(図 2) 慢性活動性 EB ウイルス感染症 (CAEBV) 診断基準
(厚生労働省研究班、2014)

4) 伝染性単核症様症状が 3 か月以上持続(連続的または断続的)
5) 末梢血または病変組織における EB ウイルスゲノム量の増加
6) T 細胞あるいは NK 細胞に EB ウイルス感染を認める
7) 既知の疾患とは異なること

以上の 4 項目をみたすこと。

補足条項

1)「伝染性単核症様症状」とは、一般に発熱・リンパ節腫脹・肝脾腫などをさす。加えて、血液、消化器、神経、呼吸器、眼、皮膚(種痘様水疱症・蚊刺過敏症)あるいは心血管合併症状・病変(含動脈瘤・弁疾患)などを呈する場合も含む。(初感染に伴う)EBV 関連血球貪食性リンパ組織球症、種痘様水疱症で皮膚症状のみのものは CAEBV には含めない。蚊刺過敏症および臓器病変・合併症を伴う種痘様水疱症は、CAEBV の範疇に含める。経過中しばしば EB ウイルス関連血球貪食性リンパ組織球症、T 細胞・NK 細胞性リンパ腫・白血病などの発症をみる。

2) PCR 法を用い、末梢血単核球分画における定量を行った場合、一般に $10^{2.5}$ (=316) コピー/ μ g DNA 以上がひとつの目安となる。定性の場合、健常人でも陽性となる場合があるので用いない。組織診断には in situ hybridization 法等による EBER 検出を用いる。

3) EB ウイルス感染標的細胞の同定は、蛍光抗体法、免疫組織染色またはマグネットビーズ法などによる各種マーカー陽性細胞解析(B 細胞、T 細胞、NK 細胞などを標識)と EBNA、EBER あるいは EB ウイルス DNA 検出などを組み合わせて行う。

4) 先天性・後天性免疫不全症、自己免疫・炎症性疾患、膠原病、悪性リンパ腫、白血病、医原性免疫不全などは除外する。鑑別診断、病型の把握のために以下の臨床検査の施行が望まれる。

a) EB ウイルス関連抗体価
蛍光抗体法による測定では、一般に VCA-IgG 抗体価 640 倍以上、EA-IgG 抗体価 160 倍以上が、抗体価高値の目安となる。加えて、VCA-IgA, VCA-IgM および EA-IgA 抗体がしばしば陽性となる。患者では抗体価が高値であることが多いが、必要条件ではなく、抗体価高値を認めない症例も存在する。

b) クロナリティの検索
1. EB ウイルス terminal repeat probe を用いた Southern blot 法
2. 遺伝子再構成検査(T 細胞受容体など)

c) 病変組織の病理組織学的・分子生物学的評価(*記載項目の変更なし、図 1 を参照)

d) 免疫学的検討(*記載項目の変更なし、図 1 を参照)

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ．研究成果の刊行物・別刷

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Review Article

Current research on chronic active Epstein–Barr virus infection in Japan

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Abstract Epstein–Barr virus (EBV) infection is usually asymptomatic and persists lifelong. Although EBV-infected B cells have the potential for 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 have 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 including hemophagocytic lymphohistiocytosis, organ failure, and malignant lymphomas. The term "chronic active EBV infection" (CAEBV) is now generally used for this disease. The geographical distribution of CAEBV is markedly uneven and most cases have been reported from Japan and other East Asian countries. Here we summarize the current understanding of CAEBV and describe the recent progress of CAEBV research in Japan.

Key words chronic active EBV infection, EBV-associated hemophagocytic lymphohistiocytosis, EBV-associated T/NK-cell lymphoproliferative disease, Epstein–Barr virus, flow-cytometric *in situ* hybridization, hydroa vacciniforme, hypersensitivity to mosquito bites, mouse model.

Epstein–Barr virus (EBV) was discovered in cultured cells of Burkitt lymphoma as the first human tumor virus.[1] Since then EBV has been found to be associated with a number of malignancies, including Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma.[2] Despite this close association with these malignancies, EBV was found to be a ubiquitous virus infecting >90% of the adult population worldwide. 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 as functions of EBV genes. EBV infection in humans is usually asymptomatic and persists lifelong as a latent infection, although primary infection later than adolescence frequently results in infectious mononucleosis (IM). IM is caused by transient proliferation of EBV-infected B cells accompanied by excessive response of EBV-specific cytotoxic T cells (CTL). The main target of EBV is B cells and epithelial cells, and EBV has a unique biological activity to transform B cells and establish immortalized lymphoblastoid cell lines. Given that EBV-transformed cells express at least nine viral proteins including the highly immunogenic EBV nuclear antigen 3 (EBNA3) and EBNA2 (the latency III type EBV gene expression), they are readily removed by the virus-specific CTL and the virus does not cause lymphoproliferative disease (LPD) in normal immunocompetent hosts.[3] In immunocompromised hosts such as transplant recipients and AIDS patients, however, EBV-transformed cells are not efficiently removed and may cause EBV-associated B-cell 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 are 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

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proliferation of EBV-infected B cells.[12] In this review, focused on CAEBV as an EBV-associated T/NK-cell LPD (EBV$^+$ T/NK-LPD), we summarize the current understanding of the disease and describe the authors' own recent work subsidized by grants from the Ministry of Health Labour and Welfare of Japan.

