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Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen Prolongs the Life Span of Primary Human Umbilical Vein Endothelial Cells

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Tumor spindle cells in all clinical types of Kaposi's sarcoma (KS) are infected with Kaposi's sarcoma-associated herpesvirus (KSHV). Although KSHV contains more than 80 genes, only a few are expressed in tumor spindle cells, including latency-associated nuclear antigen (*LANA*) and k-cyclin (*kCYC*). To assess the oncogenic potential of *LANA* and *kCYC*, primary human umbilical vein endothelial cells (HUVEC) and murine NIH 3T3 cells were stably transduced by using recombinant retroviruses expressing these genes or the known viral oncogene simian virus 40 large T antigen (*LTAg*). Interestingly, *LANA*-transduced HUVEC proliferated faster and demonstrated a greatly prolonged life span (mean ± standard deviation, 38.3 ± 11.0 passages) than untransduced cells and vector-transduced cells (<20 passages). By contrast, *kCYC*-transduced HUVEC did not proliferate faster or live longer than control cells. *LANA*- and *kCYC*-transduced HUVEC, but not *LTAg*-transduced HUVEC, retained the ability to form normal vessel-like structures in an in vitro model of angiogenesis. In cellular assays of transformation, *LANA*- and *kCYC*-transduced NIH 3T3 cells demonstrated minimal or no anchorage-independent growth in soft agar and no tumorigenicity when injected into nude mice, unlike *LTAg*-transduced NIH 3T3 cells. Lastly, gene expression profiling revealed down-regulation, or silencing, of a number of genes within *LANA*-transduced HUVEC. Taken together, these results suggest that KSHV *LANA* is capable of inducing prolonged life span, but not transformation, in primary human cells. These findings may explain why *LANA*-expressing spindle cells proliferate within KS tumors, yet most often do not demonstrate biologic characteristics of transformation or true malignant conversion.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the gammaherpesvirus subfamily, which is the group of herpesviruses known to be associated with cellular proliferation and malignancies. All tumor spindle cells in all clinical forms of Kaposi's sarcoma (KS) are infected with KSHV (4, 7, 15, 42, 62). KSHV infection is also associated with two other diseases, primary effusion lymphoma (PEL) and the plasmablastic variant of Castleman's disease (6, 15, 61). Although the KSHV genome encompasses over 80 open reading frames (ORFs) (56), the expression of KSHV genes in both KS tumor spindle cells and in B-cell lines derived from patients with PEL is highly restricted (17, 49, 58, 63, 68). Latent or nonproductive infection describes infected cells with this type of restricted pattern of viral gene expression. There are few KSHV genes expressed in latently infected cells (estimated to be less than five), yet these genes likely play critical roles in disease pathogenesis by stimulating cellular proliferation, interfering with normal tumor suppressor protein function, and blocking normal proapoptotic pathways (12, 31).

KSHV *orf73* encodes latency-associated nuclear antigen (LANA), also known as LANA-1, LNA, or LNA-1, which is a viral protein expressed in latently infected KS tumor spindle cells and PEL cells (15, 27, 28, 48). The functions of both p53 and retinoblastoma protein (Rb), two critical tumor suppressor proteins involved in tumor surveillance, are inhibited by LANA in vitro (19, 51). Within KS lesions, LANA and p53 are coexpressed within the same cells in the absence of apoptosis and p53 genes contain normal sequences, suggesting that LANA interferes with the proapoptotic function of p53 at the protein level (29). LANA also suppresses global transcriptional activity (21, 22, 32, 38, 39, 50, 59), possibly by binding to one of several multifunctional coactivators of transcription (e.g., CREB binding protein) and blocking activity. In addition, LANA is involved in tethering the KSHV genome to chromosomal DNA (3, 10, 21, 24). This function is critical for both the maintenance of the KSHV episome and the replication of KSHV DNA in dividing cells. Interestingly, Epstein-Barr virus nuclear antigen 1 (EBNA-1) performs an analogous function in cells latently infected with Epstein-Barr virus, another member of the gammaherpesvirus subfamily, although EBNA-1 and LANA share no significant sequence homology. Thus, LANA possesses several functional properties that are believed to be important in KS pathogenesis.

KSHV *orf72* encodes k-cyclin (kCYC), also known as v-cyclin, which (like LANA) is a viral protein expressed in latently infected KS tumor spindle cells (1, 13, 52). It also possesses a number of functional properties believed to be important in KS pathogenesis. kCYC is a cellular cyclin D homologue (8) that interacts with all types of cyclin-dependent kinases (Cdk's), although it prefers Cdk6 (23, 37). Unlike cellular cyclin D/Cdk complexes, kCYC/Cdk6 complexes are re-

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sistant to inhibition by Cdk inhibitors (25, 64). Furthermore, kCYC/Cdk6 complexes phosphorylate and inactivate both Rb (23) and the Cdk inhibitor p27 (Kip) (16, 40), which leads to unregulated progression through the cell cycle. In the presence of normal p53 function, kCYC also sensitizes cells to undergo apoptosis (45, 46, 65), whereas in the absence of p53 kCYC promotes cell survival (65). Thus, kCYC is believed to be a key viral protein in KSHV-mediated disease processes.

To explore the oncogenic potential of *LANA* and *kCYC* in primary human cells, we stably transduced primary human umbilical vein endothelial cells (HUVEC) by using recombinant retroviruses expressing either *LANA* or *kCYC*. HUVEC were chosen as model endothelial cells for this study because they, unlike many other types of primary endothelial cells, express VEGFR-3 (26), an antigen expressed on KS spindle cells (15). We also transduced NIH 3T3 cells, because these cells are commonly used to assess the transforming ability of genes of interest (i.e., by injection of transduced cells into nude mice). The abilities of *LANA* and *kCYC* to induce cellular changes consistent with immortalization and transformation were assessed. Cells transduced with simian virus 40 (SV40) large T antigen (*LTAg*), a known viral oncogene, were utilized as positive controls throughout the study. Interestingly, we found that *LANA* transduction increased proliferation and greatly prolonged life span in primary HUVEC, although *LANA* transduction did not induce cellular changes consistent with transformation. These results may explain why spindle cells proliferate within KS tumors yet most often do not demonstrate biologic characteristics of transformation or true malignant conversion.

