistry was thought to be the most suitable method for the analysis of complex histopathology of Hodgkin's disease (HD). Many papers were published on the immunophenotypic characteristics of RS cells[10]. Expression of T cell[11,12], B cell[13], macrophage/histiocytic as well as dendritic cell markers[14] was found and the origin of RS cells became more obscure rather than clearer. Cases with only a dendritic cell phenotype were occasionally found and suggested non-lymphocytic origin or variability of phenotypic expression of RS cells[15]. Two important observations, however, emerged from these researches. One is that RS cells, in a class of HD named nodular lymphocyte-predominant HD (LP), frequently had B-cell markers such as CD74, CDw 75[16] and were found to have the J chain of IgM. Clinically, some nodular LP cases developed into diffuse large B-cell lymphomas[17]. Based on these observations, nodular LPs were considered as one type of germinal center B-cell lymphomas as early as in 1986[18]. In the present REAL and WHO classification[19], this group of cases is separated from classical HD. Histologically, L&H cells or the so-called Popcorn cells of nodular LP are quite distinct from the classical RS cells described by Sternberg or Reed. The inadvertent inclusion of these atypical cells in the group of specific RS cells by histopathologists resulted in an unnecessary confusion in the classification of HD. Another point was the unexpectedly frequent presence of B cell markers[20,21] in diffuse LP and mixed cellularity HD (MC) cases. This finding prompted to accept later genetic analyses showing the nature of B cells (Table 1).

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Table 1. Immunophenotype of Reed-Sternberg cells in paraffine-embedded tissues. (Chiba Univ. Dept. Pathol.)

	LP	LD	MC	NS	Total
B-marker (L26; MB-1)	8/20 40.0%	1/9 11.1%	10/46 21.7%	1/16 6.3%	20/91 22.0%
T-marker (UCHL-1; MT-1)	0/19	1/9 11.1%	1/42 2.4%	0/16	2/86 2.3%
CD30 (Ber H2)	7/9	2/2	11/12	2/2	22/25 88.0%
CD15 (LeuM1)	6/18 33.3%	6/7 85.7%	25/47 53.2%	14/16 87.5%	21/88 56.0%
CD74 (LN2)	9/11 81.8%	3/6 50.0%	21/35 88.6%	14/16 87.5%	57/68 83.8%
Vimentin	7/13 53.8%	6/7 85.7%	31/42 73.8%	13/16 81.3%	57/78 73.1%

LP, lymphocyte-predomiuant HD; LD, lymphocyte depletion HD; MC, mixed cellularity HD; NS, nodular sclerosis HD

In the 1980s, Southern-blotting method was applied to study HD. Many researchers expected that this genotypic approach would finally clarify the nature of RS cells. However, the obtained results were variable. Weiss[22] and Brinker[23] found rearrangements of immunoglobulin heavy chain (IgH) genes while Griesser[24] reported rearrangements of T-cell receptor (TCR) genes in a substantial number of cases. However, Knowles[25] was unable to confirm either observations. In Japan, Shirakawa[26], as well as our group[27], reported the rearrangement of both TCRβ and IgH. The reason for the discrepancy was thought to be the paucity of RS cells in the specimen tested[28] as well as the sensitivity of the Southern-blotting. Concentration of RS cells in the test materials was tried[29,30] but the neoplastic and reactive nature of the DNA used remained unchanged, due to the unavoidable presence of non-neoplastic DNA. Highly effective PCR technique partially solved the problem and Tamaru et al[31] reported that RS cells with B-cell phenotype had rearranged IgH genes with somatic mutations of the VH segment.

