2000; Mahy et al., 2002; Sumer et al., 2003; Marenduzzo et al., 2007).

In EBV, both the latent ori-P and the lytic ori-Lyt are bound to the nuclear matrix (Jankelevich et al., 1992; Mattia et al., 1999). EBV nuclear antigen leader protein (EBNA-LP) is a nuclear matrix-associated protein, and its nuclear matrix-targeting signal is a 10-aa segment, which also functions as a NLS (Yokoyama et al., 2001). However, this is not the case for LANA, because it was reported that C-terminal truncation up to 1128 aa could not be localized in a high salt-resistant fraction (nuclear matrix fraction), but the C-terminal region (1129–1143 aa) is high salt extractable (Viejo-Borbolla et al., 2003). We previously showed that the N-terminal region up to 107 aa is localized in nucleo-cytoplasmic and chromatin fractions (Ohsaki et al., 2009). The localization to the nuclear matrix fraction of LANA might depend on the conformation of LANA or post-translational modifications.

On the other hand, a cell fractionation assay has shown that cellular pre-RC components, such as ORC2, Cdc6, and Cdt1, preferentially localize in the nuclear matrix fraction in a cell cycle-dependent manner, and LANA itself also can localize in the nuclear matrix fraction. Accordingly, TR accumulates in the nuclear matrix fraction during the late G1 phase, suggesting that LANA recruits the ori-P to the nuclear matrix, so that cellular replication machinery is abundant and available for viral DNA replication during latency (Ohsaki et al., 2009; **Figure 3**).

GENOME SEGREGATION MECHANISMS OF KSHV IN LATENCY

The same copy number of KSHV genomes appears to be maintained in daughter cells after every cell division (Ballestas et al., 1999; Ueda et al., 2006), indicating that a strict genome maintenance mechanism is working. In the KSHV-infected PEL cell lines, LANA associates with a condensed mitotic chromatin (Ballestas et al., 1999; Cotter and Robertson, 1999; Tetsuka et al., 2004). It was reported that MeCP2, a methyl CpG-binding protein, interacts with the N-terminal of LANA and that DEK protein interacts with the C-terminal of LANA. These two independent interactions are involved in the tethering of LANA to chromosomes (Krithivas et al., 2002). Various studies have reported the interaction between LANA and multiple cellular proteins associated with chromatin.

Brd4, which is a member of the BET family that carries two bromodomains and associates with mitotic chromosomes, interacts with LANA on mitotic chromosomes (You et al., 2006). It has also been reported that core histones such as H2A and H2B are essential for LANA N-terminal chromosome binding (Barbera et al., 2006a,b). Furthermore, LANA interacts with Brd2/Ring3, which is a member of the BET family of double bromodomain-containing genes and contains two tandem bromodomains (Viejo-Borbolla et al., 2005).

Xiao et al. (2010) reported that LANA is associated with centromeres via the formation of complexes with Cenp-F and Bub1, which are kinetochore-associated proteins. This suggests that LANA preferentially interacts with kinetochore-associated proteins and that its association is critical for segregation into daughter cells. Though the interaction of LANA with kinetochore factors might interfere with correct spindle formation, it suggests that LANA should support viral genome segregation along with condensed chromatin.

A NuMA plays a critical role in the nuclear architecture in the interphase. After nuclear envelope breakdown in mitosis, NuMA is hyperphosphorylated by $p34^{cdc2}$ and is distributed at spindle poles, where it remains until the anaphase and plays an essential role in tethering spindle microtubules to each pole (Merdes et al., 1996; Gehmlich et al., 2004). Although NuMA drastically alters the localization and functions in the interphase and M phase, biochemical fractionation analysis shows that NuMA is localized in

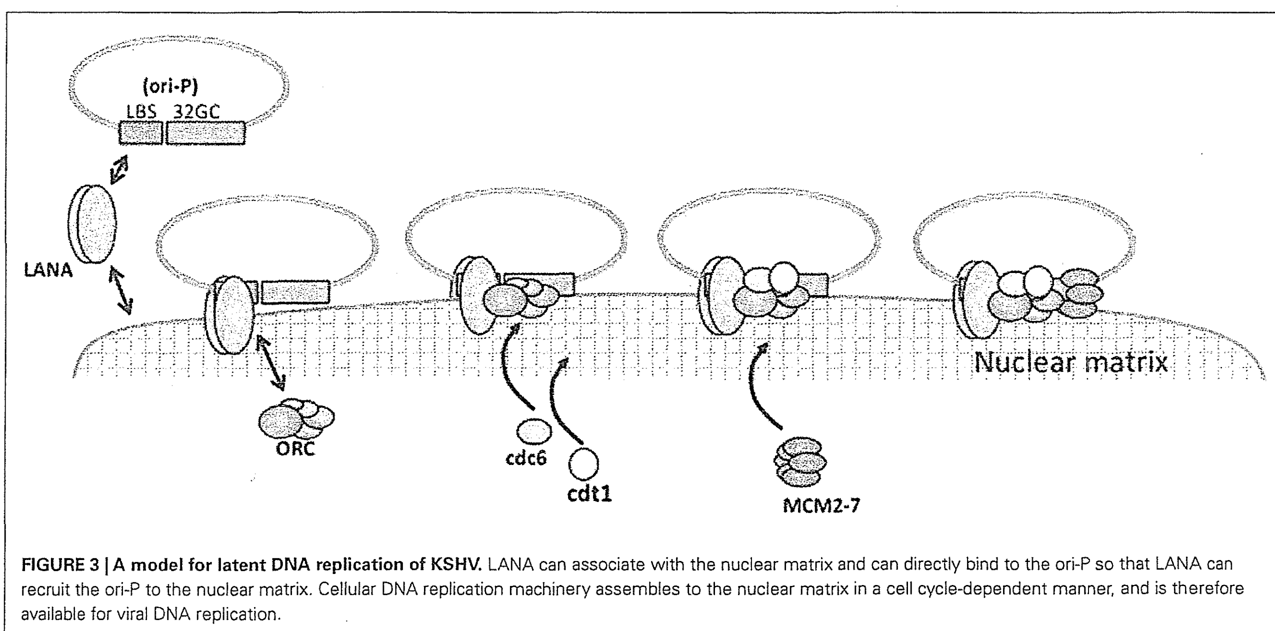

FIGURE 3 | A model for latent DNA replication of KSHV. LANA can associate with the nuclear matrix and can directly bind to the ori-P so that LANA can recruit the ori-P to the nuclear matrix. Cellular DNA replication machinery assembles to the nuclear matrix in a cell cycle-dependent manner, and is therefore available for viral DNA replication.

the insoluble nuclear matrix fraction in both phases. A recent study demonstrated that the C-terminus of LANA was co-localized with NuMA during the interphase and that the knockdown of NuMA expression caused the disruption of genome segregation and TR-containing plasmid maintenance (Bhaumik et al., 2008) and thus, KSHV genome segregation is disrupted in the absence of NuMA. Through siRNA and knockdown strategies in mice, NuMA has been shown to be an essential protein for early embryogenesis and cellular proliferation (Harborth et al., 2001; Silk et al., 2009), and it is thus unclear how the interaction with LANA actually works for viral genome segregation.

In either case, LANA has the capability of associating with various cellular proteins so that KSHV can maintain the genome stably if the cells are divided into two daughter cells. Nuclear matrix proteins function as a scaffold of DNA replication, transcription, and repair during the interphase, and also play an essential role in the segregation of condensed chromosomes in mitosis. Condensed chromosomes include, for example, NuMA, which can behave as a component of a spindle pole during mitosis, or Cenp-F, which is a nuclear matrix protein during the interphase and is distributed to kinetochores in mitosis. Taken together, the previous and present results suggest that nuclear architectures such as the nuclear matrix have essential roles not only in DNA replication and transcription but also in genome segregation during mitosis (**Figure 4**).

CONCLUSION AND PERSPECTIVES FOR FUTURE ANALYSIS

The importance of nuclear architecture is increasingly recognized as important in various nuclear events, such as DNA replication, transcription, and DNA repair. It is well accepted that the chromosomes are organized into distinct territories in the interphase. These distributions of chromosomes are closely related to the place for active or inactive transcription, the presence of DNA replication machinery, and the formation of higher-order structures of chromatin loops. DNA looping appears to be mediated by attachment to the nuclear matrix and thus achieves transcriptional control (Ostermeier et al., 2003). Other studies have suggested that the gene-rich chromosomes are frequently located in the nuclear interior. On the other hand, gene-poor chromosomes are located in the nuclear periphery (Tanabe et al., 2002; Reddy et al., 2008). A recent study shows that the transcriptional silencing might be accomplished by the binding of a specific promoter region to lamin type A (Lee et al., 2009). It is reported that replication foci at the middle to late S phase are also preferentially located toward the nuclear periphery, whereas early replication foci are located in the nuclear interior (Grasser et al., 2008) or throughout the nucleus (Izumi et al., 2004).

The spatial and temporal analyses using live-cell imaging revealed that replication forks are generated at the same origin and are closely associated during replication (Kitamura et al., 2006). Interestingly, two replication loci, located at the same distance from the origin, were in closer proximity when DNA replication took place at these loci, after which they moved apart from each other after replication. It is speculated that the replication factory anchors some region and that replicated DNA can move away from a replication factory immediately after DNA synthesis. This anchored region may be a nuclear scaffold such as the nuclear matrix.

