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Macrophage involvement in Epstein-Barr virus-related tumors

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Abstract. Epstein-Barr virus (EBV) is known as a causative agent of Burkitt's lymphoma, nasopharyngeal carcinoma and approximately 10% of stomach carcinoma cases. In other human cancers, EBV gene expression including lytic infection protein detected using *in situ* hybridization and immunofluorescence staining has been reported. Moreover, the expression and replication of EBV genes in cultured normal macrophages and in histiocytes of Langerhans' cell histiocytosis have been identified. The aim of this study was to examine EBV expression in macrophages in other EBV-associated human tumors. Forty-one cases of EBV-associated tumors, which had been confirmed to express EBV, were examined. Tissue sections after *in situ* hybridization were double-stained immunohistochemically with the monoclonal anti-CD68 antibody. EBV expression in macrophages in the lesions of nasopharyngeal carcinoma, oral cancer, thyroid carcinoma, renal cell carcinoma, testicular carcinoma, uterine carcinoma, cutaneous T-cell lymphoma and anaplastic large-cell lymphoma was identified, whereas macrophages in normal or non-cancerous lesions showed no EBV expression. Many tumor-associated macrophages in EBV-related tumors carry EBV, which appears to induce the EBV lytic infection of macrophages. Therefore, the possibility that the lytic infection of macrophages by EBV and the resulting inflammation play certain roles in the oncogenesis of EBV-associated human tumors was raised.

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Abbreviations: EBV, Epstein-Barr virus; LCH, Langerhans' cell histiocytosis; TAM, tumor-associated macrophages; NPC, nasopharyngeal carcinoma; RCC, renal cell carcinoma; CTCL, cutaneous T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; EBER1, EBV-encoded non-polyadenylated RNA-1; EBNA2, EBV nuclear antigen-2; EBNA LP, EBV nuclear antigen leader protein; BZLF1, *Bam*HIZ coding leftward reading frame-1; CIN, cervical intraepithelial neoplasia

Key words: macrophage, Epstein-Barr virus, inflammation, *in situ* hybridization

Introduction

It has been proposed that inflammation causes cancer (1,2), and that approximately 18% of the global cancer burden is attributable to infectious agents (3). Epstein-Barr virus (EBV) is a ubiquitous DNA tumor virus, infecting almost all adults worldwide. On the other hand, EBV has been thought to cause some restricted tumors such as nasopharyngeal carcinoma, which occurs frequently in Chinese populations, Burkitt's lymphoma, which occurs in children in East Africa, or approximately 10% of gastric carcinoma cases. It is difficult to explain why the widely distributed EBV causes endemic tumors in such restricted areas or races. We hypothesized that EBV causes a wider variety of human tumors more frequently than is thought at present. In studies using the mRNA *in situ* hybridization method, it has been reported that EBV genes are expressed in oral carcinoma (4), mesopharyngeal and hypopharyngeal carcinoma (5), thyroid carcinoma (6), renal cell carcinoma (7), testicular tumors (8), uterine cervical carcinoma (9-11), anaplastic large-cell lymphoma (12,13), cutaneous T-cell lymphoma (14), primary leptomeningeal lymphoma (15) and lymphoma originating in the lung (16). Most of these tumors expressed several EBV mRNAs and proteins. Other human tumors may also be associated with EBV infection. This needs to be extensively examined using several EBV mRNA probes, antibodies and primers. We frequently used EBV-encoded non-polyadenylated RNA-1 (EBER1) *in situ* hybridization in this study due to EBER expression in the nucleus and macrophage CD68 expression in the cytoplasm, and so the double staining was clearly visible. Other EBV mRNAs, for example EBV nuclear antigen-2 (EBNA2), which is an oncogene of EBV, are also important. In this study, we used four EBV probes: *Bam*HIW, EBNA2, EBV nuclear antigen leader protein (EBNA LP) and EBER1. We detected EBNA2 mRNA and protein in almost all of the tumors (4,6,7,10,14-16). In the tumor cells mentioned above, the frequency of a correlation with EBV was very high (more than 90%) in each disease. Through these studies, the expression of EBV lytic infection protein BZLF1 by means of indirect immunofluorescence staining in the tumor cells as well as infiltrating lymphocytes was frequently observed. It is generally believed that EBV-related tumor cells express limited genes which operate in tumorigenesis, but do not express lytic proteins. However, Hoshikawa *et al* reported evidence of lytic EBV infection in EBV-positive gastric carcinoma (17). Furthermore, Takasaka *et al* observed EBV

particles in established human gastric cancer cell lines by employing electron microscopy (18). These reports suggest the presence of productive EBV infection in human gastric cancer cells. Therefore, not only EBV-carrying lymphocytes, but also tumor cells may produce EBV. Lytic EBV infection of multiple tissues, may provoke a strong inflammatory response, since cell lysis induced by virus replication results in marked immune responses against viral proteins.

