Tumor Microenvironment and RIG-I Signaling Molecules in Epstein Barr Virus-Positive and -Negative Classical Hodgkin Lymphoma of the Elderly

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Classical Hodgkin lymphoma (CHL) is a B-cell neoplasm characterized by Hodgkin and Reed-Sternberg (HRS) cells. Its prevalence exhibits a bimodal pattern of peaking in young adults and the elderly. There is an association with Epstein-Barr virus (EBV) infection in about 50% of cases of CHL of the elderly, and the outcome of these patients is unfavorable. It is not well known how the latent infection of EBV is involved in the pathophysiology of CHL of the elderly. To address this issue, we examined the tumor microenvironment (TME) and the expression of molecules related to EBV infection in HRS cells in 10 EBV-positive CHL and 7 EBV-negative CHL patients older than 50 years. In EBV-positive CHL, we found an increased population of FOXP3+ cells, while that of granzyme B+ cells was reduced, compared with those in EBV-negative CHL. The expression of inhibitory chemokine CCL20 was increased in EBV-positive HRS cells compared with that in EBV-negative HRS cells. In addition, despite increased expression of a pattern recognition receptor, RIG-I, in intracellular innate immunity, there was no evidence of interferon regulatory factor 3 activation or interferon-ß induction in EBV-positive HRS cells in CHL of the elderly. The disease recurred frequently (50%) in EBV-positive CHL. The current study thus suggests the possibility that the latent infection of EBV alters the expression of chemokines and the innate immunity response in HRS cells and modulates TME to an immunosuppressive state, which may account for the unfavorable disease course in CHL of the elderly. [J Clin Exp Hematop 54(1): 75-84, 2014]

Keywords: classical Hodgkin lymphoma, Epstein-Barr virus, tumor microenvironment, RIG-I, CCL20

INTRODUCTION

Classical Hodgkin lymphoma (CHL) is a B-cell-derived malignant lymphoma that shows a bimodal age distribution with peaks in young adults and the elderly.1) The histology of CHL is characterized by the appearance of Hodgkin and Reed-Sternberg (HRS) cells, which constitute approximately 1% of the cells in the tumor.2) HRS cells are found in a tumor microenvironment (TME) that consists of lymphocytes, neutrophils, eosinophils, mast cells, macrophages and fibroblasts. Survival of HRS cells is dependent on the cytokine and chemokine signals and cell-to-cell contact in the TME.3) HRS cells can evade immune attack from the infiltrated cells in the TME. The interaction between HRS cells and TME is considered to affect the clinical behavior of CHL.

There is an association with latent infection of Epstein Barr virus (EBV) in approximately 50% of cases of CHL.4) The EBV infection in CHL varies with age, and it is more common in patients older than 50 years. The infection often results in treatment failure and a poor prognosis.5,6) It is not known, however, how EBV is involved in the pathogenesis and progression of CHL.

In the latent infection of EBV, EBV-associated molecules such as Epstein Barr nuclear antigens (EBNAs), latent membrane proteins (LMFs), EBV-encoded RNA (EBER) and BamHI-A rightward transcripts are expressed.7) These molecules induce constitutive activation of NF-κB, which stimulates the survival signals and induces the expression of cytokines and chemokines, essential to the interaction with the cells in TME. Recently, it was shown that EBER-associated molecules may also trigger intracellular innate immunity.8) It was shown that EBER interacted with retinoic acid-inducible gene-I (RIG-I), a pattern recognition receptor, that in turn activated innate immunity in a Burkitt lymphoma (BL) cell line.9,10) At present, the alteration of intracellular RIG-I signaling in HRS cells of EBV-positive CHL is not known.
The current study investigated the cellular composition of TME and the intracellular molecular alterations induced by EBV infection in CHL patients older than 50 years. We consider that an understanding of the molecular alterations would help to develop a new treatment strategy for EBV-positive CHL.

PATIENTS AND METHODS

Patients

The cases of CHL were obtained from the archives of pathology files of the Department of Pathology and Molecular Medicine, Hirosaki University. From 1995 to 2012, a total of 32 cases of CHL were procured. The age distribution was bimodal. The first peak was in the teens and the second peak was in the 60s. In the current study, 17 cases older than 50 years were used (Table 1). Hematoxylin and eosin sections of the cases were reviewed by TS, RW and NY, and the histological diagnosis was made in accordance with the criteria of the World Health Organization 2008.12 The presence of EBV was determined by in situ hybridization of EBER and immunostaining of LMP1.

