Oncogenic Herpesvirus HHV-8 Promotes Androgen-Independent Prostate Cancer Growth

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Abstract

Mechanisms underlying progression to androgen-independent prostate cancer following radical ablation therapy remain poorly defined. Although intraprostatic infections have been highlighted as potential cofactors, pathogen influences on pathways that support tumor regrowth are not known. To explore this provocative concept, we derived androgen-sensitive and -insensitive prostate epithelial cells persistently infected with human herpesvirus 8 (HHV-8), an oncogenic herpesvirus that has been detected in normal prostate epithelium, prostate adenocarcinoma, and biologic fluids of patients with prostate cancer, to explore its effects on transition to hormone-refractory disease. Strikingly, we found that HHV-8 infection of androgen-sensitive prostate cancer cells conferred the capacity for androgen-independent growth. This effect was associated with altered expression and transcriptional activity of the androgen receptor (AR). However, HHV-8 infection bypassed AR signaling by promoting enhancer of zeste homolog 2 (EZH2)–mediated epigenetic silencing of tumor-suppressor genes, including MSMB and DAB2IP that are often inactivated in advanced disease. Furthermore, we found that HHV-8 triggered epithelial-to-mesenchymal transition. Although HHV-8 has not been linked etiologically to prostate cancer, virologic outcomes revealed by our study provide mechanistic insight into how intraprostatic infections could constitute risk for progression to androgen-independent metastatic disease where EZH2 has been implicated. Taken together, our findings prompt further evaluations of the relationship between HHV-8 infections and risk of advanced prostate cancer. Cancer Res; 73(18); 1–14. ©2013 AACR.

Introduction

Prostate cancer is the most commonly diagnosed, noncurative malignancy responsible for more than 10% of all cancer-related deaths in the United States (1). In the early stages, growth and survival of prostate cancer cells relies heavily on androgens, which, upon binding to the androgen receptor (AR), activate transcription of androgen-responsive genes that drive cancer progression (2). Accordingly, the first-line standard-of-care treatment of localized early-stage disease is androgen-deprivation therapy (ADT; ref. 3). Although ADT is initially efficacious, many cases progress to the aggressive, metastatic state that is refractory to hormone deprivation, at which point the cancer becomes castration-resistant or androgen-independent. Unfortunately, it is still not possible to determine which cancers will recur and become lethal, nor do we know the pathophysiologic conditions that control transition to androgen-independent prostate cancer (AIPCa). Consequently, defining the etiologic markers of prostate cancer, as well as the mechanisms that contribute to post-ADT tumor regrowth, is an important goal that could guide development of more effective therapeutic options for treatment and clinical management of all stages of the disease.

As the prostate gland is prone to inflammatory hyperplasia, new perspectives on the etiology of AIPCa have emerged, including the provocative concept that pathogen-induced inflammation, by virtue of its ability to alter cell growth pathways, could contribute to prostate cancer initiation and/or progression (4). Indeed, many infectious agents (sexually transmitted and otherwise) have been detected in prostate epithelium and other aspects of the male genital tract (5, 6), further fueling interest in the proposition that in settings of early and/or recurrent disease, the pathogenesis of epitheliotropic infections that can establish a persistent presence within the male urogenital tract may be associated with a deregulation of the prostatic microenvironment that could promote cancer progression. Among the infectious agents whose epidemiologic association with prostate cancer has been examined, human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma–associated herpesvirus (KSHV), represents a notable example of an inherently persistent pathogen whose presence in the prostatic reservoir could modify the overall disease outcome toward an androgen-independent phenotype in high-risk individuals. The HHV-8 genome contains several open reading frames in addition to at least 17 mature
microRNAs known to alter the growth properties of infected cells through inhibition of apoptosis, modulation of immune surveillance, transformation of cells, and activation of various signaling pathways relevant to cancer histogenesis (7, 8). For example, HHV-8 encodes a viral interferon-α homolog that activates the Jak/STAT3 pathway believed to provide tricic support for transition to androgen-independent growth (9–11). HHV-8 also encodes a constitutively active viral G protein–coupled receptor that promotes angiogenesis via stimulation of VEGF, as well as a viral FLICE-inhibitory protein that activates the NF-κB pathway (12) known to regulate the progression to androgen-independent growth (13).

Because aberrant activation of the signaling pathways stimulated by many HHV-8–encoded regulatory proteins is known to occur during prostate cancer progression and in the metastatic disease, it is not improbable that unbridled activation of these proteins in the context of HHV-8 infection (outside of host regulatory controls) could exacerbate the disease outcome. It must be emphasized, however, that although no compelling evidence exists to support an etiologic role of HHV-8 in prostate cancer, there is little doubt that HHV-8 can establish a latent infection in prostate epithelial cells. Thus, viral DNA or mRNA transcripts have been detected in normal prostate (14), in prostate glands of men with Kaposi’s sarcoma (15), in prostate tissue of HIV-negative patients (16), and in semen of men with HIV-infected prostate cancer in which elevated serum antibodies to the virus correlated with disease prognosis (17, 18). Staskus and colleagues also detected the viral latency-associated T0.7 RNA transcripts in prostatic adenocarcinoma, normal portions of biopsy specimens, and within the glandular epithelium of the prostate gland (19).

Clearly, the presence of HHV-8 in normal or malignant aspects of the prostate does not imply, nor does it necessarily serve as sufficient evidence to support a pathogenetic connection with prostate cancer. Therefore, the goal of the current study was not to show such a connection; rather, we were interested in a system that would allow us to isolate virologic outcomes that overlap with markers of aggressive disease. In this effort, we were guided by the hypothesis that intraprostatic persistence of HHV-8 in a fraction of patients could alter the pathophysiology of prostate cancer by establishing conditions that drive transition to aggressive disease in those patients. To this end, we derived a panel of chronically infected lines of androgen-sensitive and -insensitive prostate epithelial cells.

