Video Article

An *In Vitro* Model for Studying Cellular Transformation by Kaposi Sarcoma Herpesvirus

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Abstract

Kaposi sarcoma (KS) is an unusual tumor composed of proliferating spindle cells that is initiated by infection of endothelial cells (EC) with KSHV, and develops most often in the setting of immunosuppression. Despite decades of research, optimal treatment of KS remains poorly defined and clinical outcomes are especially unfavorable in resource-limited settings. KS lesions are driven by pathological angiogenesis, chronic inflammation, and oncogenesis, and various *in vitro* cell culture models have been developed to study these processes. KS arises from KSHV-infected cells of endothelial origin, so EC-lineage cells provide the most appropriate *in vitro* surrogates of the spindle cell precursor. However, because EC have a limited *in vitro* lifespan, and as the oncogenic mechanisms employed by KSHV are less efficient than those of other tumorigenic viruses, it has been difficult to assess the processes of transformation in primary or telomerase-immortalized EC. Therefore, a novel EC-based culture model was developed that readily supports transformation following infection with KSHV. Ectopic expression of the E6 and E7 genes of human papillomavirus type 16 allows for extended culture of age- and passage-matched mock- and KSHV-infected EC and supports the development of a truly transformed (i.e., tumorigenic) phenotype in infected cell cultures. This tractable and highly reproducible model of KS has facilitated the discovery of several essential signaling pathways with high potential for translation into clinical settings.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54828/>

Introduction

Kaposi sarcoma (KS) is a multi-focal angioproliferative tumor affecting dermal, mucosal, and visceral sites that develops most commonly in the setting of advanced immune suppression¹. Four epidemiological forms have been described: classic, an indolent form that typically affects older people of Mediterranean and Middle Eastern heritage; iatrogenic, resulting from treatment with immunosuppressive drugs following organ transplantation; epidemic, an AIDS-defining cancer; and endemic, an HIV-independent form common in children in endemic regions in Africa. With the advent of effective combination anti-retroviral drug regimens for the treatment of HIV, epidemic KS is much less commonly diagnosed in developing countries. However, the clinically aggressive endemic and epidemic forms remain among the most commonly diagnosed cancers in many African countries^{2,3,4}. Therefore, identification of effective pathogenesis-targeted drugs for treatment of KS is a research priority.

Histologically, KS lesions are characterized by extensive but abnormal neovascularization whereby spindle cells of EC origin form discontinuous vascular networks⁵. These abnormal vessels ("vascular slits") allow extravasation of erythrocytes, which give lesions their characteristic color. Additionally, lesions contain numerous leukocytes that characterize chronic inflammation (i.e., lymphocytes, macrophages, and plasma cells). Regression of KS lesions following immune reconstitution has been described, suggesting that KS has features of both a hyper-proliferative lesion and a true tumor^{6,7,8,9}.

KS herpesvirus (KSHV), the causative agent of KS, was identified in 1994¹⁰. Since then many *in vitro* cell culture models have been developed to enable pathogenesis studies, including cells explanted from tumor biopsy material and primary or telomerase-expressing EC infected with KSHV *in vitro*^{11,12,13,14,15,16,17,18}. None of the currently available models fully recapitulates the KS tumor microenvironment, but all have contributed valuable knowledge to our understanding of the pathobiology of KSHV infection. Unlike the other known tumorigenic human herpesvirus Epstein-Barr virus (EBV), KSHV does not readily transform cells in culture following *de novo* infection^{19,20,21,22}. However, this limitation has been overcome by transducing primary human EC of either mixed microvascular or lymphatic origin with the E6 and E7 genes from human papillomavirus type 16 prior to infection with KSHV^{23,24}. Expression of these exogenous oncogenes dramatically increases the transforming potential of KSHV *in vitro* in part by providing further inhibition of the retinoblastoma protein and p53^{23,24}. This EC transduction method has allowed multiple laboratories to identify key alterations in host cell gene expression that are induced by KSHV infection and that appear to facilitate KS cell survival and proliferation^{25,26,27,28,29,30,31,32}. The protocols described herein are straightforward and highly reproducible, and will result in the generation of age- and passage-matched KSHV-infected EC and mock-infected controls that can be cultured for far longer than primary cells and will allow for the investigation of oncogenic mechanisms employed by KSHV. Although the protocol includes a method

for production of wild type KSHV from the primary effusion lymphoma cell line BCBL-1, E6/E7-immortalized EC are also highly susceptible to infection with recombinant BACmid derived KSHV-BAC16³⁰. Protocols for preparation of BAC16 are described elsewhere^{33,34}.