MATERIALS AND METHODS

Cells. Primary cultures of HUVEC were purchased from Clonetics Corp. (San Diego, Calif.), and NIH 3T3 mouse fibroblasts were purchased from American Type Culture Collection (Rockville, Md.). HUVEC were grown in endothelial cell basal medium (Clonetics Corp.) containing 2% fetal calf serum (FCS), and NIH 3T3 cells were grown in D-MEM (Sigma-Aldrich Co., St. Louis, Mo.) supplemented with 10% FCS. HUVEC were expanded and used for retrovirus transduction between passages four and five. GP2-293, a GP293-based packaging cell line that stably expresses the *gag* and *pol* genes of vesicular stomatitis virus (VSV), was purchased from Clontech (Palo Alto, Calif.).

Creation of recombinant plasmids. Two plasmids for producing infectious retrovirus, pVSV-G and pLXRN, were purchased from Clontech. pVSV-G expresses the envelope glycoprotein of VSV from the cytomegalovirus promoter. The expression plasmid pLXRN provides the virus-packaging signal ($\Psi+$), a gene to be expressed, and a G418 resistance gene (*Neo*^{r}). Full-length cDNAs for *LANA* and *kCYC* were generated by PCR amplification using DNA from the KSHV lambda phage library L54 (NIH AIDS Research and Reference Reagent Program, Rockville, Md.) as a template (56). PCR primers were as follows: *LANA* (sense), 5′-CTA GTC GAC ATG GCG CCC CCG GGA ATG CGC CTG-3′; *LANA* (antisense), 5′-AGC GTC GAC TTA TGT CAT TTC CTG TGG AGA GTC-3′; *kCYC* (sense), 5′-CCC GTC GAC ATG GCA ACT GCC AAT AAC CCG CCC-3′; *kCYC* (antisense), 5′-CAC GTC GAC TTA ATA GCT GTC CAG AAT GCG CAG-3′. PCR products were separated on 0.6% agarose gels, purified from gels using a Sephaglas BandPrep kit (Pharmacia Corp., Peapack, N.J.), digested with *Sal*I (underlined), and ligated into the unique *Sal*I cloning site of pLXRN. For subcloning of SV40 *LTAg*, a plasmid containing the whole genome of SV40 (pUCSV40-B1E) was purchased from American Type Culture Collection. A 2,941-bp fragment flanked by *Stu*I and *Apa*I restriction enzyme sites and encoding *LTAg* was excised from the parental plasmid, blunted with T4 DNA polymerase, and subcloned into the unique *Sma*I site of pUC19. The *Eco*RI site of the resultant *LTAg*-pUC19 was then changed to *Sal*I, and a 2.9-kbp *Sal*I fragment containing *LTAg* was introduced into pLXRN. To confirm results of cloning, the three pLXRN-based recombinant plasmids were sequenced by the deoxynucleotide chain termination method with an Applied Biosystems 373 automated sequencer (Foster City, Calif.) prior to transfection.

Plasmid transfection and retrovirus infection. Infectious retroviruses expressing either the G418 resistance gene alone (*Neo*^{r}), *LANA-Neo*^{r}, *kCYC-Neo*^{r}, or *LTAg-Neo*^{r} were prepared according to the manufacturer's protocol. Briefly, pVSV-G and either pLXRN, pLXRN-*LANA*, pLXRN-*kCYC*, or pLXRN-*LTAg* were cotransfected into GP2-293 by $CaPO_4$ coprecipitation (Stratagene, La Jolla, Calif.). Forty-eight hours following transfection, supernatants were collected, passed through 0.45-μm-pore-size cellulose acetate filters (Millipore Corp., Bedford, Mass.), and concentrated by ultracentrifugation as previously described (5).

HUVEC or NIH 3T3 cells were transduced with each recombinant retrovirus. Cells were first plated at a density of 3×10^5 cells per well for HUVEC and 1×10^5 cells per well for NIH 3T3 in six-well plates (Costar, Corning, N.Y.) 12 h before infection. A 2-ml aliquot of fresh medium containing the concentrated retroviruses and Polybrene (final concentration of 8 μg/ml) was then placed into each well, the plates were centrifuged at $900 \times g$ for 2 h at 4°C, and then they were incubated overnight at 37°C. Fresh culture medium containing 100 μg (HUVEC) or 500 μg (NIH 3T3 cells) of G418 (Invitrogen, Carlsbad, Calif.) per ml was then added to wells. Cells were subcultured for 10 d following infection, expanded in the presence of G418, and used directly for subsequent experiments.

Western blotting. Immunoblotting was performed as previously described (67). Briefly, cells were harvested and disrupted in 2× sample buffer containing 0.1 M Tris-hydrochloride (pH 6.8), 20% glycerol, 0.2% bromophenol blue, 4% sodium dodecyl sulfate (SDS), and 12% 2-mercaptoethanol. Solubilized proteins were resolved in SDS–7.5% or 12.5% polyacrylamide gels and transferred to nitrocellulose membranes in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were probed with antibodies directed against LANA (Advanced Biotechnologies Inc., Columbia, Md.), kCYC (Exalpha Biologicals Inc., Boston, Mass.), or LTAg (Santa Cruz Biotech, Santa Cruz, Calif.). Secondary antibodies conjugated with alkaline phosphatase were used to visualize protein-antibody complexes in a mixture of 0.016% 5-bromo-4-chloro-3-indolylphosphate and 0.033% nitroblue tetrazolium.

Immunofluorescence staining. Transduced HUVEC (5×10^4/ml) were cultured overnight on Falcon culture slides (Becton Dickinson, Franklin Lakes, N.J.). After confirming cell attachment, culture medium was carefully aspirated. As positive controls, 5×10^4 BCBL-1 cells were centrifuged onto glass slides. Slides were then placed in a methanol-acetone mixture (1:1) at −30°C for 10 min, air dried, and rehydrated with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). To block nonspecific staining, cells were incubated with PBS containing 3% BSA and 1% glycine for 30 min. Cells were then exposed to normal rabbit immunoglobulin G (IgG; R&D Systems, Inc., Minneapolis, Minn.) or rabbit serum containing polyclonal anti-LANA antibodies (a kind gift of Don Ganem, University of California at San Francisco) (diluted 1:100 in blocking buffer) for 1 h, washed twice with 4% Tween 20 containing PBS, exposed to fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:300 for 1 h, and then washed again. Slides were then mounted and analyzed using a Nikon Eclipse TE 300 microscope (Nikon, Melville, N.Y.).