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We found that under androgen-deprived conditions, HHV-8 promotes an androgen-independent growth phenotype through subversion of the AR signaling pathway. HHV-8 also establishes latency in these cells by coopting enhancer of zeste homolog 2 (EZH2), the catalytic component of the polycomb repressor complex-2 (PRC2), known to epigenetically repress the viral lytic switch protein, RTA, as well as other key tumor-suppressor genes including microseminoprotein-β (MSMB) and disabled homolog 2–interacting protein (DOC-2/DAB2) interactive protein (DAB2IP) that are often inactivated in aggressive tumors. Accordingly, the induction of viral reactiva-

ation from latency reduced EZH2 expression and consequent-

ly relieved EZH2-mediated repression of MSMB and DAB2IP in LNCP cells. HHV-8 induced neuroendocrine differentiation and epithelial-to-mesenchymal transition (EMT) under androgen-
derived conditions, marked by decreased expression of epithelial E-cadherin and increased expression of mesenchymal N-cadherin and vimentin. We also noted that HHV-8–infected cells acquired an outgrowth of dendritic spines, expressed neuron-specific enolase, and exhibited morphologic changes consistent with a neuroendocrine phenotype. Together, our results show, for the first time, that chronic HHV-8 infection of androgen-sensitive prostate cancer cells alters the AR signaling pathway and promotes an androgen-independent phenotype through virus-induced reprogramming of the host cell transcriptional profile.

Materials and Methods

Cell lines

The normal prostate epithelial cell line, RWPE2-W99 (RWPE2), and prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection. RWPE2 cells were maintained in keratinocyte serum-free growth media (K-SFM; Invitrogen), supplemented with bovine pituitary extract (0.05 mg/mL; Invitrogen) and human recombinant EGF (5 ng/mL; Invitrogen). LNCaP and PC3 cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS (HyClone Laboratories). DU145 cells were cultured in Eagle’s Minimum Essential Medium (E-MEM; Quality Biological) with 10% FBS. Infected cell lines were maintained in the appropriate medium with puromycin (0.5 μg/mL; Calbiochem, EMD Chemicals, Inc.). All cells were incubated at 37°C in 5% CO2. Androgen-deprivation experiments used RPMI-1640 supplemented with 10% charcoal-stripped FBS (CS-FBS; Invitrogen). BCBL-1, a body cavity–based lymphoma cell line that is persistently infected with HHV-8, was provided by Michael McGrath and Don Ganem, through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) and was cultured in RPMI-1640 supplemented with 10% FBS. All experiments were carried out using cells harvested at low (<20) passages.

Virus and infections

All infections were established with rKSHV.219 (20), a recombinant HHV-8 virus encoding both the GFP under the control of the constitutively active human elongation factor 1-α promoter and red fluorescent protein (RFP) driven strictly by the HHV-8 lytic polyadenylated nuclear RNA promoter. rKSHV.219 also carries a gene for puromycin resistance, which allows for selection and maintenance of the viral episome in infected cells (20). Viral inoculums were made by reactivating rKSHV.219-infected Vero cells with 1.5 mmol/L of valproic acid (Sigma-Aldrich) and monitoring for the expression of RFP. Four days later, cells were harvested and collected by centrifugation at 2,500 rpm for 10 minutes at 4°C. Supernatant with free KSHV particles was kept on ice, whereas the cell pellet was resuspended in 3 mL of Opti-MEM (Invitrogen) and frozen and thawed three times to release cell-associated virus. The lysate was then centrifuged at 2,500 rpm for 10 minutes at 4°C to
remove cellular debris and combined with the original culture supernatant, sterile-filtered through a 0.45-μm membrane filter, and stored at −80°C. Cells to be infected were seeded in 6-well plates in the appropriate growth medium. When the cultures reached 60% to 70% confluence, media was removed and 1 ml of the rKSHV.219 inoculum was added to each well. Three hours later, an additional 1 ml of growth media was added, and the cells were incubated at 37°C and monitored for GFP expression. When GFP was observed, 0.5 μg/ml of puromycin was added to the cell cultures to select for infected cells.

Microscopy
Images from immunofluorescence assays were captured using an AxiosCam MRm digital camera (Carl Zeiss North America), attached to a Zeiss Axios Observer A1 inverted fluorescence microscope (Carl Zeiss). Image coloring was carried out with the AxiosVision Release 4.6 software.

DNA isolation and PCR
DNA was isolated from infected and uninfected cell lines using the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen). PCR was conducted with the Platinum PCR SuperMix High Fidelity System (Invitrogen). PCR products were run on a 1.5% agarose gel with ethidium bromide and visualized with UV transillumination. Primer sequences used were: KS330-forward: 5'-AGC CGA AAG GAT TCC AAA TTG TGG-3', KS330-reverse: 5'-CCG TGT TGT CTA CGT CCA GAT A-3'; GAPDH-forward: 5'-GAA GGT GAA GGT CGG AGT CA-3', GAPDH-reverse: 5'-TTT ACA CCC ATG ACG AAC AT-3'.

RNA isolation and semi-quantitative reverse transcription PCR
RNA was isolated using the GenCatch Total RNA Miniprep Kit (Epoch Biolabs). Genomic DNA contamination was eliminated by treating the RNA samples with 2 U DNase for 30 minutes at 37°C (TURBO DNA-free; Ambion). Semi-quantitative reverse transcription (sqRT)-PCR was conducted using the SuperScript One-Step Kit (Invitrogen). sqRT-PCR products were run on a 1.5% agarose gel with ethidium bromide and visualized with UV transillumination. Primer sequences were: RTA-forward: 5'-GGG AGT TGC TGG TAA TGT CAG C-3', RTA-reverse: 5'-CCT CTC TTT GCT TCT CTT TCG-3'; PAN-forward: 5'-GCC AGT TGT AGC CCC CTT TTA TG-3', PAN-reverse: 5'-CCG CAC ACC ACT TTA GTC CAA TG-3'; K8.1-forward: 5'-CGT GGA TCC TTC ATC TTA GTC TGG T-G-3', K8.1-reverse: 5'-TGC GGA ATT CAT GGG TCC GTA TTT CTT CAT TGT AGT GGC-3'; GAPDH-forward: 5'-GGA GGT GAA GGT CGG AGT CA-3', GAPDH-reverse: 5'-TTT ACA CCC ATG ACG AAC AT-3'.