Protocol

NOTE: All procedures described in this protocol should be performed under BSL-2 conditions.

1. KSHV Stock Preparation

1. Prepare TNE buffer: dissolve 292.24 mg EDTA in ddH₂O, bring to 225 mL, and adjust to pH 8. Dissolve 605.7 mg Tris in ddH₂O, bring to 225 mL, and adjust to pH 8. Combine EDTA and Tris solutions, add 4.38 g NaCl, adjust final volume to 500 mL, filter sterilize, and store at 4 °C.
2. Culture the KSHV-positive, EBV-negative primary effusion lymphoma cell line BCBL-1 in a humidified incubator at 37 °C plus 5% CO₂ in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antimicrobials to approximately 1 to 1.2 x 10⁶ cells per mL.
3. To induce KSHV production, split cultures 1:2 with fresh medium and add Phorbol 12-Myristate 13-Acetate (PMA) to a final concentration of 20 ng/mL and incubate cultures for 5 days. As an alternative, treat cells with sodium butyrate (NaB; 0.1 mM) for 3 days to induce lytic viral replication.
4. To harvest viral particles, first centrifuge the culture supernatant at 300 x g for 5 min at room temperature to pellet the cells and transfer supernatants to fresh tubes.
5. To further clarify the supernatant so as to avoid transfer of cellular debris, centrifuge at 2,500 x g for 10 min at 4 °C.
NOTE: As an alternative to high speed centrifugation for clarification of culture supernatants, filtration through a sterile 0.45 µm filter can be performed. We have observed a significant drop in titer using filtration; therefore, we routinely perform the second centrifugation step instead.
6. Next, overlay 5 mL of 25% sucrose solution in TNE buffer with approximately 30 mL of the clarified culture supernatant in 6 ultracentrifuge tubes.
7. Centrifuge balanced tubes at 75,000 x g for 2 h at 4 °C under vacuum.
8. Decant culture supernatant and blot the rim of each tube to remove as much supernatant and sucrose solution as possible, then resuspend the virus pellet, which may not be visible, in 150 µL of TNE buffer.
9. Pool all resuspended virus, mix well, and freeze 25 µL aliquots at -80 °C.

2. Transduction of EC with E6 and E7 Papillomavirus Genes

NOTE: We routinely use standard tissue culture flasks for growing primary EC. However, if unsatisfactory growth of EC are obtained then the use of commercial culture flasks should be considered.

1. Culture primary human dermal microvascular or lymphatic EC in a humidified incubator at 37 °C plus 5% CO₂ in EC growth medium (EGM; contains EC basal medium [EBM] supplemented with EGM-2 BulletKit [containing FBS, hydrocortisone, human fibroblast growth factor-B, vascular EC growth factor, insulin-like growth factor-1, ascorbic acid, human epithelial growth factor, antibiotics, and heparin]) in a T75 cell culture flask to approximately 50% confluence.
2. For transduction, culture PA317 LXSN 16E6E7 cells^{24,35} in a humidified incubator at 37 °C plus 5% CO₂ in DMEM supplemented with 10% FBS and antimicrobials in a T150 cell culture flask until they are approximately 90% confluent. Then incubate overnight in 16 mL of medium.
3. Clarify the PA317 medium by centrifuging at 300 x g for 5 min at room temperature.
4. Remove the EGM from the EC culture and overlay cells with 12 mL of the clarified PA317 medium and incubate for 4 h.
NOTE: Centrifuging 16 mL of conditioned medium at 300 x g for 5 min results in a compact pellet that is not disturbed by the careful subsequent removal of 12 mL of clarified supernatant. However, if transfer of PA317 LXSN 16E6E7 cells to primary EC cultures is a concern (the packaging cell line will quickly outgrow EC), then filtration of the conditioned supernatant through a 0.45 µm filter before transfer can be performed.
5. Replace 6 mL of the PA317 medium with fresh EGM and incubate overnight.
6. Refeed EC with 12 mL fresh EGM and incubate a further 48 h.
7. To sub-culture the EC, remove medium and wash with 12 mL of PBS without cations, and add 3 mL of commercial enzymatic dissociation solution (e.g., TrypLE) by and incubate at 37 °C for 3 min.
8. Transfer cell suspension to a 15 mL conical tube and centrifuge at 300 x g for 5 min at room temperature. Resuspend the resulting cell pellet in fresh EGM and divide evenly into 3 x T75 flasks with a final volume of 12 mL EGM per flask.
9. To select for EC transduced with E6 and E7, add G418 to a final concentration of 200 µg/mL for two passages, after which the transduced EC can be plated for infection or frozen in liquid nitrogen.

