合計3名の唾液DNAのエクソーム解析を終了した。2 名の患者に共通の変異は認められなかったが、それぞれの患者から免疫関連遺伝子の variation が見つかった。また、分類不能型免疫不全症の患者から変異が見つかった遺伝子についても、homozygous あるいは heterozygous の variation が見つかった。これらの遺伝子については、さらに多くの患者においてその variation の有無を調べるために、サンガー法による遺伝子解析を開始した。また、今年度新たに 9 名の患者とその家族 15 名、合計 24 名より唾液 DNA を採取し解析を開始した。

D. 考察

分類不能型免疫不全症 CVID 患者の一部においてEBV感染TあるいはNK細胞が増殖し、CAEBV と区別できない病態を示すことが最近示されたため、遺伝子の変化に基づく何らかの免疫不全が CAEBV 発症の背景になりうると考えられるようになった。免疫グロブリン産生の低下など CVID の所見を示さない CAEBV 症例においても、何らかの遺伝子異常による微細な免疫不全が発症に関わる可能性が考えられる。

CAEBV は、発症年齢と進行のスピード、合併症の有無とその種類等に関してきわめて多様性に富む。これまでに解析された 2 症例に共通した変異あるいは variation は認められていないが、このような CAEBV の臨床的多様性からみて、複数の遺伝子が関わる（genetic heterogeneity）可能性が十分考えられる。今後は、より多くの患者 DNA を用いてエクソーム解析を行い、複数の患者に変異が認められる遺伝子に焦点を絞り検索を進めていく計画である。また、変異遺伝子がコードする蛋白質の機能解析を行う必要が生じると考えられる。

E. 結論

全エクソン配列決定による CAEBV 原因遺伝子の同定を目的として、患者 11 名およびその家族 16 名、合計 27 名の唾液から唾液を採取しエクソーム解析を開始した。このうち患者 2 名およびその家族 1、合計 3 名の DNA について解析を終了した。検出された幾つかの免疫関連遺伝子の variation について、さらに多くの患者 DNA を用いて解析を続けている。

F. 健康危機情報

該当なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

該当なし。

図　表

表 1. CAEBV 患者及びその家族からの DNA 採取の状況

症例	年齢（発症時）	年齢（唾液採取時）	性別	EBV 感染細胞	末梢血 EBV DNA 量（copies/μg DNA）	合併症	両親 DNA
1	12	21	女	NK 細胞	2.1×10^{3}	蚊刺過敏症	母
2	8	18	男	NK 細胞	1.4×10^{3}	種痘様水疱症	なし
3	7	9	男	NK 細胞	2.0×10^{6}	蚊刺過敏症	父・母・妹
4	9	13	男	γδT 細胞	6.0×10^{5}	蚊刺過敏症 種痘様水疱症	父・母
5	8	10	女	NK 細胞	1.0×10^{7}	蚊刺過敏症	母
6	9	12	男	NK 細胞	1.5×10^{4}	蚊刺過敏症	母
*7	20	23	女	NK 細胞	2.5×10^{4}	CVID	なし
*8	16	20	女	CD4T 細胞	1.9×10^{3}	CVID	なし
*9	4	6	女	NK 細胞	3.5×10^{4}	CVID	なし
10	9	13	男	γδT 細胞	2.0×10^{3}	蚊刺過敏症	父・母・妹
11	4	15	男	NK 細胞	1.5×10^{3}	蚊刺過敏症	父・母・兄・弟

*7, 8, 9 は唾液ではなく PBMC からの DNA 抽出

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Current Studies on Chronic Active Epstein-Barr virus Infection in Japan