The analysis of individual RS cells became possible with the development of techniques for the isolation of single cells from histological sections. The first effort to analyze cDNA sequences from single RS cells was done by Trumper et al[32], and they found co-expression of genes related to monocyte and lymphocyte lineages. Küppers et al[33] reported in 1994 the single cell DNA analysis of RS cells which were picked from frozen sections of HD tissue stained with CD30. The authors could demonstrate the clonal rearrangement of IgH gene in each of the nodular sclerosis HD (NS), MC and LP cases. Since then, several groups[34,35,36] tried to apply the single cell method but the results obtained did not agree with each other. Hummel et al[36] reported monoclonal and polyclonal rearrangements in 9 of their series of 12 cases with phenotypic B-RS cells, while Roth et al[35] could not find any rearrangement in 12 cases. With the refinement of the separation technique, contamination of non-neoplastic cells or their DNAs were excluded and the present consensus is that RS cells originate from the B cells of the germinal center[37].

However, questions regarding the RS cells are not completely answered yet. Rajewsky's group[38] insist that RS cells are functionally crippled in regards to immunoglobulin production because of deteriorating mutations of the IgH genes. Stein's group[39] reported that the IgH genes of RS cells are not crippled but that RS cells are defective in mRNAs for immunoglobulins (Fig. 1). Yatabe previously reported a discrepancy in the rearrangement of VDJ and its transcription in HD[40]. Although on-going somatic mutations of VH gene is generally accepted to occur in the germinal center (GC), there are evidences that it may occur outside the

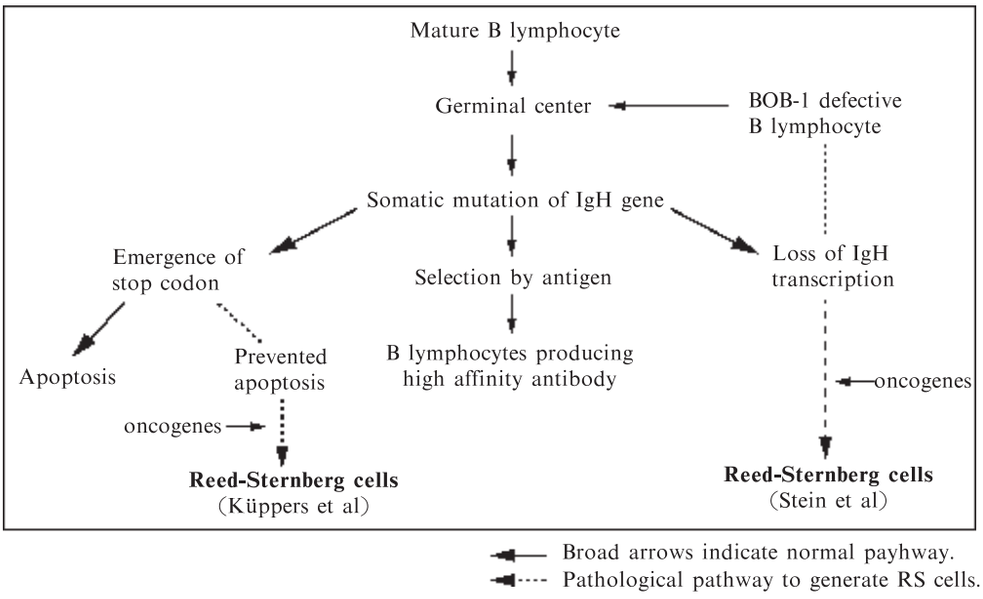

Fig. 1. Proposed mechanism of Reed-Sternberg cell development in the germinal center.

GC in mice[41]. If this is also true in humans, the origin of RS cells would be challenged again. Recently, Stein's group described two cases of HD in which RS cells had TCR$\gamma\delta$ rearrangements but had no rearrangements of IgH. They concluded that HD of T-cell origin definitely exists although the incidence may be quite low[42].

2. Physiology of Reed-Sternberg's cells

The most characteristic immunological phenotype of RS cell is the expression of CD30 on the cytoplasmic membrane and the Golgi apparatus. In the early days of its discovery, CD30 was thought to be specific to RS cells, but it was found very soon that this molecule was expressed by T or B cells when they were activated[43]. A new type of lymphoma was also found with some of the features of HD and a strong expression of CD30. This lymphoma was named Ki-1 positive anaplastic large cell lymphoma[44], and some of the cases regarded in the past as Hodgkin's sarcoma or HD, LD type may in fact belong to this lymphoma. In 1992, Durkopf[45] succeeded in cloning the CD30 gene, and established that it was a member of the tumor necrosis factor receptor (TNFR) family. Soon, a ligand was found for CD30, which allowed functions and signal pathways of this molecule to be gradually clarified.

