References

Frequent Presence of a Novel Herpesvirus Genome in Lesions of Human Immunodeficiency Virus–Negative Kaposi’s Sarcoma

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While Kaposi’s sarcoma (KS) is extremely common in homosexual men infected with human immunodeficiency virus (HIV), it is also found in several clinical settings in which HIV infection is absent. Recently, sequences from the genome of a novel member of the herpesvirus family have been identified within KS biopsies in the AIDS-associated form of the disease. The presence of these sequences was sought in 6 cases of HIV-negative KS, including 4 cases of endemic KS from Africa, and was found in 5 of the 6. These findings strengthen the association between infection with this virus and the development of KS and argue against the notion that this association is simply the result of opportunistic superinfection of profoundly immunodeficient hosts.

Kaposi’s sarcoma (KS) is now widely recognized as one of the most common neoplasms of patients with AIDS [1]. But KS has a long history, dating to well before the global pandemic of human immunodeficiency virus (HIV) infection. It was originally described in 1872 as a rare and largely indolent disease affecting Mediterranean men. Subsequently, KS was found to be much more common in African men; in some locales in Africa in the 1960s, KS was reported to represent up to 10% of neoplasms in males [1]. KS was also recognized (albeit infrequently) in the United States and Europe in the pre-AIDS era, most notably in immunosuppressed organ transplant recipients. Although some differences exist in the clinical presentations of KS in these various subgroups, in all cases the lesions are histologically similar. The tumor is composed of multiple cell types, including so-called spindle cells thought to be of endothelial or mesenchymal origin as well as slit-like neovascular structures and infiltrating inflammatory cells [2].

The biologic and clinical behavior of KS is distinct from that of many other solid tumors (reviewed in [2, 3]). The histologic complexity noted above represents an obvious difference from
traditional cancers, which derive from the clonal outgrowth of a single cell type. A growing body of experimental evidence supports the idea that KS is a disorder of growth factor production. Cultured spindle cells from AIDS-associated KS cases require a variety of exogenous cytokines and growth factors for proliferation, some of which can be supplied by activated mononuclear cells [4, 5]. In addition, the spindle cells themselves produce and respond to several growth factors, suggesting the existence of autocrine and paracrine circuits in KS proliferation [6]. These cells also produce a variety of angiogenic and proinflammatory molecules that are the presumed cause of the neovascular changes and inflammatory infiltrates that are the histologic signature of KS [2, 7, 8].

While secreted growth factors and cytokines are thought to play a key role in the pathogenesis of KS, the question remains as to how this whole cascade is initiated. Cultured AIDS-related KS cells are not infected with HIV [2]. Nonetheless, links to HIV infection have been suggested by the observation that products of the HIV tat gene can be released from infected cells and can stimulate spindle cell proliferation [9]. However, this clearly cannot be the sole inciting event in KS etiology since, as noted above, many cases of KS arise in HIV-negative hosts. In addition, strong epidemiologic data show that KS risk is not uniformly distributed among HIV-infected humans. For example, the prevalence of KS among HIV-positive homosexual men is 10-20 times that observed in equally immunodeficient HIV-positive children or hemophiliacs [1]. This has suggested that a sexually transmitted cofactor, likely another viral agent, may be required for KS development. Consistent with this are recent reports of homosexual men who have developed KS in the absence of HIV infection [10].

Recently, Chang et al. [11] identified sequences of a novel herpesvirus in biopsy material from AIDS-associated KS cases. Genomic sequences of this new virus, provisionally termed KS-associated herpesvirus (KSHV), have been found in >90% of AIDS-related KS specimens; they are also found in ~15% of lymphoid tissue specimens from HIV-positive patients lacking KS but are absent from tissues of HIV-negative hosts without KS [11]. These studies established a strong link between KSHV infection and HIV-associated KS (or the risk for KS development) but, of course, do not prove causality. Formally, it is possible that the agent is simply an opportunistic passenger that preferentially infects cell types enriched in KS lesions or that KSHV infection is a marker for another factor that is itself the proximate cause of KS. To shed light on the nature of the association of KSHV with KS, we examined HIV-negative KS cases for the presence of KSHV DNA.

Materials and Methods

Clinical samples. African study subjects were recruited from the Uganda Cancer Institute in Kampala, which is the only referral center for cancer patients in Uganda. Patients were asked to undergo biopsy of KS lesions if they were found to have a tissue diagnosis of KS, a clinical diagnosis of endemic KS, and a negative serum EIA result for anti-HIV-1 antibodies (Cambridge Bioscience, Worcester, MA). In brief, endemic KS is diagnosed if all of the following are absent: oral KS, genital KS, disseminated cutaneous KS (i.e., KS involving the face, trunk, and both extremities), diffuse lymphadenopathy, gastrointestinal KS found endoscopically or suggested by unexplained abdominal symptoms, and pulmonary KS suggested by nodules on chest radiograph. Patients underwent repeat physical examination and repeat serum antibody testing at the time of biopsy to assure that no change in clinical or HIV serologic status had occurred since their initial evaluation.