Macrophages are derived from bone marrow promonocytes, which develop into monocytes and infiltrate tissues. There, they differentiate into a specific type of resident tissue macrophage, such as microglial cells in the brain, Kupffer cells in the liver and Langerhans' cells in the skin. Their functions are to protect the host from microbial infection, to regulate tissue remodeling and to repair injury. Macrophages also comprise a major component of the inflammatory infiltrate in tumors. Such cells are termed tumor-associated macrophages (TAMs). TAMs can kill tumor cells, but they also produce growth factors, angiogenic factors and proteases which degrade the matrix. Through the action of these macrophage-derived factors, tumor cell proliferation, angiogenesis, tumor invasion and metastasis are accelerated (19).

EBV infection of a macrophage cell line was first described by Revoltella *et al* (20). Furthermore, Savard *et al* reported a lytic program of primary human macrophages induced by EBV (21). We also showed the expression and replication of EBV genes in cultured normal human macrophages (22) and abnormal histiocytes in Langerhans' cell histiocytosis (LCH) (23,24). Moreover, we revealed EBV expression in macrophages which had infiltrated primary lung lymphoma (16). EBV infects macrophages as well as B lymphocytes, T lymphocytes and epithelial cells. EBV-expressing macrophages may play important roles in cancer-causing chronic EBV infection and inflammation. To investigate the existence of EBV-expressing macrophages in several human cancers, we studied human cancer tissues that were already confirmed to express EBV oncogenes and the lytic infection protein BZLF1.

Materials and methods

Patients. Five nasopharyngeal carcinoma, 5 oral cancer, 10 thyroid carcinoma, 2 renal cell carcinoma (RCC), 2 testicular carcinoma, 11 uterine carcinoma, 4 cutaneous T-cell lymphoma, 2 anaplastic large-cell lymphoma cases and 1 case of chronic active EBV infection were examined. EBV expression was previously detected in the tumor cells. For a comparative study, 3 lichen planus, 2 Graves' disease, 2 thyroid nodular hyperplasia, 1 glomerulosclerosis case and 3 cases of normal uterine cervix were also examined. All samples used in this study have previously been described (4-16). None of the patients had a history or clinicopathological features indicative of an immunocompromised state.

Probes. *Bam*HIW probes were transcribed from 2.27-kb EBV *Bam*HIW fragments from which the '*Alu*-family'-like sequence had been deleted. The *Bam*HIW fragment of EBV is a highly repetitive sequence that contains the mRNA leader sequence for EBNAs. The fragment was cloned into the pBluescript II SK$^+$ vector. cDNA of the *Bam*HIY1Y2 (EBNA LP) region was also cloned into pBluescript II SK$^+$. The size of this cDNA was 153 bp. The sense and antisense probes were labelled with digoxigenin-11-UTP by *in vitro* transcription with T7 and T3 polymerases, respectively, using a commercial kit (Boehringer Mannheim, Mannheim, Germany). EBNA2 cDNA (14,802-48,583, including a spliced sequence) and EBER1 cDNA (6,629-6,795) were synthesized through RT-PCR according to the method reported by Tierney *et al* (25) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The sizes of these cDNAs were 386 and 167 bp, respectively. The sense and antisense RNA probes were labelled with digoxigenin-11-UTP by *in vitro* transcription with T7 and SP6 polymerases, respectively, using a commercial kit (Boehringer Mannheim). The labelled *Bam*HIW and EBNA2 riboprobes were then fragmented to ~100 bases in length by alkaline hydrolysis. The sense probe served as a negative control.

