Short Communication

High Expression of HHV-8-Encoded ORF73 Protein in Spindle-Shaped Cells of Kaposi's Sarcoma

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Human herpesvirus 8 (HHV-8) has been demonstrated previously in Kaposi's sarcoma (KS) tissues by immunohistochemistry, *in situ* polymerase chain reaction, and *in situ* hybridization. The HHV-8-encoded protein ORF73 is a 222- or 234-kd protein named latent nuclear antigen (LNA) or latency-associated nuclear antigen (LANA) that is identified in HHV-8-infected cell lines by immunofluorescence assay. In the present study, a rabbit antibody against a recombinant ORF73 protein was developed. Immunofluorescent staining of a HHV-8-infected cell line, TY-1, showed that the staining pattern of the anti-ORF73 antibody overlapped completely the LANA staining pattern obtained using KS patients' sera. Immunoblotting analysis showed that the anti-ORF73 antibody reacted specifically with 222- and 234-kd proteins that were present in TY-1 and BCBL-1 cell lysates. Immunohistochemistry using a catalyzed signal amplification system demonstrated that the anti-ORF73 antibody reacted exclusively with the majority of KS spindle-shaped cells, showing a nuclear dot-like staining pattern. Some of the ORF73 protein-positive cells also expressed CD34 and vimentin but not CD68 or factor-VIII-related antigen. These data indicate that the anti-ORF73 antibody recognizes LANA and that most KS cells are infected with HHV-8 in the latent phase. Our findings also suggest that ORF73 protein plays an important role in the pathogenesis of KS. (*Am J Pathol* 1999, 155:47-52)

Kaposi's sarcoma (KS) was first described in 1872 as a rare tumor seen in elderly men of Mediterranean descent (classic type).¹ Recently, three additional clinical types, which are histologically indistinguishable, have been recognized: the acquired immunodeficiency syndrome

(AIDS)-related, post-transplantational (iatrogenic or immunodeficient), and African (endemic) types.²

The newly identified human herpesvirus-8 (HHV-8; also called KS-associated herpesvirus, KSHV) has been demonstrated in over 90% of KS lesions by polymerase chain reaction (PCR), regardless of the human immunodeficiency virus (HIV) infection status and the clinical type.³⁻⁷ HHV-8 has also been identified in primary effusion lymphoma (PEL; also called body-cavity-based lymphoma, BCBL),^{8,9} in PEL cell lines,¹⁰⁻¹⁶ and in patients with a subset of multicentric Castlemann's disease.¹⁷

The HHV-8 genome was first detected in KS tissues, in both spindle-shaped cells and endothelial cells, by *in situ* PCR.¹⁸ *In situ* hybridization (ISH) has also been used to detect HHV-8 mRNA in KS tissues.^{7,19-25} HHV-8 was identified in the intranuclear inclusions of spindle-shaped cells and of mononuclear cells in KS tissues using a T1.1 riboprobe.²¹ HHV-8 ORF26 expression by macrophages in KS tissues has also been detected by ISH using an ORF26 riboprobe.²⁵ In these studies, however, the positive cells were few in numbers because the probes recognize early lytic or lytic genes that are expressed in the infected cells. Concerning latent genes, mRNA of ORF72 (viral cyclin D) was first detected in KS spindle cells using a radioisotope-labeled probe; however, the resolution was not enough to demonstrate the clear localization.¹⁹ Recently, mRNAs of ORF72 and ORFK12 (T0.7, kaposin) were detected in many KS spindle-shaped cells using the catalyzed signal amplification system²⁴ or riboprobes.²⁰ The sensitivity of the HHV-8-specific probes is crucial for these studies.

In immunofluorescence assay (IFA), the sera of HHV-8-infected patients react with a nuclear antigen to produce a dot-like staining pattern in latently infected PEL cell lines; the nuclear antigen is known as latency-associated nuclear antigen (LANA).^{26,27} Western blotting of nuclear extracts of the BC-1 cell line show that 222- to

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234-kd doublet bands of protein were labeled by KS patient sera, and the labeled protein has been designated as latent nuclear antigen (LNA).²⁸ It has since been shown that an antibody that was affinity purified from KS patients' sera using a recombinant ORF73 protein binds to both LNA and LANA in IFA and Western blotting, which indicates that LNA and LANA are the same protein encoded by ORF73.²⁹ It has been also indicated that the LNA was expressed in some KS tumor cells in the immunohistochemical examination using the affinity-purified patient's antibody.²⁹ Despite intensive studies, little immunohistological evidence of the expression of latent proteins has been gathered so far. In the present study, a rabbit polyclonal antibody against HHV-8 ORF73 protein was developed, and the distribution of the protein in AIDS- or immunodeficiency-associated KS tissues was investigated.