Running title: Chronic Active EBV Infection

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Abstract

Epstein-Barr virus (EBV) infection is usually asymptomatic and persists lifelong. Although EBV-infected B cells have a potential of unlimited proliferation, they are effectively removed by the virus-specific cytotoxic T cells and EBV-associated lymphoproliferative disease develops only in immunocompromised hosts. Rarely, however, individuals without apparent immunodeficiency develop chronic EBV infection with persistent infectious mononucleosis-like symptoms. These patients exhibit high EBV DNA load in the peripheral blood and systemic clonal expansion of EBV-infected T cells or natural killer (NK) cells. Their prognosis is poor with life-threatening complications includinghemophagocyticlymphohistiocytosis, organ failure, and malignant lymphomas. The term chronic active EBV infection (CAEBV) is now generally used for this disease. Geographical distribution of CAEBV is markedly uneven and most cases have been reported from Japan and other East Asian countries. Here wesummarize our current understanding of CAEBV and describe recent progress of CAEBV research in Japan.

Key words: Epstein-Barr virus, chronic active EBV infection, EBV-associated hemophagocyticlymphohistiocytosis, hypersensitivity to mosquito bites, hydroavacciniforme, EBV-associated T/NK-cell lymphoproliferative disease, mouse model, flow-cytometric in situ hybridization.

Introduction

Epstein-Barr virus (EBV) was discovered in cultured cells of Burkitt lymphoma as the first human tumor virus[1]. Since then EBV was found associated with a number of malignancies, including Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma[2]. In spite of close association with these malignancies,EBV was shown to be a ubiquitousvirus infecting more than 90% of adult population in the world_ENREF_2. EBV-associated malignancies thus develop in a restricted fraction of hosts through collective effects of various factors, including host genetic background and environmental factors, as well asfunctions of EBV genes. EBV infection in humans is usually asymptomatic and persists lifelong as latent infection, although primary infection later than adolescencefrequentlyresults in infectious mononucleosis (IM). IM is caused by transient proliferation of EBV-infected B cells accompanied by excessive responses of EBV-specific cytotoxic T cells (CTLs). The main target of EBV is B cells and epithelial cells, and EBV has a unique biological activity to transform B cellsand establishimmortalized lymphoblastoid cell lines. Since EBV-transformed cells express at least nine viral proteins including the highly immunogenic EBV nuclear antigens 3 (EBNA3s)and EBNA2 (the latency III type EBV gene expression), they are readily removed by the virus-specific CTLs and the virus does not cause lymphoproliferative disease in normal immunocompetent hosts[3]. In immunocompromised hosts like transplant recipients and AIDS patients, however, EBV-transformed cells are not efficiently removed and may cause EBV-associated B-cell lymphoproliferative disease (LPD).

Rare EBV-infected individuals without apparent immunodeficiency present with persistent or recurring IM-like symptoms including fever, hepatosplenomegaly, lymphadenopathy, and liver dysfunction, as well as high EBV DNA load in the peripheral blood[4-7]. The term chronic active

EBV infection (CAEBV) is now generally used to describe this disease. Patients with CAEBV encountered in Japan and other East Asian countries have poor prognosis and characterized by clonal expansion of EBV-infected T cells or natural killer (NK) cells[8-11]. In contrast, a similar disease with less morbidity and mortality has been reported from Western countries and it is usually associated with proliferation of EBV-infected B cells[12]. In this review, focused on CAEBV as an EBV-associated T/NK-cell lymphoproliferative disease (EBV^+ T/NK-LPD), we summarize the current understanding of the disease and describe our own recent workssubsidized by grants from the Ministry of Health Labour and Welfare of Japan.

Clinical characteristics of CAEBV and other EBV-associated T/NK-cell lymphoproliferative diseases

As described above, IM-like symptoms are the main symptoms of CAEBV[4-7]. Other clinical manifestations include thrombocytopenia, anemia, pancytopenia, diarrhea, and uveitis. Peripheral blood EBV DNA load regularly exceeds $10^{2.5}$ copies/μg DNA[13]. High-level production of various cytokines, including IL-1β, IL-10, and IFN-γ has beendetected in CAEBV patients and are thought to play important roles in inflammatory symptoms of the disease[14-16]. CAEBV can be classified into the T-cell and NK-cell types, depending on which lymphocyte subset is mainly infected with EBV. A survey of Japanese CAEBV patients revealed that the T-cell type is associated with less favorable prognosis than the NK-cell type[17, 18]_ENREF_16. CAEBV was included in the 2008 WHO classification of lymphomas as the systemic EBV^+ T-cell lymphoproliferative disease of childhood[19].