Proliferation assay. A total of 3×10^4 cells were plated into each well of six-well plates (day 0) and cultured at 37°C in complete medium. Cells from representative wells were trypsinized and counted using a hemocytometer and trypan blue exclusion daily for 5 days. Experiments were performed using HUVEC that had been passaged eight to nine times. Experiments were repeated four times.

In vitro angiogenesis assay. ECMatrix gel was purchased from Chemicon International (Temecula, Calif.) and used according to the manufacturer's protocol. HUVEC (2×10^3/well) were cultured in 96-well plates in duplicate, and the formation of tubular structures within the matrix was assessed 24 h later by using a phase-contrast microscope. Experiments were performed twice.

Assessment of clonogenicity in soft agar. For HUVEC, cells were placed in 2× M199 medium (BioWhittaker, Walkersville, Md.) with 2× endothelial growth supplement (Perimmune Inc., Rockville, Md.) and 2% FCS and mixed with equal amounts of 1.0% melted agarose. Two milliliters of the mixture was poured onto 35-mm-diameter plates to form a bottom layer. For a top layer, 2× M199 medium with 2× endothelial growth supplement and 2% FCS was mixed with equal volumes of 0.7% melted agarose; 2 ml of this mixture was then poured onto the bottom layer. For NIH 3T3 cells, cells were placed in 2× D-MEM with 20% FCS and mixed with equal amounts of 1.0% melted agarose. Two milliliters of the mixture was poured onto 35-mm plates to form a bottom layer. For a top layer, 2× D-MEM with 20% FCS was mixed with equal volumes of 0.7% melted agarose; 2 ml of this mixture was then poured onto the bottom layer. The total number of HUVEC or NIH 3T3 cells propagated was 1.5×10^4 per plate, and five separate plates were used for each type of cell. Plates were incubated at 37°C for 4 weeks. Cell colonies were stained with 1 ml of 0.005% crystal violet

(Sigma-Aldrich Co.) and counted using a phase-contrast microscope. Only colonies consisting of more than five cells were counted.

Assessment of tumorigenicity in nude mice. Female athymic nude mice (CBy.Cg-Foxn1) were purchased from Jackson Laboratory (Bar Harbor, Maine) and were used at ages 4 to 6 weeks. An equal number of HUVEC or NIH 3T3 cells (5×10^5) were harvested, suspended in 0.1 ml of PBS, and injected into subcutaneous tissue of mice. Each condition group consisted of five mice. Tumor sizes at the injected sites were assessed every other day, and tumor indices were calculated by determining the square root of $(a) \times (b)$, where a was the longest and b was the shortest axis of the tumor. Mice were euthanized when the longest axis of the tumor reached 20 mm. All experiments were reviewed and approved by the Animal Care and Use Committee of the National Cancer Institute.

cDNA microarray analyses. On two separate days, HUVEC were harvested at ~80% confluency and total RNA was extracted with an RNeasy kit (Qiagen Inc., Valencia, Calif.). Experimental procedures for preparation of the labeled cRNA probe, hybridization, and data collection were performed according to the Affymetrix GeneChip expression analysis technical manual (Affymetrix, Santa Clara, Calif.). Briefly, double-stranded cDNA was synthesized from mRNA by using the SuperScript Choice system (Gibco BRL, Carlsbad, Calif.) and a T7-$(dT)_{24}$ primer. In vitro transcription was performed on the cDNA to produce biotin-labeled cRNA by using an Enzo transcription kit (Enzo, Farmingdale, N.Y.) as described by the manufacturer. The cRNA was then purified using an RNeasy Mini kit (Qiagen), fragmented to 50 to 200 nucleotides, and hybridized to Affymetrix U95Av2 human arrays. The arrays were then processed on the Affymetrix fluidics station and scanned on an HP GeneArray scanner. The intensity for each probe set of the array was captured with the Affymetrix GeneChip software, according to standard Affymetrix procedures. To quantify RNA levels, the average differences representing the perfectly matched minus the mismatched levels for each gene-specific probe set were calculated. mRNA levels in gene-transduced cells were compared to levels in vector-transduced cells, and genes that demonstrated either twofold-higher or twofold-lower expression in each of two independent experiments were tabulated.