Immunoblotting
Whole-cell extracts were made using a high salt extraction buffer [400 mMol/L NaCl, 10 mMol/L HEPES pH 7.5, 1.5 mMol/L MgCl2, 0.1 mMol/L EDTA, 5% glycerol, 1 mMol/L diethilthreitol (DTT), 1 mMol/L phenylmethylsulfonylfluoride (PMSF), 1 mMol/L NaVO4, 1 mMol/L NaF, and 10 μL/ML Halt protease inhibitor (Thermo Scientific)]. Samples were separated on 4% to 12% NuPage Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes, and blocked in SuperBlock Blocking Buffer in TBS (Thermo Scientific) for 2 hours at room temperature. Blots were then incubated with primary antibody and 0.1% Tween-20 overnight at 4°C. The β-actin antibody (A5411) was purchased from Sigma, anti-GAPDH (L-20; 1:2,000) was purchased from Santa Cruz Biotechnology, and anti-prostate-specific antigen (anti-PSA; L500) was purchased from Dako. The following primary antibodies were purchased from Cell Signaling Technology: AR (1:1,000), p44/42 mitogen-activated protein kinase (MAPK; 1:1,000), phospho-p44/42 MAPK (1:1,000), NF-kB p65 (1:1,000), phospho-NF-kB p65 (1:1,000), 1kBoz (44D4), phospho-1kBoz-ser32 (1:4D4), EZH2 (1:1,000), SUZ12 (1:1,000), trimethyl H3K27 (1:1,000), histone H2AX (D17A5, XP), phospho-histone H2AX (ser139 (20E3), E-cadherin (24E10), N-cadherin, vimentin (D21H3), S6, p-S6, Akt, and p-Akt. Anti-AR (441) was purchased from Santa Cruz Biotechnology; MSMB (Origene Technologies), DAB2IP (ab87811; Abcam), PARP (c-2-10; Sigma), p1kBoz (Biosource), 1kBoz (Zymed/Invitrogen). Blots were incubated in secondary antibody at room temperature for 45 minutes, followed by five 10-minute washes in PBS-T and two 10-minute washes in PBS. Bands were detected with SuperSignal West Chemiluminescent Substrate (Thermo Scientific). Unless otherwise indicated, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control for all Western blot analysis conducted in this study.

Cell proliferation/viability assays
For androgen-independence studies, cells were generally seeded in a 96-well plate (total culture volume of 100 μL/well) in growth media with 10% FBS or media with 10% CS-FBS; however, because of differences in doubling times between cell lines, the following culture conditions were empirically determined to achieve comparable cell numbers at the beginning of the assay: RWPE2 and their rKSHV.219-infected counterparts (RWPE2-v219) were seeded at 0.5 × 10⁴ cells per well and assayed after 4 days; LNCaP and LNCaP-v219 cells were seeded at 1.0 × 10⁴ cells per well and assayed after 5 days; DU145 and DU145-v219 cells were seeded at 0.5 × 10⁴ cells per well and assayed after 3 days, whereas PC3 and PC3-v219 cells were seeded at 0.5 × 10⁴ cells per well and assayed after 3 days. Media was changed 48 hours after seeding, and cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. The assay uses a MTS tetrazolium compound that is reduced by viable cells to a formazan product that is directly proportional to the number of living cells in culture. Absorbance at 490 nm was measured using a Versamax microplate reader (Molecular Devices) 2 hours after addition of substrate. Background absorbance of culture media alone was subtracted from experimental values in each case.

Cloning, expression vectors, and transfection
cDNA encoding full-length MSMB was amplified by RT-PCR from LNCaP mRNA using primers: MSMB-forward: 5'-CGG GGA TCC ATG AAT GTT CTC CTG GGC AGC GTT-3', and MSMB-reverse: 5'-CTG CTC GAG TTA GTA TAT CCA TTC GAA A-3', and cloned into the eukaryotic expression
vector pcDNA3.1/Hygro (Invitrogen). The resulting construct (pcDNA-MSMB) was then transfected into prostate cancer cell lines using the jetPRIME transfection system (Polyplus-Transfection Inc.) according to the manufacturer’s instructions. Expression of recombinant MSMB was confirmed by qRT-PCR, and where possible generation of cells stably expressing MSMB was achieved by antibiotic selection with hygromycin.

Real-time quantitative reverse transcription PCR

A total of \(5 \times 10^5\) LNCaP and rKSHV.219-infected LNCaPv219 cells were seeded in either FBS- or CS-FBS-supplemented media. After 4 to 5 days of growth, RNA was isolated using the GenCatch Total RNA Miniprep Kit (Epoch Biosiol). Genomic DNA contamination was eliminated by treating the RNA samples with 2 U DNase for 30 minutes at 37°C (TURBO DNA-free; Ambion). cDNA was synthesized from 1.5 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. Each qRT-PCR reaction was carried out in triplicate in a 25 μL reaction with the SYBR GreenER qPCR SuperMix Kit (Invitrogen). The reactions were run for 15 minutes at 95°C for denaturation, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. For each experiment, a PCR reaction was run without template for a negative control. \(C_T\) values for the gene of interest (GOI) were normalized to \(β_2\)-microglobulin (\(β_2\)M) \(C_T\) (\(ΔC_T = C_T \text{GOI} - C_T \text{β}_2\)M) to generate \(ΔΔC_T\) values. RQ values to determine relative expression of each GOI were calculated by quantification cycle \((C_{\text{act}})\) for 30 seconds and 60°C for 30 seconds. For each experiment, a PCR reaction was run without template for a negative control. \(C_T\) values for the gene of interest (GOI) were normalized to \(β_2\)-microglobulin (\(β_2\)M) \(C_T\) (\(ΔC_T = C_T \text{GOI} - C_T \text{β}_2\)M) to generate \(ΔΔC_T\) values. RQ values to determine relative expression of each GOI were calculated by