Although the clinical course of CAEBV is chronic, patients often develop fatal complications such as multi-organ failure,disseminated intravascular coagulopathy (DIC), digestive tract

ulcer/perforation, coronary artery aneurysms, and malignant lymphomas, as well as EBV-associated hemophagocyticlymphohistiocytosis (EBV-HLH)[7]. HLH is a hyperinflammatory condition caused by overproduction of cytokines by excessively activated T cells and macrophages. Clinical characteristics of HLH include fever, hepatosplenomegaly, pancytopenia, hypertriglyceridemia, DIC, and liver dysfunction[20].EBV-HLH usually occurs following primary EBV infection and is itself characterized by clonal proliferation of EBV-infected T or NK cells(most often $CD8^+$ T cells)[21, 22]. EBV-HLH can also occur in association with X-linked lymphoproliferative disease (XLP) and XIAP deficiency[23].

Patients with CAEBV may have characteristic cutaneous complications, namely hypersensitivity to mosquito bites (HMB) and hydroavacciniforme (HV), that are themselves distinctEBV^+ T/NK-LPDs characterized by clonal proliferation of EBV-infected T or NK cells. Both HMB and HV can occur independently or in association with CAEBV. HV is a childhood photosensitivity disorder, characterized by necrotic vesiculopapules on sun-exposed areas[24]. EBV DNA levels are elevated in patients' peripheral blood and histochemical analysis of skin lesions revealsinfiltration of T cells expressing the EBV-encoded small RNA (EBER)[25].Although most cases of HV resolve by early adult life, cases overlapping with CAEBV may eventually develop EBV-positive malignant lymphoma that was included in the 2008 WHO classification of lymphoma as the hydroavacciniforme-like lymphoma[19, 26]_ENREF_26_ENREF_19_ENREF_19_ENREF_19_ENREF_19_ENREF_27. HMB is characterized by severe local skin reactions to mosquito bites including erythematous swelling with bullae, necrotic ulcerations, and depressed scars[27]. These local reactions may be accompanied by general symptoms such as high fever, lymphadenopathy, and liver dysfunction.Most HMB cases have EBV infection in NK cells in skin lesions and peripheral blood[28, 29]. HMB cases without

systemic symptoms may eventually develop CAEBV[28].

Prospective clinicopathologic analyses of CAEBV and other EBV⁺ T/NK-LPDs

CAEBV, EBV-HLH, HMB, and HV are thus distinct but overlapping entities categorized as EBV^+ T/NK-LPDs. Higher incidence of these diseases in East Asian countries and their occasional coincidence in a single patient imply a common pathogenesis for them[7, 30]_ENREF_7. Kimura and others performed a large-scale prospective study of Japanese EBV^+ T/NK-LPD cases[31]. 108 cases of EBV^+ T/NK-LPDs (80 cases of CAEBV, 15 cases of EBV-HLH, 9 cases of HMB, and 4 cases of HV) were analyzed. The results showed that the clinical profile of EBV^+ T/NK-LPD is closely linked with the lineage of EBV-infected cells. More than half (53%) of EBV-HLH patients had EBV in the $CD8^+$ T-cellsubset, in contrast to the low incidence of EBV infection in this subset in the other EBV^+ T/NK-LPDs. Most patients (89%) of HMB had EBV-infected NK cells, whereas majority (75%) of patients with HV had EBV-infected γδT cells. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications and 13 (12%) developed overt lymphoma or leukemia. The age of onset ≥8years and liver dysfunction were risk factors for mortality and transplanted patients had better prognosis. Patients with $CD4^+$ T-cell infection had shorter survival as compared with those with NK-cell infection. Because shorter time from onset to hematopoietic stem cell transplantation (HSCT) and inactive disease at HSCT were associated with longer survival, earlier HSCT in good condition was considered preferable. Among the 108 patients enrolled, four patients developed aggressive NK-cell leukemia (ANKL) and six patients developed extranodal NK/T-cell lymphoma (ENKL). It is thus conceivable that a certain fraction of patients with ANKL and ENKL havedeveloped these malignancies as a consequence of CAEBV[32, 33]_ENREF_34.