Diagnostic immunostaining

Immunostaining with antibodies against CD30, CD15 and LMP1 (Dako Japan, Inc., Tokyo, Japan) was carried out with Ventana GX System (Roche Diagnostics, K.K., Tokyo, Japan). The peroxidase activity was visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

in situ hybridization

Detection of EBER was carried out with Ventana HX Discovery System (Roche Diagnostics, K.K.), using the mixture of anti-sense probes of digoxigenin-labeled synthesized RNA 5'-GGU UUU GCU AGG GAG GAG AC-3' and 5'-UGA GGA CGG UGU CUG UGG UU-3'. As sense probes, synthesized RNA complementary to the anti-sense probes was used. After deparaffinization, the sections were treated with CC2 buffer and protease 2. Then, they were incubated with the probes at 70°C for 6 hr. After stringent washing with 0.1XSSC at 65°C for 6 min, the sections were incubated with alkaline phosphatase (ALP)-labeled anti-digoxigenin antibody, and ALP was visualized using BlueMap Kit (Roche Diagnostics, K.K.). The sections were then counterstained with nuclear fast red.

Morphometric analysis of cellular composition of TME

The cellular composition of TME was evaluated by morphometry on the sections subjected to triple immunostaining of FOXP3, granzyme B (GrB) and CD30. Briefly, 3.5-μm-thick sections were deparaffinized, immersed in Tris (pH9.0)/EDTA buffer at 125°C for 5 min, treated with Background Sniper (Biocare Medical, Concord, CA, USA) for 10 min and then incubated with the primary antibodies against GrB (1:500, Dako Japan, Inc.) at 37°C for 60 min.

Table 1. Clinicopathological features of the cases

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EBV-positive

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EBV, Epstein-Barr virus; EBER, EBV encoded RNA; LMP-1, latent membrane protein 1; M, male; F, female; MC, mixed cellularity; NS, nodular sclerosis; LR, lymphocyte rich
Following elimination of the endogenous peroxidase in 3% 
H2O2 in distilled water for 10 min, sections were subjected to 
MACH3 Mouse HRP Polymer Detection (Biocare Medical). 
The reaction products were visualized as black with EnzMet 
(Nanoprobes, Inc., Yaphank, NY, USA). After the treatment 
with Denaturation Solution (Biocare Medical) for 5 min and 
with citrate buffer (pH6.0) at 125°C for 3 min, the sections 
were incubated with anti-FOXP3 antibody (1:300, Abcam, 
Tokyo, Japan) at 4°C overnight. They were processed with 
MACH3 Rabbit ALP Polymer Detection (Biocare Medical). 
Labeled ALP was colorized with Vulcan Fast Red Chromogen Kit 2 (Biocare Medical). The sections were 
treated again with Denaturing Solution (Biocare Medical) and 
citrate buffer at 125°C for 2 min, and incubated with anti-
CD30 antibody (1:100, Dako Japan, Inc.) at 37°C for 75 min. 
They were processed with ALP-labeled anti-mouse IgG anti-
bodies (Nichirei Biosciences, Inc., Tokyo, Japan), which was 
colorized with Ferrangi Blue Chromogen Kit 2 (Biocare 
Medical), followed by light counterstaining with hematoxylin.

For the morphometric analysis, 10 images of the immuno-
stained sections with FOXP3, GrB and CD30, representa-
tive of a lesion containing HRS cells, were taken at a magnifi-
cation of x400 (1 image = 0.033 mm²) in each case. The 
numbers of FOXP3⁺ cells, GrB⁺ cells and HRS cells in each 
image were counted, and the numbers of FOXP3⁺ and GrB⁺ cells 
were calculated as number of cells/HRS cell. From 
these values, the FOXP3/GrB ratio was obtained.

