RAPID COMMUNICATION

Kaposi’s Sarcoma-Associated Herpesvirus (KSHV or HHV8) in Primary Effusion Lymphoma: Ultrastructural Demonstration of Herpesvirus in Lymphoma Cells


Recent molecular evidence suggests an association with a new herpesvirus, Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8), and primary effusion lymphomas (PELs). PELs have a characteristic morphology, phenotype, and clinical presentation, with malignant effusions in the absence of a contiguous solid tumor mass. We have established a cell line (KS-1) from a KSHV-positive human immunodeficiency virus (HIV)-negative patient with pleural cavity-based lymphoma that was passaged into triple-immunodeficient BNX mice. In contrast to cell lines from body cavity-based lymphomas derived from HIV-positive individuals that contain both KSHV and Epstein Barr viral genome, these cells contain only KSHV, allowing for uncontaminated virologic studies. Ultrastructural examination identified malignant cells with features of late differentiating B cells (immunoblasts). Cells with viral cytopathic effect contained typical 110-nm intranuclear herpesvirus nucleocapsids and complete cytoplasmic virions, confirming the association of PEL with KSHV.

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MATERIALS AND METHODS

Cells from the pleural fluid from a case of primary KSHV-positive effusion-based lymphoma were obtained by thoracentesis and grown in RPMI plus 20% fetal bovine serum at 37°C in 5% carbon dioxide and air. The cells began to proliferate actively within 3 days of initial in vitro culture. Cells grow nonadherently, occasionally in small clumps, and have a cellular doubling rate of about 48 to 72 hours.

Detection of KSHV sequences was performed by polymerase chain reaction (PCR) amplification of KSHV sequences and confirmed by Southern blotting of PCR products using methods previously described. Sequences of oligonucleotides used for the amplifications and hybridization were as follows: 5' primer, 5'-TGT-ATTAGCTAAACCTTCTAGCG-3'; 3' primer, 5'-TCCGTTGTTG-TCTACGTCCAG-3'; and probe, 5'-TGCAAGCAGCTGTTGGTTGACCACAT-3'. PCR reaction products were fractionated by electrophoresis, transferred to nylon membranes, and hybridized with an oligonucleotide probe for KSHV end-labeled with γ-32P

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Fig 2. Lymphoma cell from murine ascitic fluid with bilobed nucleus and prominent rope-like nucleolonema. The cytoplasm has well developed golgi apparatus and rough endoplasmic reticulum. (Uranyl acetate, lead citrate; original magnification × 15,000.)

Fig 3. Typical cytoplasmic tubuloreticular structure. (Uranyl acetate, lead citrate; original magnification × 37,000.)
Fig 4. Virus-containing cell from murine ascitic fluid with nuclear changes including fragmentation and lysis of chromatin. Herpesvirus particles are just discernible at this magnification (arrowheads). The cytoplasm contains paranuclear bundles of vimentin type intermediate filaments. (Uranyl acetate, lead citrate; original magnification × 12,600.)

(dATP) using T4 Polynucleotide Kinase (GIBCO/BRL, Gaithersburg, MD). As negative controls, we used RAJ1 lymphocytes and HL-60 myeloblasts that showed no hybridizing band.

Cells from the in vitro cultures described above were injected into the peritoneal cavity of triple-immunodeficient BNX mice (4 × 10⁶ cells total), who developed prominent ascites within 3 weeks of injection. The morphology of the cells was evaluated on giemsa-stained cytocentrifuge preparations and hematoxylin and eosin-stained sections from cell blocks. Immunophenotypic and genotypic characterization was performed as previously described. Immuno-histochemistry and/or in situ hybridization for EBV, herpesvirus (HSV-1 and HSV-2), and cytomegalovirus (CMV) was performed using modifications of methods previously described.

Electron microscopy. Fresh cells from the ascitic fluid were fixed and gelled in glutaraldehyde and 22% specific albumen (3 parts glutaraldehyde to 2 parts albumen) and immediately pelleted by centrifugation. The solidified specimen was cut into 1-mm cubes and embedded in epon for ultrathin sectioning. Sections were stained with uranyl acetate and lead citrate and evaluated with a JEOL transmission electron microscope (JEOL, Peabody, MA).

RESULTS

Clinical findings. Details of the clinical features have been reported previously. Distinctive features include the occurrence of a KSHV-positive pleural cavity-based lymphoma in an HIV-negative male patient.

Cell culture and triple-immunodeficient BNX mouse cultures. A cell line KS-1 that resembled the original lymphoma cells was established in culture and consisted of highly pleomorphic malignant lymphoid cells with prominent nucleoli and an immunoblastic appearance. Some cells had an anaplastic morphology with lobated nuclei and mark-
edly irregular nuclear outlines. Using PCR, we found a prominent 620-bp band on the ethidium bromide-stained agarose gel that hybridized with $^{32}$P-labeled KSHV sequences on Southern blot after transfer to nylon membranes. Cells from the peritoneal cavity of a triple-immunodeficient BNX mouse that developed massive ascites within 3 weeks of intraperitoneal injection had a similar morphologic appearance (Fig 1). In addition to malignant ascites, a tumor mass was present infiltrating the chest wall of the pleural cavity of the mouse. After passage in BNX triple-immunodeficient mice, the cell line KS-I remained positive for KSHV.