Clinical characteristics of CAEBV and other EBV-associated T/NK-LPD

As described in the previous section, 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 interleukin (IL)-1β, IL-10, and interferon (IFN)-γ has been detected in CAEBV patients and is thought to play an important role 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 found that the T-cell type is associated with less favorable prognosis than the NK-cell type.[17,18] CAEBV was included in the 2008 World Health Organization (WHO) classification of lymphomas as the systemic EBV$^+$ T-cell LPD 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 hemophagocytic lymphohistiocytosis (EBV-HLH).[7] HLH is a hyper-inflammatory 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 hydroa vacciniforme (HV), that are themselves distinct EBV$^+$ T/NK-LPD 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 level is elevated in patients' peripheral blood, and histochemical analysis of skin lesions indicates infiltration of T cells expressing EBV-encoded small RNA (EBER).[25]Although most cases of HV resolve by early adulthood, HV overlapping with CAEBV may eventually develop into EBV-positive malignant lymphoma, which was included in the 2008 WHO classification of lymphoma as the hydroa vacciniforme-like lymphoma.[19,26] 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 patients have EBV infection in NK cells in skin lesions and peripheral blood.[28,29] HMB patients without systemic symptoms may eventually develop CAEBV.[28]

Prospective clinicopathologic analysis of CAEBV and other EBV$^+$ T/NK-LPD

Chronic active EBV infection, EBV-HLH, HMB, and HV are thus distinct but overlapping entities categorized as EBV$^+$ T/NK-LPD. The higher incidence of these diseases in East Asian countries and their occasional coincidence in a single patient imply a common pathogenesis.[7,30] Kimura *et al.* performed a large-scale prospective study of Japanese EBV$^+$ T/NK-LPD.[31] A total of 108 cases of EBV$^+$ T/NK-LPD (80 cases of CAEBV, 15 cases of EBV-HLH, nine cases of HMB, and four cases of HV) were analyzed. They found 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-cell subset, in contrast to the low incidence of EBV infection in this subset in the other EBV$^+$ T/NK-LPD. Most HMB patients (89%) had EBV-infected NK cells, whereas the majority (75%) of HV patients 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. Age of onset ≥8 years and liver dysfunction were risk factors for mortality, and transplant 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 developed these malignancies as a consequence of CAEBV.[32,33]

Characteristics of adult CAEBV

Chronic active EBV infection has been described mainly as a disease of childhood and young adulthood; the mean age of onset was estimated to be 11.3 years.[18] Recently, however, an increasing number of adult patients fulfilling the criteria of CAEBV has been reported. This may be a true increase in the incidence of adult-onset CAEBV or reflect improved recognition of this disease by physicians. Arai *et al.* reviewed 23 cases of adult-onset CAEBV and described the characteristics.[34] In 87% of adult cases, T cells were infected with EBV, whereas in childhood-onset cases, the T- 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 a 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 prevalence of CAEBV in East Asia and in natives of Central and South America implies a genetic background for its pathogenesis. Recently HLA-A*26, a major histocompatibility complex class I allele relatively common in East Asia, was found to be associated with an increased risk for EBV$^+$ T/NK-LPD.[35] Although the possible involvement of EBV strains with increased propensity to induce T/NK-cell lymphoproliferation cannot be formally denied, it is highly unlikely because outbreaks and familial transmission of CAEBV have not been reported. Arai *et al.* reported an intriguing case of CAEBV in which the patient experienced relapse after bone marrow transplantation.[36] A 35-year-old female patient with CAEBV of the CD8 type had HSCT from an unrelated male donor following myeloablative preconditioning with total body irradiation. The serologic HLA types of the patient and the donor were identical, whereas the DNA types were different in two HLA-DR alleles. Although the peripheral blood EBV-DNA was undetectable at 1 month after HSCT and remained so for nearly 12 months, the patient's EBV-DNA load increased again and reached 1.0×10^5 copies/μg DNA. EBV was found primarily in CD8$^+$ T cells again, but the EBV-infected cells now had 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 the CD8$^+$ T cells was different before and after bone marrow transplantation, suggesting that the 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 only by chance, these findings suggest that the patient may have had a certain genetic background that exerts its direct effects on cellular lineages unrelated to hematopoietic stem cells.

Pathophysiology of CAEBV

The pathogenesis of CAEBV is not understood. Most T and NK cells do not express the EBV receptor CD21, and the 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 to 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 CAEBV patients.[38] Given that 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 in CAEBV patients and cell lines derived from them do not express the most immunodominant EBNA3 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 CTL.[3,42–45] Hosts with normal immune functions are thus expected to have 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 recognition and/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 found to have mutations in the gene encoding perforin, which has a critical role in granule-mediated killing of target cells.[49] None of the other patients with CAEBV, however, were found 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-LPD implies that these diseases have a malignant nature. CAEBV, however, is a chronic disease and patients with clonal expansion of EBV-infected T or NK cells may remain in a 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, a premalignant or smoldering phase of EBV-positive leukemia/lymphomas. Ohshima *et al.* 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 research on their pathogenesis and therapy difficult. Imadome *et al.* transplanted peripheral blood mononuclear cells (PBMC) isolated from patients with CAEBV and EBV-HLH into 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 lesions in 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 cells of 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 *et al.*, unpubl. data 2012). 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 clue to the diagnosis of CAEBV. Although elevated serum antibody titers