Expression of CC chemokine ligand 20 (CCL20) and 
molecules of RIG-I signaling

Immunostaining of RIG-I was carried out with Histofine 
SAB-PO (Nichirei Biosciences, Inc.). After blocking of en-
dogenous peroxidase in 1% H2O2 in methanol and treatment 
with normal rabbit serum, sections were incubated with anti-
RIG-I antibody (1:100, AbD Serotec, Oxford, UK) at 4°C 
overnight. They were then processed with Histofine, and 
labeled peroxidase was visualized with diaminobenzidine, 
followed by counterstaining with hematoxylin.

Expressions of CCL20, interferon regulatory factor 3 
(IRF3), phosphorylated IRF3 (pIRF3) and interferon (IFN)-β 
were examined by double immunostaining. After pretreat-
ment in Tris (pH9.0)/EDTA buffer at 125°C for 3 min, the 
sections were treated with Background Sniper (Biocare Medical) 
and incubated with the primary antibodies against 
CCL20 (1:500, Abcam), IRF3 (1:500, Santa Cruz 
Biotechnology, Inc., Dallas, TX, USA), pIRF3 (1:300, 
Thermo Scientific, Rockford, IL, USA) and IFN-β (1:500, 
Novus Biologicals, LLC, Littleton, Co, USA) at 4°C over-
night. After elimination of endogenous peroxidase, the sec-
tions were processed with MACH3 Rabbit HRP Polymer 
Detection (Biocare Medical) and visualized with EnzMet 
(Nanoprobes, Inc.). Following treatment with Denaturating 
Solution (Biocare Medical) and citrate buffer, the sections 
were incubated with anti-CD30 antibody (Dako Japan, Inc.) at 
37°C for 75 min and processed with MACH3 Mouse ALP 
Polymer Detection (Biocare Medical). The reaction products 
were visualized with Vulcan Fast Red Chromogen Kit 2 
(Biocare Medical), followed by counterstaining with hema-
toxylin.

CCL20-positive HRS cells were identified by the 
cytoplasmic-positive reaction of CCL20 (black) and the mem-
branous reaction of CD30 (red) under high magnification.
CCL20-positive reactions in HRS cells were semi-quantified 
as follows: when double-positive cells were less than 10% of 
HRS cells, the score was 0. When they exceeded 10%, the 
score was further divided into 3 grades: score 1⁺, weak but 
clearly positive, similar to the findings in inflammatory cells 
in TME; 2⁺, moderate with enhanced reaction compared with 
that of inflammatory cells; and 3⁺, strongly positive reaction 
distinct from that of inflammatory cells.

Statistical analysis

Statistical analysis was performed with JMP10 (SAS 
Institute, Inc., Cary, NC, USA). Comparison of means in two 
groups was carried out by the Mann-Whitney method. The 
frequency distributions in two groups were compared by Chi-
square test. The difference was considered significant when 
the p value was less than 0.05.

RESULTS

Clinicopathological features of the cases

The ages of the patients ranged from 50 to 86 (median 65) 
years, and the male to female ratio was 14:3 (Table 1). All 
the specimens were from the lymph nodes, and most of them 
were biopsied from the neck and mediastinum.

Eight out of 17 cases were diagnosed as mixed cellularity 
subtype, and 7 cases were nodular sclerosis (NS). Two cases 
were lymphocyte-rich. CD30 was positive in HRS cells in all 
the cases (100%) (Fig. 1), and CD15 was positive in 10 out of 
17 cases (59%). Ten out of 17 cases (59%) were positive for 
EBER, and the positive signal was localized in the nuclei of 
the HRS cells (Fig. 1). LMP1 was also expressed in these 10 
cases (59%). The frequency of EBV positivity was 75% (6/8 
cases) in mixed cellularity subtype and 43% (3/7 cases) in 
nodular sclerosis subtype.