CD30 plays multiple roles and is very important for the maturation, the functions and the proliferation of lymphocytes[46]. In mature T-lymphocytes, CD30 promotes the TCR-mediated proliferation, secretion of Th2 cytokines such as II-2, TNF-α or INF-γ. In the thymus, CD30 participates in the negative selection of thymocytes by increasing their sensitivity to CD3 -mediated apoptosis. CD 30 binds to the NFκB promoter present in the LTR of the HIV, and promotes the proliferation of HIV, thus switching from indolent to active infection. In Jurkat T-cells, binding of CD30 with CD30L, allows the cytoplasmic domain of CD30 to interact with TNFR-associated proteins (TRAF-1, 2, 3 or 5), and in turn, TRAFs activate NFκB (Fig. 2). Thus, cytokine genes under the control of NFκB, II-2, IL-6, and TNF-α, are activated. This NFκB activation is normally transient, but in RS cells, NFκB is constitutively activated and plays a central role in the clinical manifestation of HD.

NFκB is a heterodimeric protein of either two of RelA/p65, RelB, c-Rel, p105/p50 and p100/p52. These molecules have in common a so-called Rel homology domain (RHD) and constitute the Rel family. RHD is the binding site for dimerization and is also the site which binds to nuclear DNA[47]. In the cytoplasm, IκB, a molecule controlling NFκB, binds to the RHD and inhibits the transfer of NFκB into the nucleus. Ankirin repeats of IκB molecule are thought to be important to keep NFκB in the cytoplasm. When IκB is phosphorylated, it is dissociated

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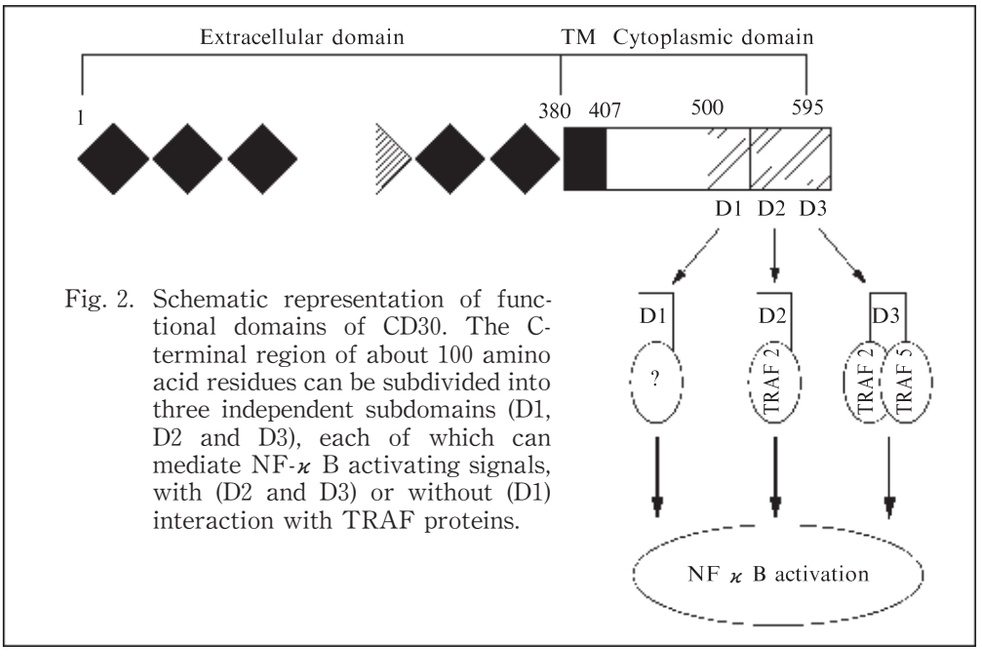

Fig. 2. Schematic representation of functional domains of CD30. The C-terminal region of about 100 amino acid residues can be subdivided into three independent subdomains (D1, D2 and D3), each of which can mediate NF-κ B activating signals, with (D2 and D3) or without (D1) interaction with TRAF proteins.