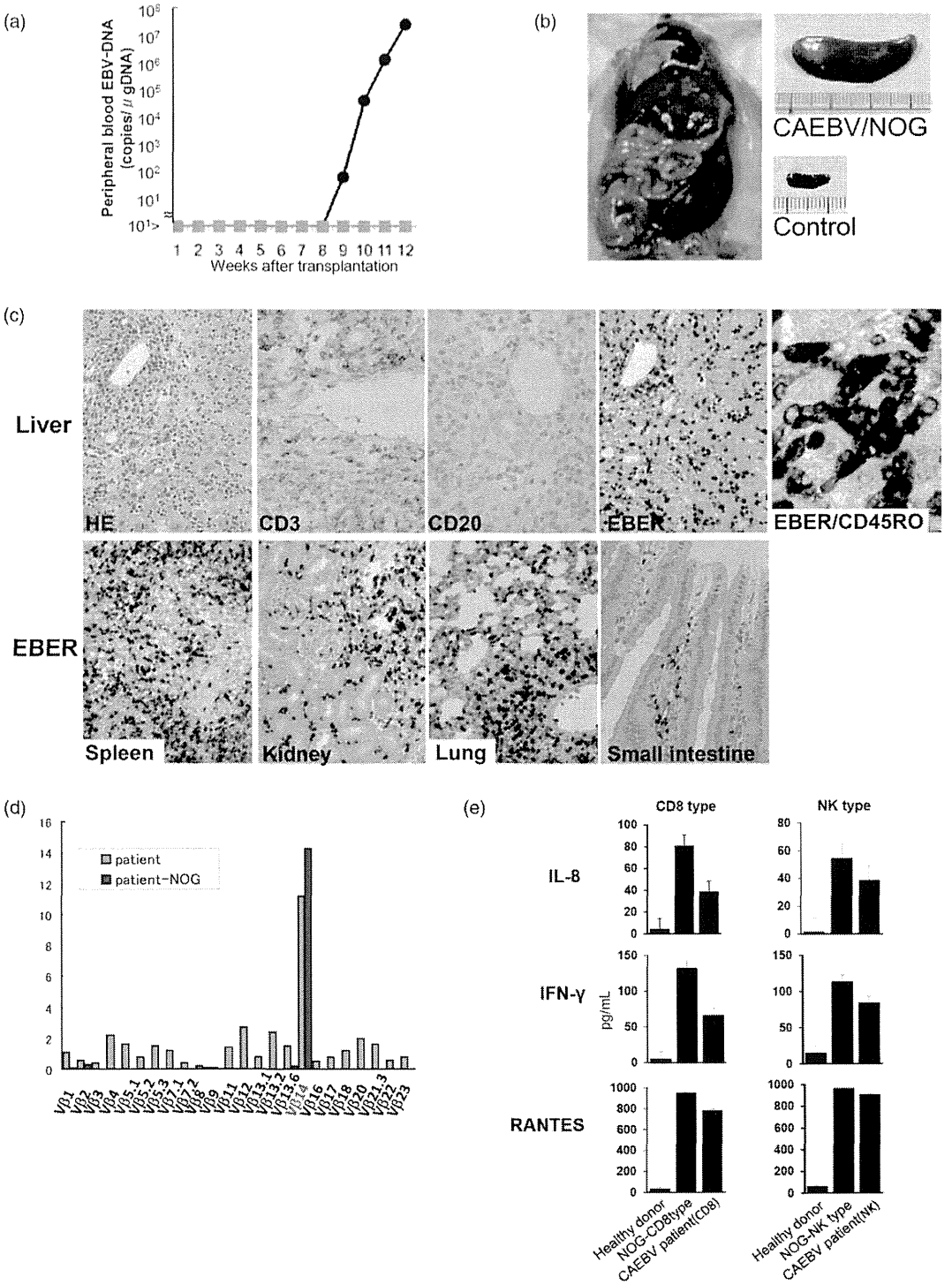

Fig. 1 Mouse xenograft model of chronic active Epstein–Barr virus infection (CAEBV). Peripheral blood mononuclear cells (PBMC) of a patient with the CD8 type CAEBV were transplanted i.v. into NOD/Shi-*scid Il2rg* ^{null} (NOG) mice. (a) Measurement of peripheral blood EBV-DNA. EBV-DNA load increased rapidly from approximately 9 weeks after transplantation, when (●) whole PBMC but not (□) isolated $CD8^+$ cells were transplanted. (b) Splenomegaly of a model mouse. (c) Pathological analysis. Histochemical analysis showed massive infiltration of EBV-encoded small RNA $(EBER)^+/CD20^-/CD3^+/CD45RO^+$ cells in most major organs including the spleen, kidneys, lungs, and small intestine. (d) T-cell receptor (TCR) repertoire analysis of peripheral blood T cells isolated from the patient and a mouse that received the patient's PBMC. An identical clone of EBV-infected T cells expressing Vβ14 is proliferating in the patient and the corresponding mouse. (e) Human cytokine levels in CAEBV model mice. Serum levels of interleukin (IL)-8, interferon (IFN)-γ, and regulated on activation, normal T-cell expressed and secreted (RANTES) were measured in mice that were transplanted with PBMC isolated from either a CD8-type or an NK-type CAEBV patient. The same set of cytokines was also quantified in the sera of the original patients and healthy donors. Modified from *PLoS Pathog.* 2011; **7**(10): e1002326.[51]

against EBV-encoded antigens are often found, this does not always occur, and normal titers of anti-EBV antibodies should not preclude the diagnosis of CAEBV.[7] Diagnostic criteria for CAEBV have been published.[13] Quantification of peripheral blood EBV-DNA is most important for diagnosis and a finding of elevation should be followed by identification of EBV-infected T or NK cells. Quantification 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 from the National Institute for Biological Standards and Controls, USA. Given that 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 clinical parameters is particularly important.