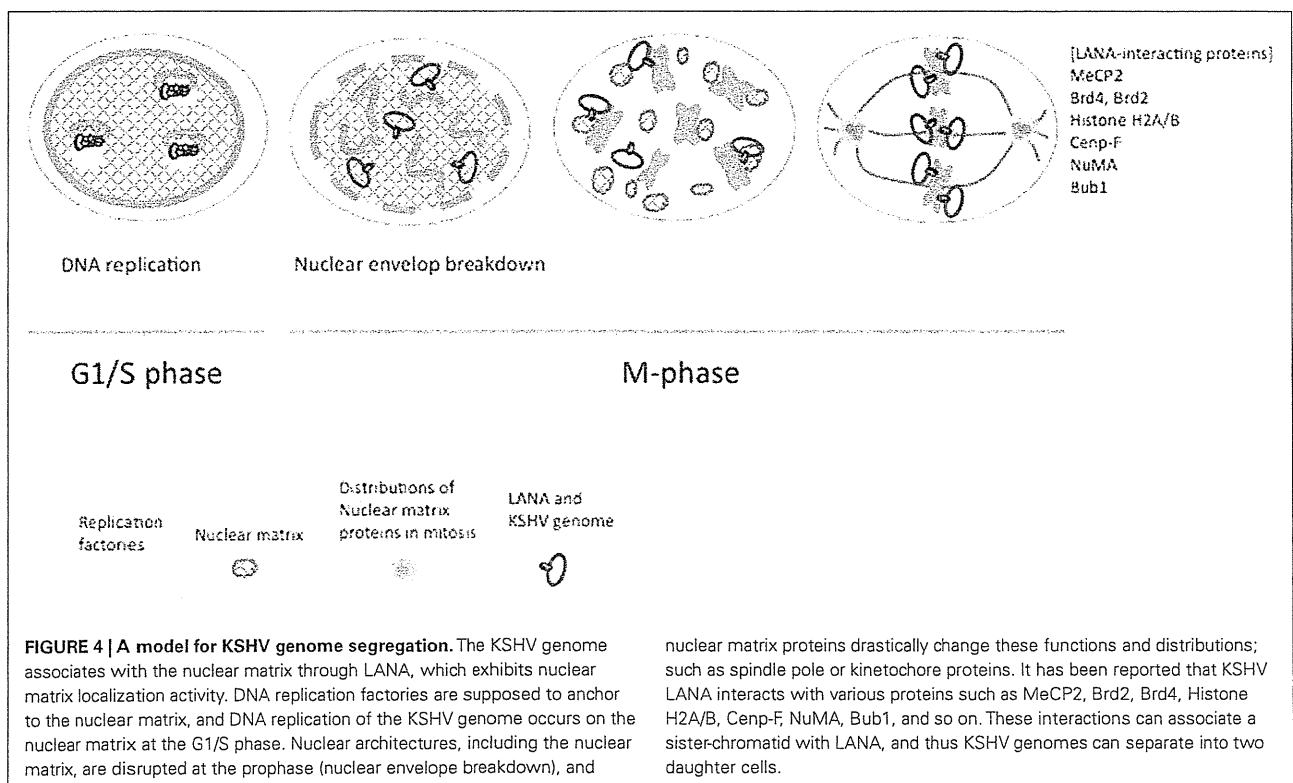

FIGURE 4 | A model for KSHV genome segregation. The KSHV genome associates with the nuclear matrix through LANA, which exhibits nuclear matrix localization activity. DNA replication factories are supposed to anchor to the nuclear matrix, and DNA replication of the KSHV genome occurs on the nuclear matrix at the G1/S phase. Nuclear architectures, including the nuclear matrix, are disrupted at the prophase (nuclear envelope breakdown), and nuclear matrix proteins drastically change these functions and distributions; such as spindle pole or kinetochore proteins. It has been reported that KSHV LANA interacts with various proteins such as MeCP2, Brd2, Brd4, Histone H2A/B, Cenp-F, NuMA, Bub1, and so on. These interactions can associate a sister-chromatid with LANA, and thus KSHV genomes can separate into two daughter cells.

The cellular environment is critical for viral survival, for which viruses make full use of the cellular machinery. The analysis of insoluble proteins, including nuclear matrix proteins, is difficult for the investigation of protein–protein and protein–DNA interactions *in vitro*, because the conditions of *in vitro* experiments always include soluble fractions. To overcome these problems, recent new approaches, such as genome-wide analysis using chromatin immunoprecipitation or live-cell imaging that reflects the *in vivo* environment, may be more powerful and accurate.

As for KSHV genome replication and maintenance, it would be first necessary to clarify how LANA is involved in both. Considering the real and actual cellular environments, we need to develop more powerful tools to know what the virus does in cells.

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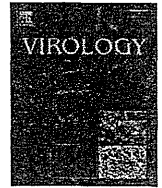

Novel monoclonal antibodies for identification of multicentric Castleman's disease; Kaposi's sarcoma-associated herpesvirus-encoded vMIP-I and vMIP-II

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ABSTRACT

Recent studies have indicated that vMIP-I and vMIP-II play important roles in the pathogenesis of Kaposi's sarcoma-associated herpesvirus (KSHV)-related diseases due to the effects of these proteins on vascularization. We developed monoclonal antibodies against KSHV-encoded viral macrophage inflammatory protein-I (vMIP-I) and vMIP-II to study these expression profiles and reveal the pathogenesis of KSHV-related diseases. The MAbs against vMIP-I and vMIP-II reacted to KSHV-infected cell lines after lytic induction. Both vMIP-I and the vMIP-II gene products were detected 24 h post-induction with 12-O-tetradecanoylphorbol-13-acetate until 60 h in the cytoplasm of primary effusion lymphoma cell lines. In clinical specimens, both vMIP-I and vMIP-II gene products were detected in the tissues of patients with multicentric Castleman's disease. On the other hand, only vMIP-II was detected in a subset of Kaposi's sarcoma. We concluded that these antibodies might be powerful tools to elucidate the pathogenesis of KSHV-related diseases.

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus originally identified in HIV-positive Kaposi's sarcoma (KS) tissues (Chang et al., 1994). KSHV is responsible for AIDS associated cancers such as Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Cesarman et al., 1995; Schalling et al., 1995; Soulier et al., 1995). As is the case for all herpesviruses, KSHV has two life cycles, one latent and the other lytic. Lytic gene expression can be induced by the treatment of latently infected cells with chemical agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate (Arvanitakis et al., 1996; Miller et al., 1997). It has been demonstrated that two KSHV-encoded chemokines, K6 (which encodes a vMIP-I) and K4 (which encodes a vMIP-II), are expressed in the course of lytic infection (Moore et al., 1996; Sun et al., 1999). Previous reports showed that both vMIP-I and vMIP-II induced Ca^{2+} signal transduction via certain chemokine receptors and the receptor-dependent migration of cells (Benelli et al., 2000; Chen et al., 1998; Endres et al., 1999; Kledal et al., 1997). In addition, in a chick chorioallantoic membrane assay, the both proteins showed strong angiogenic properties (Boshoff et al., 1997). However, little is known about the contribution of vMIPs to KSHV malignancy under physiologic conditions.

In this report, we generated new monoclonal antibodies against vMIP-I and vMIP-II, and confirmed the detection of both vMIP-I and vMIP-II in histological sections of tissues from MCD patients as well as in KSHV-infected PEL cell lines. In cases of KS, vMIP-II was detected, but not vMIP-I. These results suggest that the expression properties of vMIP-I and vMIP-II might be related to KSHV-associated diseases, and may even be involved in the generation of diseases. Thus, antiviral chemokine MAbs could potentially become useful tools for the diagnosis of KSHV-related diseases.

Materials and methods

Cells

Kaposi's sarcoma-associated herpesvirus-positive cell lines (BC-1, BC-3, BCBL-1 and TY-1 cells) and a negative cell line (BJAB cells) were

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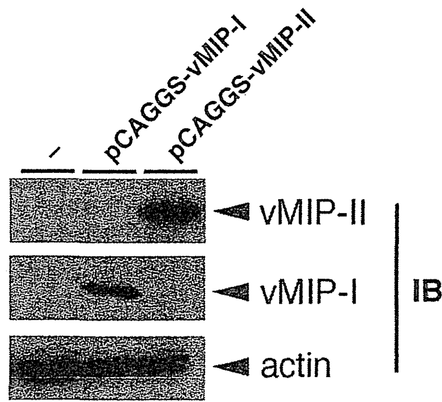

Fig. 1. Cross reactivity between anti-vMIP-I and anti-vMIP-II MAb. 293 T cells were transfected with either 2 μg of pCAGGS- vMIP-I or 2 μg of pCAGGS-vMIP-II plasmids. Forty-eight hours after transfection, the cells were harvested and expression of vMIP-I or vMIP-II was tested by Western blot analysis using the anti-vMIP-I or -vMIP-II MAb, respectively. Actin was also probed with anti-actin monoclonal Ab as a loading control.

obtained from the American Type Culture Collection (ATCC) (Manassas, VA). These cells were grown in RPMI 1640 (Nakalai Tesque, Inc., Kyoto, Japan) supplemented with 10 IU/ml penicillin G, 10 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 5% CO_2 atmosphere. In addition, 293 T and 293/EBNA (Clontech) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Nakalai Tesque, Inc.) supplemented with 10 IU/ml penicillin G, and 10 μg/ml streptomycin, 10% FBS, and 200 mM L-glutamine.