Materials and Methods

Synthesis and Purification of Recombinant HHV-8 ORF73 Protein

A DNA fragment corresponding to amino acids 35 to 291 of the ORF73 gene (GenBank accession number KSU75698) was amplified from the total DNA of TY-1 cells¹⁵ by PCR. The forward primer sequence was 5'-CTC gga tcc TTG GCG ATG ACC TAC ATC TA-3', and the reverse primer was 5'-CTC gaa ttc TGC AAT CTC CGC AAG GAG CAC-3' (the cloning sites are underlined and lowercase).³⁰ The TY-1 cell line is HHV-8 positive and Epstein-Barr virus (EBV) negative. It was established from an AIDS patient with PEL.¹⁵ After purification by agarose gel electrophoresis, the PCR product was cloned into the *Bam*HI and *Xho*I sites of the bacterial expression vector *pGEX5X-2* (Pharmacia, Uppsala, Sweden). The expression vector produced 257 amino acids of ORF73 as a fusion protein with glutathione-S transferase (GST) in *Escherichia coli* (JM109, Toyobo, Toyama, Japan). The fusion protein (GST-ORF73) was affinity purified using glutathione-Sepharose as described previously.³¹ The purity and concentration of the eluted protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Bradford assay (Protein Assay, BioRad, New York, NY), respectively.

Production of an Anti-ORF73 Antibody (PA1-73N)

Purified GST-ORF73 in Freund's complete adjuvant (Difco Laboratories, Detroit, MI) was used to immunize New Zealand White rabbits to produce anti-ORF73 polyclonal antibodies. Immune sera were collected from the rabbits and partially purified twice by ammonium sulfate precipitation, and the precipitates were dissolved in PBS. The protein solution was passed through a GST-Sepharose column to remove the anti-GST antibodies before the desired antibody was affinity purified using GST-ORF73 coupled to activated CH-Sepharose (Pharmacia).

The purified antibody, designated PA1-73N, was used in the following experiments.

Immunofluorescent Assay (IFA) Using PA1-73N and Sera from KS Patients

To detect the LANA protein of HHV-8, IFA of cell smears was carried out as described previously.²⁶⁻²⁸ Sera from AIDS-KS patients and PA1-73N were mixed and diluted to 1:200 in PBS/1% bovine serum albumin (BSA) and used as the primary antibodies. TY-1 cells smeared on spotted slides and fixed in acetone were incubated with the primary antibodies for 60 minutes at 37°C and then washed extensively in PBS. The secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Tago Immunologicals, Camarillo, CA) and Texas-red-conjugated goat anti-rabbit IgG (Cappel, Organon Teknika Co., Durham, NC), were applied at a dilution of 1:50 in PBS for 30 minutes. The slides were then washed extensively in PBS and mounted with buffered glycerin. Imaging was performed using a confocal microscope equipped with an argon-krypton laser (LSM-MicroSystem, Zeiss, Germany). Texas red was excited at 568 nm, and FITC was excited at 488 nm. The emission patterns of the two fluorescent labels were collected separately, and the data were overlaid by a computer to create two-color images.

Western Blot Assay

Western blotting was carried out using PA1-73N and sera from AIDS-KS patients. The cell lines examined for GST and GST-ORF73 expression were BCBL-1 (HHV-8 positive and EBV negative, kindly provided by Dr. Brian Herndier),¹⁶ TY-1 (HHV-8 positive, EBV negative)¹⁵, Raji (HHV-8 negative, EBV positive), and Molt-4 (HHV-8 negative, EBV negative). To assess the specificity of PA1-73N, excess GST-ORF73 (100 µg/ml) was added to the dilution buffer.

Immunohistochemistry

To investigate the expression of ORF73 protein in tissues, 26 KS biopsy specimens from 25 cutaneous lesions and one lymph node, together with 30 specimens from non-KS tissues, were fixed in 10% buffered formalin and embedded in paraffin. Twenty-one of the cutaneous KS specimens were in the nodular stage and four were in the patchy stage. Sections (4 µm) were deparaffinized by sequential immersion in xylene and ethanol and rehydrated in distilled water. They were then irradiated for 15 minutes in a microwave oven for antigen retrieval. Endogenous peroxidase activity was blocked by immersing the sections in methanol/0.6% H₂O₂ for 30 minutes at room temperature. Affinity-purified PA1-73N antibody, diluted 1:3000 in PBS/5% BSA, was then applied, and the sections were incubated overnight at 4°C. After washing in PBS twice, the second and third reactions and the am-

