JOURNAL OF VIROLOGY, Jan. 1997, p. 715–719 Vol. 71, No. 1
0022-538X/97/$04.00+0

NOTES

Kaposi's Sarcoma-Associated Herpesvirus Gene Expression in Endothelial (Spindle) Tumor Cells

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Received 10 July 1996/Accepted 23 September 1996

The recent discovery of DNA sequences of a new human herpesvirus in Kaposi's sarcoma (KS) has fueled speculation that this virus might cause KS. The mere presence, however, of a virus in a complex multicellular tumor like KS could just as well be construed as evidence of a passenger agent. We sought stronger evidence linking the KS-associated herpesvirus (KSHV) to tumor formation by using in situ hybridization to investigate the specificity, constancy, and timing of KSHV gene expression in KS tumor cells. Here we document expression of a 700-nucleotide viral RNA in every KS tumor examined, from the earliest histologically recognizable stage to advanced tumors in which the vast majority of identifiable spindle tumor cells contain this transcript. Two other KSHV RNAs were also detected in a smaller fraction of the tumor cells in all but the earliest lesion. These viral RNAs were expressed to relatively low levels in this subset; because one of these RNAs encodes a major viral capsid protein, these cells may be producing KSHV. We did not find these KSHV genes expressed in a variety of other tumors and proliferative processes, but we did detect viral gene expression in prostatic tissue, supporting a possible mechanism for sexual transmission of KSHV. The close relationship between KS and KSHV gene expression is consistent with the hypothesis that KSHV is directly involved in the etiology and pathogenesis of KS.

The striking increase in Kaposi's sarcoma (KS) in human immunodeficiency virus (HIV)-infected homosexual men (3), together with other epidemiological factors pertaining to this risk group (4, 23), has stimulated the search for a sexually transmitted agent that might cause KS. While HIV infection strongly predisposes to KS, HIV itself is not a likely candidate for this agent because KS occurs in individuals who are not infected with HIV (11, 25). HIV infection, however, likely plays a major role in the pathogenesis of KS by promoting tumor formation through the release of cytokines and growth factors from infected lymphocytes or macrophages (1, 8) and by compromising immune surveillance mechanisms.

Another possible etiological agent has been identified by representational difference analysis of nucleic acids extracted from KS tumors (7) which revealed DNA sequences of a novel herpesvirus in both classical and AIDS-associated lesions (2, 7, 12, 16, 22, 26) as well as a rare body cavity-based lymphoma (BCBL) in HIV-infected individuals (6). The discovery of herpesvirus DNA, however, in the complex multicellular KS lesion in and of itself supports a number of equally viable hypotheses. The virus might cause KS, or it could be a mere passenger in one of the many other kinds of cells in the tumor. To discriminate between these possibilities, we sought evidence for the expression of KS-associated herpesvirus (KSHV) genes in the tumor cell itself, from the earliest stages of tumor formation onward. Using in situ hybridization with KSHV-specific probes, we now demonstrate a remarkable and constant correlation of KSHV gene expression with endothelium-derived KS tumor cells in a spectrum of AIDS-associated KS tumors.

We recently identified two major viral transcripts with sizes of 0.7 and 1.1 kb that are detectable in RNA extracted from KS tumors (28). These KSHV RNAs are also the predominant viral transcripts found in a BCBL-derived cell line, BCBL1, that is latently infected with KSHV (21). Upon treatment with 12-*O*-tetradecanoyl phorbol-13-acetate, these cells are induced to enter a more complex transcriptional program that results in the production of virus particles. Full-length cDNAs for the 0.7- and 1.1-kb transcripts have been cloned and characterized. One RNA (T0.7) likely encodes a small (60 amino acids) membrane protein, while the other (T1.1) represents an abundant nuclear RNA of uncertain coding potential (28). We have also identified and characterized a genomic clone that encodes the major viral capsid protein, MCP (open reading frame 25 [17, 29]), a known viral structural protein with homology to Epstein-Barr virus BCLF-1 and herpesvirus saimiri MCP, which is expected to be expressed principally in cells involved in productive rather than latent viral infection. We synthesized strand-specific RNA probes from these clones to investigate by in situ hybridization the cellular patterns of KSHV gene expression.