Quantitative real-time RT-PCR. RNA was extracted from HUVEC as described above, and 750 ng of total RNA was reverse transcribed in a volume of 100 μl using TaqMan reverse transcription (RT) reagents (Applied Biosystems), including 10 μl of 10× RT buffer, 22 μl of $MgCl_2$ (25 mM), 20 μl of deoxynucleoside triphosphates (10 mM), 5 μl of random hexamers (50 μM), 5 μl of oligo$(dT)_{16}$ (50 μM), 2 μl of RNase inhibitor (20 U/μl), and 3 μl of reverse transcriptase (50 U/μl). After incubation for 30 min at 48°C, reactions were stopped by heating for 5 min at 95°C. Quantitative real-time RT-PCR was then performed in a total volume of 50 μl, including 25 μl of SYBR Green PCR Master Mix (Applied Biosystems), 1.5 μl of each primer (50 μM), and 3.3 μl of cDNA (7.5 ng/ml). Thermal amplification was performed using the following linked profile: 10 min at 95°C, 40 cycles of denaturation (95°C for 15 s), and annealing-extension (60°C for 1 min) in a model 7700 sequence detection system (Applied Biosystems). Primer sequences used were as follows: G_0/G_1 switch 2 (*G0S2*) (sense), 5′-AAG GAG ATG ATG GCC CAG AA-3′; *G0S2* (antisense), 5′-GAG CAC CAC GCC GAA GAG-3′; DNA polymerase delta 1 (*POLD1*) (sense), 5′-GCC CTA CGA GGC CAA CGT-3′; *POLD1* (antisense), 5′-CAG TTG CAG CCG ACG ATG T-3′; kinesin-like 4 (*KNSL4*) (sense), 5′-AGA CAC AGT CTC CGC ACT CAA CT-3′; *KNSL4* (antisense), 5′-TCT CAT TGG TAA AAG GCC GAT T-3′; carbohydrate sulfotransferase 1 (*CHST1*) (sense), 5′-AGA CCC GCG ATT AAA CCT CAA-3′; *CHST1* (antisense), 5′-TGC GCG AAG CCA GAA TG-3′; matrix metalloproteinase 1 (*MMP1*) (sense), 5′-TGA GCT CAA TTT CAT TTC TGT TTT CT-3′; *MMP1* (antisense), 5′-TGT CGG CAA ATT CGT AAG CA-3′; integrin α2 (*ITGA2*) (sense), 5′-GAG GTG ACC AGA TTG GCT CCT A-3′; *ITGA2* (antisense), 5′-AAG AGC ACG TCT GTA ATG GTG TCT T-3′; cyclin D2 (*CCND2*) (sense), 5′-GCT GCT GGC TAA GAT CAC CAA-3′; *CCND2* (antisense), 5′-GCA CCG CCT CAA TCT GCT-3′; ephrin-A1 (*EFNA1*) (sense), 5′-AGG TGC GGG TTC TAC ATA GCA-3′; *EFNA1* (antisense), 5′-AGT CCA GGC AAG TGG GAA GA-3′; *CD24* (sense), 5′-TCC AAC TAA TGC CAC CAC CAA-3′; *CD24* (antisense), 5′-GAC CAC GAA GAG ACT GGC TGT T-3′. mRNA was extracted from stably transduced cells twice on different days, and RT-PCR was performed four times for each mRNA sample. For each experiment, a threshold cycle value, or C_t, was calculated for each gene by determining the point at which PCR product fluorescence exceeded a threshold limit. The relative expression level of each gene compared to glyceraldehye-3-phosphate dehydrogenase (GAPDH) expression was calculated as $2^{-[(Ct \text{ of gene}) - (Ct \text{ of GAPDH})]}$. After normalization to *GAPDH* expression in this manner, fold differences in gene expression between *LANA*-transduced HUVEC and vector-transduced HUVEC were assessed.

RESULTS

Protein expression in transduced cells. Cells infected with recombinant retroviruses were cultured in the presence of G418. In general, stably transduced G418-resistant cells were obtained following three to five passages. Protein expression of *LANA*, *kCYC*, and SV40 *LTAg* was confirmed by immunoblotting and (for LANA) by immunofluorescence staining. As expected, proteins with molecular masses of 222/234 kDa (doublet bands), 28 kDa, and 94 kDa were observed in *LANA*-, *kCYC*-, and *LTAg*-transduced cells, respectively (Fig. 1A). The sizes of LANA and kCYC proteins were the same in both HUVEC and NIH 3T3 cells, as well as in BCBL-1 cells used as positive controls (Fig. 1A and data not shown). Specific bands for LANA were slightly darker when BCBL-1 cells were used as a source for protein, compared to results with transduced HUVEC (data not shown). No specific protein bands were observed by immunoblotting in HUVEC or NIH 3T3 cells stably transduced with vector alone (Fig. 1A and data not shown). By immunofluorescence, all *LANA*-transduced HUVEC and NIH 3T3 cells showed the expected intranuclear speckled pattern, confirming both the proper location of protein expression and the homogeneity of the stably transduced cells (Fig. 1B and data not shown).

Increased proliferation and prolonged life span of *LANA*-transduced HUVEC. The growth rates of stably transduced HUVEC (in the presence of 100 μg of G418/ml) were determined. Compared to cells stably transduced with vector alone, cellular proliferation was consistently increased in *LANA*-transduced HUVEC (at day 5, $P = 0.029$ by the exact Wilcoxon rank sum test, with the P value being two-sided and unadjusted for multiple comparisons), although proliferation was not as great as that observed in *LTAg*-transduced cells (Fig. 2). By contrast, stable transduction with *kCYC* did not increase proliferation rates in HUVEC ($P > 0.05$ at day 5) (Fig. 2).

Primary HUVEC normally senesce within 10 to 15 passages in cell culture. Interestingly, three HUVEC cultures stably transduced with *LANA* were passaged 26, 42, and 47 times before senescing (mean ± standard deviation [SD], 38.3 ± 11.0 passages) (Fig. 3). Additional HUVEC transduced with *LANA* at a more recent time have currently been passaged 14 times and continue to grow well in culture. Strikingly, HUVEC initially transduced with *LTAg* have not yet died, with the passage number being greater than 88 at the time of this study (Fig. 3). By contrast, two HUVEC cultures stably transduced with vector alone survived only 17 and 21 passages, whereas HUVEC stably transduced with kCYC survived 11 passages in culture (Fig. 3).

***LANA*- and *kCYC*-transduced HUVEC retain the ability to form normal vessel-like structures in an in vitro angiogenesis assay.** HUVEC, as well other types of normal cultured endothelial cells, possess the ability to form vessel-like tubular structures in vitro. To test this function in endothelial cells expressing LANA or kCYC, transduced cells were cultured overnight in a commercially available extracellular matrix gel solution. Cultures of *LTAg*-transduced HUVEC, but not cultures of *LANA*- or *kCYC*-transduced HUVEC, were impaired in their ability to form normal vessel-like structures (Fig. 4). In fact, cultures of *LANA*- or *kCYC*-transduced HUVEC were