Figure 1. Immunofluorescence assay using a rabbit antibody to recombinant ORF73 protein, PA1-73N, and AIDS-KS serum. Double labeling of IFA was carried out on TY-1 cells by using PA1-73N (a) and AIDS-KS serum (b). Texas-red-conjugated anti-rabbit and FITC-conjugated anti-human IgG antibodies were used as secondary antibodies. c: A dual image indicated that the staining of PA1-73N was overlapped completely on that of AIDS-KS serum.

plification procedure were performed using kits according to the manufacturer's instructions (catalyzed signal amplification system kit, DAKO, Copenhagen, Denmark). The signal was visualized using 0.2 mg/ml diaminobenzidine and 0.015% H_2O_2 in 0.05 mol/L Tris-HCl, pH 7.6. Double immunostaining was performed as described previously.³² Mouse monoclonal antibodies against CD34 (DAKO), factor-VIII-related antigen (DAKO), vimentin (DAKO), and CD68 (KP-1, DAKO) were each used as the first primary antibody followed by PA1-73N. To detect one of the lytic proteins of HHV-8, a rabbit polyclonal antibody against an HHV-8-encoded ORF59 protein was used.^{33,34}

Results

IFA of HHV-8-Infected Cells

IFA of the HHV-8-infected cell line TY-1 using PA1-73N (the anti-ORF73 antibody) produced a dot-like pattern of nuclear staining (Figure 1a). This staining pattern overlapped the staining pattern produced by sera from patients with KS (Figure 1, b and c). Close observation showed that the staining pattern of the KS sera was broader than that of PA1-73N because patient sera recognized other HHV-8 antigens in addition to LANA. These data suggest that PA1-73N recognizes LANA.

Figure 2. Western blot analysis of PA1-73N and AIDS-KS serum. Lane 1, BCBL-1 (HHV-8-positive, EBV-negative cell line); lane 2, TY-1 (HHV-8-positive, EBV-negative cell line); lane 3, Raji (HHV-8-negative, EBV-positive B-cell line); lane 4, Molt-4 (HHV-8-negative, EBV-negative T-cell line); lane 5, GST protein (26 kD, open arrowhead); lane 6, recombinant GST-ORF73 protein (approximately 53 kD, closed arrowhead). a: PA1-73N recognized several proteins ranging from 120 to 234 kD. These bands were detected in the cell lysates of two HHV-8-infected PEL cell lines, BCBL-1 and TY-1, but not in two HHV-8-negative cell lines, Raji and Molt-4. The recombinant GST-ORF73 was stained with PA1-73N, whereas GST protein was not stained. b: The recombinant GST-ORF73 protein blocked the reaction of anti-ORF73 rabbit antibody with the 53-kD protein and recombinant GST-ORF73 protein completely. c: AIDS-KS serum reacted with several proteins, including 120- to 234-kD proteins in BCBL-1 and TY-1 cells. AIDS-KS serum also reacted with a recombinant GST-ORF73 protein.

Figure 3. Immunohistochemistry of ORF73 in KS. **a:** Hematoxylin and eosin staining of a biopsy specimen of nodular KS. **b:** Demonstration of the HHV-8 lytic protein. ORF59 antigen is detected in a small number of KS cells. **c and d:** Stained with affinity-purified rabbit antibodies to ORF73. A large proportion of KS tumor cells expressed ORF73 antigen in their nuclei. **e and f:** ORF73 antigen is demonstrated exclusively in KS lesion in an inguinal lymph node. Any lymphocytes do not express ORF73 antigen.

Western Blot Analysis

PA1-73N reacted with GST-ORF73 and with several proteins, ranging from 120 to 222 and 234 kd, that were present in the lysates of both BCBL-1 and TY-1 cells (Figure 2a). PA1-73N did not recognize GST or any proteins from the HHV-8-negative cell lines Raji and Molt-4 (Figure 2a). Preincubation with excess GST-ORF73 blocked these positive reactions completely (Figure 2b). By contrast, AIDS-KS sera reacted with many protein bands from 120 to 222 and 234 kd. These findings indicate that PA1-73N identified LNAs that are present in BCBL-1 and TY-1 cells and that these proteins are specific to HHV-8-infected cells.