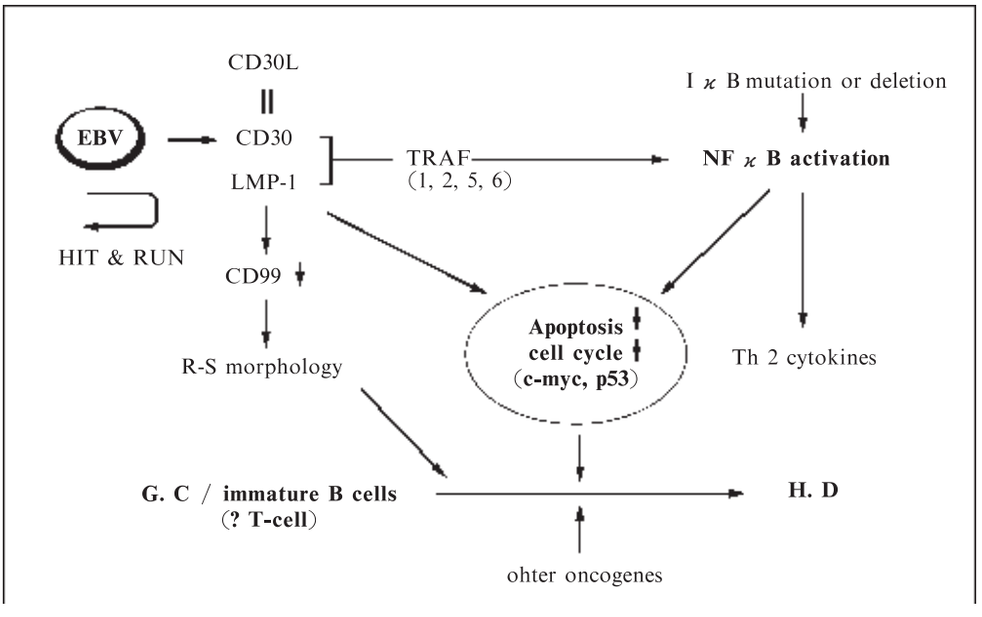

Fig. 3. NF κ B activation in Reed-Sternberg cells.

from NFκB, and NFκB can move into the nucleus to promote the transcription of certain genes such as GM-CSF, IL-6, IL-8, IL-2R, VCAM-1, E-selectin, ICAM and IFN-β. It is also related to the activation of c-myc and the regulation of p53, thus contributes to the cell proliferation.

In 1996, Bargou and his colleagues[48] found that NFκB was constitutively and strongly expressed in RS cells of HD cell lines and also in RS cells freshly isolated from pleural effusion. NFκB of RS cells were dimers of p50 and p65/RelA, while those in activated B-cells simultaneously studied were dimers composed of p50 and c-Rel. They also found that high level of expression of IκB mRNAs was not accompanied by a corresponding protein level, and suggested that the deficit in the translation of IκB is the cause for the constitutivelyhigh expression of NFκB in RS cells. In 1998, Wood[49] underscored this

assumption by demonstrating that IκB gene was not translated in the KM-H2 lines. Similarly, Emmerich et al. [50] reported that mutation of pos893 resulted in a stop-codon in L428 cells, deletion of two segments of pos509 to 613 and pos 618 to 640 in KM-H2 cell line, and occurrence of two allelic stop-codon in RS cells freshly prepared from HD tissues. The abnormal expression of NFκB, for which the causes can be found in several pathways including defective IκB, EBV infection and CD30 expression, seems to be the central event in the physiology of RS cells (Fig. 3).