Results

HHV-8 can establish chronic, latent infections in prostate epithelial and prostate cancer cell lines

Although others have detected HHV-8 in human prostate specimens, a basis for examining the role of this virus as a potential risk factor for cancer progression requires demonstration of its ability to establish a persistent infection in both normal prostate epithelial cells and in prostate cancer-derived cell lines. Toward this goal, we infected the normal prostate epithelial cell line RWPE2, the androgen-sensitive prostate cancer cell line LNCaP, and the androgen-independent prostate cancer cell lines, DU145 and PC3 with rKSHV.219 (20), a recombinant virus that constitutively expresses GFP under the control of the human elongation factor-α promoter, as well as GFP under the viral lytic poly-adenylated nuclear transcripts (PAN) promoter, allowing us to monitor not only de novo infection and subsequent establishment of latency based on GFP expression, but also the switch to the lytic cycle based on GFP expression. rKSHV.219 also carries a puromycin resistance gene for selection and enrichment of long-term chronically infected cells. rKSHV.219 was able to establish infection in all prostate cell lines tested, as depicted by long-term GFP expression under puromycin selection (Fig. 1A), and presence of viral DNA in infected cells (Fig. 1B). Infection was also confirmed by immunofluorescence assay designed to detect the viral latency-associated nuclear antigen (LANA), which maintains viral latency in part by tethering the viral episome to the host chromosome (21); the characteristic punctate foci of nuclear LANA staining in chronically infected cells can be seen in LNCaP-v219 cells (Fig. 1C) and in DU145-v219 cells (Supplementary Fig. S1A) that support robust virus reactivation upon treatment with a subtoxic dose of n-butyrate (Supplementary Fig. S1B). Therefore, contrary to a previous report (22), HHV-8 is capable of establishing a latent infection in both androgen-dependent and -independent cell lines, which is important as a basis for testing whether this oncogenic virus can skew androgen-dependent prostate cancer cell growth toward an androgen-independent state.

HHV-8 confers enhanced viability to RWPE2, PC3, and DU145 cells, whereas it induces an androgen-independent growth phenotype in LNCaP cells

To determine whether long-term HHV-8 infection influences growth of both androgen-dependent and -independent cell lines, we used the Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay to measure proliferation and/or viability of uninfected and rKSHV.219-infected AR-positive (AR+ ) LNCaP and RWPE in comparison with androgen-independent PC3 and DU145 cells. As shown in Fig. 2A, HHV-8 slightly enhanced growth of RWPE2 cells that are derived from normal prostate epithelium and do not require androgens for growth. Remarkably, this prosurvival effect was even more pronounced in the androgen-insensitive DU145 and PC3. In contrast, there was no significant increase in the viability of LNCaP cells, which was initially perplexing because we also observed that infected LNCaP cells displayed elevated AR (mRNA and protein) in conjunction with a virus-induced increase in expression of proinflammatory mediators such as

Statistical analyses

Error bars in graphical data represent mean ± SD. Unless otherwise noted in the figure legends, Student’s t test was used to calculate the statistical significance between groups, with \(P < 0.05\) considered to reflect a high probability of occurrence beyond chance. Image analysis of Western blot analysis data was conducted using the Open-source ImageJ suite (version 1.38) available from the U.S. NIH (http://rsb.info.nih.gov/ij/).
HHV-8 induces androgen-independent growth of LNCaP cells concomitant with repression of AR transcriptional activity

In the majority of cases, AR signaling is fundamental to prostate cancer progression to a castration-resistant phenotype (23). Therefore, to determine whether HHV-8 promotes androgen-independence by modulating AR signaling, we measured expression of AR and its transcriptional target gene, PSA, in LNCaP versus infected LNCaP-v219 cells cultured in FBS- or CS-FBS media. Consistent with the data in Fig. 2C and D, both AR and PSA were greatly reduced in LNCaP cells grown without androgens (Fig. 3A, compare lanes 1 and 3). However, in normal conditions (i.e., when androgens are present), infected LNCaP-v219 cells exhibited increased AR expression (analogous to our initial observation in Fig. 2B) but surprisingly had reduced PSA (Fig. 3A, compare lanes 1 and 2). In contrast, when cultured in androgen-deprived (−) media, LNCaP-v219 cells had a pronounced reduction in both AR and AR-controlled genes, including PSA protein (Fig. 3A) and mRNA (Fig. 3B). Moreover, qPCR profiling of a selected panel of AR-controlled genes further confirmed that nearly all AR-client genes tested (KLK2, KLK3, NKX3.1, ODC1, PMEPA1, and SCL-65k3) were downregulated in infected LNCaP-v219 cells cultured in the absence of androgens (Fig. 3C), further supporting the conclusion that AR-mediated transcription is indeed inhibited by the virus in this setting. These complementary findings provoke new thinking, as they underscore a profound virologic effect on AR transcriptional activity that illustrates a paradigm, whereby HHV-8 upregulates AR when androgens are present but severely alters its transcriptional activity so that PSA and other AR-controlled genes are not expressed, whereas it promotes cell viability in the absence of androgens presumably by activating alternative, AR-independent pathways that promote cell growth through mechanisms that either do not require, or are potentially uncoupled from the AR signaling pathway. Identification of these AR-independent pathways is an essential step toward understanding the pathophysiologic etiology of aggressive disease that does not require androgens for growth.