Immunohistochemistry of KS Tissues

The expression of ORF73 protein in KS tissues was examined by staining tissue sections with PA1-73N. Spe-

cific positive reactions were noted in the nuclei of KS cells (Figure 3c). High-power views showed that PA1-73N produced a dot-like nuclear staining pattern on over 90% of the spindle-shaped cells (Figure 3d). A positive signal was not observed in non-tumor endothelial cells, fibroblasts, or macrophages or in lymphocytes infiltrating in the KS lesions. On the other hand, ORF59 protein, which is a lytic protein encoded by HHV-8, was expressed by only a small proportion of the KS tumor cells (Figure 3b). Furthermore, the ORF59-positive cells did not react with PA1-73N (data not shown). The PA1-73N nuclear staining pattern was similar in all 24 KS tissue specimens from AIDS-KS patients and in the two specimens from KS patients without HIV infection, whereas PA1-73N did not label the normal tissues of various organs (skin, gastrointestinal tract, liver, lung, kidney, and spleen) or non-KS lesions (angiosarcoma, hemangioma, scar tissue, and granulation tissue; data not shown). The KS330₂₃₃³ or KS330₁₅₃³⁵ DNA products were amplified successfully

by PCR from the total DNA of each immunohistochemically positive case (data not shown). The lymph node specimen showed ORF73 antigen exclusively in the nuclei of the KS tumor cells (Figure 3, e and f). Double staining of the KS sections showed that a small number of the ORF73-positive cells also expressed CD34 and vimentin. However, most of the KS cells did not express any of the cell-specific markers tested: CD34, vimentin, CD68, and factor-VIII-related antigen (data not shown). These data indicate that ORF73 protein is specifically expressed by most KS spindle-shaped cells, which suggests that the majority of KS spindle-shaped cells are infected with HHV-8 in the latent phase.

Discussion

The present study demonstrated clearly that HHV-8-encoded ORF73 protein was highly expressed in most KS spindle-shaped cells. An association between KS and HHV-8 infection has been shown previously by Southern blot hybridization, PCR, *in situ* PCR, ISH, and electron microscopy.^{3,18-25,35,36} The distribution of HHV-8-encoded proteins, however, has not been reported in detail. As specific, high-affinity antibodies or probes for latent proteins or their genes have not been available, the previous immunohistochemical or ISH studies examined mainly the lytic proteins and their genes. Nonetheless, a few reports about the expression of the latent proteins have been published.^{19,20,29} Rainbow et al²⁹ reported that the ORF73 antigen is expressed in KS tissues, but they used affinity-purified AIDS-KS sera as the primary antibody. Dittmer et al²⁰ detected ORF72 and ORF-K12 mRNAs by ISH, but they needed a long exposure to amplify the signals. To our knowledge, therefore, this is the first immunohistochemical study to have detected a HHV-8-encoded latent protein in KS tissue sections using a rabbit polyclonal antibody.

We have confirmed that PA1-73N binds specifically to ORF73 protein: 1) PA1-73N IFA staining completely overlapped the LANA distribution pattern in TY-1 cells, 2) similar results were obtained when Western blotting was performed using PA1-73N and the serum of a patient with AIDS-KS, and 3) PA1-73N labeled only KS tumor cells, not other cell types.

In our Western blot analysis PA1-73N recognized not only the 222- or 234-kd proteins of LNA but also several proteins ranging from 120 to 220 kd in size, which were also recognized by sera from patients with AIDS-KS. Two groups^{20,37} reported the presence of mRNA splice variants that are produced using a common promoter by a cluster of latently expressed genes encoding ORF K13, ORF72, and ORF73. ISH has shown that these RNAs are present in at least 70% of the spindle-shaped cells in KS tumors.²⁰ Although the sizes of these mRNAs were reported as 1.7, 5.4, and 5.7 kb, no splicing site was detected in ORF73. Therefore, we think that the small sizes of bands from 120 to 222 kd were generated by degradation of LNA, which differ in size from the reported ones (222 and 234 kd).

The results of our immunohistochemical examination indicate that ORF73 protein is specifically expressed in KS spindle-shaped cells and not in non-tumor endothelial cells or macrophages in KS lesions. Some previous studies have demonstrated that macrophages in KS lesions were infected with HHV-8,²⁵ but we could not obtain any evidence for the infection to macrophages in the present study. Generally, immunohistochemical localization is superior to and far cleaner than ISH studies. Thus, the present study clearly confirmed that HHV-8 infected spindle tumor cells and was not detected in non-tumor endothelial cells or the majority of macrophages infiltrating KS lesions. It is known that some KS tumor cells are positive for CD34, CD68, factor-VIII-related antigen, vimentin, and smooth muscle actin. However, these antigens are not specific to KS and cannot be used as markers of this disease.² Our immunohistochemical data suggest that the ORF73 protein can be used as a diagnostic marker for KS and that the antibody PA1-73N is a useful tool for detecting HHV-8-infected cells in the latent phase.

The findings of the present study indicate that HHV-8 infects most KS cells. Based on the findings for other oncogenic viruses, ie, EBV, human papillomavirus, human T-cell leukemia/lymphoma virus type 1, hepatitis B virus, and hepatitis C virus, we believe that the association of KS with HHV-8 infection has been confirmed by this immunohistochemical study of KS tumor cells.

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