Messenger RNA in situ hybridization. Paraffin sections were prepared from formalin-fixed tissues from biopsied or surgically resected materials. Serial sections were cut to 3-5 μm. After dewaxing and dehydration with graded ethanol, slides were treated with 0.2 N HCl for 15 min at room temperature (RT) and rinsed with phosphate-buffered saline (PBS) for 5 min also at RT. They were then treated with 50 μg/ml proteinase K in PBS for 15 min at 37˚C and immersed in 2 mg/ml glycine in PBS for 10 min at RT. Subsequently, the sections were refixed with 4% paraformaldehyde in PBS for 15 min and washed twice with PBS for 3 min at RT, then treated with 0.1 M triethanolamine (pH 8.0) for 10 min at RT. After washing with PBS, the sections were dehydrated with ethanol and hybridized for 40 h at 37˚C for EBNA2 and EBER1, 39˚C for *Bam*HIY1Y2 (EBNA LP) and 45˚C for *Bam*HIW in 4X SSC, 50% formamide, 1X Denhardt's solution, 5% dextran sulfate, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA and 10 mM dithiothreitol. After hybridization, the sections were washed twice with 2X SSC for 30 min and then twice with 0.5X SSC for 20 min with gentle shaking at RT. They were subsequently blocked with 1% skim milk (Difco) in 100 mM Tris and 0.15 M NaCl (pH 7.5) for 30 min at RT. Next, they were reacted with 1:100 (for EBNA2) and 1:200 diluted (for *Bam*HIW, *Bam*HIY1Y2 and EBER1) alkaline phosphatase-labelled anti-DIG antibody (Boehringer Mannheim) in blocking buffer for 2 h at RT. After washing, the sections were incubated with nitroblue tetrazolium and X-phosphate (Boehringer Mannheim) in buffer containing 0.1 M Tris, 0.1 M NaCl, 0.005 M $MgCl_2$ and 1 mM levamisole (pH 9.6) for 16 h at RT. The reaction was stopped with EDTA, and then the slides were then dehydrated with graded ethanol and xylene and sealed with malinol. These methods are a modified version of those we previously reported (4-16).

Double staining with mRNA in situ hybridization for EBV and immunostaining against macrophages. Tissue sections that had been prepared in the previous studies, after *in situ* hybridization, were immersed in 100% xylene, and the cover glasses were removed. After rehydration with a series of descending concentrations of ethyl alcohol and distilled water, the sections were exposed to microwaves (500 W) for 5 min in 10 mM Tris and 1 mM EDTA (pH 9.0) and digested with 0.05% trypsin in

Table I. Summary of results.

Disease	Histology	Case	Probe for ISH	Double-stained/CD68-positive[a]	Rate	Average[b]
NPC	SCC	1	*Bam*HIW	9/35	0.26	
	SCC	2	*Bam*HIW	6/28	0.21	
	SCC	3	*Bam*HIW	7.5/31.5	0.24	
	SCC	4	*Bam*HIW	9/29	0.31	
	SCC	5	*Bam*HIW	3/28	0.11	0.226
Oral cancer	SCC	1	EBER1	27/59.5	0.45	
	SCC	2	EBER1	12/30.6	0.39	
	SCC	3	EBER1	4.5/14	0.32	
	SCC	3	EBNA2	10/36	0.28	
	SCC	4	EBNA2	11/41	0.27	
	SCC	5	EBNA2	0/0	0	0.285
Lichen planus		1	EBER1	0/67	0	
		2	EBNA LP	0/24	0	
		3	EBNA LP	0/73	0	0
Thyroid ca.	Pap. ca.	1	EBER1	5/20	0.25	
	Pap. ca.	2	EBER1	3/20	0.15	
	Pap. ca.	3	EBER1	7/34	0.21	
	Pap. ca.	3	*Bam*HIW	2/18	0.11	
	Pap. ca.	4	*Bam*HIW	1.5/14	0.12	0.168
	SCC	1	EBER1	6/53	0.11	0.11
	Undiff. ca.	1	EBER1	12.5/97.5	0.13	
	Undiff. ca.	2	EBER1	12.7/43	0.29	
	Undiff. ca.	3	EBER1	9.5/93	0.10	
	Undiff. ca.	4	EBER1	10/36	0.28	
	Undiff. ca.	5	EBER1	9/19.3	0.47	0.248
Graves' disease		1	*Bam*HIW	0/12	0	
		2	*Bam*HIW	0/13	0	
Nodular hyperplasia		1	EBER1	0/45	0	
		2	EBER1	0/51	0	0
RCC	Clear cell	1	*Bam*HIW	1/9	0.11	
	Clear cell	1	EBNA LP	15/65	0.23	
	Clear cell	2	*Bam*HIW	21/40	0.47	
	Clear cell	2	EBNA LP	30/70	0.43	0.308
Glomerulosclerosis		1	EBER1	0/4	0	0
Testicular ca.	Seminoma	1	*Bam*HIW	2/12	0.17	
	Seminoma	2	*Bam*HIW	8/43	0.19	0.185
Uterine CIN3	SCC	1	EBER1	1/3.7	0.27	
	SCC	1	EBNA2	1/1.7	0.59	
	SCC	2	EBER1	5.5/10.75	0.51	
	SCC	2	EBNA2	3.7/15.7	0.24	
	SCC	3	EBER1	11/33.4	0.32	
	SCC	3	EBNA2	9.5/67.5	0.14	0.387
Invasive uterine cervical ca.	SCC	1	EBNA2	39/52	0.75	
	SCC	2	EBNA2	12/37	0.32	
	SCC	3	EBNA2	20.5/40.5	0.51	
	SCC	4	EBNA2	4/17	0.24	
	SCC	5	*Bam*HIW	26/46.5	0.56	
	SCC	6	EBER1	23.7/78.7	0.30	
	SCC	6	EBNA2	20.4/60.6	0.34	0.432