HHV-8 induces expression of polycomb group proteins in androgen-deprived LNCaP cells, resulting in an increase in H3K27me3 deposition and transcriptional repression of MSMB and DAB2IP tumor-suppressor proteins

The human EZH2 is the catalytic component of the multimeric PRC2, a core of polycomb group (PcG) proteins that also includes embryonic ectoderm development (EED) and suppressor of Zeste 12 (SUZ12). EZH2 is essential for the initial
HHV-8 promotes viability of RWPE2, DU145, and PC3 cells, whereas it induces an androgen-independent growth phenotype in LNCaP cells. A, a cell viability assay of uninfected (gray) versus rKSHV.219-infected (black) RWPE2, LNCaP, DU145, and PC3 cells was assayed using the Promega CellTiter 96 AQueousOne Solution Cell Proliferation Assay as described in Materials and Methods. B, left, RT-PCR products from total RNA isolated from uninfected (−) and rKSHV.219-infected (+) LNCaP cells, using primer sets designed to amplify mRNA for human AR and the indicated inflammatory mediators (i.e., cellular IL-6, IL-8, and COX-2). Viral genomic (episomal) DNA from the HHV-8-infected cells was detected by PCR using primer sets designed to amplify a fragment of the viral minor-capsid protein (49), whereas DNA from BCBL-1 cell line was used as a positive control for virus detection. GAPDH and 18S were used as two-tier positive controls, respectively. Right, Western blot analysis for AR expression in uninfected (−) and rKSHV.219-infected (+) LNCaP cells correlates with enhanced AR mRNA expression in infected cells. β-actin was used as a loading control. C, HHV-8 enhances LNCaP cell viability in androgen-deprived conditions. Approximately, 1 × 10⁴ uninfected (white) LNCaP or HHV-8-infected (black bars) LNCaP-v219 cells were seeded in a 96-well plate and cultured with (−) or without (−) androgens, and cell viability was assayed 4 days postseeding. Values are the mean of six samples ± SD. **, P < 0.0001. D, progressive (time course) increase in the viability of uninfected (gray bars) versus HHV-8-infected (black bars) LNCaP cells cultured without (−) androgens. A total of 1 × 10⁴ uninfected LNCaP (gray) and infected LNCaP-v219 cells (black) were seeded in CS-FBS-supplemented media, and incubated at 37°C, and the assay was conducted at 3, 5, and 7 days postseeding. Values are the mean of six samples ± SD for each time point. Statistical analysis was by two-way ANOVA with Bonferroni posttest; **, P < 0.0001. In all cases, data are typical values from at least three independent experiments. *, P = 0.005; **, P < 0.001 (A and C).

binding and maintenance of the transcriptional repressive state of promoters that control expression of tumor suppressors and other target genes via trimethylation of histone H3 on lysine 27. Accordingly, overexpression of EZH2 occurs frequently during cancer development and confers a poor prognosis in many aggressive cancers including prostate and breast cancers (24). Because HHV-8 latency also requires EZH2-mediated suppression of viral lytic genes (25), we hypothesized that viral cooption of the EZH2 methyltransferase activity may contribute to epigenetic modifications that in turn alter the normal transcriptional program of early-stage tumors, leading to the establishment of a state of androgen-independent growth in latently infected LNCaP-v219 cells. In line with this hypothesis, both EZH2 and SUZ12 were increased coincident with deposition of the H3K27me3 mark (Fig. 4A). To more fully determine the biologic consequence of EZH2-upregulation by HHV-8, we used microarray analysis to profile virus-induced changes in the transcriptional program of infected LNCaP-v219 grown in CS-FBS. Results from this analysis revealed significant virus-induced downregulation of known EZH2 targets in androgen-depleted (CS-FBS) media, including MSMB, MT1G, and CDKN2C (Supplementary Table S1). We then validated this observation by Western blot analysis, which showed a direct correlation between EZH2 expression and a corresponding reduction in expression of MSMB and DAB2IP (another tumor-suppressor gene that is epigenetically repressed by EZH2 (26, 27); Fig. 4B, lanes 3 and 4) that is corroborated by qPCR analysis (Fig. 4C).

Virus-induced repression of MSMB in infected LNCaPv219 cells under androgen-deprived conditions is associated with increased expression of parathyroid hormone–related protein

Because HHV-8–induced upregulation of EZH2 directly impacts expression of MSMB in infected LNCaP-v219 cells under androgen-deprived (CS-FBS) conditions (Fig. 4C, left), and as MSMB limits tumor growth and metastasis in part by directly inhibiting expression of parathyroid hormone-related protein (PTHrP; ref. 28), we measured expression of PTHrP as a biologic readout of the consequence of virus-induced EZH2-
mediated repression of MSMB under these conditions. As shown in Fig. 4C, complete abrogation of MSMB resulted in a greater than 5-fold increase in PTHrP expression. Conversely, overexpression of recombinant MSMB in infected LNCaP-v219 cells dramatically reversed the effect of HHV-8 and reduced PTHrP expression to virtually undetectable levels (Fig. 4D, right), consistent with previous reports of an inverse relationship between MSMB and PTHrP expression (28). Given that PTHrP is expressed by prostatic neuroendocrine cells and acts as a growth factor for prostate cancer cells that are directly involved in advanced skeletal metastases (29), we propose that the loss of MSMB in infected LNCaP-v219 cells, putatively via a virus-induced upregulation of EZH2, represents an alternative virus-induced mechanism that contributes to androgen-independent growth of an otherwise androgen-dependent cell line by enhancing PTHrP expression.

Ectopic expression of MSMB induces apoptosis and reverses the virus-induced androgen-independent growth of infected LNCaPv219 cells

Garde and colleagues recently showed that MSMB inhibits prostate cell proliferation in part by inducing apoptosis (30). In light of the fact that MSMB is drastically impacted by HHV-8 under androgen-deprived conditions (Fig. 4B and C), we hypothesized that HHV-8 downregulation of MSMB protects cells from cell death during androgen-deprivation. To test this hypothesis, we first confirmed the apoptotic function of recombinant human MSMB in the androgen-insensitive PC3 cells, in which MSMB has already been shown to suppress cell growth by inducing apoptosis (30). Overexpression of MSMB in PC3 and their infected PC3-v219 counterparts augmented MSMB expression several-fold above endogenous levels (Supplementary Fig. S2A); and, in agreement with previous findings (30), PC3 cells overexpressing MSMB quickly died (Supplementary Fig. S2B), ostensibly due to MSMB-induced apoptosis, as evidenced by cleavage of nuclear PARP [an early marker of apoptosis (Supplementary Fig. S2C)]; also evident in MSMB-overexpressing cells is a slight indication of phosphorylated H2A.X, an early sensor of double-stranded DNA breaks in response to genomic stress such as during apoptosis (31).