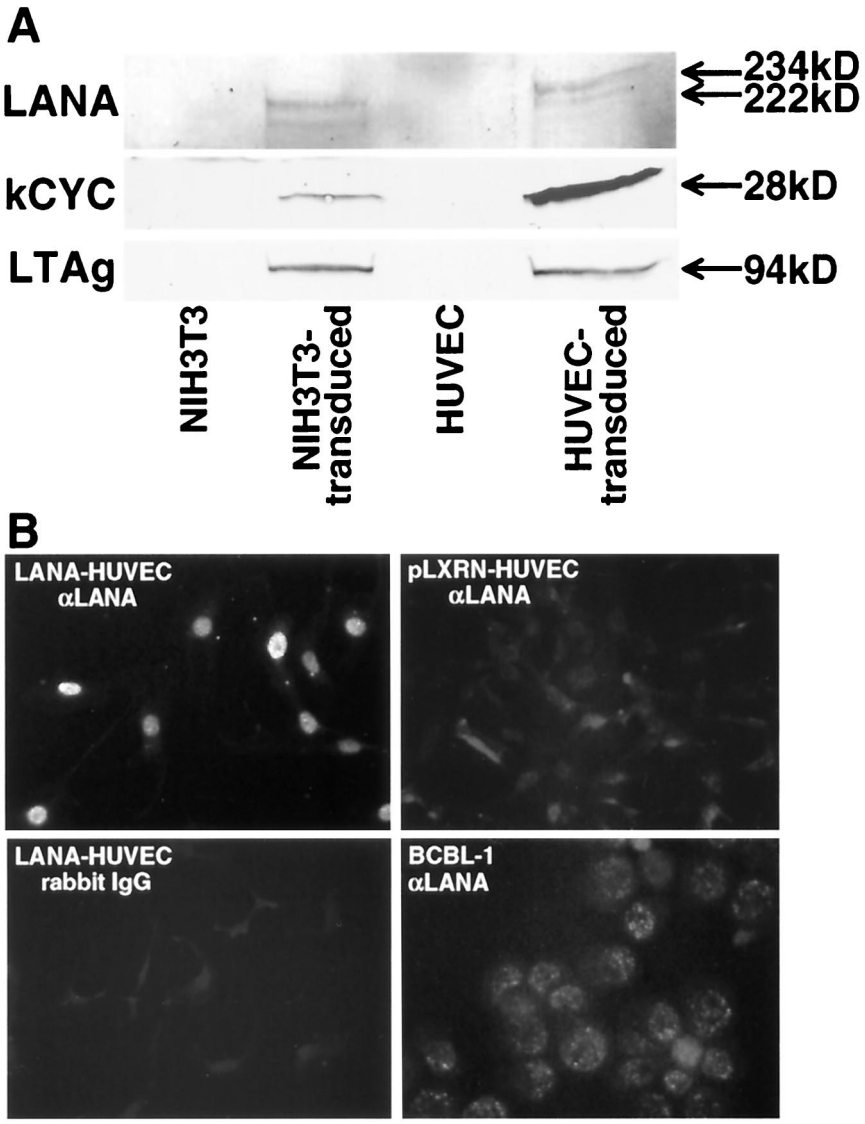

FIG. 1. LANA, kCYC, and LTAg protein expression in cells following infection with recombinant retroviruses and selection in the presence of G418. (A) Proteins were extracted from either transduced or untransduced cells and immunoblotted with specific antibodies as indicated. (B) LANA protein expression within all nuclei of *LANA*-transduced and G418-selected HUVEC (upper left), but not in vector (*pLXRN*)-transduced HUVEC (upper right). *LANA*-transduced HUVEC stained with rabbit IgG (lower left) and the KSHV-infected PEL cell line BCBL-1 stained with anti-LANA antibodies (lower right) were used as additional negative and positive controls, respectively.

indistinguishable from cultures consisting of untransduced or vector-transduced HUVEC (Fig. 4).

Neither *LANA*- nor *kCYC*-transduced cells are transformed. One important characteristic of cells that have undergone transformation is their ability to form colonies in soft agar (i.e., to grow in an anchorage-independent manner). Compared to untransduced or vector-transduced NIH 3T3 cells, cells transduced with *kCYC* did not form colonies in soft agar. Cells transduced with *LANA* formed some small, but few, colonies in soft agar (Fig. 5). This was most evident in *LANA*-transduced NIH 3T3 cells. By contrast, both *LTAg*-transduced NIH 3T3 cells and *LTAg*-transduced HUVEC readily formed soft agar colonies (Fig. 5).

Transduced cells were also tested for their ability to form tumors in nude mice (another measure of cellular transformation). Neither *LANA*- nor *kCYC*-transduced NIH 3T3 cells were able to form tumors in nude mice, unlike *LTAg*-transduced NIH 3T3 cells (Fig. 6). None of the transduced HUVEC (including *LTAg*-transduced HUVEC) formed tumors in nude mice (data not shown), consistent with previous oncogene studies using primary cells. Taken together, these data indicate that neither *LANA* nor *kCYC* alone transforms cells.

Gene expression analyses in *LANA*-transduced HUVEC using cDNA microarrays and quantitative real-time RT-PCR. To begin to elucidate the mechanisms underlying the ability of *LANA* to induce prolonged life span in primary cultures of HUVEC, gene expression analyses using cDNA microarray technology were performed. Using total RNA extracted on two

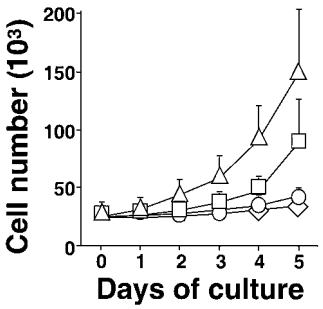

FIG. 2. *LANA*-transduced HUVEC proliferate faster than *kCYC*- or vector (*pLXRN*)-transduced HUVEC. A total of 30×10^3 stably transduced HUVEC were subcultured on day zero, and absolute cell counts were determined daily for the next 5 days by using a hemocytometer. Triangles = *LTAg*-transduced cells; squares = *LANA*-transduced cells; circles = *kCYC*-transduced cells; diamonds = vector (*pLXRN*)-transduced cells. Values shown represent the means plus SD of four separate experiments. $P = 0.029$ when comparing *LANA*-transduced HUVEC to vector-transduced cells at day 5.

separate days and analyzed separately, three genes were up-regulated and 29 genes were down-regulated in *LANA*-transduced HUVEC compared to vector-transduced HUVEC (Table 1). By contrast, using identical methods, 60 genes were up-regulated and 76 genes were down-regulated in *LTAg*-transduced HUVEC compared to vector-transduced HUVEC (data available upon request). Of note, all three genes up-regulated and 18 of the 29 genes down-regulated in *LANA*-transduced HUVEC were differentially regulated in the same manner in *LTAg*-transduced HUVEC. Interestingly, *G0S2* was at the top of both lists, being dramatically up-regulated in both *LANA*- and *LTAg*-transduced HUVEC.