Table I. Continued.

Disease	Histology	Case	Probe for ISH	Double-stained/CD68-positive[a]	Rate	Average[b]
Normal cervix		1	EBER1	0/0	0	
		2	EBER1	0/3	0	
		2	EBNA2	0/7.5	0	
		3	EBER1	0/13.5	0	
		3	EBNA2	0/7	0	0
Uterine cp. ca.	Adenoca.	1	EBNA2	26.5/64	0.41	
	Adenoca.	2	EBER1	0/0	0	0.205
CTCL		1	EBER1	4.5/33	0.13	
		2	EBER1	8.6/22	0.39	
		3	EBNA2	10/37	0.27	
		4	EBNA2	12.5/34	0.37	0.29
ALCL		1	*Bam*HIW	6/40	0.15	
		2	*Bam*HIW	4/39	0.10	0.125
Chr. ac. EBV infect.		1	EBNA LP	2/2	1	

[a]Average of 3-5 photographs at x400 magnification. [b]Average rate of the disease. ISH, *in situ* hybridization; NPC, nasopharyngeal carcinoma; SCC, squamous cell carcinoma; *Bam*HIW, *Bam*HIW fragment of EBV DNA; EBER1, EBV-encoded small non-polyadenylated RNA-1; EBNA2, EBV nuclear antigen-2; EBNA LP, EBV nuclear antigen-leader protein; Thyroid ca., thyroid carcinoma; Pap. ca., papillary carcinoma; Undiff. ca., undifferentiated carcinoma; RCC, renal cell carcinoma; Clear cell, clear cell carcinoma; Testicular ca., testicular carcinoma; CIN3, cervical intraepithelial neoplasia 3; ca., carcinoma; cp. ca., corpus carcinoma; Adenoca., adenocarcinoma; CTCL, cutaneous T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; Chr. ac. EBV infect., chronic active EBV infection.

PBS at 37˚C for 60 min. They were subsequently immersed in 3% H_2O_2 in methanol, washed with PBS and treated with x25 diluted anti-CD68 mouse monoclonal antibody (Dako, Glostrup, Denmark) for 60 min at RT. Finally, the tissue sections were treated with a staining kit (Histofine Simple Stain NAX-PO(M); Nichirei BioScience, Tokyo, Japan) for 30 min.