Tissue collection. For the Ugandan cases, nodular lesions were selected for biopsy to assure adequate tissue for analysis. The skin was prepared with chlorhexidine gluconate or povidone iodine, and local anesthesia was established with subcutaneous lignocaine (2%). Two to four nodules were excised with overlying skin from each patient. Each biopsy was divided: One part was fixed in formalin for histologic confirmation of KS by the Department of Pathology at New Mulago Hospital, which adjoins the Uganda Cancer Institute, and the remaining tissue was immediately frozen in liquid N₂ and shipped to the United States for analysis.

Biopsies from cases 5 and 6 were available only as formalin-fixed tissue blocks.

DNA extraction. Ugandan skin tumor samples were crushed with separate mortars and pestles under liquid nitrogen, then incubated in 10 vol of 10 mM TRIS-HCl, pH 8.0, 100 mM EDTA, 1% SDS, and 0.5 mg/mL proteinase K at 55°C overnight. After extraction with phenol-chloroform and precipitation with ethanol, the pellets were resuspended in 2 mL of 250 μg/mL RNase A and 5 mM EDTA at 37°C for 1 h. The reactions were adjusted to 20 mM TRIS-HCl, pH 8.0, 50 mM sodium acetate, pH 7.0, and 0.1 mg/mL proteinase K and incubated at 55°C for at least 1 h. After extraction with phenol-chloroform and precipitation with ethanol, the pellets were resuspended in a small volume of 10 mM TRIS-HCl, pH 8.0, with 1 mM EDTA and quantitated by spectrophotometry.

DNA from a lymph node involved with KS from an HIV-seropositive man was extracted in a similar manner but in a different laboratory. Before DNA from any of the tumors was extracted, DNA was extracted from the peripheral blood leukocytes of a control HIV-seronegative man without KS or risk factors predisposing to KS.

DNA from the 2 HIV-seronegative American men was extracted from blocks of paraffin-embedded skin. The tissue was sliced thinly with sterile scalps and extracted three times with xylene and twice with ethanol. After drying in a SpeedVac, tissue slices were incubated in 100 mM TRIS-HCl, pH 8.0, 40 mM EDTA, 10 mM sodium chloride, 1% SDS, and 0.5 mg/mL proteinase K at 55°C overnight. Solutions were adjusted to 2% SDS and 1 mg/mL proteinase K and incubated at 55°C for an additional 24 h. After extraction with phenol-chloroform and precipitation with ethanol, pellets were resuspended in 50 μL of 10 mM TRIS-HCl, pH 8.0, and 1 mM EDTA.

Aerosol-barrier pipette tips were used to prepare all solutions used in the DNA extractions and analysis. To further minimize the potential for contamination, the relevant target sequences were not amplified by polymerase chain reaction (PCR) in the laboratory before these DNA samples were extracted and used.
Table 1. Clinical and laboratory features of 6 men with KS.

<table>
<thead>
<tr>
<th>Patient, nationality</th>
<th>Age</th>
<th>Date of KS diagnosis</th>
<th>Date of biopsy</th>
<th>HIV serology</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>5, American</td>
<td>61</td>
<td>12/1990</td>
<td>12/1990</td>
<td>(-) 7/1992</td>
<td>Nodular KS lesion on right leg</td>
</tr>
</tbody>
</table>

NOTE. PCR, polymerase chain reaction.

Amplification and analysis. PCR was done using 100 ng of DNA from Ugandan tumors or 5 μL (of 50 μL total) of extracted DNA from American tumors, 1× PCR buffer (Perkin-Elmer Cetus, Norwalk, CT; 1.5 mM magnesium), 100 μM dNTPs, 1 U of Taq polymerase, and 50 pmol of each primer in a 25-μL volume. The primers used were as follows: KS3, 5'-GATCGAATTCTCG-GGTAGGTCTGGAGACAAA-3'; KS4, 5'-GATCGTACCACGAGGCGATCACCCACCGTC-3'; KS5, 5'-GATCCCTCGAGATGGCACTCAGACAGATGTC-3'; KS6, 5'-GATCCCTACGAGATGGCACTCAGACAGATGTC-3'; β-globin 1, 5'-CAACTTCATCCCGTGCTACCC-3'; and β-globin 2, 5'-GAAGAGCCAAGGGTAC-3'. The reactions were incubated in a thermocycler (model 480; Perkin-Elmer) set for 94°C for 1 min, 58°C for 1 min, and 72°C for 35 cycles followed by 72°C for 5 min.