We first examined an advanced KS tumor from the tongue of

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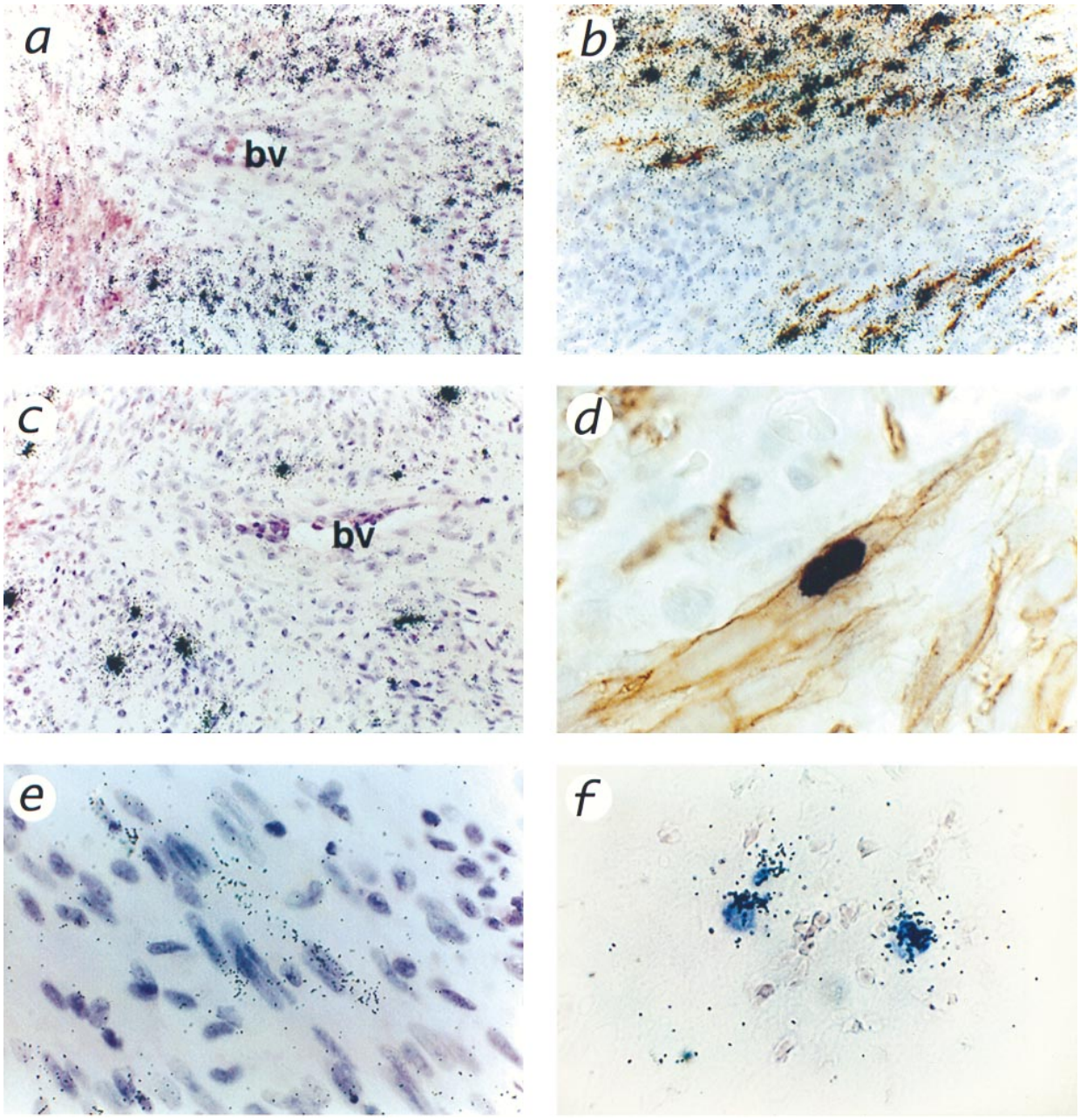