3. HD and Epstein-Barr virus infection

Since early 1960, infectious mononucleosis and HD were thought to be related in some way. Although epidemiologic researches pointed the possible etiological relationship of Epstein-Barr virus (EBV) to HD[51], we had to wait for a clear evidence until 1989 when Weiss et al[52] demonstrated the presence of EBV genome in RS cells by *in situ* hybridization. As an in situ hybridization method utilizing EBER-1 probe becomes popular, many papers concerning EBV in HD appeared from all parts of the world[15,53,54,55,56], and it became a general consensus that 40 to 50% of HD in developed countries and 70 to 80% of HD in developing countries are EBV-related[20]. In the report on the new WHO classification of lymphomas, it is suggested that the frequency of EBV-positive HD is proportional to the incidence of EBV infection in the general population of the area in which the HD study is performed[19]. The monoclonal nature of the infecting EBV indicated that the infections were inversely related to the occurrence of HD. Although EBV infection may terminate without leaving any evidences (the Hit and Run theory[57]), the existence of EBV-negative cases indicates that EBV is not the sole etiology for the development of HD. RS cells express LMP-1 protein at variable levels[58], whose gene is encoded in the EBV genome, which has the ability to transform fibroblasts and epithelial cells, and therefore, considered to be an oncogene[59]. It is also dispensable for the transformation of B lymphocytes. Introduction of LMP-1 gene into mononuclear RS cells lacking expression of LMP-1, is accompanied by a multiple nucleation and an increase in the size of mononuclear RS cells[60]. Down-regulation of CD99 by LMP-1 is reported to be important for this morphological transformation[61]. LMP-1 also activates the bcl-2 gene and rescues RS cells from apoptosis. Recently, it was reported that TRAF-1 is over-expressed in EBV-positive HD and in EBV-related proliferation of lymphocytes[62]. Most probably, proteins of TRAF members bind to LMP-1 and/or to intracytoplasmic domain of CD30, and this results in the escape from apoptosis. As most of RS cells do not express BCL-2 protein[63], this assumption is more reasonable to explain the non-apoptotic nature of RS cells. If LMP-1 is thus essential for oncogenesis of HD or RS cells, what is the agent playing a comparatively important role in the EBV-negative HD or RS cells? Or does EBV escape from RS cells after its transformation? Discovery of Akata strain and subsequent observation on another cell line of Burkitt lymphoma, in which the EBV genome disappeared during a long-term culture and still kept neoplastic its nature[64] may speak to the latter possibility.

4. Epidemiology of HD and Japanese contribution

In Japan, because of the rarity of the disease, only sporadic researches on HD has been published in the past. The main interest in HD has been focused on the nature of the disease, whether HD resulted from neoplastic or inflammatory processes, and if neoplastic, whether HD was a type of reticulum cell sarcoma or a distinct lymphocytic tumor. Akazaki[65] classified the reticulum cell sarcoma into undifferentiated, differentiated, pleomorphic and transitional/transforming types, and only Hodgkin's sarcoma was included in the last subtype. HD was popularly considered to be an inflammatory process in which occasional malignant transformation occurs resulting into Hodgkin's sarcoma. The view of Sternberg's lymphogranuloma probably prevailed among Japanese pathologists before 1950. Aoki[66] proposed possible etiologic relation between Brucella Bang and Hodgkin's disease based on the positive reaction of Ascolli's antigen precipitation test of Hodgkin's disease tissues. This was the only scientific-minded research on this subject while most others discussed only in morphological terms[67,68]. Among recent efforts, Kamesaki's work[69] deserves special attention in that he established a genuine RS cell line and, the

KM-H2 cells were since then popularly used for the genetic molecular and cytological studies of RS cells.