Plasmids

In order to express vMIP-I and vMIP-II, the ORFs were cloned into the pCAGGS eukaryotic expression vector, and pCAGGS-vMIP-I and pCAGGS-vMIP-II were established. The plasmid vector, pCAGGS was kindly provided by Dr. J. Miyazaki of Osaka University (Niwa et al., 1991). Briefly, fragments including vMIP-I and vMIP-II ORFs were amplified by PCR using the following primer sets: vMIP-I-Met (5′-CGGTACCGAATTCTCCAGATGGCC-3′) and vMIP-I-Ter (5′-ACTCGA-GAATTCTACTTGTCATCGTCGTCCTTGTAGTCGGAAGCTATGGCAGGCAG-3′); and vMIP-II-Met (5′-AGGTACCGAATTCAGTTATGGACACCAAGGGC-3′) and vMIP-II-Ter (5′-ACTCGAGAATTCTACTTGTCATCGTCGTCCTTG-TAGTCGGAGCGAGCAGTGACTGG-3′). The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. After digestion with *Eco*RI, the fragments were ligated into the *Eco*RI site of the pCAGGS vector. Then, the DNA fragments encoding vMIP-I and vMIP-II were liberated by *Eco*RI, and were inserted into pCAGGS to generate the expression vectors pCAGGS-vMIP-I and -vMIP-II, respectively. vMIP-I (pGEX-vMIP-I) and vMIP-II (pGEX-vMIP-II) were also generated using PCR-based technology using BCBL-1 genomic DNA as a templete. The coding region, without a signal peptide, was amplified with vMIP-I-Eco (CAGAATTCGCGGGGTCACTCGTGTCG-3′), vMIP-I-Sal (CTGTCGACCGTC-TAAGCTATGGCAGG-3′), vMIP-II-Eco (5′-CGGAATTCGCGTCCTGGCATA-GACCG-3′), and vMIP-II-Sal (5′-GGGTCGACATTCTTCAGCGAGCAGTG-3′). The amplified vMIP-I and the vMIP-II fragments were digested with *Eco*RI and *Sal*I and inserted downstream of the GST coding of pGEX-5X-1 (GE Healthcare, Uppsala, Sweden) at the *Eco*RI and *Sal*I sites to construct pGEX-vMIP-I and pGEX-vMIP-II. To express a full-length and the deletion mutants of the GST-vMIP-I and GST-vMIP-II fusion protein, the genes for GvM1-Full, GvM1-D1, GvM1-D2,

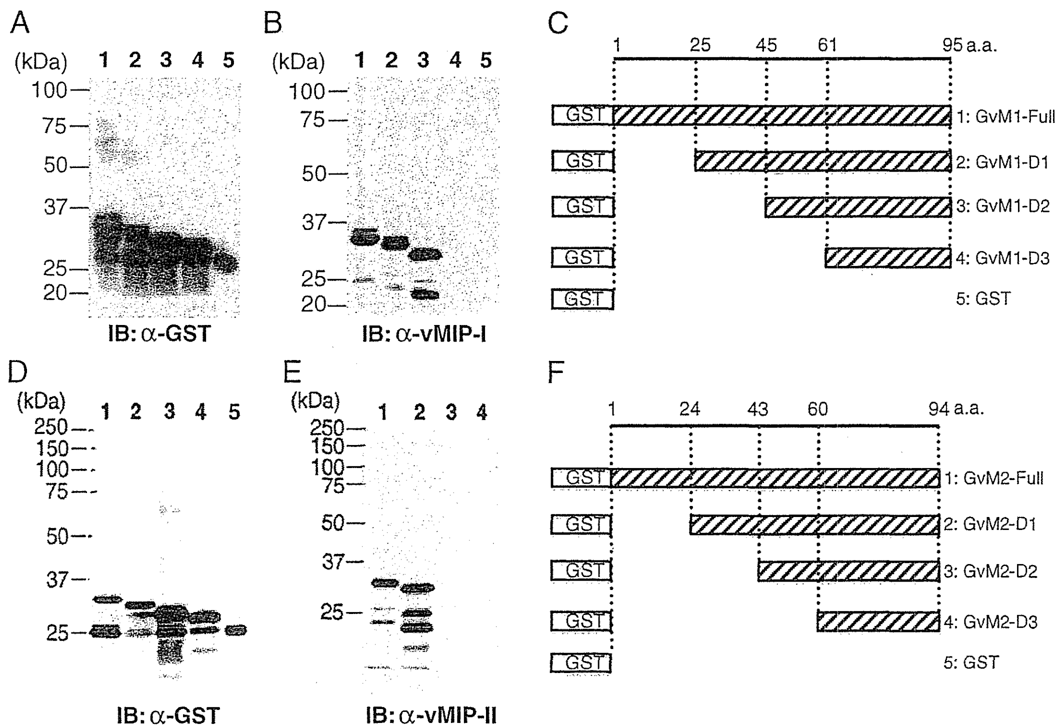

Fig. 2. Epitope mapping of the anti-vMIP-I and the anti-vMIP-II MAbs. To map the regions of vMIP-I and vMIP-II recognized by the anti-vMIP-I and anti-vMIP-II antibody, a series of GST-vMIP-I and GST-vMIP-II fusion proteins containing the individual regions of vMIP-I and vMIP-II were constructed as described in Fig. 2C and F, and the proteins were expressed in *E. coli*. The lysates of the fusion proteins, vMIP-I and vMIP-II, and its deletion mutants were immunoblotted with an anti-GST antibody (A and D) and an anti-vMIP-I (B) and an anti-vMIP-II antibody (E) to detect GST-vMIP-I or GST-vMIP-II fusion proteins. Lane 1, GvM1-Full; lane 2, GvM1-D1; lane 3, GvM1-D2; lane 4, GvM1-D3; lane 5, GvM1-D4; lan3 6, GST in Fig. 2A and B. Lane 1, GvM2-Full; lane 2, GvM2-D1; lane 3, GvM2-D2; lane 4, GvM2-D3; lane 5, GST (in D only) in Fig. 2D and E. Summary of GST-vMIP-I (C) and GST-vMIP-II (F) deletion mutants. Individual regions of vMIP-I and vMIP-II were cloned in-frame into the pGEX-5X-1 vector to generate GST-vMIP-I and GST-vMIP-II fusion proteins, respectively. The boxes at left indicate GST, and the white boxes with slashed lines indicate individual domains of vMIP-I and vMIP-II. 1, GvM1-Full(1-95a.a.); 2, GvM1-D1(25-95a.a.); 3, GvM1-D2(45-95a.a.); 4, GvM1-D3(61-95a.a.) in Fig. 2C, and 1, GvM2-Full(1-94a.a.); 2, GvM2-D1(24-94a.a.); 3, GvM2-D2(43-94a.a.); 4, GvM2-D3(60-94a.a.) in Fig. 2F.

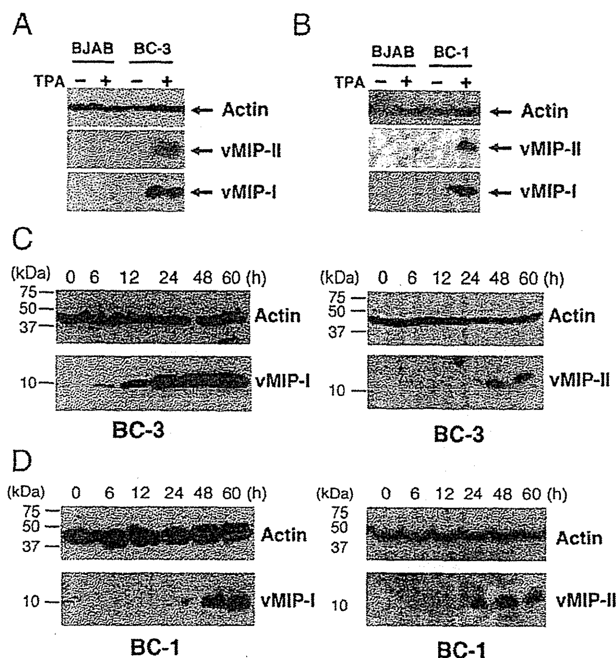

Fig. 3. Detection of vMIP-I and vMIP-II gene products in a KSHV-infected PEL cell line. BC-1 and BC-3 cells were treated with TPA for the indicated number of hours, and the whole-cell extract was prepared after the indicated time post-induction. vMIP-I and vMIP-II were detected by Western blotting and IFA with anti-vMIP-I and -vMIP-II antibodies. Western blot analysis of protein extracted from BC-3 and BJAB cells (A), and BC-1 and BJAB cells (B) with either the anti-vMIP-I or the anti-vMIP-II MAb. Arrows indicate actin, vMIP-I, and vMIP-II proteins. As expected, the estimated sizes of the vMIP-I and vMIP-II proteins, based on comparisons with the migration of molecular size markers, was around 10 kDa. Expression kinetics of vMIP-I (left panel) and vMIP-II (right panel) in TPA-treated BC-3 (C) and BC-1 (D) cells by Western blot analysis. BC-1 and BC-3 cells were harvested after 6, 12, 24, 48, and 60 h post-induction. The lysate was subjected to Western blot analysis as in (A).

GvM1-D3, GvM2-Full, GvM2-D1, GvM2-D2, and GvM2-D3 genes were generated by PCR using the following primer sets: vMIP-I-1F (5′-ATGAATTCCAGATGGCCCCCGTCCAC-3′) and vMIP-I-5R (5′-CCGTGTCGACCGTCTAAGCTATGGCAGGCAGC-3′); vMIP-I-2F (5′-ATGAATTCGCGGGGTCACTCGTGTCG-3′) and vMIP-I-5R; vMIP-I-3F (5′-ATGAATTCCCGCCCGTCCAAATTC-3′) and vMIP-I-5R; vMIP-I-4F (5′-ATGAATTCCCAAAACCCGGAGTTATTTTGC-3′) and vMIP-I-5R; vMIP-II-1F (5′-CGGAATTCGTTATGGACACCAAGGGC-3′) and vMIP-II-5R (5′-GGCAGTCGACTCTTCAGCGAGCAGTGACTG-3′); vMIP-II-2F (5′-GGGAATTCCTGGGAGCGTCCTGGCATAGAC-3′) and vMIP-II-5R; vMIP-II-3F (5′- AAGAATTCTTACCACAGGTGCTTCTGTCC-3′) and vMIP-II-5R; and vMIP-II-4F (5′-TGGAATTCAAGCCGGGTGTGATATTTTTG-3′) and vMIP-II-5R. The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and confirmed by sequencing. The products were digested with the *Eco*RI and *Sal*I restriction enzymes and were cloned into pGEX-5X-1 (GE Healthcare). The PCR conditions for all products were as follows: 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min in a TP480 PCR thermal cycler (Takara Shuzo, Kyoto, Japan).