Next, confirmation of a subset of these *LANA*-HUVEC microarray data was assessed using quantitative real-time RT-PCR. Out of nine genes selected for testing by real-time RT-PCR, five had definite patterns of expression that corresponded to the cDNA microarray data (Table 1). Specifically, *G0S2* and *POLD1* were confirmed to be up-regulated, whereas *CHST1*, *MMP1*, and *CCND2* were confirmed to be down-regulated in *LANA*-transduced HUVEC compared to vector-transduced HUVEC.

DISCUSSION

In this report, we studied the ability of two purported KSHV oncogenes, *LANA* and *kCYC*, to induce cellular immortalization and transformation. The most striking observations we made were that primary HUVEC stably transduced with *LANA* proliferated faster and lived much longer than untransduced or vector-transduced cells (Fig. 2 and 3). *LANA*, however, did not induce cellular changes consistent with transformation or malignant conversion (Fig. 5 and 6). Gene expression analyses suggested that LANA induced silencing (rather than up-regulation) of many cellular genes (Table 1). We suggest that these particular cellular effects may explain why *LANA*-expressing tumor spindle cells within KS lesions proliferate yet do not form true cancers in most cases of KS.

Some investigators have previously ascribed transforming capabilities to KSHV in endothelial cells infected with whole virus. In initial studies, KSHV infection of endothelial cells as well as a variety of other primary cells and cell lines in vitro was difficult to establish (47, 54). Cesarman and coworkers first described "transformation" of primary endothelial cells in cultures where approximately 5% of the cells were infected with KSHV (18). Moses et al. demonstrated biologic features of transformation in KSHV-infected dermal microvascular endothelial cells that also expressed the human papillomavirus oncoproteins E6 and E7 (43). By contrast, Hayward and colleagues successfully infected dermal microvascular endothelial cells by using infectious supernatants containing both KSHV and Epstein-Barr virus and described induction of spindle cell morphology, yet no transformation (9). Ganem and colleagues recently described KSHV infection in telomerase-immortalized dermal microvascular endothelial cells, although these cells also did not demonstrate features of transformation (33). Sakurada et al. (57) and Dezube et al. (14) have also recently described KSHV infection of endothelial cells in vitro without evidence of transformation. Thus, our results that demonstrate no transforming capability of either *LANA* or *kCYC*, as well as most of the previous infection studies cited here, suggest that KSHV alone does not transform endothelial cells.

The oncogenic potential of single genes encoded by KSHV has also been examined. *K1* transforms rodent fibroblasts, immortalizes marmoset lymphocytes, and induces lymphoma formation in marmosets (34). Cells transduced with viral G-protein coupled receptor (*vGPCR*) prolong survival in primary endothelial cells (41) and form tumors when injected into nude mice (2), results that implicate *vGPCR* as an oncogene. Similarly, viral interferon regulatory factor 1-transduced NIH 3T3 cells demonstrate tumorigenicity in nude mice (20). The relevance of these particular studies to KS pathogenesis has been questioned, however, since these KSHV genes are predominantly lytic-cycle genes and thus are not expressed in the majority of KS tumor spindle cells (17, 30, 49, 58, 63).

Immortalization and transformation capabilities of KSHV latent-cycle genes have also been reported and perhaps offer more insight into KS pathogenesis, since these genes are highly expressed within KS tumors. Kaposin has been reported to have transforming ability (44). As well, Radkov et al. reported that *LANA*, in cooperation with the known oncogene *Hras*, could cause cellular transformation when expressed in primary

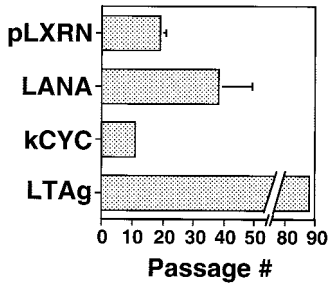

FIG. 3. *LANA*-transduced primary HUVEC have a prolonged life span. Primary HUVEC were transduced as indicated and cultured under similar standard conditions. Data represent means ± SD for *pLXRN*- and *LANA*-transduced cells. Transduction of HUVEC with *kCYC* and *LTAg* was performed only once. LANA protein expression was periodically confirmed in *LANA*-transduced cells by immunofluorescence antibody staining (data not shown).

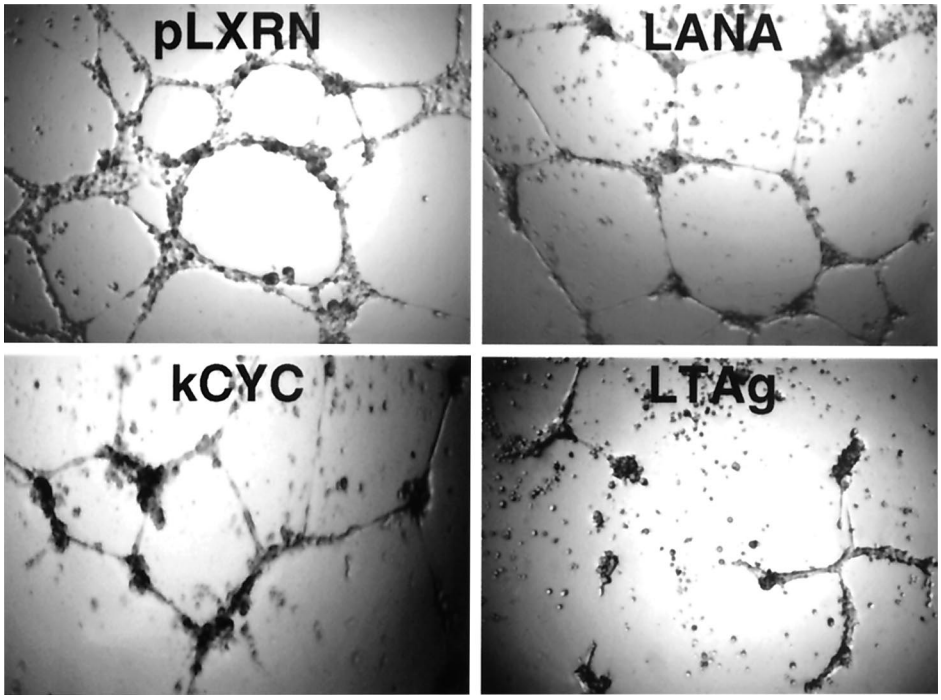

FIG. 4. *LANA*- and *kCYC*-transduced HUVEC retain the ability to form normal vessel-like structures in an in vitro angiogenesis assay, unlike *LTAg*-transduced HUVEC. Representative fields are shown.

