Acta Medica Okayama

Volume 50, *Issue* 5 1996 *Article* 6

OCTOBER 1996

Variable expression of Epstein-Barr virus latent membrane protein I in Reed-Sternberg cells of Hodgkin's disease.

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Abstract

Reed-Sternberg cells (RS cells) of Hodgkin's disease (HD) are frequently infected with Epstein-Barr virus (EBV) and express EBV-encoded nonpolyadenylated RNA transcripts (EBER)-1. EBV latency has been classified into three distinct forms: Latency I, expressing only one of the latent proteins, EBV nuclear antigen (EBNA)-1, latency II, coexpressing EBNA-1 and LMPs, and latency III, expressing all latent viral proteins. RS cells express LMP-1 in addition to EBNA-1 and are considered to be EBV latency II frequently encountered in nasopharyngeal carcinoma. We examined 13 cases of EBV-infected HD by combined EBER-1 in situ hybridization and immunostaining for LMP-1. All of the RS cells expressed EBER-1, but a substantial number of EBER-1+ RS, cells were negative for LMP-1. The percentage of LMP-1+ RS cells out of EBER-1+ RS cells varied from 7% to 100% (average 69%). In this study, we showed that all EBV-infected RS cells were not restricted to latency II, and some belonged to latency I.

KEYWORDS: in situ hybridization, EBER-1, immunohistochemistry, latecy

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*PMID: 8914680 [PubMed - indexed for MEDLINE]

Acta Med Okayama 1996; 50(5): 267-270

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Reed-Sternberg cells (RS cells) of Hodgkin's disease (HD) are frequently infected with Epstein-Barr virus (EBV) and express EBV-encoded nonpolyadenylated RNA transcripts (EBER)-1. EBV latency has been classified into three distinct forms: Latency I, expressing only one of the latent proteins, EBV nuclear antigen (EBNA)-1, latency II, coexpressing EBNA-1 and LMPs, and latency III, expressing all latent viral proteins. RS cells express LMP-1 in addition to EBNA-1 and are considered to be in EBV latency II frequently encountered in nasopharyngeal carcinoma. We examined 13 cases of EBV-infected HD by combined EBER-1 *in situ* hybridization and immunostaining for LMP-1. All of the RS cells expressed EBER-1, but a substantial number of EBER-1$^+$ RS cells were negative for LMP-1. The percentage of LMP-1$^-$ RS cells out of EBER-1$^+$ RS cells varied from 7% to 100% (average 69%). In this study, we showed that all EBV-infected RS cells were not restricted to latency II, and some belonged to latency I.

Key words: *in situ* hybridization, EBER-1, immunohistochemistry, latency

The Epstein-Barr virus (EBV) usually persists latently in lymphocytes except under certain conditions (1, 2). Rowe *et al* identified three distinct forms of latent infection and termed them latency I, latency II and latency III (3): in latency I, EBV-infected cells express only EBV nuclear antigen (EBNA)-1 and EBV-encoded nonpolyadenylated RNA transcripts (EBER)-1 and -2; in latency II, EBV-encoded latent membrane proteins (LMP)-1, -2A and -2B are also expressed; and in latency III, a full spectrum of EBV latent genes including EBNA-2, -3a, -3b, -3c, -LP are expressed (1-4). This classification has been used to describe the types of latent infection (1-4); latencies I-III are typically encountered in Burkitt's lymphoma cells, nasopharyngeal carcinoma cells and *in vitro*-immortalized lymphoblastoid cell lines, respectively.

Reed-Sternberg cells and their variants (RS cells) of Hodgkin's disease (HD) are frequently infected by EBV (1, 4-9) and express EBNA-1, LMP-1, 2s and EBER-1, 2 (2, 4, 7, 9, 10). Therefore, RS cells in EBV-infected HD have been regarded as indicating latency II (4). LMP-1 is a transforming gene product which is able to transform the EBV-infected cells (11, 12). LMP-1 induces activation antigens such as CD23 (13) and also upregulates expression of the Bcl-2 protein which can protect cells from apoptosis (14). Therefore, expression of LMP-1 in RS cells has been regarded as evidence that EBV is etiologically closely associated with HD (7, 8, 10, 15, 16). In this study, we investigated the expression of LMP-1 in RS cells of EBV-infected HD using a double labeling method by immunohistology and EBER-1 *in situ* hybridization (EBER-ISH), to clarify the etiological role of LMP-1 in HD.