**Immunophenotypic characterization.** Immunophenotypic studies showed the cells to be positive for CD45 and CD20, but negative for surface Igs, for T-cell markers (CD3 and CD5), for CD56 (N-CAM), and for CD79a. The B-cell lineage was confirmed by the demonstration of clonal rearrangements of the Ig gene in Southern blots, as previously described. Cells from the murine ascitic fluid stained positively for cytoplasmic $\lambda$ light chains. Cells were negative for EBV by immunohistochemistry for EBV latent membrane protein (LMP-1) and in situ hybridization for EBER. Cells were also negative for CMV by in situ hybridization and for herpesvirus 1 and 2 by immunohistochemistry.

**Ultrastructural examination.** Ultrastructural features were similar in cells from the in vitro cultures and from the murine ascitic fluid. The cells were large with lobated nuclei containing marginated heterochromatin and prominent rope-like nucleolonemas (Fig 2). The cytoplasm was moderate in amount and exhibited short blunt surface projections. Cytoplasmic organelles included a prominent golgi apparatus, numerous mitochondria, rough endoplasmic reticulum, and numerous polyribosomes. Tubuloreticular structures were rarely observed in the cytoplasm (Fig 3).

The majority of the cells appeared viable, and viral particles could not be identified in these cells. Occasional malignant cells showed evidence of cellular injury in the form of condensation of chromatin near the nuclear envelope, nuclear degeneration, clumping and lysis of chromatin, and nuclear fragmentation (karyorrhexis; Fig 4). Electron lucent areas presumably resulted from degradation of the DNA. Within nuclei of these cells, typical herpesvirus particles were identified with capsids that measured approximately...

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**Fig 5.** Nuclear herpesvirus particles (cells from murine ascites) with capsids measuring approximately 110 nm that contain central nucleoids of varying electron density. Some capsids contain internal proteins arranged as a ring. (Uranyl acetate, lead citrate; original magnification $\times 69,000$.)
110 nm in diameter, with many containing central cores or nucleoids (Fig 5). Incomplete particles without cores were also found amongst the nuclear granular material. The cores appeared to be round, polygonal, or bar-shaped. Some nucleoids had electron dense dark centers (Fig 6), whereas other centers appeared empty, possibly representing different stages of DNA condensation. Some capsids contained internal proteins arranged as a ring. Enveloped viral capsids were identified within the rough endoplasmic reticulum and perinuclear cisterna (Fig 7). The number of viral particles increased with serial passage in mice.

DISCUSSION

PELS appear to be a distinct clinicopathologic entity based on their characteristic morphology, phenotype, and growth pattern. Although the association with human herpesvirus HHV-8 or KSHV has been shown by molecular techniques, replicating viral particles have only recently been identified. Herpes-type virus particles have been localized within the nucleus of KS cells from patients from Africa and with acquired immunodeficiency syndrome (AIDS), but these may be coinfectected with EBV or CMV. Similarly, KSHV-positive lymphomas have occurred in HIV-positive patients who are coinfectected with EBV. The occurrence of this type of lymphoma in an HIV-negative patient is rare, but in this case, permitted virologic studies without contamination by other herpesvirus, including EBV. As in patients with KS, KSHV may be associated with effusion-based lymphomas in both HIV-seropositive and HIV-seronegative individuals. Not all primary effusion lymphomas are associated with herpesvirus-like DNA sequences, and diagnosis of PEL should depend on the presence of characteristic clinical, cytological, phenotypic, and molecular findings. Two previous cell lines (BC-1 and BC-2) have been developed from KSHV-containing HIV-related lymphomas. Viral particles have been identified by negative staining in pellets from these cells, but, because these cell lines are coinfectected with EBV, it is difficult to determine whether these particles represent EBV or KSHV. Although the evidence strongly suggests that the virus replicating in cell line KS-1 derived from an HIV-negative, EBV-negative patient represents KSHV, without immunoelectronmicroscopy, we cannot totally exclude coincidental infection by an as yet unidentified member of the Herpes virus family.

Malignant cells from this lymphoma cell line had ultrastructural features consistent with terminal B-cell differentiation, including the presence of well-developed rough endoplasmic reticulum. Cytoplasmic staining for light chains in cells from murine ascites with absence of surface Ig is also in accordance with a late stage of B-cell differentiation. Although we were unable to detect virions in healthy appearing proliferating cells, the search for cells with features of nuclear degeneration, particularly lysis of chromatin and karyorrhexis, showed the presence of nuclear viral particles. Their presence in only a few cells showing nuclear changes may explain the previous inability to detect these particles, because in healthy viable cells, virus appears to be present only in the episomal form. Increased particles were...
Fig 7. (A) Electron micrograph showing nuclear viral capsids and an enveloped viral particle in the perinuclear cisterna (arrowhead). The envelope appears to derive from the inner nuclear membrane, whereas the outer nuclear membrane is intact. (Uranyl acetate, lead citrate; original magnification × 56,000.) (B) Lymphoma cell with nuclear capsids and a cytoplasmic virion within the dilated endoplasmic reticulum (arrowhead). (Uranyl acetate, lead citrate; original magnification × 44,000.)
identified in later passages in BNX triple-immunodeficient mice, suggesting possible adaptation by the virus. The viral particles have the typical appearance of herpesvirus with central cores (nucleoids) and 110 nm capsids. Production of complete virions and viral shedding may occur with lytic infection and destruction of the host tumor cells. The ultrastructural identification of herpesvirus particles supports the association of KSHV with effusion-based malignant lymphomas.

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Kaposi’s sarcoma-associated herpesvirus (KSHV or HHV8) in primary effusion lymphoma: ultrastructural demonstration of herpesvirus in lymphoma cells

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