FIG. 1. In situ localization of KSHV T0.7 and T1.1 RNA transcripts in an advanced KS tumor of the tongue. (a) Hybridization with a ^{35}S-labeled riboprobe identifies KSHV T0.7 RNA-containing cells (clusters of silver grains over nuclei and cytoplasm) surrounding a blood vessel (bv) in the central region of the tumor (3-day exposure; original magnification, ×64). (b) Combined in situ hybridization with ^{35}S-labeled T0.7 riboprobe and immunohistochemistry with anti-CD34 shows that a majority of the brown-staining CD34-positive tumor cells contain KSHV RNA (5-day exposure; original magnification, ×80). (c) Hybridization of ^{35}S-labeled riboprobe specific for KSHV T1.1 RNA to a tissue section subjacent to that shown in panel a shows that the highly nuclear transcript is present in a higher copy number per cell than T0.7 but is in only a fraction of the T0.7-positive cells (20-h exposure; original magnification, ×64). (d) Combined in situ hybridization and immunohistochemistry shows that the spindle-shaped KS tumor cells reacting with antibody to CD34 (brown peroxidase-3,3′-diaminobenzidine reaction product) are the cells that contain the highly abundant and highly nuclear KSHV T1.1 transcripts detected here by hybridization with a digoxigenin-labeled riboprobe (dark purple alkaline phosphatase–nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium reaction product) (original magnification, ×202). The majority of the cells in the section, with or without CD34 staining, do not contain detectable levels of KSHV T1.1 RNA (pale blue hematoxylin-stained nuclei). (e) Hybridization with a ^{35}S-labeled riboprobe specific for KSHV MCP shows that these transcripts are distributed throughout the faintly pink eosin-stained cytoplasm along the length of the spindle-shaped tumor cell paralleling the shape of the CD34-stained spindle cell in panel d (5-day exposure; original magnification, ×161). (f) Double-label in situ hybridization with ^{35}S-labeled MCP riboprobe and digoxigenin-labeled T1.1 riboprobe demonstrates colocalization of these transcripts in three cells which may represent a population of cells in the tumor that are producing low levels of KSHV (20-h exposure; original magnification, ×202).

an HIV-infected individual with AIDS. Following protocols previously described (10), 6- to 8-μm sections of formalin-fixed paraffin-embedded tissues attached to silanized slides were deparaffinized, pretreated, and hybridized for 16 to 20 h at 45°C with ^{35}S-labeled riboprobes (specific activity, ~1×10^9 dpm/μg, 1×10^5 dpm of probe/μl of hybridization mix). After a posthybridization wash (19), the slides were coated with Kodak NTB-2 photographic emulsion, exposed for designated

periods of time, developed, and stained with hematoxylin and eosin. Using a radiolabeled T0.7-specific antisense probe, we detected viral RNA in the nucleus and cytoplasm of a large number of cells (Fig. 1a) in the central region of the tumor nodule, identified by characteristic vascular slits and lakes and palisading arrays of spindle-shaped cells (9). Since (i) the nucleic acid within the tissue was not denatured prior to hybridization and (ii) there was no detectable hybridization with the sense probes derived from the same clones (not shown), it is viral RNA rather than viral DNA that is specifically detected with the antisense probes.

Many of the hybridization-positive cells could be identified as spindle-shaped KS tumor cells based on their morphology and position within the tumor. Because the cellular morphology varies with the plane of the section, and because the tumor mass is composed of a complex mixture of transformed and normal cells, we combined in situ hybridization for KSHV and immunohistochemistry for specific cellular antigens to identify the types of infected cells unambiguously. CD34 is a hematopoietic antigen expressed to relatively high levels in many KS tumor cells (18, 20), but it cannot be used as the sole criterion for the identification, since it is present in many normal cells, including vascular endothelium, from which the tumor is thought to be derived. By combining, however, three criteria—(i) immunohistochemical staining with anti-CD34, (ii) spindle morphology, and (iii) localization within a well-defined tumor mass, we could unequivocally identify spindle tumor cells. For combined in situ hybridization and immunohistochemistry, deparaffinized tissue sections were rehydrated, placed in 10 mM citrate buffer (pH 6.0) (24) (five slides/70 ml in a plastic coplin jar), and microwaved for 10 min at the medium-high setting of a 1,200-W microwave oven, stopping every 1.5 min to replace evaporated liquid with distilled water. The slides were cooled to room temperature, acetylated, and hybridized with ^{35}S-labeled riboprobe. After the posthybridization wash, the sections were subjected to immunohistochemistry with a monoclonal antibody to CD34 (1:50 dilution; QB-END/10; Vector Labs, Inc.) and avidin-biotin peroxidase detection with 3,3′-diaminobenzidine (ABC *elite*; Vector Labs, Inc.), dehydrated through graded alcohols containing 0.3M NH_4 acetate, coated with emulsion, exposed, developed, and lightly counterstained with hematoxylin. By this approach, the majority of tumor cells in this and other well-defined lesions where these criteria could be applied were determined to be KSHV-infected and transcribing KSHV RNA (Fig. 1b). In five advanced lesions, an average of at least 85% (range, 80 to 92%) of the cells that fulfilled these criteria were positive for KSHV RNA. This is a conservative estimate, because tumor cells with lower levels of viral gene expression could not be scored. These cells can only be detected with autoradiographic exposure times that increase the signal over cells with higher levels of expression to the point that morphologically distinguishing features and cellular borders are obscured. Other normal cell types did not hybridize to the T0.7 antisense probe (not shown). These cells were intermixed with the tumor cells and were identified with antibodies specific for T (CD3) and B (CD20) lymphocytes, macrophages (CD68), dendrocytes (factor XIIIa), and normal endothelium (factor VIII-related antigen).