Materials and Methods

Combined EBER-1 in situ hybridization and LMP-1 immunostaining of Hodgkin's disease. Thirteen cases of EBV-infected HD, which included 11 cases of mixed cellularity and 2 cases of lymphocyte predominance, were selected from the files of the Department of Pathology, Okayama University Medical School (Okayama, Japan). Formalin-fixed, paraffin-embedded tissue sections cut at 4 μm were placed

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onto glass slides that had been pretreated with 3-aminopropyltriethoxysilane and baked for more than 2 h at 47°C. After deparaffinization, the sections were treated with 70% ethanol for 5 min and dehydrated in 100% ethanol. Digestion by proteinase K and other pretreatments were not done since they are not essential for EBER-1 detection using an oligonucleotide probe. Hybridization was performed at 37°C overnight by applying 0.5 ng/μl of FITC-labeled antisense EBER-1 oligonucleotide probe in hybridization buffer (45% formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% sodium pyrophosphate) as described by Chang *et al* (17). A G-C content-matched control probe in hybridization buffer was also used. After hybridization, probes were washed in 50 mM Tris-HCI (pH 8.0) containing 150 mM NaCl and 0.1% Tween 20. Signal detection was performed with an *in situ* detection kit (K046, Dakopatts, Kyoto, Japan). Positive signals indicated by as a dark blue or black color on nuclei with 5-brom-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride. After *in situ* hybridization for EBER-1, the sections were treated with 3% H_2O_2 for 5 min and with 0.1% trypsin for 30 min. They were then incubated with anti-LMP-1 which was a pool of four monoclonal antibodies (mAb), CS1-4 (1:30, Dakopatts) (18) for 1 h at 37°C. After the usual avidin-biotin-complex procedures, LMP-1 was indicated by a reddish-brown color on the cytoplasm and/or membrane with 3-amino-9-ethyl carbazole. Some HD that had been proven to be negative for LMP-1 and EBER-1 were used as a negative control (Fig. 1D). EBER-1$^+$, LMP-1$^+$ RS cells (latency II RS cells) and EBER-1$^+$, LMP-1$^-$ cells (latency I RS cells) were counted in each case in a blind fashion until all the RS cells were counted or the sum of the number exceeded 100. Cells that were morphologically too small to be RS cells were not counted even if they were positive for EBER-1.

Results

Almost all RS cells of HD studied expressed EBER-1. Some of these EBER-1$^+$ RS cells also showed membranous or both cytoplasmic and membranous staining with anti-LMP-1 (Fig. 1). All of the LMP-1$^+$ cells also expressed EBER-1. The percentage of LMP-1$^+$ RS cells out of EBER-1$^+$ RS cells was variable (from 7 to 100%, average 69%) (Table 1 and Fig. 1). EBER-1$^+$ RS cells were not scattered uniformly, but the percentage of latency I RS cells and latency II RS cells did not greatly change depending on the location of the lesions. The cytologic appearance of the latency I RS cells and latency II RS cells was the same (Fig. 1 A and B). Some EBER-1$^+$ cells were smaller than RS cells and were

Table I Number of Reed-Sternberg cells of Hodgkin's disease double-labeled with EBER-I *in situ* hybridization and immunostaining for LMP-I

Case	Subtype	No. of RS cells[a] counted		Latency II ratio (%)[b]
		EBER$^+$, LMP-I$^+$	EBER$^+$, LMP-I$^-$	
1	Lymphocyte predominance	81	9	90
2	Lymphocyte predominance	61	34	64
3	Mixed cellularity	96	4	96
4	Mixed cellularity	34	32	52
5	Mixed cellularity	91	9	91
6	Mixed cellularity	76	47	62
7	Mixed cellularity	22	18	55
8	Mixed cellularity	40	10	80
9	Mixed cellularity	26	0	100
10	Mixed cellularity	16	11	59
11	Mixed cellularity	71	35	67
12	Mixed cellularity	7	93	7
13	Mixed cellularity	66	22	75

a: RS cells, Reed-Sternberg cells and their variants; and *b*: Percentege of EBER-I$^+$, latent membrane protein (LMP)-I$^+$ Reed-Sternberg cells out of all Reed-Sternberg cells.