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

Diagnosis of CAEBV requires exact phenotyping of EBV-infected cells. This has usually been done with immunobead sorting of PBMC into lymphocyte subsets, followed by measurement of EBV-DNA in each subset using quantitative polymerase chain reaction. These processes are, however, time-consuming and require specific skills. Kimura *et al.* developed a new method termed "flow-cytometric *in situ* hybridization" (FISH) to phenotype EBV-infected cells (Fig. 2).[53,54] They utilized a fluorescence-labeled peptide nucleic acid (PNA) probe complementary to EBER and succeeded in detecting EBER on flow cytometry. Following reaction with antibodies specific to surface markers, PBMC were permeabilized and subjected to *in situ* hybridization with the PNA probe. EBER probes and surface-bound antibodies were then detected simultaneously on flow cytometry. EBV-infected cells with a certain phenotype can be directly counted using FISH, which is less laborious than the current method. They showed that FISH can be applied for the diagnosis of EBV^+ T/NK-LPD, and that EBV infects mainly γδT cells in HV.[53–55]

MicroRNA as a potential biomarker of CAEBV

MicroRNA (miRNA) is a small non-coding RNA of 18–25 nucleotides that 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–*Bam*HI fragment H rightward open reading frame 1 (miR-BHRF1) and miR–*Bam* HI A region rightward transcripts (miR-BART).[58] Kawano *et al.* reported that plasma levels of miR-BART 1-5p, 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 compared to those with inactive disease. miR-BART 13 level could differentiate patients with active disease from those with inactive disease, with a clear cut-off. Similarly, plasma miR-BART 2-5p and 15 levels could clearly differentiate patients with complete remission from others. Importantly, plasma EBV-DNA level did not show any significant correlation with these clinical parameters. These results suggest that EBV-encoded miRNA in plasma may be a useful biomarker for the diagnosis and monitoring of CAEBV.

Therapy of CAEBV

Various therapies have 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] The current event-free survival rate for CAEBV patients following HSCT is estimated to be 0.561 ± 0.086.[61]Very recently, Kawa *et al.* reported excellent results of HSCT following non-destructive pretreatment (reduced intensity hematopoietic stem cell transplantation; RIST).[62] For 18 pediatric patients with CAEBV who were treated with RIST, 3 year event-free survival was $85.0 \pm 8.0\%$ and the 3 year overall survival rate was $95.0 \pm 4.9\%$. HSCT is thus the therapy of choice for CAEBV, but HSCT is still accompanied by substantial risk and CAEBV patients have high risk 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.

Bortezomib, known as an inhibitor of 26S proteasome,[63] also has an inhibitory effect on the cellular transcription factor NF-κB. Because the survival and proliferation of EBV-transformed B cells are critically dependent on NF-κB activity, bortezomib has been shown to induce apoptosis in these cells.[64] Iwata *et al.* investigated the effect of bortezomib on EBV-infected T-cell lines including those derived from CAEBV.[65] Bortezomib induced apoptosis in all human T-cell lymphoma cell lines examined, whether or not they were infected with EBV. In addition, bortezomib induced the expression of EBV lytic-cycle genes *BZLF1* and *gp350/220*, as has been reported for EBV-infected B-cell lines.[66] Bortezomib also induced apoptosis specifically in EBV-infected T or NK cells cultured *ex vivo* from patients with EBV^+T/NK-LPD.

Valproic acid is a widely used anti-epileptic drug and is also known as a potent histone deacetylase (HDAC) inhibitor. HDAC inhibitors have potent anticancer activities with proven efficacy in various human malignancies. Valproic acid induces lytic infection in EBV-infected B-lymphoblastoid and gastric carcinoma cell lines and thereby potentiates the effects of chemotherapeutic agents both *in vitro* and *in vivo*.[67] Iwata *et al.* examined the effect of valproic acid on EBV-infected T and NK cell lines.[68] They found that this agent induces apoptosis in human EBV-infected T and NK cells. Use of the drug with the NF-κB inhibitor bortezomib had an additive effect. In contrast to the previous results with EBV-infected B-cell lines, valproic acid did not induce lytic infection in the virus-infected T- and NK-cell lines, indicating that the apoptosis-inducing effect of valproic acid is not dependent on induction of EBV lytic cycle.