We then expressed recombinant MSMB in LNCaP and infected LNCaP-v219 cells and sought to correlate cell viability with the apoptotic effect of MSMB in these cells, which demonstrably correlated with the level of MSMB expression (Fig. 5B). As shown in Fig. 5A, however, MSMB-transfected LNCaP cells did not survive in culture (not shown), whereas...
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...androgen-independent and if virus-induced upregulation of EZH2 represents one motivator that regulates the viral lytic switch protein RTA (25), H3K27me3 deposition is sufficient to silence the HHV-8 pro-viral promoter, which may reveal new perspectives on the molecular link between HHV-8 reactivation and progression. Further investigation of this aspect is warranted.

HHV-8 reactivation relieves epigenetic repression by EZH2/SUZ12 and upregulates MSMB and DAB2IP expression in infected LNCaPv219 cells

If EZH2 histone methyltransferase activity that results in H3K27me3 deposition is sufficient to silence the HHV-8 promoter that regulates the viral lytic switch protein RTA (25), and if virus-induced upregulation of EZH2 represents one of the mechanisms that contributes to androgen-independent growth of latently infected LNCaP cells, it is reasonable to predict that induction of a molecular switch from latency to the lytic phase should relieve the repressive H3K27me3 mark and allow expression of EZH2-repressed genes. Indeed, reactivation of HHV-8 with valproic acid, a histone deacetylase-1 (HDAC1) inhibitor, led to the expression of RTA, PAN, and the two splice variants of the strictly lytic mature virion enveloped-associated glycoprotein K8.1A/B (Supplementary Fig. S3B). Consistent with our prediction, virus reactivation was accompanied by a dose-dependent relief of EZH2-mediated transcriptional repression of DAB2IP (Supplementary Fig. S3C) and MSMB (Supplementary Fig. S3D). Accordingly, virus reactivation reduced EZH2 and SUZ12 expression, resulting in a progressive reduction in the H3K27me3 mark (Fig. 6A, lane 2) probably reflecting an initial burst in PRC2 stability as an adaptive response to changes in the landscape of acetylation spots at the EZH2/SUZ12 promoters, whereas the subsequent decline in EZH2/SUZ12 expression at 72 and 96 hours likely reflects the effect of achieving a threshold increase in Nafl-induced expression of viral RTA molecules that then mask access of specific histone demethylases to the EZH2...
promoter. Such a paradigm needs to be investigated further but it would be analogous to viral K-bZip inhibition of specific demethylases such as JMJD2A that potentially promotes virus replication through removal of the H3K27-me3 mark (32).

The surprisingly convergent role of EZH2 in establishment of HHV-8 latency and in prostate cancer progression was also illustrated by the fact that in presence of androgens, virus reactivation reduced MSMB and DAB2IP expression in a time- (Fig. 6B) and dose-dependent (Fig. 6C and D) manner, whereas in androgen-deprived conditions, the effect of an intrinsically high expression of EZH2 (reflected by the low level expression of MSMB and DAB2IP) is overcome only at high concentrations (2.5 mmol/L) of the reactivating drug (Fig. 6D), which was also clearly evident in cell culture (data not shown). Whether this phenomenon is related to RTA promoter regulation by acetylation is not clear, but it underscores the possibility that depending on the replicative context of the viral genome in relation to host chromatin, EZH2-mediated regulation of HHV-8 latency probably operates in concert with epigenetic cis-regulatory mechanisms not necessarily required for maintaining the mark on hypermethylated promoters (33).

**Markers of EMT are overexpressed in HHV-8–infected LNCaP-v219 under androgen-deprived conditions**

Recent advances have brought into focus the correlation between EMT and the development of aggressive malignancies, including androgen-independent prostate cancer progression and metastasis (34). This process occurs when epithelial cells acquire a migratory and invasive mesenchymal phenotype characterized by "cadherin-switching"—the coordinated loss of epithelial markers (e.g., E-cadherin), gain of mesenchymal markers (e.g., N-cadherin and vimentin), and expression of transcriptional repressors of E-cadherin, including Twist, Snail, Slug, and Zeb1/2 (35). It was recently shown that HHV-8 can induce endothelial-to-mesenchymal transition (EndoMT) during Kaposi’s sarcoma histogenesis (36, 37). Therefore, to determine whether virus-induced androgen-independent growth and deregulation of AR transcriptional activity in infected LNCaP-v219 cells occurs via a cellular dedifferentiation process reminiscent of EMT, we assessed expression of EMT-relevant genes by Western blot and microarray analysis. Remarkably, we found that LNCaP-v219, but not uninfected LNCaP, undergo “cadherin-switching” (34), marked by decreased expression of E-cadherin and increased N-cadherin expression at both mRNA (Fig. 7A, left two graphs) and protein levels (Fig. 7B). We also detected an increase in mRNA for the mesenchymal marker, vimentin, and the transcription factors Snail and Zeb1 (Fig. 7A, last three graphs), confirming that HHV-8 infection of prostate cancer cells is indeed associated with transition to a promesenchymal phenotype, an established pathologic feature of aggressive, castration-resistant prostate cancers. And, as shown in Supplementary Table S2, the HHV-8–downregulated genes represent a family of membrane-bound and other cell-adhesion molecules that generally antagonize EMT, whereas the set of genes that are upregulated by HHV-8 (e.g., extracellular matrix–modifying metalloproteases and growth factor receptors) have been shown to promote processes that support EMT.

We were intrigued that HHV-8 also induced "cadherin-switching" in the AR+ RWPE2 cell line, as marked by a significant decrease in E-cadherin and an increase in N-cadherin expression (Fig. 7B and C, right). It is, of course, noteworthy that RWPE2 is a normal, nontumorigenic, Ki-Ras–expressing cell line derived from the peripheral zone of a
histologically normal adult human prostate; although these cells are AR⁺, they are maintained in K-SFM containing essential nonsteroidal supplements. Therefore, the apparent ability of HHV-8 to induce promesenchymal characteristics in LNCaP and RWPE2 cells as well, implies that the virologic effects of this oncogenic virus may be broadly relevant in settings where androgens are not present. An important point revealed by this analysis is that the tumorigenic pathways at play both during histogenesis of Kaposi's sarcoma and during transition to hormone-refractory prostate cancer seem to converge at nodes that anchor promesenchymal outcomes during the pathogenesis of these two human cancers.