In situ hybridization with a radiolabeled probe to KSHV T1.1 sequences revealed transcripts in a subset of about 10% of the cells in which we could detect T0.7 RNA (Fig. 1c). From the levels of hybridization signal, the short exposure times required, and the specific activity of the probe, we estimate that there are at least 10,000 copies of the T1.1 RNA per cell. By using combined in situ hybridization with digoxigenin-labeled T1.1 riboprobe (detected with alkaline phosphatase-conjugated antidigoxigenin and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium substrate; Boehringer Mannheim) and immunohistochemistry with antibody to CD34, we verified that the highly nuclear T1.1 RNA was present in the spindle tumor cells (Fig. 1d). KSHV MCP RNA was detected primarily in the cytoplasm of spindle-shaped tumor cells (Fig. 1e) in the subset which contained T1.1 RNA (Fig. 1d). This was shown by colocalizing the viral RNAs by double-label in situ hybridization with a digoxigenin-labeled probe to reveal T1.1 RNA and a radiolabeled probe for MCP RNA (Fig. 1f). Hybridization mixture containing both ^{35}S- and digoxigenin-labeled riboprobes was applied to pretreated slides. After hybridization and a posthybridization wash, the slides were processed to detect the digoxigenin-labeled probe, dehydrated through graded alcohols, and then coated with emulsion. Although the level of expression of MCP is relatively low, its cytoplasmic localization and sequence homology to a structural protein may indicate low levels of virus production in these cells or, alternatively, an abortive replication cycle.

TABLE 1. In situ hybridization survey of KS and non-KS tissue specimens for the presence of KSHV RNA-containing cells[a]

Tissue specimen	No. of specimens with detectable KSHV T0.7/T1.1 RNA	No. of specimens
Kaposi's sarcoma (cutaneous and visceral)		
Very early patch	1/0	1
Patch/plaque	4/4	4
Nodule[b]	5/5	5
Angiomatous lesions and processes (hemangioma, hemangiosarcoma, hemangiopericytoma, granulation tissue, placental villi)	0/0	17
Tumors and proliferative processes (adenocarcinomas of colon and prostate, glioblastoma, epithelioid sarcoma, seborrheic keratosis, hairy leukoplakia, anal condyloma)	1/0	10

[a] All specimens (formalin fixed and paraffin embedded) were sectioned and hybridized with ^{35}S-labeled riboprobes to both KSHV T0.7 and T1.1 RNAs independently, exposed to emulsion for 5 to 7 days, developed, counterstained, and evaluated for the presence of hybridization-positive cells.

[b] Mean percentage of CD34-positive spindle-shaped cells in tumor nodules that contained KSHV T0.7, 85% (range, 80 to 92%).

We investigated the constancy of association and timing of KSHV viral gene expression in 10 KS lesions, ranging from early (patch/plaque stage) to advanced lesions (tumor nodule) originating from a variety of sites (dermis, lymph node, gut, and tongue), by using the antisense probe for the most widely expressed RNA, T0.7 (Table 1). In a blinded study, we detected the KSHV T0.7 transcript in all of the KS specimens at all stages. Detection of viral gene expression at the earliest histological stage of tumor formation satisfies the prediction that a tumorigenic agent is present ab initio. We could not estimate the percentage of KSHV-positive tumor cells in these early lesions, because there are no clearly defined masses of tumor cells and individual tumor cells cannot be confidently distinguished from the many normal cells in the lesion that are either spindle shaped and/or CD34 positive. Although T0.7 RNA was detected in cells in early patch KS lesions, the T1.1 and MCP RNAs were not detectable (not shown).