Immunization and generation of monoclonal Abs against vMIP-I and vMIP-II

In mice, anti-vMIP-I and -vMIP-II antibodies were raised against the GST-vMIP-I and GST-vMIP-II fusion protein, respectively. These GST fusion proteins were purified on a glutathione-Sepharose 4B column (GE Healthcare), and the GST-vMIP-I and the GST-vMIP-II fusion proteins were conjugated to keyhole limpet hemocyanin KLH (Calbiochem. Co., La Jolla, CA). Mice were initially immunized with 250 μg each of the purified GST-vMIP-I or -II fusion protein in Freund's complete adjuvant administered to the peritoneal cavity, and 200 μg of the antigen in Freund's incomplete adjuvant were injected again 14 and 28 days after the first injection. The mice were exsanguinated 7 days after the last injection. To generate MAbs against vMIP-I and vMIP-II, hybridomas were established by fusing splenocytes from the hyperimmune mice using a nonproducing myeloma cell line, Sp-2/0-Ag14 (ATCC, Manassas, VA). After selection in medium containing hypoxanthine-aminopterin-thymidine, cells secreting MAbs were screened by immunofluorescence assays (IFA). The TPA-induced and -uninduced BCBL-1 cells were fixed in acetone and exposed to supernatants of the hybrid cells. Clones secreting antibodies reactive with TPA-stimulated BCBL-1 cells were expanded and isolated by limiting dilutions.

Transfection analysis of vMIP-I and vMIP-II

To express the vMIP-I and vMIP-II proteins, 293/EBNA cells were transfected with pCAGGS-vMIP-I and -vMIP-II plasmids using TransIT-LT1 (Mirus Bio LLC, Madison, WI). The transfected cells were incubated for 48 h in DMEM supplemented with 10% FCS. The cells were harvested and lysed with lysis buffer (0.05 M Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium-dodecyl sulfate [SDS]). The cell lysate was fractionated by electrophoresis on 16% polyacrylamide gel as described below.

Antibodies and Western blotting

The expression of vMIP-I and vMIP-II in BC-3 cells stimulated with TPA was determined with MAbs against vMIP-I and vMIP-II, respectively, as noted above. The concentration of proteins extracted from BC-3 cells was normalized using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). The samples were subjected to SDS-15% polyacrylamide gel electrophoresis under reducing conditions, and were electrophoretically transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h while being shaken at room temperature in PBS containing 0.05% Tween 20 and 5% w/v nonfat skim milk. The membranes were incubated with a primary antibody and were then incubated for 1 h with an appropriate dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). The primary antibody against actin, anti-actin (Ab-1) mouse MAb, was purchased from Merck (Merck KGaA, Darmstadt, Germany). The bound HRP-labeled antibodies were detected with a West Pico substrate kit for horseradish peroxidase (Thermo Fisher Scientific Inc).

IFA

BC-3 cells (10^7 cells) in RPMI 1640 medium with supplements were induced with 25 ng/ml TPA (Sigma Chemical Co., St. Louis, MO). The cells were collected after 0, 4, 8, 12, 24, 48, and 60 h for analysis of the expression kinetics, and for cellular localization analysis 48 h after exposure to TPA. The cells were washed in phosphate-buffered saline (PBS), pH 7.4, and spotted on glass slides. The spots were air-dried, then fixed in ice-cold acetone for 10 min. The cells were then washed with a washing buffer (PBS supplemented with 0.1% Triton X-100) for 15 min, and incubated with either an anti-vMIP-I or an anti-vMIP-II MAb (diluted 1:100 in IFA dilution buffer [PBS containing 2% bovine serum albumin, 0.2% Tween-20, and 0.05% NaN_3]) for 1 h at 37 °C. Then, the slides were washed with the washing buffer, and incubated for 1 h at room temperature with a pre-standardized diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago Immunologicals, Camarillo, CA). The slides were washed and stained with 4′, 6′-diamidino-2-phenylindole (DAPI) to detect nuclei and were mounted with 50% (v/v) glycerol in PBS. For formalin-fixed paraffin-embedded tissues, antigen retrievals were performed on the deparaffined sections using citrate buffer. Alexa 488 or 568-conjugated

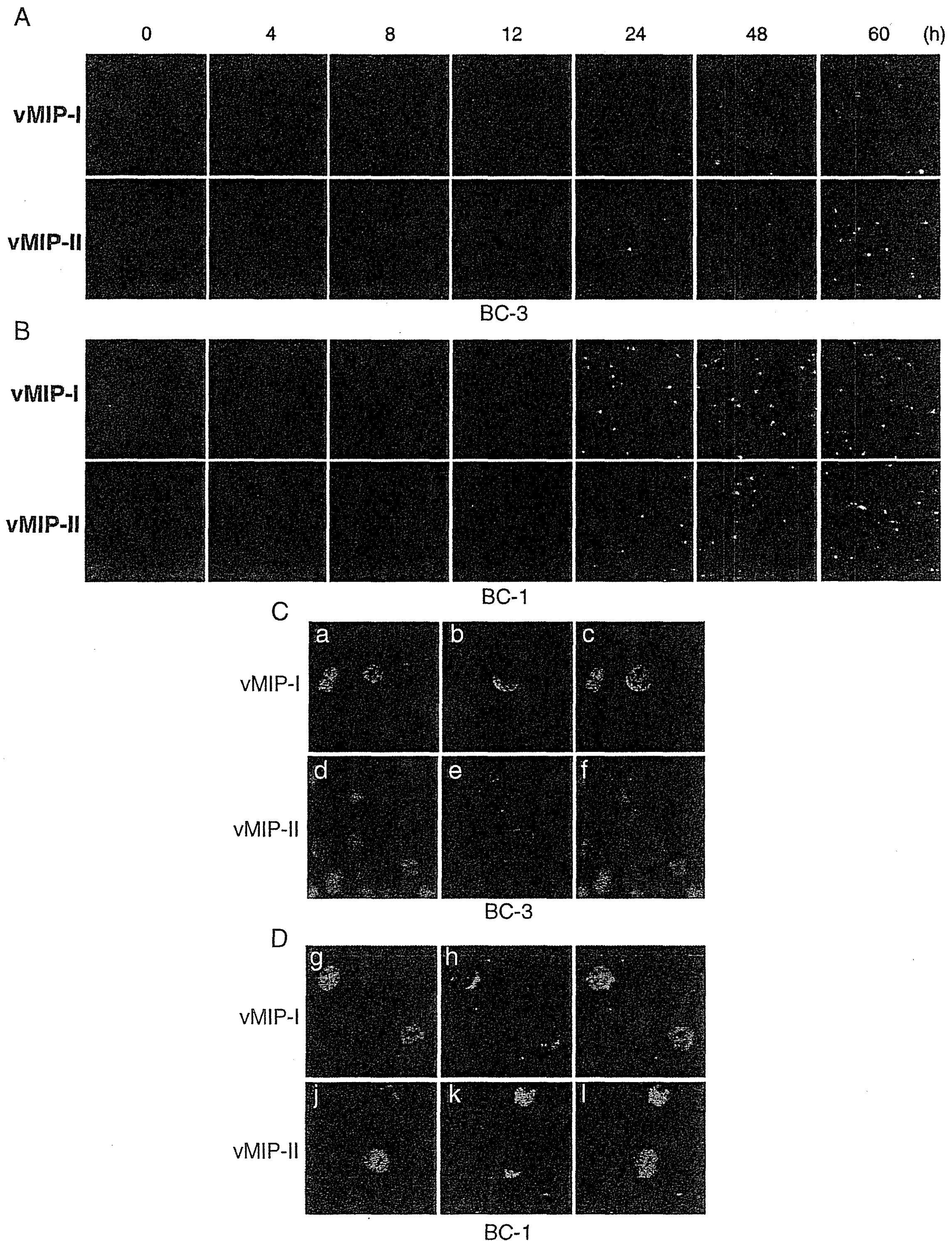

Fig. 4. Expression of vMIP-I and vMIP-II in BC-3 and BC-1 cells by IFA. After 4, 8, 12, 24, 48, and 60 h, BC-3 (A) and BC-1 (B) cells were labeled either with the anti-vMIP-I (upper) or the anti-vMIP-II (lower) MAb followed by goat anti-mouse FITC-conjugated Abs. FITC photomicrographs showing anti-vMIP-I and anti-vMIP-II immunoreactivity in BC-3 and BC-1 cells treated with TPA. (C) Cellular localization of vMIP-I and vMIP-II in BC-3 (C) and BC-1 (D) cells. The cells were stained with DAPI (a, d, g and j), and the localization of vMIP-I and vMIP-II was visualized by IFA with anti-vMIP-I or -vMIP-II MAbs (b, e, h and k); panel a and b, d and e, g and h, and j and k were merged (c, f, i and l). Fluorescence photomicrographs revealed anti-vMIP-I and -vMIP-II immunoreactivity using FITC-conjugated anti-mouse IgG MAb.

anti-mouse or rabbit antibodies (Invitrogen) were used as the secondary antibodies. Confocal microscopic analysis was performed (FV-1000, Olympus, Tokyo, Japan), and the contrast was adjusted before the images were exported as TIFF files to Adobe Photoshop.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues from KS and MCD patients, and those from an animal model of KSHV-associated solid lymphoma