Characteristics of adult CAEBV cases

CAEBV has been described mainly as a disease of children and young adults; the mean age of onset was estimated to be 11.3 years[18]_ENREF_25. Recently, however, increasing number of adult patients fulfilling the criteria of CAEBV_ENREF_34are reported. This may be a true increase in the incidence of adult-onset CAEBV or reflect improved recognition of this disease by physicians. Arai and others reviewed 23 cases of adult-onset CAEBV and described their characteristics[34]_ENREF_34. In 87% of adult cases, T cells were infected with EBV, whereas in childhood-onset cases, theT- and NK-cell types were equally frequent. Adult-onset cases appeared rapidly progressive and more aggressive, although the number of patients analyzed was limited. Further investigation with larger number of patients is required to elucidate the characteristics of adulthood CAEBV and its relation to the childhood counterpart.

Recurrence of CAEBV with EBV-infected, donor-derived T cells following HSCT

The relative prevalenceof CAEBV in East Asia and natives of Central and South America implies a genetic background for its pathogenesis. Recently HLA-A*26, an MHC class I allele relatively common in East Asia, was found to be associated with an increased risk for EBV^+ T/NK-LPD[35]_ENREF_37. Although possible involvement of EBV strains with increased propensity to induce T/NK-celllymphoproliferation cannot be formally denied, it is highly unlikely because outbreaks and familial transmission of CAEBV havenot been reported. Arai and others reported an intriguing case of CAEBV who experienced a relapse after bone marrow transplantation[36]. A 35 year-old female patient with CAEBV of the CD8 type had HSCTfrom an unrelated male donor following myeloablative preconditioning with total body irradiation. The serologic HLA types of the patient and the donor were identical, whereasthe DNA typeswere different in two HLA-DR alleles.Although the peripheral blood EBV DNA was undetectable at one month after HSCT and remained so for nearly twelve months, her EBV DNA load increased again

and reached 1.0×10^5 copies/μg DNA. EBV was found primarily in $CD8^+$ T cells again. However, her EBV-infected cells had now an XY karyotype,clearly indicating their donor origin. Sequencing analysis of the variable region of the EBV-encoded *LMP1* gene showed that the virus strain infecting $CD8^+$ T cells were different before and after BMT, suggesting that her repeated episodes of CAEBV were not caused by a rare EBV strain with an unusual biological activity. If we do not suppose that these two consecutive episodes of CAEBV in a single patient occurred just by chance, these findings may suggest that she has a certain genetic background that exerts its direct effectson cellular lineages unrelated to hematopoietic stem cells.

Pathophysiology of CAEBV

Pathogenesis of CAEBV is not understood. Most T and NK cells do not express the EBV receptor CD21 andthe mechanism of their infection with EBV is not clear. Transfer of CD21 from B cells to NK cells through immunological synapse may render the latter cells accessible for EBV[37]. The mechanism by which EBV induces proliferation of T and NK cells is not known either. EBV-induced expression of CD40 and its engagement by CD40L may have a role in the survival of EBV-infected T and NK cells of CAEBVpatients[38]. Since EBV-positive T or NK cells have been occasionally found in the tonsil and peripheral blood of IM patients, ectopic EBV infection in T or NK cells does not necessarily lead to the development of CAEBV[39-41]. Although EBV-infected T and NK cells inCAEBV patients and cell lines derived from them do not express the most immunodominant EBNA3s and EBNA2, they express EBNA1, latent membrane protein 1 (LMP1) and LMP2 (the latency II type EBV gene expression) that are frequently recognized by EBV-specific CTLs[3, 42-45]._ENREF_40 Hosts with normal immune functions are thus expected tohave the capacity to recognize EBV-infected T and NK cells. It is thus conceivable that patients