To further assess the specificity of the association of KSHV gene expression with the KS endothelial-derived tumor cell, we examined a variety of non-KS tumors and tissues with proliferating cells and/or prominent neoangiogenesis. In four cases, we analyzed pairs of tissues in which the tissues of a pair came from the same HIV-infected individual with AIDS. One spec-

TABLE 2. Detection of KSHV RNA in prostatic tissue

Patient	Age (yr)	Diagnosis[a]	Status[b]	
			HIV	KSHV
1	0.5	NI	?	−
2	21	NI	−	−
3	32	NI	+	−
4	36	NI	?	+
5	36	NI	+	+
6	39	Prostatitis	+	+
7	45	NI	+	+
8	50	NI	−	+
9	51	Ca	?	+
10	65	Ca	?	+
11	69	Ca	?	+
12	70	?	−	−
13	72	?	?	+
14	74	BPH	?	+
15	75	BPH	?	+
16	77	Ca	−	+

[a] Histopathological diagnosis of specimen. NI, normal prostatic tissue, specimens obtained at autopsy; Ca, adenocarcinoma of the prostate, specimen obtained by surgical excision; BPH, benign prostatic hypertrophy, specimen obtained by surgical excision; ?, could not be ascertained from available information.

[b] For HIV status, + indicates infection diagnosed by serology, and ? indicates unknown. For KSHV status, + indicates detection of KSHV RNA in cells in the specimen by in situ hybridization with KSHV T0.7 antisense riboprobe.

imen was KS, and the other was either granulation tissue or a hemangioma from another site. We detected KSHV T0.7 RNA only in the KS lesions. With one exception, we did not detect viral gene expression in the tissues from the angiogenic and other proliferative conditions (Table 1).

The one exception was a prostatic adenocarcinoma from an HIV-negative individual in which we detected the T0.7 RNA in the noncancerous portion of the specimen in cells lining the glands. Because excretion of virus or infected cells from the male genitourinary tract could account for the apparent sexual transmission of KS, we undertook a preliminary survey of the prevalence of KSHV RNA in a randomly selected set of prostate specimens from HIV-positive and -negative individuals. Although we did not find KSHV RNA in prostatic tissues obtained at autopsy from an infant or most of the younger individuals with normal prostatic histology, we did find KSHV T0.7 RNA in many of the abnormal tissues obtained at autopsy and surgically from both HIV-infected and uninfected individuals (Table 2). In most cases, the hybridization-positive cells were localized to the glandular epithelium (Fig. 2a), the identity of which was confirmed in double-label experiments with antibodies to cytokeratins that discriminate between luminal columnar epithelium (human cytokeratin 8, 1:100 dilution, 35βH11; Dako Corp.) (Fig. 2b) and basal epithelium (high-molecular-weight human cytokeratin, 1:100 dilution, 34βE12; Dako Corp.) (Fig. 2c) (27). We did not detect the T1.1 transcript in these cells or in occasional, unidentified, stromal cells that were positive for KSHV T0.7 RNA in some specimens (not shown) and, based on the correlation between T1.1 and late gene (MCP) expression shown above (Fig. 1f), suggest that infection is primarily latent in the prostatic epithelium.

We were unable to ascertain critical epidemiological information about HIV status or other risk factors in many individuals in which we documented KSHV infection in the prostate (Table 2), and therefore we wish to emphasize that our data are insufficient at this stage to draw any conclusions about the prevalence of KSHV infection of the genitourinary tract in the general population. While our finding is consistent with a recent report (15) describing PCR detection of KSHV in prostate and semen from a significant number of HIV-negative and immunocompetent men, forthcoming seroepidemiological sur-