Follow-up data were available in 10 out of 17 patients. 
Among 6 cases of EBV-positive CHL that were followed up, 
recurrence was noted in 3 cases (50%; Nos. 2, 4 and 5). Two 
patients are alive without disease (Nos. 6 and 9) and one 
died of pancreatic cancer (No. 1). Among 4 cases of EBV- 
negative CHL that were followed up, there was no recurrence 
(0%) and only one died of progressive disease (No. 13).
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Three patients are alive (Nos. 11, 14 and 15)

**Cellular composition of TME**

The cellular composition of TME was examined by triple immunostaining of FOXP3 (red), GrB (black) and HRS cells (blue) (Fig. 2A, 2B). CD56-positive NK cells were scarcely observed in TME of either EBV-positive or -negative CHL (data not shown). FOXP3⁺ and GrB⁺ cells were distributed diffusely in TME in both EBV-positive and -negative CHL (Fig. 2A, 2B). In EBV-positive CHL, FOXP3⁺ cells appeared to surround HRS cells (Fig. 2A, arrowheads).

Morphometry revealed a significant increase in the proportion of FOXP3⁺ cells in EBV-positive CHL compared with...
that in EBV-negative CHL (Fig. 2C & Table 2), and the GrB+ cells were significantly reduced in EBV-positive CHL (Fig. 2D & Table 2). FOXP3/GrB ratio was therefore significantly higher in EBV-positive CHL than in EBV-negative CHL (Fig. 2E & Table 2).

Expression of CCL20

CCL20 was expressed in HRS cells, adjacent small lymphocytes and neutrophils (Fig. 3). The intensity of CCL20 reactions in HRS cells was significantly enhanced in EBV-positive CHL compared with that in EBV-negative CHL (Tables 2 & 3).

Expression of RIG-I signaling molecules

RIG-I was expressed in all the cases of EBV-positive CHL (Fig. 3 & Table 2). The positive reaction was noted in the cytoplasm of the HRS cells, and the intensity appeared to vary from cell to cell in each case. In EBV-negative CHL, the expression of RIG-I in HRS cells was not apparent.

IRF3 was expressed in HRS in both EBV-positive and...
negative CHLs (Fig. 3, Tables 2 & 3). Small lymphocytes were also positive for IRF3. The nuclear positivity of pIRF3 was not found in EBV-positive CHL (0/9, 0%), whereas it was detected in 2 out of 7 cases (29%) of EBV-negative CHL (Table 3). The expression of IFN-β was absent in all cases of EBV-positive CHL (Fig. 3); in EBV-negative CHL, IFN-β was expressed in the cases in which nuclear translocation of pIRF3 was detected (Fig. 3).

DISCUSSION

The current study on lesions of CHL showed an increase in FOXP3+ cells and a decrease in GrB+ cells in EBV-positive CHL of the elderly. In previous studies, when subjects were included from all age groups, FOXP3+ regulatory T cells (Tregs) dominated the TME of EBV-positive CHL, as shown in the current study.13-15 However, in pediatric patients younger than 10 years, there was no difference in the proportion of Tregs between EBV-positive and negative cases, while GrB+ CTL was increased in EBV-positive CHL.16 These studies indicate the importance of age in the involvement of EBV in CHL. However, the topographic correlation between EBV-positive HRS cells and surrounding cells has not been clear.15-16 The current study is the first to show the significance of EBV infection in the modulation of TME in CHL of the elderly. Our results of quantification of FOXP3+ cells and GrB+ cells per HRS cell on triple immunostained sections may reflect a close interaction between EBV-positive HRS cells and surrounding inflammatory cells.

The constitutive activation of NF-κB in HRS cells plays an important role in the pathogenesis of CHL.4 The activation of NF-κB is critical for the survival of HRS cells, and it also induces the expression of immunosuppressive cytokines.

Table 2. The cellular composition of tumor microenvironment and expression of molecules in classical Hodgkin lymphoma

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FOXP3 and GrB are expressed as positive cells/HRS cells.

GrB, granzyme B; RIG-I, retinoic acid-inducible gene-I; IRF3, interferon regulatory factor 3; pIRF3, phosphorylated IRF3; IFN-β, interferon-β; ETV, Epstein-Barr virus; nd, not done

Table 3. Expression of retinoic acid-inducible gene-I (RIG-I), phosphorylated interferon regulatory factor 3 (pIRF3), interferon (IFN)-β and CCL20 in Hodgkin and Reed-Sternberg cells