with CAEBV have a certain defect in immunologic functions that causes inefficient recognitionand/or killing of EBV-infected latency II cells. Indeed, deficiency in cellular immune responses to EBV has been detected in patients with CAEBV[46-48].The defect in T-cell responses to LMP2A might be particularly relevant to this issue[47]. Interestingly, a patient with clinical manifestations similar to CAEBV, although the virus was found in his B cells, was shown to have mutations in the gene encoding perforin, that has a critical role in granule-mediated killing of target cells[49]. None of other patients with CAEBV, however, were shown to have a mutation in the*perforin* gene. Mutations of the genes responsible for XLP, XIAP deficiency, and familial HLH (except for the type 2 that is caused by mutations of *perforin*) have not been reported for patients with CAEBV[7].

Clonal proliferation of EBV-infected T or NK cells in CAEBV and other EBV^+ T/NK-LPDs implies that these diseases have amalignant nature. However, CAEBV is a chronic disease and patients with clonal expansion of EBV-infected T or NK cells may stay in stable condition for years without treatment[18]. Overt malignant lymphoma occurs usually after a long course of disease. Therefore CAEBV may represent, at least in its early phase,premalignant or smoldering phase of EBV-positive leukemia/lymphomas_ENREF_46. Ohshima and others proposed a pathological categorization of CAEBV into a continuous spectrum ranging from a smoldering phase to overt leukemia/lymphoma[50]. Clonality of EBV-infected T or NK cells in CAEBV may not necessarily indicate a malignant phenotype; acquisition of clonality might be a result of other selective processes such as immune escape.

Mouse xenograft models for EBV^+ T/NK-LPD

Animal models for EBV^+ T/NK-LPD have not been available, rendering researches on their pathogenesis and therapy difficult. Imadome and others transplanted peripheral blood

mononuclear cells (PBMC) isolated from patients with CAEBV and EBV-HLH to immunodeficient mice of the NOD/Shi-*scid*/IL-2Rγ^{null}(NOG) strain, and successfully reproduced major features of these diseases including systemic monoclonal proliferation of EBV-infected T or NK cells and hypercytokinemia (Fig. 1)[51]. Although many features were common to CAEBV and EBV-HLH model mice, hemorrhagic lesionsin the abdominal and thoracic cavities and extreme hypercytokinemia were unique to the latter model, indicating that these mouse models reflect the differences in the pathophysiology of the original diseases. Importantly, these models revealed an essential role of $CD4^+$ T cells in the engraftment of EBV-infected T and NK cells. In vivo depletion of $CD4^+$ T cells following transplantation effectively prevented the engraftment of EBV-infected cellsof not only the $CD4^+$ lineage but also the $CD8^+$ and $CD56^+$ lineages. Furthermore, OKT-4 antibody given after engraftment was also effective to reduce EBV DNA load in the peripheral blood and major organs (Imadome and others, unpublished results). These results suggest that therapeutic approaches targeting $CD4^+$ T cells may be possible.