Results

To clarify the presence of EBV expression in TAMs, we carried out immunohistochemical staining using the anti-CD68 monoclonal antibody in sections already hybridized with EBV mRNA by *in situ* hybridization. Tissue specimens of 5 nasopharyngeal carcinoma, 5 oral cancer, 10 thyroid carcinoma (involving 4 cases of papillary carcinoma, 1 of squamous cell carcinoma and 5 of undifferentiated carcinoma), 2 renal cell carcinoma, 2 testicular carcinoma, 3 uterine cervical intraepithelial neoplasia, 8 invasive uterine carcinoma (involving 6 cases of cervical squamous cell carcinoma and 2 of corpus adenocarcinoma), 4 cutaneous T-cell lymphoma, 2 anaplastic large-cell lymphoma cases and 1 case of chronic active EBV infection were prepared. For a comparative study, oral mucosal tissues of 3 cases of lichen planus, thyroid tissues of 2 cases (each of Graves' disease and nodular hyperplasia), kidney tissue of 1 case of glomerulosclerosis and mucosal tissue of 3 cases of normal cervix were also prepared. Hybridization signals appeared blue-purple in the nucleus with the EBER1 probe and in the cytoplasm with the others (*Bam*HIW, EBNA2 and EBNA LP), whereas immunohistochemical staining with CD68 appeared dark brown in the cytoplasm. The results of double staining with EBV mRNA *in situ* hybridization and CD68 immunostaining are summarized in Table I and representative images are shown in Fig. 1. Double-stained TAMs were detected in almost all tissues of the EBV-associated neoplasms examined. Generally, the more macrophages were detected in the tissue, the more they were double-stained. Although the number of TAMs was variable between cases, the ratio of double-stained macrophages to all macrophages was highest in the uterine cervical carcinoma, then oral cancer, undifferentiated thyroid carcinoma and nasopharyngeal carcinoma, when restricted to diseases for which we examined more than 5 cases (Table I). In most cases, macrophages also infiltrated into each comparative non-cancerous tissue, whereas they were not double-stained (Table I, Fig. 1c). In the nasopharyngeal carcinoma of case 1, tissue around the cancer showed many EBV-expressing epithelial cells (small arrowhead), a moderate number of infiltrating macrophages (large arrowhead) and several double-stained macrophages (double arrowhead). Since both signals of *Bam*HIW and CD68 staining were expressed in the cytoplasm, the color of double-stained cells appeared black (Fig. 1a). In the oral cancer case 1, EBER1 signals were expressed in the nucleus and CD68 in the cytoplasm (Fig. 1b). In the case of lichen planus, a non-cancerous oral disease, there were many macrophages stained with CD68; however, no double-stained macrophage was observed (Fig. 1c). In the thyroid carcinomas, undifferentiated carcinoma expressed more EBV RNA than the papillary carcinoma cases (6);