Discussion

Castration-resistant, androgen-independent prostate cancer remains a significant clinical challenge due to lack of information on the biologic events that control transition to this fatal form of the disease. Although it is generally accepted that constitutive activation of the AR signaling pathway is necessary for prostate cancer progression, only a fraction of cells in metastatic, castration-resistant cancer overexpress AR (38), implying that a subset of prostate cancers may progress to become completely independent of the AR signaling pathway, giving rise to disease that is not responsive even to some of the latest AR-inhibiting drugs. This challenge has consequently inspired the attitude that effective treatment options for advanced disease ought to include strategies that target pathways other than those involved in AR signaling (39), but the identity of such pathways or the types of cancer whose progression depends on AR-independent mechanisms remain largely unknown.

Chronic infections (particularly viruses) have been implicated in a number of cancers and in most cases they have taught us many lessons in tumor biology by serving as in vivo probes into mechanisms of cancer development. Here, we derived androgen-dependent prostate epithelial cell lines chronically infected with HHV-8 as a model system for probing mechanisms of cancer progression to the androgen-independent phenotype. Our rationale for this approach was based on a number of factors: (i) the infectious process of HHV-8 in prostate cancer cells includes a latency phase in which oncogenic viral genes are expressed; (ii) HHV-8 has evolved numerous homologs of cellular genes known to control cell growth pathways that overlap with those already implicated in prostate cancer progression, and (iii) HHV-8 has been detected in prostate cancer adenocarcinoma, prostate cancer biopsies, and in semen and plasma of a fraction of patients with prostate cancer, indicating that it can establish a chronic infection in the male urogenital tract. On the basis of these factors, we reasoned that although HHV-8 prevalence may be rare in the

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**Figure 6.** A, expression of the polycomb group proteins EZH2 and SUZ12, and the trimethylated H3K27me3 mark was assessed by Western blot analysis in whole-cell lysates of infected LNCaP-v219 cells treated with 2 mmol/L sodium butyrate (NaB) for 48, 72, and 96 hours; GAPDH was used as a loading control. B, qRT-PCR analysis of fold changes in mRNA expression levels of DAB2IP and MSMB in uninfected LNCaP (light gray) or HHV-8–infected LNCaP-v219 cells (dark gray) treated with 2 mmol/L sodium butyrate (NaB) for 0, 24, 48, and 72 hours. C and D, qRT-PCR analysis of dose-dependent changes in DAB2IP and MSMB mRNA expression in HHV-8–infected LNCaP-v219 cells cultured in media containing (+) androgens (C) or CS-FBS media without (−) androgens (D) in presence of increasing concentrations (0, 1, or 2.5 mmol/L) of sodium butyrate (NaB). All reactions were carried out in triplicate and normalized to an internal β2m control, as described in Materials and Methods.
general population (within available detection limits), expression of one or more of its cell-transforming genes (and/or microRNAs) in a chronically infected cross-section of patients with prostate cancer could negatively impact the overall disease prognosis in these patients.

The virologic outcomes of HHV-8 infection in androgen-dependent prostate epithelial cell lines revealed a paradigm also exposed by other systems, in which transition to AIPCa seems to be uncoupled from the AR signaling mechanisms that are at play in the early stages of disease. We found, for example, that when androgens are present, HHV-8 induces AR gene amplification, whereas under conditions of androgen-deprivation, the virus promotes androgen-independent growth by causing a reduction in AR and several key AR-controlled genes. At the same time, the virus induces phenotypic and transcriptional changes mechanistically distinct from the androgen-regulated program seen in early disease. Although understanding the mechanism(s) by which HHV-8 alters AR signaling activity is an important goal for ongoing studies, several important clues have emerged from this study.

First, it is noteworthy that the epigenetic repression catalyzed by EZH2 is also important for establishment of HHV-8 latency (25, 40), implying that, as in Kaposi's sarcoma (the cancer with which HHV-8 is most famously associated), the viral latency program is tightly linked to induction of a pathologic outcome in prostate cells as well. HHV-8 elevates EZH2/SUZ12–mediated methyltransferase activity in settings of limited or no androgens, resulting in an increase in the trimethylated H3K27me3 mark that concomitantly represses transcription of EZH2 target genes, including MSMB and DAB2IP that are frequently inactivated in advanced cancer. MSMB expression is high in benign tissue, reduced in malignant tissues, and progressively declines as prostate cancers evolve to the more aggressive tumors (41). Several tumor-suppressor functions have been identified for MSMB, including modulation of apoptotic pathways, inhibition of neo-angiogenesis, and impairment of prostate cancer bone metastases (28, 30). Interestingly, a genetic risk allele potentially underlying a predisposition to poor cancer outcomes has also been described for MSMB, further supporting this protein as a strong prognostic marker that could explain the disparity in disease burden among certain racial groups (42).

Second, the frequent loss of MSMB in high-grade, advanced cancers suggests that some prostate cancer cells do undergo dedifferentiation, and that by downregulating MSMB, HHV-8 may also promote cellular dedifferentiation. Thus, infected LNCaP-v219 cells in which MSMB is severely reduced exhibit a corresponding increase in PTHrP expression, whereas ectopic MSMB expression strongly blocked PTHrP expression and further limited the proliferative ability of these cells, implying that viral suppression of MSMB directly contributes to androgen-independent proliferation through modulation of PTHrP expression. As PTHrP is a known growth factor for prostate
cancer cell lines (29), future studies should aim to define the role of PTHrP and other neuroendocrine peptides in the processes that support the ability of HHV-8–infected LNCaP-v219 cells to switch to an androgen-independent cell proliferation program.