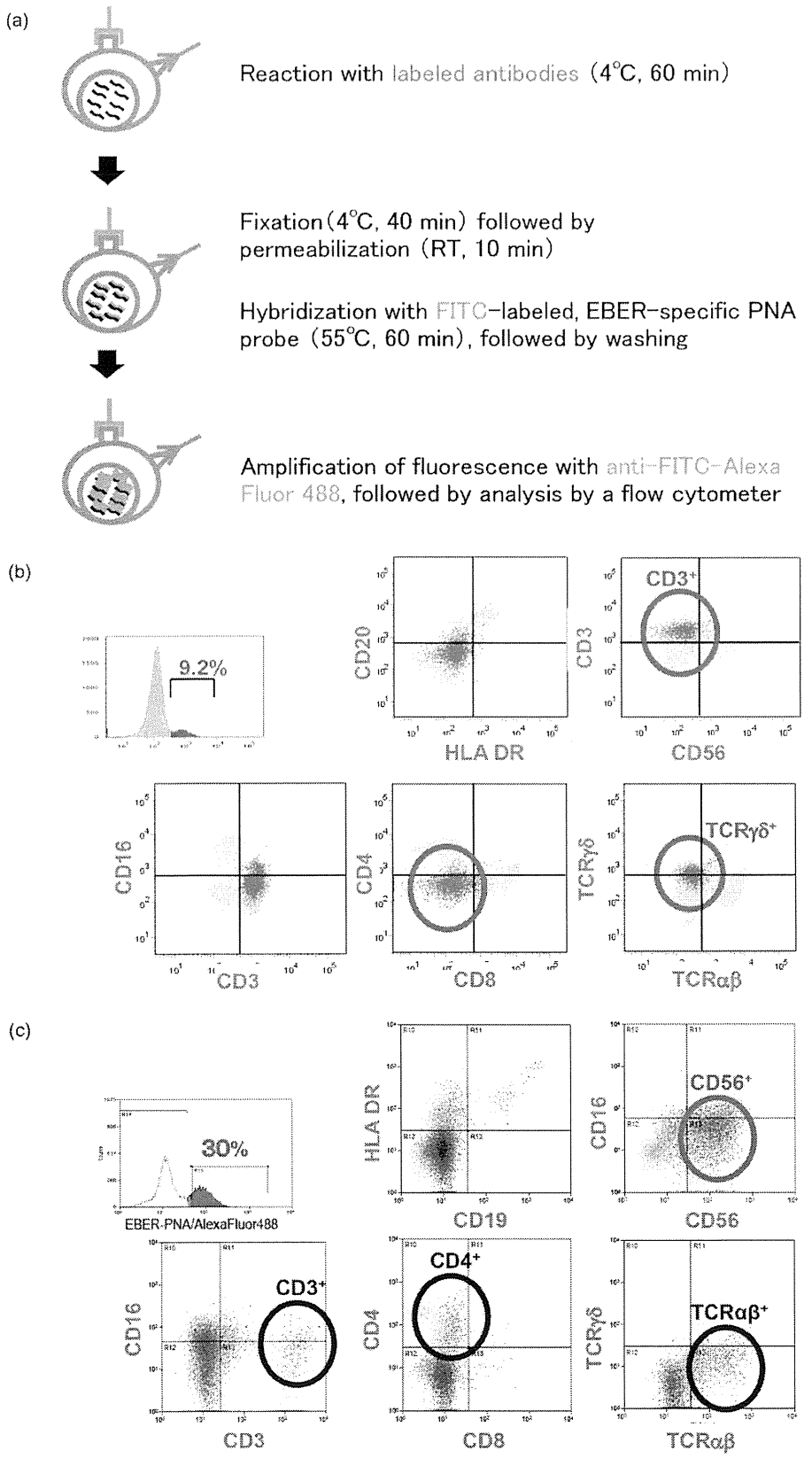

Fig. 2 Flow-cytometric *in situ* hybridization (FISH). (a) Protocol of FISH. (b) Results of FISH in a patient with hydroa vacciniforme. Red, EBER-positive cells; blue, EBER-negative cells. Most EBV-infected cells in the peripheral blood of this patient had the phenotype $CD3^+/CD4^-/CD8^-/TCR\gamma\delta^+$. (c) Results of FISH in a patient with the NK-cell type chronic active Epstein–Barr virus infection. Red, EBER-positive cells; blue, EBER-negative cells. The majority of EBV-infected cells in the peripheral blood of this patient were $CD56^+$ NK cells. Also, a small proportion of $TCR\alpha\beta^+/CD3^+/CD4^+$ cells also contained EBV. EBER, Epstein–Barr virus-encoded small RNA; FITC, fluorescein isothiocyanate; PNA, peptide nucleic acid; RT, reverse transcription.

Perspective

Significant progress has been made in the research of many aspects of CAEBV, including pathophysiology, diagnosis, monitoring, and therapy, but the fundamental cause of the disease has not been elucidated. The recent development of novel technologies for genetic analysis, including new-generation sequencing, may enable identification of genetic alterations responsible for CAEBV. Given that CAEBV is an uncommon disease, it may sometimes take years for the correct diagnosis to be reached. The advanced techniques required for this also make the diagnosis of CAEBV difficult. Although there is a consensus that early HSCT produces a better result, the decision to have HSCT is often difficult, especially when the patient is in a stable condition without severe symptoms. Establishing a standard clinical guideline for the diagnosis and treatment of CAEBV will alleviate these problems and facilitate quick and accurate diagnosis, followed by timely intervention with the right choice of treatment.