Diagnosis and monitoring of CAEBV

Prolonged or relapsing symptoms of IM are the major cluetothe diagnosis of CAEBV. Although elevated serum antibody titers against EBV-encoded antigens are often found, this does not occur always and normal titers of anti-EBV antibodies should not preclude the diagnosis of CAEBV[7]. Diagnostic criteria for CAEBV has been published[13]. Quantitation of peripheral blood EBV DNA is most important for diagnosis and a finding of elevated value should be followed by identification of EBV-infected T or NK cells. Quantitation of EBV DNA is however influenced by many factors and the results can vary in different laboratories[52]. Recently, therefore, an international standard EBV DNA sample for normalization became available fromthe National Institute for Biological

Standards and Controls, USA. Since CAEBV is a chronic disease that may progress to overt malignancy and early HSCT in a better clinical condition is recommended, precise monitoring of patient's clinical parameters is particularly important.

Flow-cytometric in situ hybridization for identification of EBV-infected cells

Diagnosis of CAEBV requires exact phenotypingof EBV-infected cells. This process has been usually performed by immunobeads sorting of PBMC into lymphocyte subsets, followed by measurement of EBV DNA in each subset by quantitative PCR. These processesarehowever time-consuming and require specific skills. Kimura and others developed a new method termed flow-cytometric in situ hybridization (FISH) to phenotype EBV-infected cells (Fig. 2)[53, 54]. They utilized a fluorescently labeledpeptide nucleic acid (PNA) probe complementary to EBER and succeeded in detecting EBER by flow cytometry. Following reaction with antibodies specific to surface markers, peripheral blood mononuclear cells were permeabilized and subjected to in situ hybridization with the PNA probe. EBER probes and surface-bound antibodies were then detected simultaneously by flowcytometry. EBV-infected cells with a certain phenotype can be directly counted by FISH that is less laborious thanthe current method. They demonstrated that FISH can be applied for the diagnosis of EBV^+ T/NK-LPD and showed that EBV infects mainly γδT cells in HV[53-55].

miRNA as a potential biomarker of CAEBV

microRNA (miRNA) is a small non-coding RNA of 18-25 nucleotides and plays a critical role in the regulation of cellular proliferation, differentiation, and apoptosis through negatively regulating mRNA translation[56]. miRNAs are encoded not only by cells but also by viruses; EBV is actually the first virus shown to encode miRNAs[57]. Two clusters of EBV-encoded miRNAs have been identified; miR-BHRFs and miR-BARTS[58]. Kawano and others reported that plasma levels of

miR-BART1-5p, 2-5p, 5, and 22 are significantly higher in patients with CAEBV than in those with IM and healthy controls[59]. Plasma miR-BART 2-5p, 4, 7, 13, 15, and 22 levels were significantly elevated in CAEBV patients with active disease than in those with inactive disease. miR-BART13 levels could discriminate between patients with active disease and those with inactive disease with a clear cut-off value. Similarly, plasma miR-BART2-5p and 15 levels could clearly discriminate between patients with complete remission from others. Importantly, plasma EBV DNA levels did not show any significant correlation with these clinical parameters. These results suggest that EBV-encoded miRNAs in plasma may be a useful biomarkerfor the diagnosis and monitoring of CAEBV.

Therapy of CAEBV

Various therapeuticshave been tried for the treatment of CAEBV, including antiviral, chemotherapeutic, and immunomodulatory drugs, with only limited success. These regimens induced sustained complete remission in only exceptional cases and HSCT is at present the only curative therapy for CAEBV[60]. Current event-free survival rate for CAEBV patients following HSCTis estimated to be 0.561 ± 0.086[61].Very recently, Kawa and colleagues reported an excellent results of HSCT following non-destructive pretreatment (RIST)[62]. For18 pediatric patients with CAEBV who were treated with RIST, three-year event-free survival was 85.0±8.0% and three year overall survival rate was 95.0±4.9%. HSCT is thus the therapy of choice for CAEBV. However, HSCT is still accompanied by substantial risk and CAEBV patients have high risks for transplantation-related complications[18]. It is therefore desirable to develop novel therapies that do not depend on HSCT. Preclinical studies of two candidate drugs for CAEBV have been carried out recently and gave hopeful results.