rat embryo fibroblasts (51). This report contrasts somewhat with our finding that *LANA* transduction did not induce clonogenicity (Fig. 5) or tumorigenicity (Fig. 6) in NIH 3T3 cells. These differences may be due to a protransforming influence of *Hras*, which was not present in our *LANA*-transduced cells. In a recent report, *kCYC* induced proliferation, prolonged life span, and triggered tumor-forming ability in p53-deficient cells but not in cells with wild-type p53 (65). Those results are similar to the findings that we report here, where we used cells with intact p53 function. Thus, the role that *kCYC* plays in KS

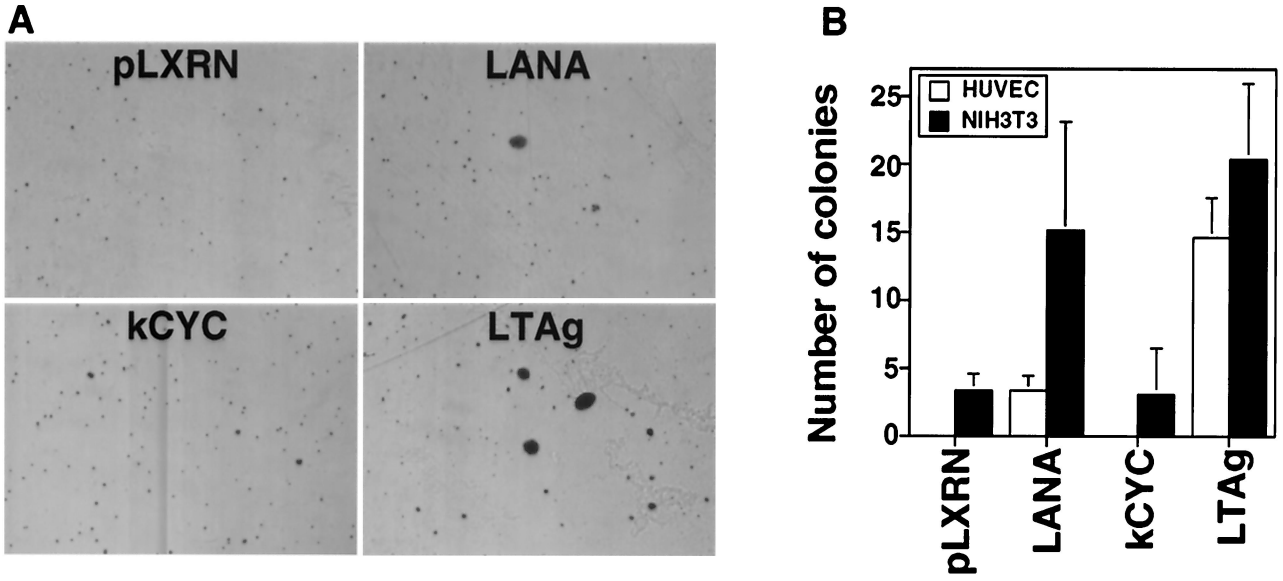

FIG. 5. No anchorage-independent growth of *kCYC*-transduced cells and minimal anchorage-independent growth of *LANA*-transduced cells in soft agar. (A) Representative photographs of soft agar plates 4 weeks following culture of transduced HUVEC, demonstrating no colony formation by *pLXRN*- and *kCYC*-transduced cells and rare colony formation by *LANA*-transduced HUVEC. (B) Mean colony number in five separate soft agar plates ± SD 4 weeks following culture of either transduced HUVEC (open bars) or transduced NIH 3T3 cells (closed bars). Only colonies consisting of more than five cells were counted.

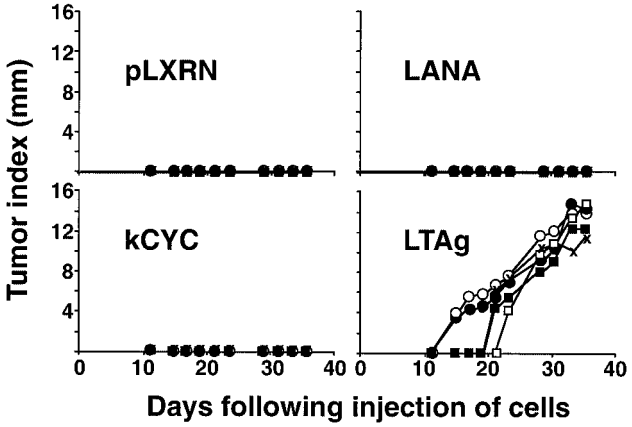

FIG. 6. *LANA*- and *kCYC*-transduced NIH 3T3 cells do not form tumors when injected into nude mice. A total of 5×10^5 transduced NIH 3T3 cells were subcutaneously injected into nude mice on day zero, and tumor sizes were assessed at regular intervals. There were five mice in each group. Each mouse is represented by a different symbol. Transduced HUVEC (including *LTAg*-transduced HUVEC) injected subcutaneously on day zero did not form tumors (data not shown).

pathogenesis may be critically dependent upon whether p53 is damaged or lost within KS tumors. In this regard, studies examining p53 function in KS, although limited, suggest that p53 mutations are not common in KS tumors (29, 36, 60), unlike in many other human cancers.