As to epidemiology of HD, incidence in Japan is very low, occupying only 10 to 15% of all malignant lymphomas, while in European and North American countries, HD is the most common type of lymphomas. Relation of HLA and HD was studied on the occasion of 11th International HLA Workshop and Conference held at Yokohama, in 1991[70]. Peripheral blood lymphocyte DNAs from 741 cases of HD were collected from all over the world as well as 686 healthy subjects of corresponding races or ethnic groups. Genotypes of HLA were analyzed using type-specific oligonucleotide probes, and its was found that Oriental and Caucasian HD patients differed in the incidence of HLA DPB1 *0301 and DPB1*0401 loci.

Analyses of these data revealed the possibility that HD susceptible gene reside close to DPB1*0401 in Caucasians while Orientals lack this gene having a potential resistant gene close to DPB1*0301. Interestingly, malignant tumors so far proven to have a definite relation with HLA are cervical carcinomas and nasopharyngeal carcinomas, and both are also known to be causally related to virus infection. Most probably, immunological reactions of the host regulated by HLA play an important role in provoking virus induced malignancies.

The age distribution of HD patients in Japan has been considered to be monophasic with a single peak in elderly persons and lacked the younger peak that constitutes biphasic pattern of civilized countries. Akazaki and Wakasa[71] reported that the lack of younger peak corresponded to the scarcity of NS cases in Japan. MC subtype accounted for 30–50% of HD cases in most of series published during 1970 to 1980[72,73]. More recently, Aozasa[74] reviewed HD cases collected in the Osaka area and concluded that NS cases increased to 32.2% in 1975–1985 compared with 22.6% in the period of 1964–1974. In 55 cases treated between 1994 and 1999, NS subtype occupied 34.6% of the total and in this series, the typical biphasic age distribution was also found[75]. Our recent studies on the incidence of EBV infection in HD cases revealed that about 40% of the cases were positive for EBV with EBER-1 ISH methods[76]. Nakamura *et al*. reported 51.6% cases positive for EBV with the EBER-1 ISH methods[76]. and 49.4% with LMP-1 immunostaining[15]. This is compatible with reports from most of the advanced countries. There are also some evidences that EBV infection would negatively monitor IgH gene expression[77].

As mentioned in the previous chapter, although rare, there are reports of RS cell lines and HD cases in which RS cells show T-cell surface makers or TCR rearrangements[11,42,78]. In Japan, some of the adult T cell lymphoma (ATLL) cases were misdiagnosed in the past as HD. After the discovery of ATLL, cases of HD-like morphology tended to be classified as ATLL if RS-like cells showed T-cell surface makers. Occurrence of HD-like lymphadenopathy in the early stage of ATLL was reported by Kikuchi et al[79]. The boundaries between HD and T cell lymphomas, including avaplastic large-cell lymphoma (ALCL) or HD-like ALCL, must be further defined in morphological terms.

5. Questions and possible future development

To summarize the results of recent works, Reed-Sternberg cells of HD are reported to be derived from the germinal center B-cells, from T-cells or from follicular dendritic cells. If we consider that HD is a kind of syndrome or a group of tumors defined by specific histological manifestations, then the normal counterpart of HD tumor cells could well be more than one. Then, the most compatible idea to the present various data is that HD is a group of GC tumors originating from either B-cells, T-cells or dendritic cells of the GC with a common NFκB dysfunction and a specific morphology (Fig. 4). To verify this hypothesis, characteristics of the GC T-cells must be well categorized and found in RS cells supposedly originating from T-cells.