Third, the observed HHV-8–induced upregulation of EZH2 also resulted in reduced expression of DAB2IP, a Ras GTPase-activating protein known to suppress prostate cancer cell growth and metastasis (43). Like MSMB, DAB2IP has several tumor-suppressor functions, including suppression of the Ras signaling pathway and inhibition of the NF-kB transcriptional activity, which effectively limits prostate cancer growth (43). Accordingly, there is an inverse relationship between DAB2IP expression and tumor grade (i.e., low DAB2IP expression is predictive of disease progression and poor prognosis). Therefore, in light of the known tumor-suppressor functions of MSMB and DAB2IP, reduced expression of these EZH2-regulated genes in HHV-8–infected cells underscores the central role played by EZH2 not only in the pathogenesis of HHV-8 but also in the complex pathobiology of prostate cancer.

At a genetic level, a significant number of prostate cancers accumulate chromosomal rearrangements that result in fusion of the androgen-regulated transmembrane protease, serine 2 (TMPRSS2) promoter to a member of the ETS transcription factor family, most commonly ERG. This fusion results in ERG overexpression that directly contributes to prostate tumorigenesis or progression (44). Interestingly, pathologic effects of the prostate cancer–specific TMPRSS2–ERG oncogenic gene fusion seem to be more profound during the early stages of disease while the tumor is still dependent on androgens. This is supported by the fact that TMPRSS2–ERG disrupts AR signaling in androgen-sensitive prostate cancer cell lines, such as LNCaP, leading to cellular dedifferentiation ostensibly via repressive epigenetic programs that depend on activation of EZH2 (45). A remarkably similar theme is emerging in our model as well, whereby HHV-8 skews the pathophysiologic networks associated with AR signaling by upregulating EZH2, which consequently induces a stem cell–like transcriptional program through epigenetic repression of genes that promote cellular differentiation and tumor metastasis. Whether HHV-8 can promote or at a minimum increase the frequency of TMPRSS2–ERG fusions under androgen-deprived conditions is an exciting area of ongoing study, but available evidence has now revealed that some of the most significant pathobiologic consequences of this genetic rearrangement (e.g., dysregulation of AR signaling and EZH2 overexpression) are remarkably analogous to the impact of HHV-8 on prostate cancer cells under these conditions. Although this level of pathogenetic triangulation involving (i) viral latency through polycomb, (ii) AR transcriptional activity, and (iii) epigenetic regulation via EZH2 methyltransferase activity may be unprecedented, it predicts that HHV-8 persistence in androgen-dependent prostate epithelial cells has the potential to either directly promote or mimic the same integrated network of TMPRSS2–ERG–induced, EZH2-mediated epigenetic reprogramming that occurs during prostate cancer progression to the metastatic, castration-resistant phenotype (46).

Through EMT, loss of normal cellular polarity and the deconstruction of cell–cell adhesions collectively confer invasive and metastatic phenotypes to tumor cells. In this study, we have also presented the first evidence that HHV-8 can induce pro-EMT conditions in androgen-deprived prostate cancer cells as well, accompanied by "cadherin switching" and increased expression of EMT-promoting transcription factors (34). Given that the loss of DAB2IP in prostate cancer is also linked to induction of EMT (47), the significant HHV-8–induced reduction of DAB2IP in our system adds to the emerging list of potential mechanistic portals by which HHV-8–induced EMT would favor transition to the dedifferentiated, late-stage androgen-independent disease. Collectively, these findings give us reason to believe that virus-induced upregulation of promesenchymal markers occurs concurrently with promotion of an androgen-independent phenotype resembling the castration-resistant disease that progresses independent of the AR signaling pathway.

In summary, we have shown that under androgen-deprived conditions, HHV-8 alters the AR signaling pathway, induces EMT, and reprograms the host cell transcriptional profile of otherwise androgen-sensitive cells by upregulating EZH2, whose methyltransferase activity epigenetically represses the viral lytic switch protein as well as other key tumor-suppressor genes that are frequently inactivated in advanced cancers. Remarkably, we have also found that at least two HHV-8 microRNAs expressed during the latent phase directly target a family of Jumonji demethylases for degradation (Supplementary Fig. S5A #3, our unpublished data), and that this targeting operates in concert with the elevated EZH2 methyltransferase activity. When the virus is reactivated from latency, however, loss of EZH2 and SUZ12 destabilizes PRC2, resulting in a loss of the trimethylated mark at H3-Lysine 27 and relief of transcriptional repression, which liberates tumor-suppressor proteins such as MSMB and DAB2IP (Supplementary Fig. S5). Collectively, these findings not only underscore the central role of EZH2-mediated epigenetic modifications in maintenance of viral latency, but also support a model, whereby prostatic infection by HHV-8 could constitute risk for progression to an androgen-independent phenotype that mimics the EZH2–mediated transcriptional profile of poorly differentiated prostate tumors.

Although the strength of our findings is premised on chronic presence of HHV-8 in the prostate, we are mindful of a few epidemiologic undercurrents: (i) the measurable HHV-8 infection rates worldwide do not track with the epidemiology of prostate cancer in general, and (ii) others failed to detect viral nucleic acids or antibodies in prostate tissues or serum of some patients with prostate cancer (48), although contemporary detection tools now available are likely to capture a more accurate picture of current prevalence rates. Nonetheless, it is arguable that the "randomness" with which androgen-independent disease emerges among posttherapy patients may be controlled by equally random degrees of susceptibility to modifying influences such as infections and/or environmental risk factors that could impact various nodes along the temporal evolution of the cancer toward the definitive androgen-independent phenotype. Considered in this context,
parameters that control differential susceptibility to such infections should be exploited to probe pathologic networks through which an infectious agent capable of stimulating the same pathways as those revealed by our study could promote prostate cancer progression. Derivative insights from these efforts will be valuable in achieving the important goal of isolating relevant biomarkers and correlates of cancer progression that will then guide development of effective therapies or methodologies for better diagnosis and stratification of prostate cancer cases potentially at high risk for treatment-resistant disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Oncogenic Herpesvirus HHV-8 Promotes Androgen-Independent Prostate Cancer Growth

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