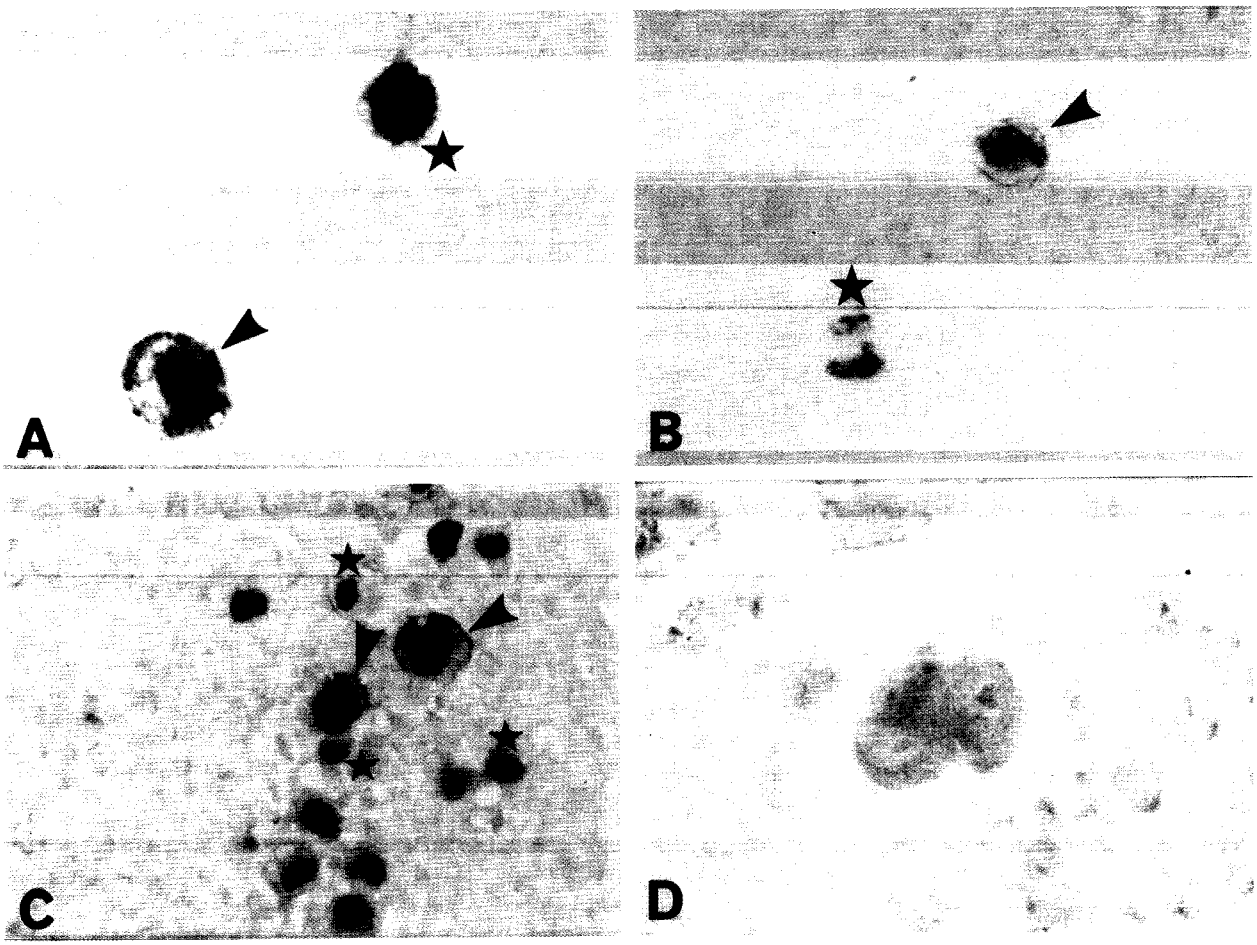

Fig. 1 LMP-1 expression of EBER-1$^+$ Reed-Sternberg cells and their variants (RS cells) in a case of Hodgkin's disease (HD), mixed cellularity. Combined LMP-1-immunostaining and EBER-1 *in situ* hybridization. **A** and **B**: Both LMP-1 and EBER-1 are detected on some RS cells (arrowheads), while the RS cells marked with asterisks are positive only for EBER-1. **C**: EBER-1$^+$ cells marked with asterisks are not considered to be RS cells. Note that they have much smaller nuclei than the neighboring RS cells (arrowheads) and do not express LMP-1. **D**: Neither LMP-1 nor EBER-1 is detected on RS cells in a control case of EBV-negative HD. (**A**, **B** and **D**, original magnification × 1000; **C**, original magnification × 400).

considered to be small lymphocytes or immunoblasts (Fig. 1C), and were not counted as RS cells. Such smaller cells never expressed LMP-1.

Discussion

The mRNA of LMP-1 is known to be the most abundantly translated of all the latency genes of EBV (18). LMP-1 was detected easily in the cytoplasm and on the membranes of latency II RS cells in paraffin sections (4, 7, 10, 15). EBV-infected HD examined in this study expressed LMP-1 in 7 % to 100 % of the EBER-1$^+$ RS cells. We used formalin-fixed, paraffin-embedded specimens; therefore, it is possible that LMP-1 reactivity was underestimated. However, this may not be the case since the anti-LMP-1 mAbs used in this study are known to be available to routinely processed tissues and gave a strong reaction (8, 19). It is also possible that latency I RS cells are not true RS cells but rather activated blastic lymphocytes. Indeed, small non-neoplastic cells interspersed among the RS cells frequently express EBER-1 (4, 8, 20). We also found some EBER-1$^+$ small lymphocytes and blastic cells which were negative for LMP-1. It is truly difficult to distinguish each RS cell from activated large