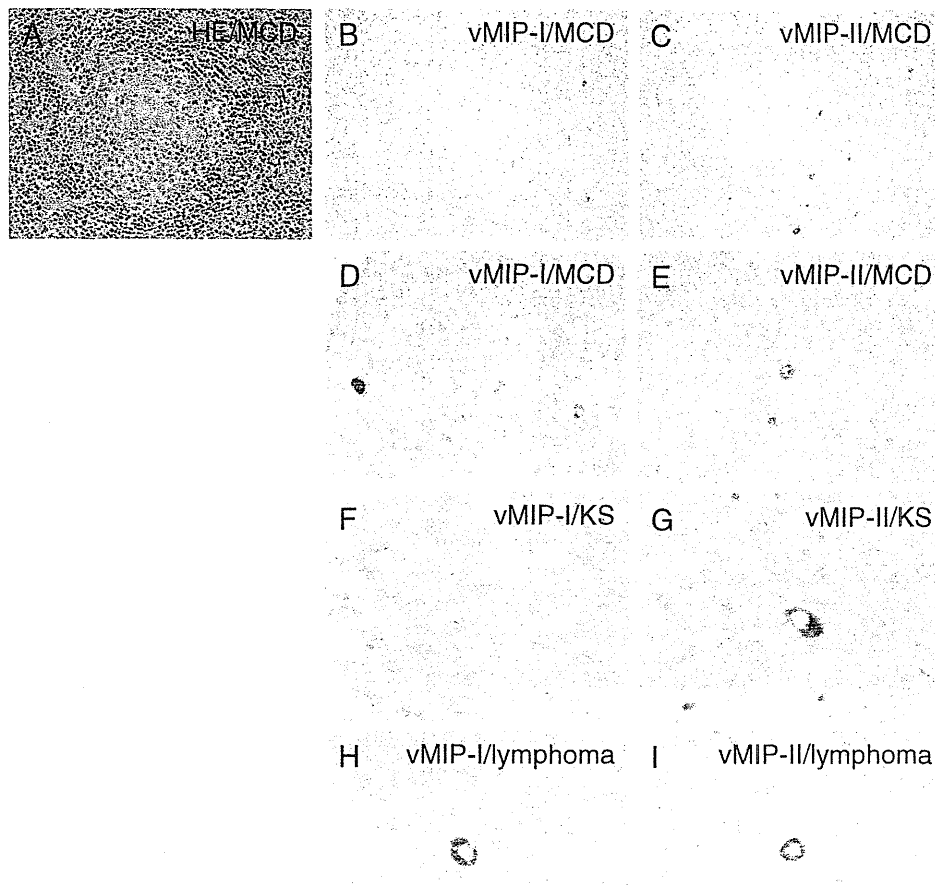

Fig. 5. Expression of vMIP proteins in KSHV-associated diseases. (A–C) Hematoxylin and eosin staining and immunohistochemistry for vMIPs in serial sections of a tissue sample from a patient with MCD. Brown stains indicate positive signals. The nucleus was counter-stained by hematoxylin. (D and E) Higher magnification view of vMIPs expression in an MCD case. Some large lymphocytes in the mantle zone were stained. (F and G) vMIP-I and vMIP-II expression in a KS sample. (H and I) Expression of vMIPs in an animal model of KSHV-associated lymphoma in SCID mice.

were sectioned and stained with hematoxylin and eosin (H&E). Immunohistochemistry of the serial sections was performed with either the anti-vMIP-I or -II MAb. For the second- and third- phase reagents used for immunostaining, a CSAII kit (DAKO, Copenhagen, Denmark) was used. An animal model of KSHV-associated solid lymphoma, which was established as described previously (Katano et al., 2000b), was also subjected to immunohistochemical analysis. Briefly, TY-1 cells were inoculated into the subcutaneous tissue of mice with severe combined immunodeficiency (SCID). One month after inoculation, lymphomas appeared in the subcutaneous region at the inoculation site. Lymphoma cells contained the KSHV genome, and expressed various viral proteins of KSHV (Katano et al., 2000b).

Table 1
Expression of vMIP-I and vMIP-II in MCD and KS tissue samples.

	KSHV proteins, (+)/total	
Cases	vMIP-I	vMIP-II
MCD	(3)/3	(3)/3
KS	(0)/5	(2)/8

Chemotaxis assays

Chemotaxis assays were performed as described previously (Nakano et al., 2003). Briefly, THP-1 cells were washed twice with chemotaxis buffer, 0.5% bovine serum albumin, 20 mM HEPES, pH 7.4, in RPMI 1640. Migration of cells was assessed in a cell culture chamber (Costar, Cambridge, MA), with the upper and lower compartments separated by a 3 µm pore size polycarbonate filter (???). The lower compartment of the chamber was filled with dilutions of vMIP-I, vMIP-II (R&D Systems, Minneapolis, MN) or with PBS alone, and/or with each 10 µg/ml anti-vMIP-I or -vMIP-II MAbs at a volume of 600 µl. The upper compartment contained 100 µl of THP-1 cell suspensions in chemotaxis buffer (10^5 cells/well). The chambers were then incubated for 4 hours at 37 °C, 5% CO_2, and spun at 300 x g, 4 °C, for 5 min. Finally, the cells from the lower compartment were counted.

Results

Specificity of the anti-vMIP-I MAb and the anti-vMIP-II MAb

In order to check specificity of the MAbs, we transfected vMIP-I and vMIP-II expression vectors (pCAGGS-vMIP-I, and -II) into 293/EBNA

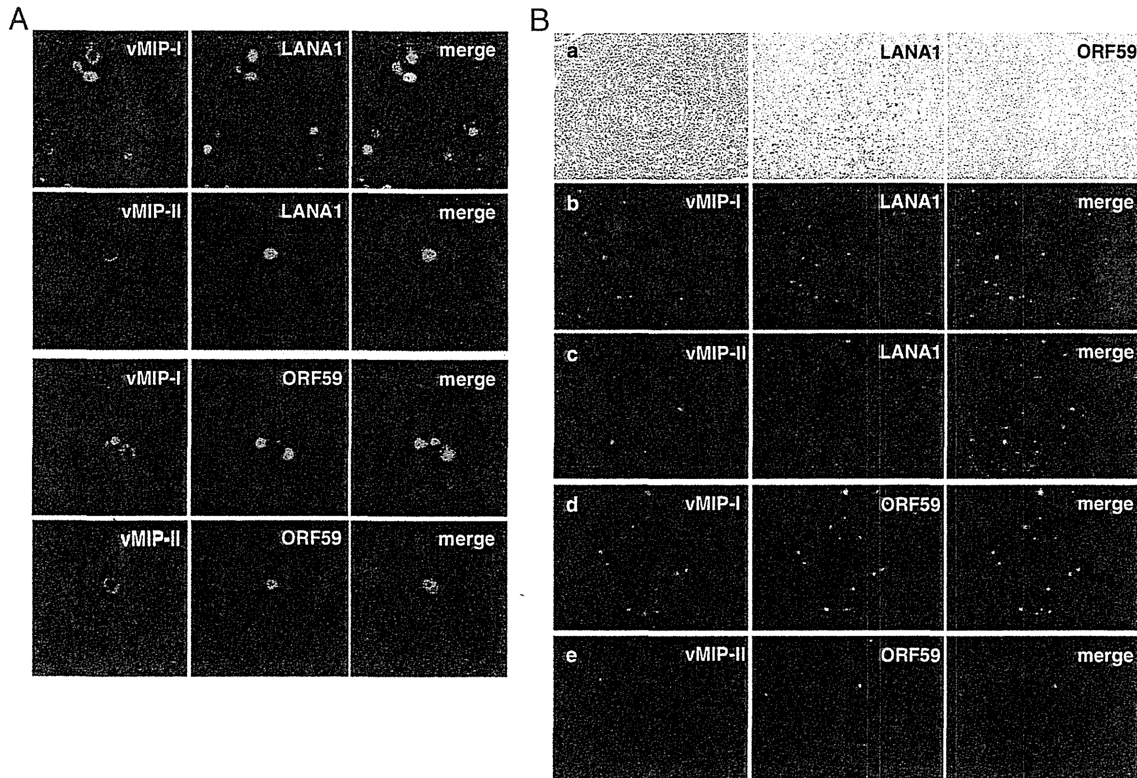

Fig. 6. (A) Expression of vMIPs. LANA1 and ORF59 in the animal model of KSHV-associated solid lymphoma by confocal microscopy. vMIPs were labeled with Alexa 488 (green). LANA1 (upper panels) and ORF59 (lower panels) were labeled with Alexa 568 (red). (B) Expression of vMIPs in MCD. (a) HE staining and immunohistochemistry of LANA1 and ORF59. (b–e) Immunofluorescence assay on MCD lesion. A germinal center is shown in the center of each panel. This case is KSHV-positive large B cell lymphoma arising in MCD.

cells, respectively. The total lysate of the transfected cells was subjected to Western blot analysis. vMIP-I and vMIP-II proteins were detected with anti-vMIP-I or vMIP-II MAbs, respectively (Fig. 1). These antibodies did not show cross-reactivity each other.

Epitope mapping of the anti-vMIP-I and anti-vMIP-II MAbs

We established hybridoma clones secreting MAbs against vMIP-I and vMIP-II, respectively. To map the regions of vMIP-I and vMIP-II where anti-vMIP-I and anti-vMIP-II antibody reacted, a series of GST-fused vMIP-I and vMIP-II deleted proteins were constructed as described in Fig. 2C and F, and used for Western blot analysis with an anti-GST antibody (Santa Cruz Biotechnology Inc), (Fig. 1A, D) and the anti-vMIP-I or the anti-vMIP-II (Fig. 1B, E) antibody, respectively. The results showed that all GST-vMIP-I and GST-vMIP-II fusion proteins interacted with the anti-GST antibody (Fig. 2A, D) and showed that GvM1-Full, GvM1-D1, and GvM1-D2 reacted with the anti-vMIP-I antibody, whereas GvM1-D3 did not (Fig. 1B), and GvM2-Full and GvM2-D1 reacted with the anti-vMIP-II antibody, whereas GvM2-D2, and GvM2-D3 did not (Fig. 2E). Thus, these results demonstrated that an anti-vMIP-I MAbs was successfully generated and suggest that the amino acid residues 61 to 95 of vMIP-I could be a major epitope reacted with the anti-vMIP-I antibody. On the other hand, the amino acid residues 24 to 42 of vMIP-II could be an epitope reacted with the anti-vMIP-II antibody.