Although EBV is considered to be etiologically related to HD, no evidence of viral participation is obtained in more than half of all HD cases. Is there any other virus present to substitute to EBV's role in these EBV-negative cases? Or, does EBV disappear without any evidences of infection after HD developed? The present trend favors the latter possibility and the scenario is known as the "Hit and Run" theory. EBV oncogenesis is well established in Burkitt lymphoma, but some of the cultured Burkitt cell lines become EBV-negative while still keeping the characteristics of Burkitt's lymphoma[64]. This

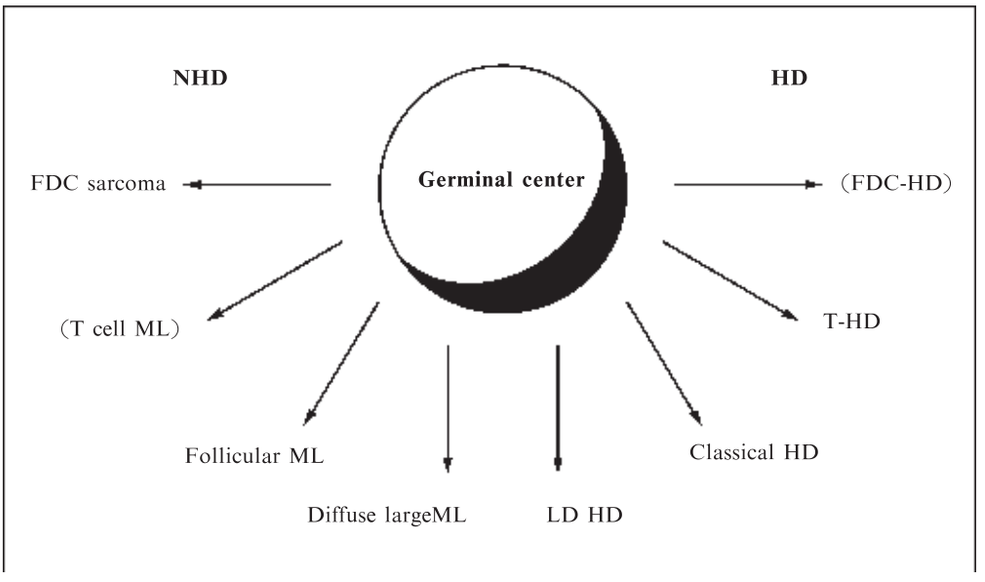

Fig. 4. Hodgkin's disease as a histologically defined germinal center tumor. NHD, non-Hodgkin's lymphoma ; HD, Hodgkin's disease ; FDC, follicular dendritic cell ; ML, malignant lymphoma

fact supports the "Hit and Run" theory by Sixbey. However, as LMP-1 protein is usually absent in EBV-negative RS cells, other viral oncogenic products must be identified to confirm this theory. In this regard, further detailed analysis of gene construction and gene function of the EBV is essential.

In many hematological conditions and lymphomas, specific cytogenetic abnormalities are defined. Cytogenetic studies were not well performed because of the difficulties to isolate and culture RS cells[80,81]. We now have potent methods such as FISH to analyze interphase nuclei. In the near future, with the use of various chromosome markers, it will be possible to demonstrate specific translocations or deletions in RS cells.

Mutation of IκB gene and resulting abnormal expression of NFκB appears to be the central event in the function of RS cells. There may be more dysfunction in many aspects of RS cell physiology. Single-cell separation coupled with DNA microarray technique would further clarify the gene expression in and the behavior of RS cells, and clinical manifestation of HD.

HD is regarded as a curable disease, but some are therapy-resistant[82]. What defines the reactivity to multi-drug chemotherapy is unknown. For these therapy-resistant cases, immunotoxin or gene therapy should be developed. So far, CD25 and CD30 were selected as targets of the immunotoxin treatment[83,84]. Immunotoxin or vaccine produced by transfected bacteria of reconstructed anti-CD30-globulin-CDR3-Pseudomonas-exotoxin genes[85] are now available and await clinical trials. NFκB and IκB genes can be candidates for gene therapy. Bargue et al[86] demonstrated that transfection of a mutated IκB gene, IκB$\alpha\Delta$N, to the RS cell lines effectively prohibited the growth of the cells and cell-line derived transplant tumors. Kitajima et al[87] reported that anti-sense NFκBp65 eradicated fibrosarcoma induced by HTLV-1 tax gene. Studies along these lines must be pursued for the effective management of HD patients. In the near future, we may understand HD better than ever, and treat the patients more successfully.

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