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ORIGINAL RESEARCH

Role of latent membrane protein 1 in chronic active Epstein–Barr virus infection-derived T/NK-cell proliferation

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Keywords
AKT, CAEBV, dominant negative, LMP1, NFκB

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Abstract

Epstein–Barr virus (EBV) predominantly infects B cells and causes B-cell lymphomas, such as Burkitt lymphoma and Hodgkin lymphoma. However, it also infects other types of cells, including T and natural killer (NK) cells, and causes disorders, such as chronic active EBV infection (CAEBV) and T/NK-cell lymphoma. The CAEBV is a lymphoproliferative disease with poor prognosis, where EBV-positive T or NK cells grow rapidly, although the molecular mechanisms that cause the cell expansion still remain to be elucidated. EBV-encoded latent membrane protein 1 (LMP1) is an oncogene that can transform some cell types, such as B cells and mouse fibroblasts, and thus may stimulate cell proliferation in CAEBV. Here, we examined the effect of LMP1 on EBV-negative cells using the cells conditionally expressing LMP1, and on CAEBV-derived EBV-positive cells by inhibiting the function of LMP1 using a dominant negative form of LMP1. We demonstrated that LMP1 was responsible for the increased cell proliferation in the cell lines derived from CAEBV, while LMP1 did not give any proliferative advantage to the EBV-negative cell line.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous human virus that belongs to the γ-herpesvirus subfamily. A primary, acute infection of EBV in adolescence can cause infectious mononucleosis, and the virus is also associated with many types of tumor, including Burkitt lymphoma, nasopharyngeal carcinoma (NPC), and posttransplant lymphoproliferative disorder [1, 2]. EBV generally infects B cells via CD21 on the cell surface [3, 4], but it can also rarely infect T or natural killer (NK) cells by unknown, CD21-independent mechanisms [5]. The infection of T or NK cells can cause diseases with poor prognosis, including chronic active EBV infection (CAEBV), extranodal NK/T-cell lymphoma (ENKTL), nasal type and aggressive NK-cell leukemia (ANKL) [5].

CAEBV occasionally results in severe, chronic or recurrent infectious mononucleosis-like symptoms, such as

fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, and pancytopenia, and has high mortality [6]. Despite its severity, the therapeutic options are limited and adequate therapies are not yet established; patients are currently treated with anti-cancer or immunoregulatory drugs and/or a bone marrow transplant. Recent reports have suggested that bone marrow transplants give promising results, but this is a high-risk procedure and so the development of safe, effective, and specific alternative therapies remains important. The development of novel drugs for the treatment of CAEBV has been hampered by a limited number of cases, and also by a poor understanding of the disease pathogenesis. Therefore, understanding the molecular mechanisms of the dysregulated cell proliferation in CAEBV is critical. The clonal expansion of EBV-infected T or NK cells during the development and maintenance of CAEBV is the major factor that contributes to poor prognosis. We are thus determined to identify the factors responsible for the dysregulated cell division of T or NK cells in this lymphoproliferative disorder.

In cells transformed by EBV, the virus exists in a latent infection state which is characterized by a limited expression of viral proteins and RNAs [1]. Neoplasms such as Burkitt lymphoma or gastric carcinoma typically express only the EBV-encoded RNAs (EBERs) and EBV nuclear antigen 1 (EBNA1) (latency type I), whereas some Hodgkin lymphoma, NPC, and T/NK lymphomas produce EBERs, EBNA1, and latent membrane protein 1 (LMP1) and LMP2 genes (latency type II). In addition to type II genes, EBNA2, EBNA3, and EBNA-LP are also expressed in most cases of immunosuppression-related lymphomas and lymphoblastoid cell lines (LCLs) (latency type III) [1, 5].

One of the EBV-encoded genes expressed in the latency II or III state, LMP1 is an oncogene that plays a well-established role in B lymphocytic tumors and NPC [2, 7, 8]. LMP1 can transform a several cell types by mimicking the activated form of human CD40 [9, 10]. LMP1 is a membrane protein with tandem six transmembrane domains and C-terminal signaling domains (please see Fig. 4A). The six transmembrane domains are responsible for the oligomerization of LMP1, which is prerequisite for its precise function. The C-terminal regions contain two functional domains: transformation effector site 1 (TES1)/C-terminal activating region 1 (CTAR1) and TES2/CTAR2. Both domains promote cell proliferation via the NFκB, AKT, and c-Jun N-terminal kinase (JNK) signaling pathways. TES1 activates NFκB and AKT signaling pathways via tumor necrosis factor receptor (TNFR)-associated factors (TRAFs), whereas TES2 induces the activation of NFκB and JNK/AP1 activations by stimulating the indirect assembly of TRAFs mediated by receptor-interacting protein (RIP1), TNFR-associated death domain (TRADD), and BS69. Then LMP1 is thought to be the possible cause of the increased cell proliferation in CAEBV, although there is still no direct evidence to support this.

In this study, we investigated the effect of LMP1 on an EBV-negative (Jurkat) and CAEBV-derived T (SNT16), NK (KAI3) cell lines. First, we developed two Jurkat-derived cell lines that conditionally express LMP1 and examined the effect of LMP1 on EBV-negative T cells. Then we assessed whether proliferation of T/NK cells in CAEBV requires LMP1 by inhibiting endogenous LMP1, using a dominant negative (DN) form of the protein. Although the exogenous expression of LMP1 failed to enhance proliferation in Jurkat cells, we confirmed contribution of the membrane protein to enhanced cell proliferation of T/NK cells isolated from a CAEBV patient.

Material and Methods

Plasmids and cell lines

LMP1 DNA derived from the B95-8 strain was subcloned into the *Hind*III and *Eco*RV sites of pTRE-Tight expression vector. The vector overexpressing DN-LMP1 was kindly provided by J. S. Pagano [11]. The DN effects of this mutant LMP1 have been confirmed previously [12, 13].

