Transformation of Endothelial Cells by Kaposi's Sarcoma-Associated Herpesvirus

by

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School of Medicine

CERTIFICATE OF APPROVAL

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ABSTRACT

The outgrowth of truly transformed clonal tumors is thought to require significant alterations of multiple cellular processes including growth controls, survival, angiogenesis, and tissue invasiveness. Within proliferating populations of pre-malignant cells, these characteristics arise from alterations in genetic code. Such alterations that confer a replicative or survival advantage are maintained in a manner formally similar to Darwinian evolution. Some viruses, including herpesviruses, can contribute to tumorigenesis by inducing some of these tumor cell characteristics, either by the direct actions of viral genes or indirectly by inducing changes in cellular gene expression.

Kaposi's sarcoma (KS) is the most common AIDS-associated malignancy and is characterized by dysregulated angiogenesis, proliferation of spindle cells, and extravasation of inflammatory cells and erythrocytes. Early stage KS lesions are typically polyclonal, reflective of the hyperproliferative nature of KS. However, lesion progression is associated with the outgrowth of transformed spindle cell clones resulting in oligo- or monoclonal advanced stage lesions. Kaposi's Sarcoma-associated herpesvirus (KSHV; also human herpesvirus-8) is implicated in all clinical forms of KS as well as the lymphoproliferative disorders primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). However, as the seroprevalence of KSHV far exceeds the incidence of these diseases (even in populations known to be at higher risk), we can conclude that KSHV is necessary but not sufficient for the

development of disease; indeed, KS most frequently occurs in immunocompromised individuals, most notably those infected with HIV. Endothelial cells harbor the KSHV genome *in vivo*, are permissive for virus infection *in vitro*, and are thought to be the precursors of spindle cells. Because spindle cells are rare in early patch-stage KS lesions but become the predominant cell type in later plaque- and nodular-stage lesions, alterations in spindle cell physiology that induce proliferation and survival are thought to be important in disease progression and may represent potential therapeutic targets. That other factors besides KSHV infection is required for KS is reflected by *in vitro* studies demonstrating that KSHV inefficiently transforms cultured endothelial cells. Like the closely related tumorigenic herpesvirus Epstein-Barr virus, KSHV contains genes that interfere with cellular growth restriction and apoptotic signals; the combined effect of these genes is thought to drive the proliferation and survival of virally-infected lesional spindle cells. However, large scale gene expression analyses have revealed that KSHV infection induces dramatic reprogramming of DMVEC gene expression. These changes in cellular gene expression may contribute to the pro-growth and pro-survival phenotype observed in KS spindle cells. Thus, identification of cellular genes upregulated following KSHV-infection of DMVEC and subsequent validation of gene expression in KS lesional tissue is a valid approach to identifying important changes in KS spindle cell physiology that can be exploited therapeutically.

I have used a previously established *in vitro* KS model for many of the studies presented in this dissertation. The salient features of this model include recapitulation of KS cell physiology including spindle cell formation, loss of contact inhibition and

anchorage-dependent growth restriction; long term propagation of predominantly latently-infected cells; and the ability to generate age- and passage-matched KSHV-infected and uninfected cultures. This system has been amenable to gene expression profiling by cDNA microarrays proteomics methods. I show in chapter 2 that heme oxygenase-1 (HO-1), the inducible enzyme responsible for the rate-limiting step in heme catabolism, is upregulated in KSHV-infected endothelial. This alteration of endothelial physiology results in enhanced proliferation of infected cells in the presence of free heme, a compound known to be present within the KS lesional microenvironment. This proliferative response to heme can be blocked with HO-1 inhibitors, suggesting that HO-1 is a potential therapeutic target for KS that warrants further study. I show in chapter three that matrix metalloproteases-1 (MMP-1) is likewise upregulated by KSHV infection. MMP-1 is a member of a family of enzymes that degrade extracellular matrix components and are involved in a numerous normal and pathological processes; targeted inhibition of MMP-1 may, therefore, be a useful treatment option for KS.

In addition to these gene expression profiling studies, I present my work concerning the control of lytic cycle induction in KSHV-infected PEL cells. KSHV genes are expressed in two patterns, latent and lytic. Latent gene expression predominates and is characterized by expression of a limited subset of viral genes. Production of infectious progeny virions requires expression of lytic cycle genes, which include regulatory as well as structural genes. Though lytic genes are expressed by only a minority of infected cells within KS lesions, some lytic gene products are thought to be essential for lesion progression by stimulating cellular

proliferation, preventing apoptosis, and evading the host immune system. In chapter 3 I show that lytic cycle is maximally induced by phorbol ester treatment during S phase of the cell cycle, a finding that may aid studies of lytic cycle genes within the context of whole virus as well as increasing the yield of infectious virus for stock preparation.