To begin to elucidate how *LANA* prolonged the life span of HUVEC, cDNA microarray analyses were performed using total RNA extracted from stably transduced cells. We found relatively small numbers of genes to be either up-regulated or down-regulated at least twofold in *LANA*-transduced HUVEC compared to vector-transduced HUVEC (Table 1). Interestingly, down-regulated genes far outnumbered up-regulated genes. This pattern is consistent with previous studies showing silencing of many genes by *LANA* (21, 22, 32, 38, 39, 50, 59). By contrast, expression of a large number of genes was either up-regulated or down-regulated at least twofold in *LTAg*-transduced HUVEC compared to vector-transduced HUVEC (data available upon request). Gene expression patterns of a subset of the genes differentially regulated in *LANA*-transduced HUVEC were confirmed by real-time RT-PCR (Table 1), further strengthening the validity of our microarray data. Of note, Renne et al. previously reported on cellular gene expression using cDNA microarrays in BJAB cells (an Epstein-Barr

TABLE 1. Summary of differentially regulated genes in LANA-transduced HUVEC

Gene	Microarray[a]	RT-PCR[b]	Comments[e]
G0S2	4.6, 6.3	20.5	Cell cycle regulation
POLD1	2.5, 3.1	1.3	DNA polymerase activity
KNSL4	2.2, 3.6	−1.1	Microtubule and DNA binding, chromosome movement during mitosis and meiosis
KIAA0758[c]	−2, −2.1	ND[d]	Tumor rejection antigen (gp96) pseudogene 1
CHST1	−2, −2.4	−2.7	Carbohydrate (chondroitin 6/keratan) sulfotransferase 1
AL049338[c]	−2.1, −2.5	ND	Unknown function
PTPRM	−2.3, −2.9	ND	Signal transduction, growth control
MMP1	−2.3, −2.8	−2	Cleavage of collagen types I, II, III, VII, and X
M64936[c]	−2.4, −2.5	ND	Retinoic acid-inducible endogenous retroviral DNA
NUDT4	−2.4, −4	ND	Nucleoside diphosphate-linked moiety X-type motif 4
KIAA0960[c]	−2.4, −2.7	ND	Tumor rejection antigen (gp96) pseudogene 1
M64936[c]	−2.5, −4.5	ND	Retinoic acid-inducible endogenous retroviral DNA
LDB2	−2.5, −4.9	ND	LIM domain binding protein 2
PPP1R16B	−2.5, −5	ND	Possible downstream target for TGF-β1 signaling in endothelial cells
PTPRM	−2.5, −3	ND	Signal transduction, growth control
U66680[c]	−2.6, −3.8	ND	Unknown function
DEPP	−2.6, −4.2	ND	Progesterone-induced decidual protein
MAF	−2.6, −2.1	ND	Musculoaponeurotic fibrosarcoma oncogene homologue
ITGA2	−2.7, −2.9	1.1	Integrin α2, cellular adhesion, receptor for laminin, collagen, fibronectin, and E-cadherin
SATB1	−2.7, −4.4	ND	DNA binding at special AT-rich sequences within nuclear matrix
AL049949[c]	−2.8, −2.3	ND	Unknown function
CCND2	−2.9, −3.9	−11.1	Key controller of cell cycle at G_1 to S transition
EFNA1	−2.9, −3.3	1.3	Eph-related receptor tyrosine kinase ligand 1 (TNF-α-induced protein 4)
W07033[c]	−3.1, −3.4	ND	Unknown function
AI651024[c]	−3.4, −2.2	ND	Unknown function
CD24	−3.5, −5.7	1.1	Modulation of B-cell activation, proliferation, and differentiation
PTPRM	−3.5, −2.3	ND	Signal transduction, growth control
KIAA0378[c]	−3.6, −3.3	ND	Unknown function
KLRG1	−4, −2.9	ND	Killer cell lectin-like receptor subfamily G
AL080076[c]	−4.1, −4.2	ND	Unknown function
AA128249[c]	−4.1, −7.2	ND	Unknown function
RDC1	−5.3, −.27	ND	Chemokine orphan receptor 1

[a] Fold differences in gene expression between LANA-transduced HUVEC and vector-transduced HUVEC, using Affymetrix cDNA microarrays. Results from two independent experiments are shown.

[b] Fold differences in gene expression between LANA-transduced HUVEC and vector-transduced HUVEC, using real-time RT-PCR. The means from four independent experiments are shown.

[c] Gene accession number rather than gene name.

[d] ND, not determined.

[e] Abbreviations: TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor alpha.

virus-negative Burkitt's lymphoma cell line) transiently transduced with *LANA* (53). Our cDNA microarray data differ from their data, perhaps because of differences in the cell type used (BJAB versus HUVEC) and/or differences in the expression system (transient transfection versus stable transduction using recombinant retroviruses).

G0S2 and *POLD1* were confirmed to be up-regulated in *LANA*-transduced HUVEC by both methods, with results for *G0S2* being much more striking (Table 1). *G0S2* is involved in pushing resting cells into the G_1 phase of the cell cycle (11, 55), and thus increased expression of this gene would be expected in cells with increased proliferation and prolonged life span. *LANA* has been shown to stimulate DNA replication, although the mechanisms of this function are unclear. We suggest that *POLD1* may play an important role in this function, since the main function of *POLD1* is to promote DNA replication (66). It is also possible that up-regulation of *POLD1* in *LANA*-transduced HUVEC may be secondary to impaired p53 function, since wild-type p53, but not mutated p53, is a known suppressor of *POLD1* transcription (35). Further studies are required to test these hypotheses.

In summary, using cells that are relevant to KS pathogenesis, we have shown that *LANA*, a KSHV gene expressed within tumor spindle cells, increased proliferation and greatly prolonged life span of primary HUVEC. Thus, expression of a single latent-cycle KSHV gene is capable of enhancing life span in primary human cells. We believe these findings are consistent with a model that proposes that KSHV causes KS (at least in part) by immortalizing, yet not transforming, tumor spindle cells. Further testing of this model will be the focus of future studies, work that will likely involve increasingly complex in vitro cell culture and animal systems.

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