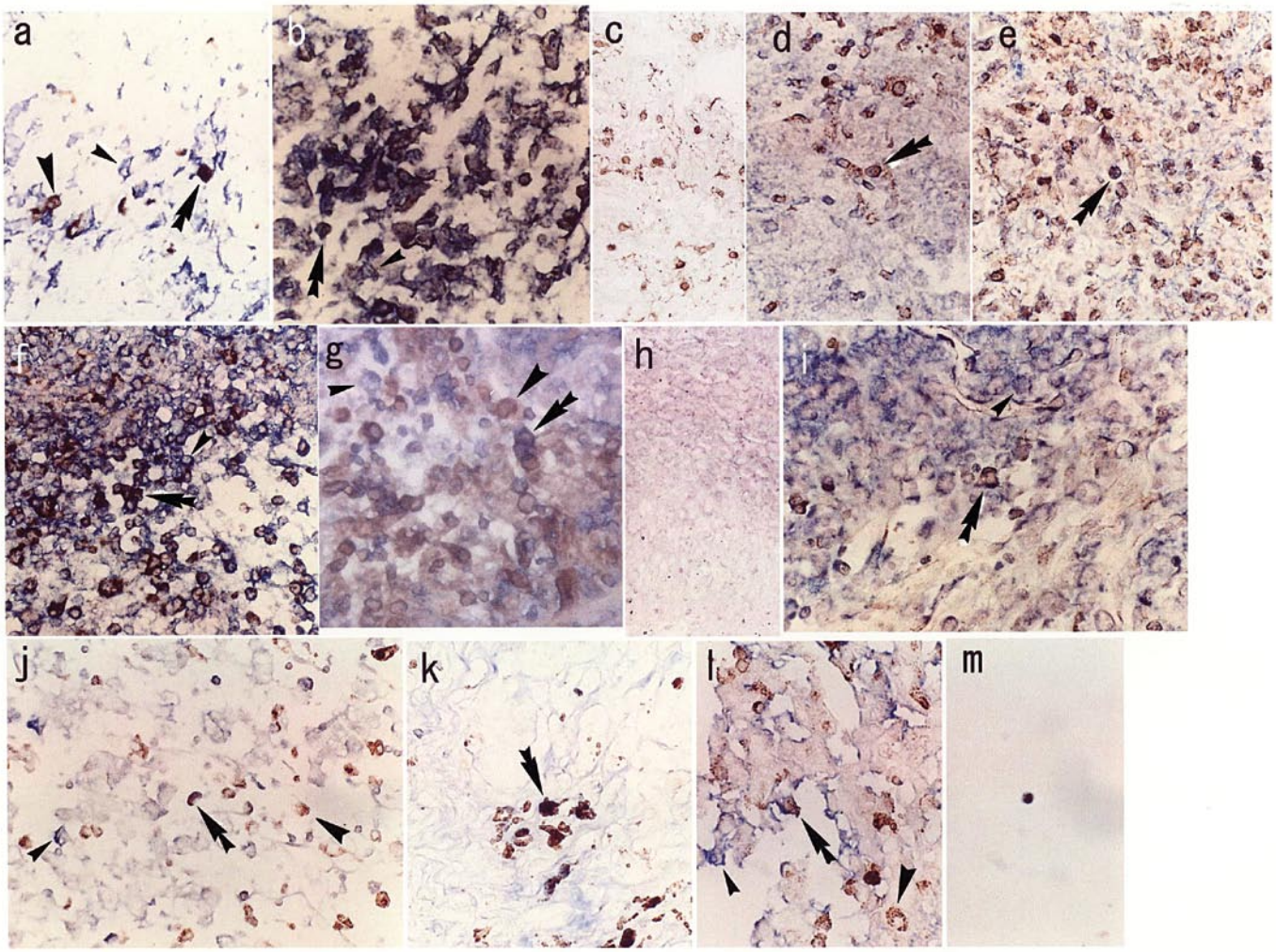

Figure 1. Results of the double staining of EBV mRNA on *in situ* hybridization and immunostaining with CD68. EBV-expressing cells are indicated by the small arrowhead; macrophages are indicated by the large arrowhead; double-stained macrophages are indicated by the double arrowhead. (a) Nasopharyngeal carcinoma, case 1, *Bam*HIW antisense probe, x40; (b) oral cancer, case 1, EBER1 antisense probe, x40; (c) lichen planus for non-cancerous control of oral cancer, case 1, EBER1 antisense probe, x20; (d) thyroid papillary carcinoma, case 3, EBER1 antisense probe, x40; (e) thyroid undifferentiated carcinoma, case 1, EBER1 antisense probe, x40; (f) uterine cervical carcinoma, case 1, EBNA2 antisense probe, x40; (g) uterine corpus carcinoma, case 1, EBNA2 antisense probe, x40; (h) normal cervix, case 1, EBER1 antisense probe, x20; (i) renal cell carcinoma, case 2, *Bam*HIW antisense probe, x40; (j) testicular carcinoma, case 2, *Bam*HIW antisense probe, x40; (k) cutaneous T-cell lymphoma, case 4, EBNA2 antisense probe, x40; (l) anaplastic large-cell lymphoma, case 1, EBNA2 antisense probe, x40; (m) chronic active EBV infection, case 1, EBNA LP antisense probe, x40.

however, the number of double-stained macrophages was not significantly higher in the undifferentiated than in the papillary carcinomas (Table I). The number of TAMs that had infiltrated the uterine tissue was higher in the uterine cervical than in the uterine corpus carcinoma cases, and double staining was clearer in the former than in the latter (Fig. 1f and g). EBV expression of CIN was similar to invasive cervical carcinoma (9), and the number of double-stained macrophages was not significantly different between CIN and invasive carcinoma (Table I). The normal cervix showed few macrophages in the tissue (Fig. 1h). TAMs dually expressing EBV and CD68 were observed in the renal cell (Fig. 1i) and testicular (Fig. 1j) carcinomas. Moreover, in the tissues of the cutaneous T-cell lymphoma and anaplastic large-cell lymphoma, dually expressed TAMs were detected (Fig. 1k and l). Bone marrow macrophages derived from chronic active EBV infection also showed double staining for EBV and CD68 (Fig. 1m).