Jurkat is an EBV-negative T cell line derived from a human acute T-cell leukemia. The cells were cultured at 37°C with 5% CO_2 in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 4 mmol/L L-glutamine, and 100 U/mL penicillin and streptomycin. Jurkat Tet-On (JT) is a Jurkat subclone that expresses the doxycycline (Dox)-regulated transactivator, Tet-On, and was purchased from Clontech. Cell clones that conditionally express LMP1 by the addition of Dox were constructed by stable transformation of the plasmid vector described above into JT cells. Transfection was carried out by electroporation using the Invitrogen Neon transfection system following the manufacturer's recommendations. For the cloning of cell lines, the transformed cells were diluted to 0.3 cells per well and incubated in 50% conditioned medium prepared from JT cell cultures at the proliferative stage. Two JT cell lines that express LMP1 in response to Dox, JTL1-1, and JTL1-2, were successfully isolated by limited dilution. These cells were cultured with 100 μg/mL G418.

SNT16 and KAI3 are an EBV-positive T and NK cell line, respectively, derived from patients of CAEBV [14, 15]. EBV shows latency type II in SNT16 and KAI3, and these cells expresses LMP1 endogenously [16]. SNT16 and KAI3 cell

lines were cultured in RPMI1640 supplemented with 10% FBS, 4 mmol/L L-glutamine, 100 U/mL penicillin and streptomycin, and 100 U/mL interleukin-2 (Primmune Inc., Kobe, Japan).

One-step multiplex real-time RT-PCR

RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Approximately 400 ng RNA was obtained from 5×10^5 cells. LMP1 mRNA samples were quantified by one-step multiplex real-time RT-PCR using a Quantitect multiplex RT-PCR kit (Qiagen) and an Mx3000P real-time PCR system (Stratagene, La Jolla, CA) with primers and probes, as described previously [17]. All samples were analyzed in triplicate. The expression of LMP1 mRNA was determined by comparing the expression of LMP1 to β2 microglobulin (β2m) mRNA as the endogenous control.

Western blotting

Cell extracts were diluted in sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol, and 0.0025% bromophenol blue) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were loaded at the same protein concentration for each experiment. The primary antibodies used were anti-LMP1 antibody (S12) at 1:50, anti-actin antibody (AC-74, Sigma, St. Louis, MO) at 1:5000, anti-phospho-AKT antibody (#4058, Cell Signaling Technology, Danvers, MA) at 1:1000, anti-AKT antibody (#9272, Cell Signaling Technology) at 1:1000, anti-NFκB (p65) antibody (610868, BD Biosciences, Franklin Lakes, NJ) at 1:250, anti-IκBα antibody (#4814, Cell Signaling Technology) at 1:1000, anti-caspase-3 antibody (#9662, Cell Signaling Technology) at 1:1000, and anti-poly(ADP-ribose) polymerase (PARP) antibody (C-2-10, Sigma) at 1:2000. The secondary antibodies used were Goat Anti-Mouse Ig's HRP Conjugate (AMI3404, BioSource International, Camarillo, CA) and HRP-Goat Anti-Rabbit IgG (H+L) (656120, Invitrogen, Carlsbad, CA). The bands were visualized using WESToneTM Western Blot Detection System (iNtRON Biotechnology, Seongnam, Korea) or Chemi-Lumi One Super (Nacalai tesque, Kyoto, Japan).

Cell proliferation

Cells (2×10^5 per mL) were cultured for 4 days in the presence of each concentration of Dox as indicated. Live cells were counted on a hematocytometer using trypan blue exclusion at the indicated days.

Cell cycle analysis

After the treatment with 0 or 1000 ng/mL Dox for 2 or 3 days, JT and JTL1-2 cells were fixed with 70% ethanol, and then washed with phsophate buffered saline (PBS). The fixed cells were treated with RNase, stained with 50 μg/mL propidium iodide for 15 min, and then analyzed by flow cytometry using flow cytometry (FACS) Calibur (Becton Dickinson, Franklin Lakes, NJ) and ModFit LT software (Verity Software House, Topsham, ME).

Apoptosis assay

Apoptosis was assessed by flow cytometry using a PE Annexin V Apoptosis Detection Kit I (BD Pharmingen Biosciences, Franklin Lakes, NJ) according to manufacturer's instructions. Briefly, JT and JTL1-2 cells were treated with or without 1000 ng/mL Dox for 48 h, and washed with PBS. Then they were resuspended in binding buffer, incubated in the presence of Annexin V and 7-AAD for 15 min in the dark, and then analyzed using a FACSCanto II flow cytometer and Cell Quest software (Becton Dickinson).

Transient transfection

SNT16, Jurkat, and KAI3 cells were transfected with empty pcDNA3 vector or vector expressing DN LMP1 (DN-LMP1) under the control of CMV promoter. The DN-LMP1 has point mutations that change the PXQXT motif in the TES1 domain to AXAXT, and YYD in the tail of TES2 to IID, dysregulating its signaling activity [11]. The original DNA sequence is derived from EBV B95-8 strain. The Neon transfection system (100 μL Kit) (Invitrogen) was used for electroporation following the manufacturer's protocols.