Expression of vMIP-I and vMIP-II in the KSHV-infected PEL cell line

We tested vMIP-I and vMIP-II expression in KSHV and Epstein Barr virus (EBV) dually infected PEL cell lines (BC-1), KSHV infected PEL cell lines (BC-3) and in non-infected Burkitt's lymphoma cell line (BJAB), and detected them in TPA-stimulated BC-3 and BC-1 cells with developed antibodies, but not in BJAB cells non-stimulated BC-3 or BC-1 cells (Fig. 2A, B). In a KSHV infected PEL cells, BC-1 and BC-3, vMIP-I and vMIP-II were detected around at 10 kDa, which matches the size deduced from amino acids length (Fig. 3C, D). Actually, vMIP-I was detected from 6 hours post induction and vMIP-II was at 24 hours in BC-3 cells (Fig. 3C), and vMIP-I and vMIP-II were detected at 24 h in BC-1 cells (Fig. 3D). In the immunofluorescence microscopy, the number of vMIP-II expressing cells seemed to be more than that of vMIP-I in BC-3 cells (Fig. 4A, B). In order to analyze the cellular localization of vMIP-I and vMIP-II protein, BC-3 and BC-1 cells stimulated with TPA were doubly labeled with DAPI (Fig. 4C, a, d and D, g, j), and either the anti-vMIP-I MAb (Fig. 4C, b and D, h) or the anti-vMIP-II MAb (Fig. 4C, e and D, k). Merged images were shown in Fig. 4C, c, f, and D, i, l). The vMIP-I and the vMIP-II clearly showed cytoplasm and possibly membranes in TPA-induced BC-3 and BC-1 cells (Fig. 4C, b, e, and D, h, k).

Expression of vMIPs in KSHV-associated diseases

To know the expression of vMIPs in KSHV-associated diseases, immunohistochemistry for vMIPs was performed on pathological samples of eight KS cases, three MCD cases, and the animal model of KSHV-associated solid lymphoma (Fig. 5). Immunohistochemistry demonstrated that vMIP-I and vMIP-II were detected in some cells in the mantle zone of germinal center and the interfollicular zone in KSHV-positive MCD samples (Fig. 5A to E). Both vMIP-I and vMIP-II were detected predominantly in the cytoplasm of large lymphocytes. The numbers of positive cells varied among three MCD cases examined. On the other

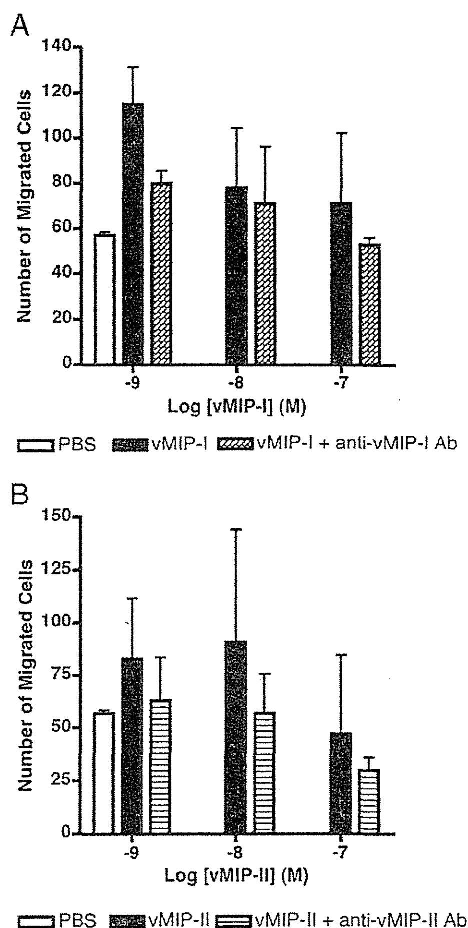

Fig. 7. Neutralizing activity of anti-vMIP-I and -vMIP-II MAbs. THP-1 cell migration in response to increased concentrations of vMIP-I and vMIP-II (1, 10, 100 nM), and the neutralizing activity of 10 μg/ml anti-vMIP-I and -vMIP-II MAbs against vMIP-I and vMIP-II were measured, as outlined in Materials and Methods, by using the transwell migration assay system. Various doses of vMIP-I and vMIP-II were tested for their ability to induce the chemotaxis of THP-1 cells. The data presented are from one experiment, and are representative of the triplicate experiments performed. The error bars indicate the standard deviations of three independent experiments.

hand, any positive signal of vMIP-I was not observed in all KS cases (Fig. 5F, G). vMIP-II was rarely detected in the cytoplasm of spindle cells in two KS cases at the nodular stage out of eight KS cases. In the samples of animal model of KSHV-associated solid lymphoma, both vMIP-I and vMIP-II were detected in the cytoplasm of a part of lymphoma cells (Fig. 5H, I). These data showed that vMIP-I and vMIP-II were expressed in cells in MCD and KSHV-associated lymphoma, but vMIP-II was rarely in KS (Table 1). To know the association of vMIPs expression with expression of other KSHV-encoded proteins, we examined immunofluorescence assay on KSHV-associated diseases. Since, all KSHV-infected cells express LANA1, vMIPs-positive cells were positive for LANA1. However, expression pattern of LANA1 showed diffuse nuclear staining in vMIPs-positive cells in the animal model of KSHV-associated solid lymphoma (Fig. 6A). Confocal microscopy revealed that vMIP-I stain showed usually cytoplasmic pattern, but rarely diffuse nuclear staining pattern *in vivo*. Almost all cells with vMIPs expression were also positive for ORF59 protein, a lytic protein of KSHV. IFA also demonstrated that vMIPs-positive cells expressed LANA1 at various levels in MCD clinical samples (Fig. 6B, a to c). A large portion of vMIPs-positive cells also expressed ORF59 protein in MCD (Fig. 6B, d, e). These data suggest that vMIPs are expressed by cells with KSHV-lytic infection in KSHV-associated MCD and lymphoma.

Neutralization of vMIP-I and vMIP-II by anti-vMIP-I and anti-vMIP-II MAbs

We examined whether the anti-vMIP-I and anti-vMIP-II MAbs could neutralize the chemoattractant of vMIP-I and vMIP-II to induce the migration of THP-1 cells. As expected, vMIP-I and vMIP-II induced migration of THP-1 cells (Fig. 7A, B), but not with PBS alone. However, anti-vMIP-I and anti-vMIP-II MAbs inhibited respective vMIP-I and vMIP-II-induced cell migration of THP-1 cells at 10 μg/ml final concentration.

Discussions

It was known that KSHV encodes three chemokine genes of the so-called viral macrophage inflammatory proteins: *vMIP-I*, *vMIP-II*, and *vMIP-III* in the genome. Analysis of the translated amino acid sequence indicate that the vMIP-I and vMIP-II gene have four conserved cysteines capable of forming two essential disulfide bonds (first cysteine and third cysteine, and second cysteine and fourth cysteine). The family of chemokines comprises CC, CXC, C, and CX_3C subfamilies. The vMIP-I and vMIP-II have four cysteines, the first two of which are found in the sequence of CC, which correspond to the CC profile. These gene products were expressed in the phase of KSHV lytic infection (Moore et al., 1996; Sun et al., 1999). Both vMIP-I and vMIP-II were expressed in a KSHV-infected cell lines, BC-3, which had been treated with TPA. Mono-specific polyclonal Abs against vMIP-I and vMIP-II have been described in previous studies that investigated the localization of vMIPs in PEL cells (Nakano et al., 2003). In the present study, we developed the respective MAbs that reacted either with KSHV vMIP-I or vMIP-II. We first applied these MAbs against KSHV vMIP-I and vMIP-II to detect KSHV-infected BC-3 and BC-1 cells by Western blotting and immunofluorescence assay. The Western blot analysis revealed that both the anti-vMIP-I and the anti-vMIP-II MAbs reacted to the 10-kDa proteins considered specific to the respective vMIP protein. The anti-vMIP-I MAb was shown to be reactive with the epitopes in the middle of the protein (sequence, PPVQILKEWYPTSPAC), and the epitope of the anti-vMIP-II MAb was shown to be reactive at the N-terminal end (sequence, LGASWHRPDKCCLGYQKRP). Further immunofluorescence analysis of the cellular localization of both vMIP-I and vMIP-II with anti-vMIP-I and anti-vMIP-II MAb showed a cytoplasmic pattern of expression in BC-3 and BC-1 cells. As the results indicated that these gene products were expressed in the cytoplasm, it might be located at the KSHV-infected BC-3 or BC-1 cells membrane prior to secretion. An investigation of the antigenic specificities of MAbs against KSHV vMIP-I and vMIP-II in MCD and KS patients has not yet been reported. Here, immunohistochemical analysis detected only vMIP-II in samples from both KS and MCD patients, but vMIP-I was not detected in KS cases: however, both vMIP-I and vMIP-II proteins were expressed in some cells in the interfollicular zone of MCD tissues. Lytic proteins of the KSHV such as K8, RTA, and ORF59 have been detected in large lymphocytes in the mantle zone of MCD cases (Dupin et al., 1999; Katano et al., 2000a). The expression of vMIPs showed a similar pattern to that of the lytic proteins in MCD tissues. In contrast, lytic protein expression, including that of vMIPs, was rare in the KS lesions (Abe et al., 2006). In the present study, we demonstrated that vMIPs were expressed in the cells expressing ORF59 protein. Thus, our data clearly indicated that the expression of vMIPs is associated with lytic infection in individual cells affected by KSHV-associated diseases. Human monocytic cell line THP-1 respond to various chemokines suggesting that they express receptors for these chemokines (Wang et al., 1993). Previous study, vMIP-I and vMIP-II were shown chemotaxis in THP-1 cells (Nakano et al., 2003). It has been reported that vMIP-I acts as a specific agonist for CC chemokine receptor 8 (CCR8) (Dairaghi et al., 1999; Endres et al., 1999) and vMIP-II shows a Ca^{2+} flux as a specific agonist for CCR3 (Boshoff et al., 1997). Our data showed anti-vMIP-I and anti-vMIP-II MAbs were able to neutralize vMIP-I- and vMIP-II-mediated chemotaxis in THP-1 cells. However, neutralizing activities

of anti-vMIP-I MAb was apparently low, even the addition of 10 μg/ml MAbs. These findings support the assumption that anti-vMIP-I and -vMIP-II MAbs-blocked chemotaxis in THP-1 cells act through binding to the certain amino acid residue of vMIP-I and vMIP-II.

In summary, MAbs developed specifically for this series were used to detect vMIP-I and vMIP-II in MCD and KS tissues, which may account for certain clinical features of MCD and KS. To gain a better understanding of these important viral genes, additional studies will be needed that focus on revealing vMIP-I and vMIP-II expression profiles during lytic infection. Taken together, these studies provide an insight into the pathogenesis of the contribution of vMIP-I and vMIP-II to the lytic induction of KSHV. These MAbs could serve as useful tools to clarify the pathogenesis of KSHV-related diseases.