immunoblasts, especially in *in situ* hybridization and immunostaining. However, we counted only the atypical cells which were large enough to be distinguished from immunoblasts as shown in Fig. 1. This suggested that LMP-1 expression in RS cells of EBV-infected HD may be quite unstable, from undetectable to abundant levels, or that latency I and latency II RS cells were of different clones. The latter possibility seems to be less plausible since latency I and latency II RS cells were admixed in the same lesions. Switching of latency is mediated by using the promoters of EBNAs; Fp for latency I and II, and Cp and Wp for latency III (1, 2, 4). Latency I and latency II share the Fp promoter, and LMPs are additionally expressed in latency II (1, 2, 4). EBV need not switch its EBNA promoter when EBV latency changes from latency I to latency II. Therefore, the switching between latency I and latency II may be easier than that between latency I/II and latency III. Not all the RS cells in EBV-infected HD expressed EBER-1 (4, 8, 15). Murray *et al* reported that 41–97 % of RS cells in EBV-infected HD expressed LMP-1 (10). That is to say, RS cells in EBV-infected HD consist of non-infected RS cells, latency I RS cells, and latency II RS cells. The mode of EBV latency *in vivo* may be influenced by the immunological milieu since latency genes other than EBERs and EBNA-1 are strongly antigenic (1, 2). LMP-1 is reported to behave as a transforming gene product (11). However, the variable expression of LMP-1 in EBV-infected HD reported here casts doubt on the role of LMP-1 in maintaining a neoplastic state in HD even if it contributes to the development of HD.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture of Japan. We wish to thank Miss Ayumi Torigoe for preparation of the manuscript.

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Received July 17, 1996; accepted August 1, 1996.