Results

Isolation of two T-cell clones that conditionally express LMP1

In order to evaluate the effects of the viral oncogene, LMP1, on cell proliferation in T cells, we prepared cell clones from the Jurkat background that express LMP1 by the addition of Dox. Two clonal cell lines (JTL1-1 and JTL1-2) conditionally expressing LMP1 were obtained successfully by electroporation and limiting dilution as mentioned in the Material and Methods. The induction of LMP1 by Dox was confirmed using RT-PCR and western blotting (Fig. 1). Each clone expressed LMP1 in the Dox dose-dependent manner. The LMP1 mRNA expression in JTL1-1 and JTL1-2 was comparable to the expression in

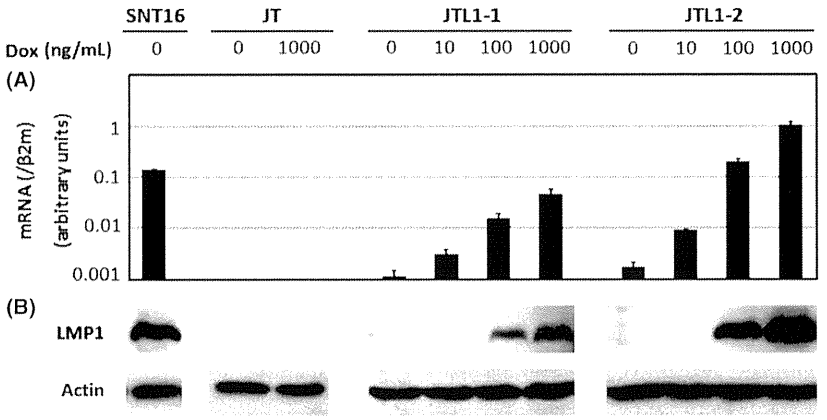

Figure 1. Constructions of LMP1-expressing Jurkat cells. The conditional expression of LMP1 was induced by treatment with 0, 10, 100, or 1000 ng/mL doxycycline (Dox). Cell extracts from JT, JTL1-1, and JTL1-2 cells were harvested from each experiment 2 days after the addition of Dox. (A) The quantification of LMP1 mRNA by RT-PCR. The relative expression of LMP1 mRNA is shown after normalization to β2 microglobulin (β2m) mRNA. (B) The expression of LMP1 protein was analyzed, along with actin, by western blotting.

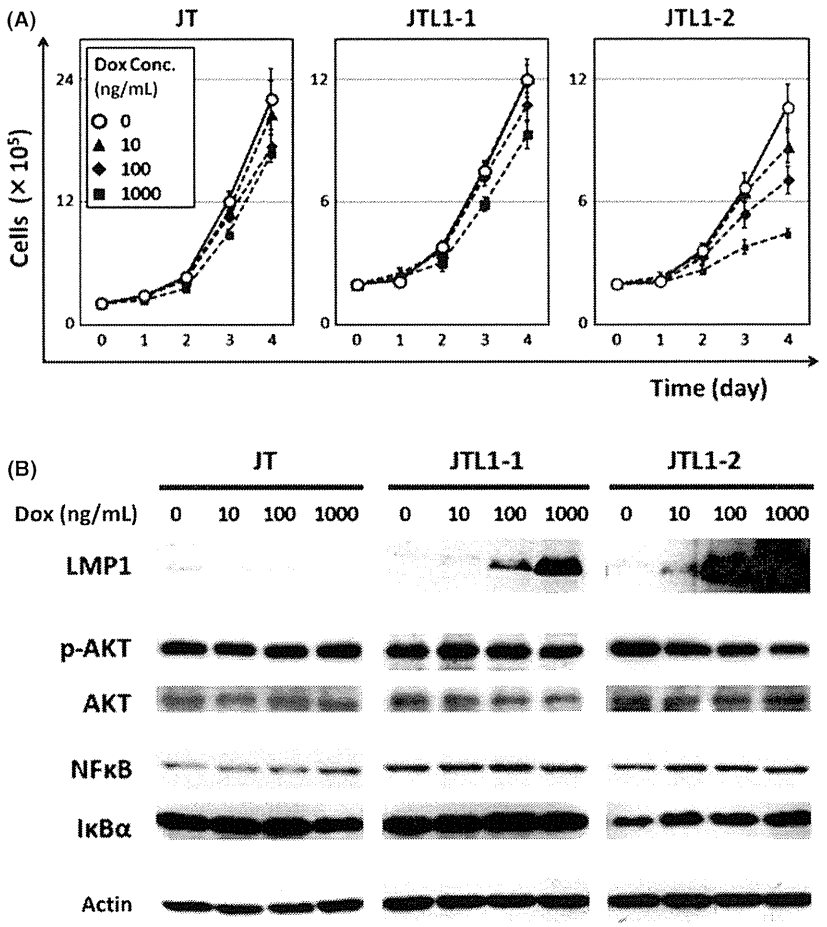

Figure 2. Cell proliferation and levels of signaling molecules in JT, JTL1-1, and JTL1-2 cells. (A) Cell proliferation was assessed by trypan blue staining, followed by cell counting, at days 0, 1, 2, 3, and 4 after the induction of LMP1 with 0, 10, 100, or 1000 ng/mL Dox. Experiments were performed in triplicate, and standard errors and means are shown. (B) Cell extracts harvested 2 days after Dox induction (0, 10, 100, 1000 ng/mL) were analyzed by western blotting. AKT and NFκB signalings were assessed by AKT phosphorylation and the expression of NFκB (p65) and IκBα, respectively.