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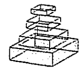

For the future studies of Kaposi's sarcoma-associated herpesvirus

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It is 18 years since Kaposi's sarcoma-associated virus (KSHV), also called human herpesvirus 8 (HHV-8), was found from Kaposis' sarcoma (KS) by Chang et al. (1994). More than 8, 000 reports have been published so far and we have learned many things from this virus. I would like to say it is about time to look back previous studies and to think what to study next on the virus, and planed a topic to think what to study next on the virus for future.

Herpesviruses have relatively big genomes and encode a 100 genes or so. Thus, the virion assembly/structure, gene expression regulation and attachment/entry are complicated and have known only an iceberg of them. Studying the details how the viruses run their life cycles and cause diseases in their processes will lead to exploring new therapeutic drugs/methods.

A viral life cycle starts from attachment on the susceptible cells and then, entry into the cells, followed by the viral gene expression, the genome replication, the particle assembly and finally the daughter viruses egress out of the cells. This process is skillfully built and all the viral genes are required for the process, though there are essential genes and non-essential ones. Viral pathogenesis could be established during this process by the interaction between viruses and host cells, and individual host systems such as immune system. In this topic, although I would like to cover all the processes, thankfully, 15 specialists in each field have contributed for this topic.

Polizzotto et al. (2012) described clinical manifestations of KSHV-associated diseases. So far, there were few reports on clinical manifestations of primary KHSV infection. In this term, KSHV inflammatory cytokine syndrome (KICS) is a new concept and we might have been looked over an important disease sign on KHSV infection. We will have to be more careful about what happens in primary KSHV infection than before.

Fukumoto et al. (2011) describe KSHV infection from a pathologist's points of view. Pathologic study is very important to know what happens in the lesions. Currently, we are able to know what is going on only in the KSHV associated lesions such as Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphomas (PEL) of human samples suffered from KSHV infection, but once an infection model is established, chronological pathologic studies will provide a lot of information on how KSHV-associated diseases are formed.

Chakraborty et al. (2012) review the entry mechanism of KSHV into cells. In general, herpesviruses can infect various kinds of cells *in vitro* including non-human cell lines, but the infectivity to B lymphocyte originated cells is very inefficient. Their report will give us a hint why such phenomenon happens.

An immediate early gene, *RTA* (reactivation and transcription activator) is very important for the viral lytic replication induction and shows multifunctions. We still have not understood how the factor functions. Guito and Lukac (2012) and Tsai et al. (2012) review or report mechanistic regulation of this strong transactivator, respectively.

Jackson et al. (2012) describe ORF57, which is also an interesting and multifunctional protein. This is involved in post-translational processes of the viral gene expression as sumoylation and ubiquitination described by Campbell and Izumiya (2012) and Ashizawa et al. (2012) respectively. We had believed that K-bZIP, a homolog of Epstein-Barr virus Zta was a transactivator and origin recognition factor in the lytic replication. K-bZIP, however, has other important roles for KSHV lytic replication. In latency, metabolism of LANA (latency-associated nuclear antigen) could be critical for KSHV-induced tumor formation and/or its phenotype.

Viral particle assembly is virologically an exciting and interesting field. There have been few reports on this, Sathish et al. (2012) try to search this issue.

The detail replication mechanism of KSHV in both lytic and latent phase has been still unclear. In latency, the virus is supposed to utilize host replication machinery including pre-replication complexes (pre-RC) for the viral replication initiation in the presence of LANA. The viral factor, LANA, is an essential factor, but its necessity has not been elucidated well. LANA binds with LANA-biding sites (LBS) and recruits origin recognition complexes (ORCs) on the viral replication origin (ori-P), which cannot account for necessity of the GC-rich element followed by LBS. Ohsaki and Ueda (2012) will give us a hint about this question.

Viral immune evasion system is very tactic to maintain its latency in case of herpesviruses. The maintenance of latency is then critical for the virus to wait for reactivation to produce daughter viruses, whose transition may a step for the viral oncogenic process. Lee et al. (2012) summarize KSHV immune evasion strategy and make a comment on the future landscape.

Kaposi's sarcoma-associated virus mediated tumorigenesis including PEL and KS has been still unclear, though there are many reports on individual viral putative oncogenes. KSHV has not been reported to infect and immortalize and/or transform endothelial cells or peripheral blood mononuclear cells *in vitro*. And thus, we have not known how the viral genes with oncogenic potentials such as *vFLIP*, *vCYC*, *vGPCR* and so on in addition to *LANA* cooperate in the viral oncogenic process. DiMaio and Lagunoff (2012) address on this issue and look forward for this field.

MicroRNA is one of the hottest research fields even in KSHV. This kind of small RNA molecule seems to have profound effects on cellular processes and then viral activities but their details have not been elucidated totally. KSHV lytic and latent phases are regulated by viral but also cellular microRNAs. Two specialists; Liang et al. (2011) and Gottwein (2012) reveals the microRNA world of KSHV.

And finally, we have to think about treatment of KSHV-associated tumors such as KS, PEL and a lympho-proliferative disease, multicentric Castleman's disease. It should be very hard to treat these tumors in the immunodeficient setting. It will be desirable if KSHV specific strategy is designed, since these tumors are very tightly linked with KSHV infection. Dittmer et al. (2012) contribute for this theme and discuss about it.

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I would like to thank all contributors, and hope that this topic will be useful for the future study of KSHV.

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Editorial Open Access

Kaposi's Sarcoma-Associated Herpesvirus Induced Tumorigenesis; How Viral Oncogenic Insults are Evaded

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Some viral infections in human are strongly related to cancer formation. Apart from retrovirus induced cancer formation seen in rodents and avian, virus induced cancer formation in human seems to be very complicated. In human, mainly DNA viruses such as papillomavirus, hepatitis B virus (HBV), Epstien-Barr virus (EBV), and Kaposi's sarcoma-associated herpes virus (KSHV) are etiological agents and some RNA viruses such human T-cell leukemia virus and hepatitis C virus (HCV) are involved in their specific cancer formation. It takes long time for the viruses to cause cancers and we do not have good systems to observe how the viral infection leads to cancer formation.

KSHV is belonging to gamma-herpesviridae and an agent involved in the formation of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castelman's disease (MCD).

The virus infection has a very strong link with these cancers. The mechanism how the virus causes such cancers is, however, still enigmatic and remains to be elucidated. KSHV latent infection should be important in terms that this type of infection provides with an origin of the related cancers. But, many genes with oncogenic activity of this virus are lytic genes, which are expressed only in the lytic phase.

As mentioned above, virus induced carcinogenesis is very complicated and is attractive to take an insight how the virus causes related cancers [1].

KSHV expresses an extremely limited number of viral genes such as latency-associated nuclear antigen (LANA), viral cyclin (v-cyc), viral FLICE inhibitory protein (vFLIP), kaposin and viral interferon regulatory factor-3 (vIRF-3) and 17 viral microRNAs in latency. The genes build an active gene locus in the KSHV genome in latency.

Among them, v-CYC, a homolog of cellular D-type cyclins, functions as an oncogene to deregulate cellular proliferation which leads to DNA damage response (DDR) and p53 induced apoptosis. Normal cells respond to oncogenic insults and cannot be easily transformed by choosing suicide pathway through p53 [2]. If the virus survives this situation, there must be a mechanism and this is one of ways how KSHV causes cancers.

In this point a recent report from Leidal et al. [1] is attractive for an insight to link the v-CYC induced oncogenic insult with subversion of this activity by vFLIP and how the virus causes related cancers. They found that v-CYC caused autophagy induced senescence and/or apoptosis. On the other hand, vFLIP is known for an autophagy inhibitor as well as an NF-kB activator [3,4]. And thus, vFLIP functions to evade from v-CYC induced oncogenic insult/senescence and make a direction of KSHV induced carcinogenesis.

However, we should be careful whether such a pathway happens in the natural infection course, since this kind of experiment is usually performed in over-expression system. Actually, vFLIP expression at the protein level has not been confirmed even in KSHV infected PEL cell lines and thus it is unclear whether such vFLIP activity is seen in the native situation.

In summary, a report from Liang seems to be very important to explain how KSHV causes cancers by connecting oncogene (v-CYC in this case) induced apoptosis and/or senescence [5]. Although there is no related report about the other human virus induced carcinogenesis, similar mechanisms might be stealthing.

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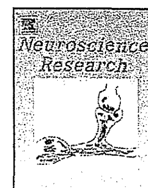

Overexpression of HGF attenuates the degeneration of Purkinje cells and Bergmann glia in a knockin mouse model of spinocerebellar ataxia type 7

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ABSTRACT

Spinocerebellar ataxia type 7 (SCA7) is an autosomal dominant disorder associated with cerebellar neurodegeneration caused by expansion of a CAG repeat in the ataxin-7 gene. Hepatocyte growth factor (HGF), a pleiotrophic growth factor, displays highly potent neurotrophic activities on cerebellar neurons. A mutant c-met/HGF receptor knockin mouse model has revealed a role for HGF in the postnatal development of the cerebellum. The present study was designed to elucidate the effect of HGF on cerebellar neurodegeneration in a knockin mouse model of SCA7 (SCA7-KI mouse). SCA7-KI mice were crossed with transgenic mice overexpressing HGF (HGF-Tg mice) to produce SCA7-KI/HGF-Tg mice that were used to examine the phenotypic differences following HGF overexpression. The Purkinje cellular degeneration is thought to occur *via* cell-autonomous and non-cell autonomous mechanisms mediated by a reduction of glutamate transporter levels in Bergmann glia. The Purkinje cellular degeneration and reduced expression of glutamate transporters in the cerebellum of SCA7-KI mice were largely attenuated in the SCA7-KI/HGF-Tg mice. Moreover, phenotypic impairments exhibited by SCA7-KI mice during rotarod tests were alleviated in SCA7-KI/HGF-Tg mice. The bifunctional nature of HGF on both Purkinje cells and Bergmann glia highlight the potential therapeutic utility of this molecule for the treatment of SCA7 and related disorders.