3. Infection of Transduced EC with KSHV

1. On the day prior to infection harvest EC by enzymatic digestion using the commercial enzymatic dissociation solution as described above. Resuspend cell pellet in 1 mL of EGM. Use 5 µL of cells to prepare a 1/10 dilution in Trypan blue. Count live cells using a hemocytometer and seed 2.5 x 10⁵ live cells in 2 mL of EGM per well in 6 well plates.
2. On the day of infection, remove EGM and wash cells with 3 mL of PBS with calcium and magnesium per well, and then add 2 mL EBM.
NOTE: It is essential to use EBM rather than EGM during infection, as the heparin in EGM will inhibit binding of viral particles to and subsequent infection of target EC.
3. To infect cells with KSHV, add 5 to 20 µL virus stock to each well and swirl plate to mix. For mock-infected cells add an equal volume of TNE buffer to each well.
4. Centrifuge plates at 400 x g for 30 min at room temperature and then incubate the plates at 37 °C for 90 min.
5. If the goal of the study is to investigate early events requiring a synchronous viral infection, e.g., early events in *de novo* infection, remove the viral inoculum at this point. Rinse and refeed the cells with 2 mL fresh EGM. Otherwise, add 2 mL EGM and incubate cultures overnight.

6. Refeed cells with 2 mL EGM the day after infection and then every other day.
7. When cells are approximately 90% confluent, harvest by enzymatic digestion using commercial enzymatic dissociation solution as described above, then pool the cells from three wells into a T75, noting both the total passage number and the passage post-infection.
8. Expand mock- and KSHV-infected EC with 1:3 splits for at least two more passages at which time cells can be used in experiments or frozen in liquid nitrogen.
NOTE: As with splitting of EC transduced with E6 and E7, maintenance cultures of mock- and KSHV-infected cells should be split before reaching confluence (~85 to 90%).

4. Confirming Infection with KSHV by Immunofluorescence

NOTE: Detection of the KSHV latency-associated nuclear antigen (LANA-1/ORF73) provides a reliable quantitative measure of infection. Anti-LANA antibodies are commercially available.

1. The day prior to staining, plate two wells each with mock- or KSHV-infected EC on a 24-well plate at 1×10^5 cells per well in 1 mL EGM and incubate overnight.
2. Wash cells twice with 500 μ L of PBS plus calcium and magnesium. Wash for 30 s on a rocking platform.
3. In an externally-ventilated fume hood, fix cells with 500 μ L of 4% paraformaldehyde for 15 min at room temperature.
4. Wash cells three times with 500 μ L of wash buffer (0.1% Triton X-100 plus 0.02% goat serum in PBS plus cations).
5. Block cells with 500 μ L of 2% goat serum in wash buffer for 30 min at room temperature.
6. Wash cells three times with 500 μ L of wash buffer.
7. Label one well of mock- or KSHV-infected EC with 200 μ L of primary antibody diluted 1:100 in wash buffer for 30 min at room temperature on a rocker. Incubate the remaining two wells in 200 μ L of wash buffer only.
8. Wash cells three times with 500 μ L of wash buffer.
9. Label all four wells with 200 μ L of secondary antibody diluted 1:100 in wash buffer for 30 min at room temperature on a rocker.
10. Wash cells three times with 500 μ L of wash buffer.
11. To each well apply 20 μ L of mounting medium containing DAPI and a coverslip and evaluate staining using an inverted fluorescent microscope.
12. Determine the percent infection of KSHV-infected cells by counting the number of LANA-positive nuclei in at least 200 cells. Infection with recombinant KSHV-Bac16 can also be monitored via observation of cultures for expression of GFP.