Discussion

In the present study, double-stained TAMs were detected in almost all tissues of the EBV-associated neoplasms examined. Tissues from normal controls or those from non-cancerous disease cases sometimes contained many macrophages; however, they were not TAMs and were never double-stained. On the other hand, macrophages in a case with chronic active EBV infection (without any neoplasms) were also double-stained (Fig. 1m). We previously reported EBV expression in cultured macrophages from normal tissues of the bronchus and testis, and in cultured epididymitis macrophages (22). In the present study, however, macrophages in the normal or non-cancerous tissues did not show EBV expression. This may have been due to the selection of EBV-carrying macrophages, which is very rare in normal or non-cancerous tissues, but has a growth advantage in the process of cell culture. We previously reported EBV-expressing macrophages in the

parotid tumor, non-Hodgkin's lymphoma (22), LCH (23,24) and primary lung lymphoma (16). These results indicate that TAMs of EBV-related neoplasms as well as macrophages infiltrating tissues with chronic active EBV infection express EBV mRNA. In the case of chronic active EBV infection, the selection of EBV-carrying macrophages, which is similar to cell culture, may occur in the bone marrow.

Furthermore, double-stained TAMs were detected in the thyroid papillary carcinoma and CIN at almost the same level as in the thyroid undifferentiated and invasive cervical carcinomas, respectively. This suggests the earlier association of TAMs in the process of EBV oncogenesis.

As mentioned previously, most of these tumors were already confirmed to express EBV oncogene EBNA2 and lytic infection protein BZLF1; therefore, it can be said that TAMs in EBV-related tumors involve EBV-carrying macrophages, and may also produce EBV. We reported that childhood LCH expressed high levels of EBV lytic infection protein, and that in one case the administration of acyclovir resulted in complete remission (23). If inflammation caused by EBV production in the tumor tissue is always as intense as we observed, we can expect that the administration of an anti-herpesvirus drug will be more effective and safer than the usual anti-cancer chemotherapy. These lytic infections of TAMs may also be a target of inflammation. We observed that the more macrophages were detected in the tissue, the more they were double-stained by *insitu* hybridization and CD68 immunostaining. This suggests a strong correlation between EBV-carrying TAMs and inflammation. Notably, a correlation between infiltrating macrophages and the risk or poor prognosis of cervical intraepithelial neoplasia (26), uterine endometrioid adenocarcinoma (27) and RCC (28) was reported, although it is unknown whether EBV infection is associated with these macrophages.

Chemical agent-associated chronic inflammation with oxidative and nitrative DNA damage was reported by Kawanishi *et al*. They described 8-nitroguanine as a potential biomarker for evaluating the risk of inflammation-related carcinogenesis (29). The correlation between 8-nitroguanine and EBV has been studied in cases of nasopharyngeal carcinoma (30) and oral cancer (31). Moreover, Ma *et al* showed that the cells responsible for the reaction are macrophages (30).

Recently, the suppression of HIV replication by human herpesvirus 6 (32) or 7 (33) was reported. Furthermore, it was reported that latently infected murine-γ herpesvirus 68, which is genetically very similar to EBV, confers resistance against *Listeria monocytogenes* and *Yersinia pestis* in mice (34). Such virus-virus or virus-microbe interactions may be important when considering oncogenesis due to inflammation caused by viral infection. We previously reported that EBV genes of *Bam*HIW (9), EBNA2 (10) and EBNA LP (11) were expressed in uterine cervical carcinoma tissue. The frequency of the correlation was higher with EBV than with human papillomavirus (HPV) (9). Almost all cervical carcinoma samples also carried the HPV16 gene (9), and E6-E7 proteins of HPV16 were reported to induce uncontrollable cell growth (35). Through this study, TAMs in CIN as well as cervical and a part of corpus carcinoma were shown to express EBV mRNA. Therefore, we hypothesize that EBV infection may synergistically act with HPV to cause the development or progression of cancer through the long-term inflammation induced by infiltrating macrophages carrying EBV. EBV-associated tumors other than uterine carcinoma were also suspected to be caused by long-term inflammation with EBV alone or EBV and another unidentified virus or microbe. Moreover, the role of macrophages, not only in inflammation but also in the interaction between viruses or viruses and microbes, should be clarified. Through these studies of tumor and virus-related inflammation involving macrophages, it may be possible to fully elucidate the dynamic mechanism of EBV oncogenesis.

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