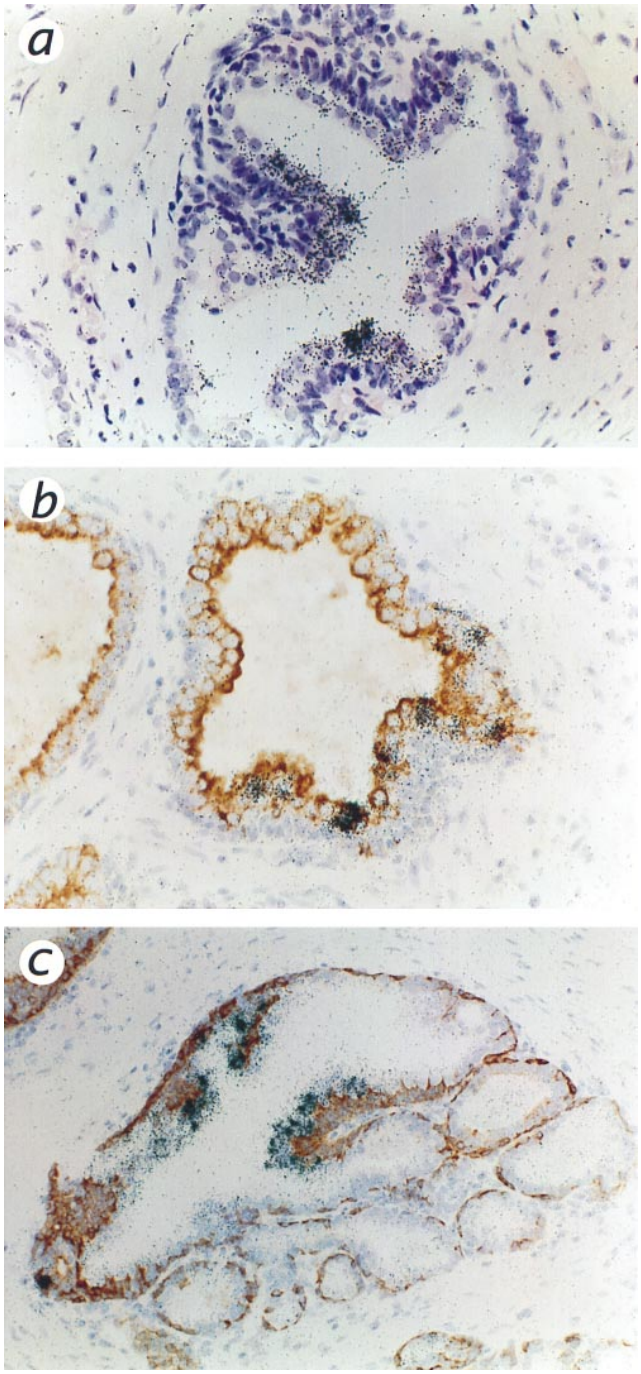

FIG. 2. Expression of KSHV in male prostate. (a) In situ hybridization of ^{35}S-labeled T0.7 riboprobe to prostatic tissue from an elderly HIV-negative individual reveals the presence of KSHV-infected cells in the glandular epithelium (16-h exposure; original magnification, ×80). (b) Combined in situ hybridization with radiolabeled riboprobe and immunohistochemistry with a monoclonal antibody to human cytokeratin 8 shows that the majority of these infected cells are within the columnar epithelium lining the tubules (4-day exposure; original magnification, ×64) as opposed to the basal epithelium, shown in the double label in panel c, decorated with monoclonal antibody to the high-molecular-weight cytokeratins 1, 5, 10, and 14 (3-day exposure; original magnification, ×41).

veys (13) in fact suggest that KSHV infection is not widespread but occurs more frequently in individuals with histories of sexually transmitted diseases. Nevertheless, what our findings do clearly demonstrate is that prostatic cells can be, and in some cases are, latently infected with KSHV and have the potential to sexually transmit infection in prostatic secretions. For example, periodic reactivation to productive infection could result in the shedding of virus into semen, or, alternatively, latently infected cells sloughed into semen could undergo lytic reactivation in the recipient genital tract. If this occurred in an immunocompromised individual, the unchecked spread of KSHV in the recipient could progress to development of KS at multiple sites.

The discovery of KSHV DNA in KS in HIV-infected and uninfected individuals and in the peripheral blood of HIV-infected individuals who have or are likely to develop KS (26) has fueled speculation that KSHV is involved in tumorigenesis. This possibility has been further supported by the recent demonstration, by in situ hybridization (14) and in situ PCR (5), of KSHV DNA in KS tumor cells in classical and AIDS-associated KS. We now show that there is a constant and specific association of KSHV gene expression with the KS spindle tumor cells of endothelial origin from the earliest stages of the tumor. This documented linkage between KSHV and the KS tumor cell is compatible with KSHV as the etiological agent of this enigmatic neoplasm.

We thank T. Reinhart for critical reading of the manuscript and C. O'Neill and T. Leonard for preparation of the manuscript and figures.

We also thank the NIH for support (B.H.; CFAR NIH AI27763).

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