Representative Results

The morphology of primary EC is classically described as "cobble stone", and this morphology is not altered by expression of the papillomavirus E6 and E7 genes (**Figure 1A**). Expression of the E6 and E7 genes alone does not induce a transformed phenotype; thus, cells are susceptible to contact inhibition and will cease dividing upon reaching confluence in culture. The cells will however proliferate and regrow to confluence upon trypsinization and replating at a lower density, allowing for the maintenance of age- and passage-matched cultures for use as mock-infected controls.

Infection of E6/E7-transduced EC with KSHV causes dramatic morphological changes in culture that are reminiscent of the "spindle cell" phenotype observed in KS lesions (**Figure 1B**). A transformed phenotype is also induced upon infection with KSHV, which is manifest in part by loss of contact inhibition when cells are cultured post-confluence (**Figure 1C**) and anchorage-independent growth in soft agar (**Figure 2**). Loss of both contact inhibition and the dependence on extra-cellular matrix interactions (anchorage) during oncogenesis are two of the hallmarks of cellular transformation³⁶. It is important to note that primary EC are also susceptible to infection with KSHV and primary EC systems have been extremely valuable for elucidating diverse aspects of the virus-host interaction, and its consequences. Such studies are well-represented in the literature and several examples are cited herein^{37,38,39,40,41,42,43,44}.

Immunofluorescent staining demonstrates that the spindle morphology of KSHV-infected cells is associated with expression of the viral protein LANA-1/ORF73 (**Figure 3A and 3B**, red). As the concentration of virus in stocks will vary between preparations, the volume of virus added per well should be adjusted so that KSHV-infected cultures reach >90% infection within one or two passages. With serial passage the percentage of infection will approach 100% and will be maintained for the duration of the culture (approximately 20 passages). Lower amounts of virus can be used if a study of paracrine influences is intended, or if adjacent uninfected cells are desirable as controls (e.g., for immunofluorescence studies involving other proteins of interest). Infected cultures also support expression of lytic viral proteins but, as is observed in KS lesions, only a minority of cells in culture will spontaneously undergo lytic reactivation (**Figure 3B**, green). Cells infected with KSHV-BAC16, which is tagged with GFP, also develop a spindle morphology (**Figure 3C and 3D**). When KSHV-BAC16 is used, viral titers can be obtained by infecting cells with a serially-diluted concentrated virus stock and evaluating cells for GFP expression at 48 h post infection using flow cytometry³⁰.

For both recombinant and WT virus, viral genomes in stock preparations can also be measured by purifying DNA from infected cells followed by PCR quantification of KSHV DNA levels; however, one should not assume that all genomes are infectious⁴⁵. If the study of the KSHV lytic cycle is intended, virus reactivation can be achieved by stimulating EC with inducing agents as described above for BCBL-1 cells, although the efficiency of reactivation is lower²³. The lytic cycle can also be induced by transduction of EC with the KSHV transactivator protein ORF50⁴⁶. Consistent with findings in other cell culture models of KS, both spontaneous expression and chemical induction of KSHV lytic genes in E6/E7-expressing EC decreases over time despite maintenance of a stable latent infection^{11,16}.

Figure 1: Morphology of mock- and KSHV infected EC. (A) Mock-infected EC monolayers exhibit the classic cobble stone appearance by phase contrast vital microscopy. Without the actions of KSHV genes these cells are not transformed and will therefore cease dividing and exhibit contact inhibition upon reaching confluence in culture. (B) KSHV-infected cells, in contrast, develop an elongated "spindle cell" morphology reminiscent of KS spindle cells. This panel shows the morphology of KSHV-infected cells at day 5 PI upon reaching confluence. (C) In KSHV-infected cells, extensive changes in host cell gene expression lead to cellular transformation, which becomes evident when KSHV-infected cells are cultured without passage. Under such conditions, KSHV-infected cells continue proliferating even after reaching confluence leading to the development of multi-layered foci of cells. This panel shows the morphology of KSHV-infected cells at day 14 PI, cultured for 9 days post-confluence. [Please click here to view a larger version of this figure.](#)