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EBV, Epstein-Barr virus; ns, not significant
Fig. 3. CCL20 expression and retinoic acid-inducible gene-I (RIG-I) signaling in Epstein-Barr virus (EBV)-positive classical Hodgkin lymphoma (CHL) (case 7) and EBV-negative CHL (case 16). RIG-I expression was visualized using diaminobenzidine (as brown), and the HRS cells are indicated with arrowheads. The expression was up-regulated in EBV-positive CHL. CCL20, interferon regulatory factor 3 (IRF3), phosphorylated IRF3 (pIRF3) and interferon-β (IFN) are shown in black, and Hodgkin and Reed-Sternberg cells were stained with CD30 (red). Phosphorylation and nuclear translocation of pIRF3 were found in HRS cells of EBV-negative CHL.
chemokines and growth factors for the cellular interactions and formation of TME suitable for the survival of HRS cells (Fig. 4). In EBV-positive CHL, the expression of interleukin-10, an immunosuppressive cytokine, is increased, and LMP1 may directly induce the migration of Tregs. The current study suggests the additional mechanisms by which HRS cells interact with TME in EBV-positive CHL.

EBNA1 is an essential factor for the replication of EBV, and it is expressed under the latent infection of EBV in CHL. It was shown that EBNA1 induces the expression of CCL20 and recruits Tregs in vitro; however, the association of CCL20 expression with Treg infiltration in the primary tumors of CHL was not demonstrated in vivo. In this study, we confirmed the enhanced expression of CCL20 in EBV-positive HRS cells and its association with infiltration of FOXP3+ cells. Hence, it is likely that infiltrating FOXP3+ cells further inhibit the migration and differentiation of GrB+ cells in TME (Fig. 4). The current study unveiled the up-regulation of RIG-I in EBV-positive HRS cells. This result is consistent with the up-regulation of RIG-I (DDX58) mRNA in EBV-positive CHL using microarray in a previous study (Ref. 20, in supplementary data). RIG-I is a pattern recognition receptor against double-stranded RNA, and its interaction with EBER was shown in an EBV-positive BL cell line. The interaction activates NF-κB and IRF3 and up-regulates the type I IFN in BL cells (Fig. 4). Since it was shown that NF-κB is constitutively activated in EBV-positive CHL, activation of RIG-I signaling may in part contribute to NF-κB activation (Fig. 4). In this study, however, we could find neither phosphorylation of IRF3 nor induction of IFN-β in EBV-positive HRS cells, in spite of RIG-I up-regulation. The defect in type I IFN induction in EBV-positive HRS cells may interfere with the expansion and differentiation of memory T cells in TME, leading to the immune evasion of EBV-infected HRS cells (Fig. 4).

The mechanism by which the post-RIG-I signal in EBV-positive HRS cells was down-regulated remains unclear. The difference from the BL cell line may be accounted for by the difference in latency of EBV infection between BL and CHL. In addition, the intracellular signaling may be different between in vitro and in vivo conditions, in the latter of which signals are influenced by neighboring inflammatory cells. The post-RIG-I signaling may also be interfered with by molecules encoded by EBV. It was shown that the immediate-early gene BRLF1 reduced the expression levels of IRF3 and IRF7 and that virion-associated kinase BGLF4 suppressed the transcription of IFN-β through the aberrant phosphorylation of IRF3. These considerations are mostly speculative, and further investigation is required to elucidate the inhibitory mechanism of post-RIG-I signaling in EBV-positive CHL.
positive CHL.

Our results may provide new insight into the therapeutic strategy for CHL. Although CHL is basically curable, complete eradication of neoplastic cells is difficult, and treatment failure is not uncommon in EBV-positive CHL of the elderly. Currently, clinical trials of antiproliferative and proapoptotic agents, which target NF-κB, Akt/mTOR and bcl2 in HRS cells, are in progress. EBV-associated molecules or their downstream molecules could be a novel therapeutic target for EBV-positive CHL. Inhibition of CCL20 by targeting EBNA1 may be expected to reduce Treg infiltration. The activation of IRF3 could up-regulate the expression of type I IFN, which in turn induces expansion and differentiation of memory T cells against EBV-infected HRS cells. Induction of apoptotic cell death of HRS cells could also be anticipated. Consequently, the decrease in the dose of chemotherapeutic agents and the reduction of adverse reactions such as severe immunosuppression or the occurrence of infectious diseases can be achieved.

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