After electrophoresis, the 614- and 936-bp bands were also purified using Qiaex (Qiagen) and partially sequenced using the CircumVent thermal cycle DNA sequencing kit (New England Biolabs, Beverly, MA) per the manufacturers' protocols.

Results

Table 1 summarizes the clinical and laboratory features of the 6 patients whose KS biopsies were studied. Four of the patients were Ugandans who had typical endemic KS [12]. All were negative for anti-HIV antibodies by ELISA testing. The remaining 2 patients were homosexual white men of North American origin; neither was known to have resided in or traveled to Africa. From both of them, multiple ELISAs for anti-HIV antibody were negative, as was examination of peripheral blood mononuclear cells and plasma for HIV sequences by PCR. All patients had biopsy-confirmed KS. In the case of patient 4, a known KS lesion was biopsied after chemotherapy had resulted in marked clinical involution; pathologic examination of the tissue immediately adjacent to the sample we extracted revealed no residual KS histologically. DNA was extracted from biopsy specimens and examined for KSHV sequences by PCR, using two different sets of primers. One set corresponded to KSHV sequences homologous to the minor capsid protein of herpesvirus saimiri (HVS), the other to the KSHV homolog of a tegument protein (open-reading frame [ORF] 75) of HVS. As controls for the adequacy of genomic DNA extraction, samples were also examined for human β-globin DNA sequences by PCR; in all cases, efficient amplification of globin DNA was observed. As shown in figure 1, all 4 African KS specimens (including the 1 that had undergone involution) and 1 of the 2 North American specimens revealed the presence of KSHV-specific amplification products of the expected sizes. In all cases, both sets of KSHV-specific primers gave concordant results, and negative control specimens yielded no KSHV amplification products. To confirm that the observed products were of viral origin, amplified DNA from each African specimen was purified and the DNA sequence of a limited subregion directly determined without intervening molecular cloning (data not shown). Over a 277-nt region corresponding to the minor capsid ORF, all PCR products were identical in sequence to the published sequence of KSHV [11]. In the PCR products corresponding to the HVS ORF 75 (tegument protein) homolog, all Ugandan samples contained a single missense mutation; in addition, 1 of the 4 patients harbored an additional silent base substitution (not shown).

Discussion

These data indicate that sequences of KSHV are frequently found in forms of Kaposi’s sarcoma that are not related to HIV infection and further strengthen the association of this new agent with the disease. Five of the 6 biopsies we examined harbored KSHV sequences; we do not know whether the lesion of patient 5 was truly uninfected or whether the negative PCR result was...
**Figure 1.** Electrophoresis of polymerase chain reaction products from HIV-seronegative Ugandan and American patients with KS. 

A, KS3 and KS4 primers, which flank 594-bp region within 631-bp fragment that shares homology with open-reading frame (ORF) 75 of herpesvirus saimiri and BNRF-1 of Epstein-Barr virus; B, KS5 and KS6 primers, which border 916-bp ORF similar to ORF 26 of herpesvirus saimiri and BDLF-1 of Epstein-Barr virus; C, β-globin 1 and 2 primers, which amplify 268-bp fragment from human β-globin gene. Primers encode restriction sites; consequently, actual size of products of KS3 and KS4 is 614 bp and of KS5 and KS6 is 936 bp. In each panel, lanes m are 100-bp molecular weight ladder; 1–6, DNA from KS tumors of patients 1–6; 7, DNA from KS tumor of HIV-seropositive American man; 8, DNA from HIV-seronegative man without KS; 9, no template.

obtained reflects sampling error or an extremely low level of infection in this specimen. In any case, the frequency with which KSHV sequences were found in these HIV-negative tumors approaches that with which they are found in AIDS-associated KS [11]. While this report was in preparation, several other groups have independently reported the presence of KSHV sequences in KS specimens from HIV-uninfected persons [13, 14]; our results confirm and extend these observations.

Taken together, these findings strengthen the association of KSHV with this unusual neoplasm. While not sufficient to establish causation, these results render unlikely the proposition that the association of KSHV sequences with KS can be simply attributed to the acquisition of the virus as an opportunistic infection of systemically immunodeficient hosts. None of the patients in the present study were clinically immunodeficient; in patient 6 (the only KSHV-positive case for which such information was available), the levels of circulating CD4 cells were normal (800/mm$^3$). The finding of KSHV sequences in forms of KS unlinked to HIV also supports the notion that all clinical forms of the disease are related, an inference consistent with their shared and highly distinctive histologic features. Of course, firm proof of an etiologic role for KSHV in KS will require extensive seroepidemiologic and clinical investigation. The identification and cloning of KSHV genomic sequences [11] now puts that task within experimental reach.

References