Figure 2: Anchorage independent growth. Untransformed cells typically undergo apoptotic cell death following loss of contact with a substrate, a process called anoikis. (A) When E6/E7-transduced mock-infected EC, which are not transformed, are suspended in soft agar and cultured for two weeks they do not form colonies. (B) KSHV-infected EC, which are virus-transformed, are resistant to anoikis and will form colonies in soft agar. [Please click here to view a larger version of this figure.](#)

Figure 3: Demonstration of viral protein expression. (A) The spindle morphology is evident following infection with KSHV (40X magnification). (B) Immunofluorescent staining of the same field shown in (A) demonstrates that the spindle morphology is associated with expression of the viral latency protein ORF73 (red). Infected cells support lytic viral replication as well, as demonstrated by a small percentage of cells expressing the viral processivity factor ORF59 (green). (A and B modified from reference²⁶.) The spindle morphology of cells infected with KSHV-BAC16 virus (C) is associated with expression of GFP (D) (20X magnification). [Please click here to view a larger version of this figure.](#)

Discussion

Oncogenesis is a multistep process that circumvents important safeguards within an organism³⁶. As KS lesions exist along a spectrum of chronic inflammation to true sarcomas, elucidation of certain pathophysiological processes mediated by KSHV requires that some studies be conducted in cell culture models that support transformation⁹. It should be noted that loss of contact inhibition and anchorage-dependent growth, phenotypes indicative of cellular transformation, do not readily develop following infection of primary EC or EC immortalized by exogenous expression of telomerase. Therefore, while the *in vitro* model of KS described here does not recapitulate all aspects of the KS tumor microenvironment or behavior of explanted KS cells, it is uniquely suited for studying changes in host cell gene expression induced by infection with KSHV that contribute to KS tumorigenesis.

The first reported gene expression profiling study using the culture system described herein identified the receptor tyrosine kinase c-Kit as a contributor to the transformed phenotype of KSHV-infected cells²⁵. Knock down of c-Kit expression using siRNA or inhibition of c-Kit signaling using a dominant negative construct or tyrosine kinase inhibitor STI 571 (Imatinib) interfered with the transforming ability of KSHV manifest by loss of foci formation after prolonged culture of infected cells. Subsequent clinical evaluation of Imatinib has demonstrated efficacy of this drug in patients with epidemic KS^{47,48,49}. Other potential treatment targets have also been identified, including heme oxygenase-1^{26,50}, CXCR7²⁷, and the beta adrenergic receptors²⁸, all of which play a role in the growth or transformation of EC *in vitro*. Confirmation of these gene expression patterns in primary EC as well as KS biopsy tissue confirms the physiologic relevance of this system and underscores its value as a pre-clinical model for KS therapeutics^{25,26,51,52,53,54,55}.

Another strength of the E6/E7 transduction system is that the extended life span of immortalized as compared to primary EC enables the long-term maintenance of age and passage-matched mock-infected control cells for comparative evaluation of the outcomes of KSHV infection. It is critical to note that in order to maximize the number of passages of EC transduced with E6 and E7 it is critical to split cultures before reaching confluence (~85 to 90%). Furthermore, these cells tolerate serum-free conditions for extended periods of time. Primary EC rapidly undergo programmed cell death upon growth factor withdrawal and therefore cannot be cultured for long periods without serum or recombinant growth factors^{56,57}. This dependence makes autocrine signaling pathways induced by infection with KSHV difficult to identify and study, as prior to full transformation KSHV-infected cells will also undergo programmed cell death upon growth factor withdrawal (unpublished observation). The presence of the E6 and E7 proteins in this model, however, prevent programmed cell death following growth factor withdrawal and allow for prolonged (*e.g.*, 48 h) culture in low serum or serum free medium^{25,26,28}.

KS is a complex and unusual disease with features of both chronic inflammation and cellular transformation. The clinical need for novel therapeutic strategies is great, and the cell culture model described herein has unique properties that allow assessment of candidate drug targets.

Disclosures

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