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1. Introduction

Spinocerebellar ataxia type 7 (SCA7) is a progressive inherited disorder characterized by ataxia and neurodegeneration of the cerebellum and retina (Ieraci et al., 2002). The disease is the result of an abnormal CAG repeat expansion in the ataxin-7 gene. SCA7 patients display ataxia in addition to neurodegeneration and neuronal death of Purkinje cells (Ieraci et al., 2002). SCA7 knockin mice also show a neurodegeneration of Purkinje cells (Yoo et al., 2003). Therefore, it is thought that the protection of Purkinje cells may represent a therapeutic strategy to combat SCA7.

Additionally, following findings suggest that Bergmann glial cells have been considered as another therapeutic target of the disease. Bergmann glia are cerebellum-specific astrocytes that are located around synapses between Purkinje cells and cerebellar granule cells or climbing fibers. The glial cells play a role in removing excess glutamate from synapses *via* two primary glutamate transporters, glutamate/aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) (Huang and Bordey, 2004). A mouse model that expresses expanded ataxin-7 specifically in Bergmann glia displays a neurodegeneration of Purkinje cells, indicating that a dysfunction of Bergmann glia contributes to the degeneration of Purkinje cells in SCA7 mice and thereby progression of the disease in a non-cell autonomous manner (Custer et al., 2006). Therefore, prevention of cell degeneration and concomitant increase of glutamate transporter function may represent a valid therapeutic strategy for SCA7.

Hepatocyte growth factor (HGF), which was first identified as a potent mitogen for mature hepatocytes (Nakamura et al., 1984, 1989), exhibits neurotrophic activities in a wide variety of neurons in the hippocampus, the cerebral cortex, the cerebellum, the brainstem (midbrain dopaminergic neurons) and the spinal cord (sensory and motor neurons) (Funakoshi and Nakamura, 2011). Recent experiments have indicated that HGF exerts neuroprotective effects on various neurons in animal models of cerebral ischemia, amyotrophic lateral sclerosis (ALS) and spinal cord injury (Funakoshi and Nakamura, 2011; Sun et al., 2002; Ishigaki et al., 2007; Kitamura et al., 2011; Miyazawa et al., 1998). In the cerebellum, HGF is expressed in Purkinje cells and granular cells, and plays a role in the cerebellum during both developmental and adult

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stages (Honda et al., 1995; Ieraci et al., 2002). In a mutant with a partial loss of Met function, the cerebellum was smaller than in controls and showed abnormal foliation (Ieraci et al., 2002). In addition to the cell growth and development, HGF exhibits neuroprotective effects for mature granule cells in primary cerebellar neuron culture (Zhang et al., 2000; Hossain et al., 2002). Moreover, overexpression of HGF not only attenuates the degeneration of motor neurons as a neurotrophic factor but also maintains adequate levels of the astrocytic glutamate transporter GLT-1 in a transgenic mouse model of ALS (Sun et al., 2002). This evidence led us to hypothesize that HGF may have a therapeutic potential on cerebellar neurons and Bergmann glia, cerebellar astrocyte subpopulations, in a valid mouse model of SCA7 in which a targeted 266 CAG repeat segment (a length known to cause infantile disease onset) of ataxin-7 is knocked into the mouse *ATXN7/Sca7* locus. These mice show features, which resemble those observed in an infantile SCA7 patient (Yoo et al., 2003).

The purpose of this study was to examine the effect of HGF on the Purkinje cells and Bergman glia of SCA7-KI mice. For this purpose, transgenic mice overexpressing HGF in a neuron-specific manner (HGF-Tg mice; Sun et al., 2002) were crossed with SCA7 knockin mice (SCA7-KI mice; Yoo et al., 2003) and phenotypic comparisons were made in wild-type (WT), HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice. Overexpression of HGF attenuated the shrinkage of Purkinje cells and prevented reduction of glutamate transporters in Bergmann glia and improved motor performance during the rotarod test in SCA7-KI mice.

2. Materials and methods

2.1. Animals

The SCA7 knockin (Sca7$^{266Q/5Q}$: SCA7-KI) mouse is a knockin mouse, which is a valid model of SCA7 that contains a targeted insertion of 266 CAG repeats (a number that causes infantile-onset disease) into the mouse *Sca7* locus. The mice were generously provided by Dr. Huda Zoghbi from the Baylor College of Medicine, Houston, TX (Yoo et al., 2003). Neuron-specific enolase (NSE)-promoter driven HGF transgenic (HGF-Tg) mice were generated and maintained as previously described (Sun et al., 2002). Heterozygous SCA7-KI male mice were crossed with heterozygous HGF-Tg female mice, which had been back-crossed with C57BL/6J mice for more than seven generations, to generate WT, heterozygous HGF-Tg, heterozygous SCA7-KI, and heterozygous SCA7-KI/HGF-Tg mice. Mouse genotypes were determined by dot blot hybridization or by polymerase chain reaction (PCR) using forward (5′-TTGTAGGAGCGGAAAGAATGTC-3′) and reverse (5′-CCACCCACAGATTCCACGAC-3′) primers for SCA7-KI and with forward (5′-CCAAACATCCGAGTTGGTTACT-3′) and reverse (5′-ATTACAACTTGTATGTCAAAAT-3′) primers for HGF-Tg mice. Experimental protocols were approved by the Animal Experimentation Ethics Committee of Asahikawa Medical University and Osaka University Graduate School of Medicine. All efforts were made to minimize animal discomfort and the number of animals used.

2.2. Cerebellar neuronal culture

Sixteen-day-old mouse embryos (E16) were obtained from timed pregnant C57BL/6J females (Japan SLC, Hamamatsu, Japan) that had been deeply anesthetized with isoflurane and euthanized *via* decapitation. Routinely, two pregnant females were processed in parallel. Immediately after euthanasia, uteri containing the embryos were removed and transferred into a sterile 100 mm tissue culture dish that was kept on ice and filled with ~20 ml ice-cold Leibovitz's L-15 medium. The cerebella were dissected using a stereomicroscope. After removing the meninges, the isolated cerebellar primordia were minced and transferred to a 15-ml Falcon tube containing L-15 medium. The supernatant was replaced with a pre-warmed 0.25% trypsin solution and the cerebella were incubated for 4–5 min at 37 °C with gentle shaking. Incubation was terminated by the addition of fetal bovine serum (JRH Biosciences, Brooklyn, Australia). Following the addition of DNase I and centrifugation, cells were dissociated by repeated pipetting and separated from non-dissociated tissue by sedimentation. The cells were seeded in plates precoated with poly-L-ornithine (500 μg/ml) at 2.5×10^5 cells/cm^2. Cultures were grown in neurobasal medium (Gibco Invitrogen, Grand Island, NY) supplemented with B27 (Gibco Invitrogen), 2 mM GlutaMaxI (Gibco Invitrogen), 1 mM adenine, 3 mM KCl, 1% heat-inactivated horse serum (Gibco Invitrogen), and a mixture of penicillin–streptomycin (100 U/ml and 100 μg/ml; Nacalai Tesque, Kyoto, Japan). From 2 days after seeding, 10 μM triiodothyronine (T3) and 1 μM Ara-C were added in order to mature Purkinje cells and to prevent the proliferation of non-neuronal cells. Half of the medium was replaced with fresh medium every 2 days. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air. The cells cultured for 14 days were washed with phosphate buffered saline (PBS) and fixed with 10% formalin in PBS.

2.3. Tissue preparation

Animals (WT, HGF-Tg, SCA7-KI, and SCA7-KI/HGF-Tg mice) at 10 weeks of age ($n = 3$ each) were deeply anesthetized with sodium pentobarbital and transcardially perfused with ice-cold PBS followed by ice-cold 4% paraformaldehyde in PBS. The cerebella were excised and immersed in the same fixative for several hours at 4 °C. Fixed tissues were immersed in 10% sucrose in PBS overnight at 4 °C, followed by 20% sucrose in PBS for 6 h at 4 °C, after which they were subsequently frozen in powdered dry ice or CO_2 gas. Frozen tissues were cut into either 16-μm or 40-μm thick sagittal sections using a Leica CM3050 S or CM1900 cryostat (Leica Microsystems GmbH, Wetziar, Germany).

2.4. Immunocytochemistry and immunohistochemistry

Formalin-fixed cerebellar neurons or cryosections were incubated in blocking buffer consisting of 10% normal goat serum (S26-100 mL, CHEMICON, Temecula, CA) and 0.3% Triton X-100 in PBS for an hour at room temperature followed by one or two of the following primary antibodies for 20 h at 4 °C: (1) mouse monoclonal anti-calbindin D28K antibody (1:250; 300, Swant, Marly, Switzerland); (2) mouse monoclonal anti-GFAP (glial fibrillary acidic protein) antibody (1:250; MAB3402, CHEMICON); (3) rabbit polyclonal anti-GFAP antibody (1:10; N150687, DAKO, Glostrup, Denmark); (4) rabbit polyclonal anti-c-Met antibody (1:50; SP260, Santa Cruz Biotechnology, Santa Cruz, CA); (5) rabbit polyclonal anti-rat HGF antibody (Ohya et al., 2007; Yamada et al., 1995) (6) guinea-pig polyclonal anti-GLAST antibody (1:500; AB1782, CHEMICON); (7) guinea-pig polyclonal anti-GLT-1 antibody (1:600; AB1783, CHEMICON). For immunostaining of phospho-c-Met, sections were incubated with Blocking One Histo (Nacalai Tesque) for an hour at room temperature, and then immunoreacted with rabbit polyclonal anti-phosphorylated c-Met antibody (1:200; C7240, Sigma, St. Louis, MO) in Signal Enhancer HIKARI B Solution (Nacalai Tesque) for 20 h at 4 °C. After washing the sections with PBS, immunoreactivity was visualized by incubating them further for 20 min at room temperature with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 546 or Alexa Fluor 647 diluted 1:600 (Invitrogen, Carlsbad, CA). Fluorescence-immunostained sections were observed under an Olympus FV1000

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