Chapter One

Introduction

1. Description and classification of Kaposi's sarcoma

Moritz Kaposi, a dermatologist at the University of Vienna, described the case histories of five middle aged and elderly men who presented with "multiple idiopathic pigmented sarcomas of the skin" in 1872 [1]; this disease was named Kaposi's sarcoma (KS) a few years later, but remained a medical rarity in the developed world for the next hundred or so years. In 1981 an aggressive variant of KS became one of the sentinel diseases of the AIDS pandemic[2]. The poor prognosis of AIDS-associated KS led one playwright to describe the disease as "the wine-dark kiss of the angel of death."[3].

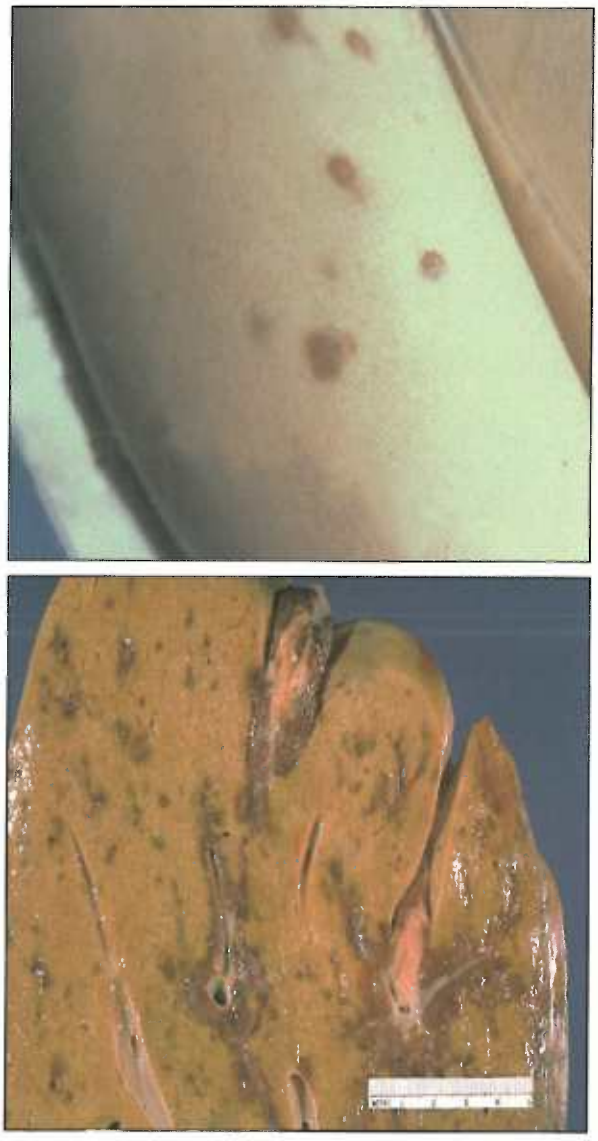
Four distinct epidemiological forms of KS are now recognized [4] and are distinguished by relative severity, HIV serostatus, and geographic distribution: classical, the variant first described in elderly Mediterranean men; AIDS-KS, severe and often fatal, the most common AIDS-associated neoplasm; endemic, an aggressive but non-HIV-associated variant common in sub-Saharan Africa; and iatrogenic, a form occasionally complicating organ transplantation. In all forms, KS usually presents as multiple reddish-purple dermal or mucosal lesions that may be flat or

raised (figure 1). Lesions may coalesce as disease progresses, becoming nodular and even ulcerated. Dissemination to visceral organs, including the lungs, liver, lymph nodes, and gut may also occur and is associated with poor prognosis. The four epidemiological forms of KS are histologically indistinguishable, characterized by disorganized networks of abnormal microvasculature composed of spindle-shaped cells[5](figure 2); these cells do not maintain the integrity of microvascular channels, accounting for lesional edema and abundant extravasation of inflammatory cells and erythrocytes. Spindle cells, thought to be of endothelial lineage, are present in early patch-stage lesions but become the predominant cell type in later plaque- and nodular-stage lesions [6-13]. Patch-stage lesions are typically polyclonal, but oligoclonal and monoclonal later-stage lesions have been described[14-16]. Thus KS exhibits characteristics of both a reactive hyperproliferative lesion and a truly transformed sarcoma.

FIG. 1. Kaposi's sarcoma. (A) Plaque stage dermal lesions (top panel) and late stage visceral involvement; in this instance hepatic (lower panel). (B) Advanced African KS of the lower extremities. KS in Africa is associated with significant morbidity and mortality.

Figure 1

a

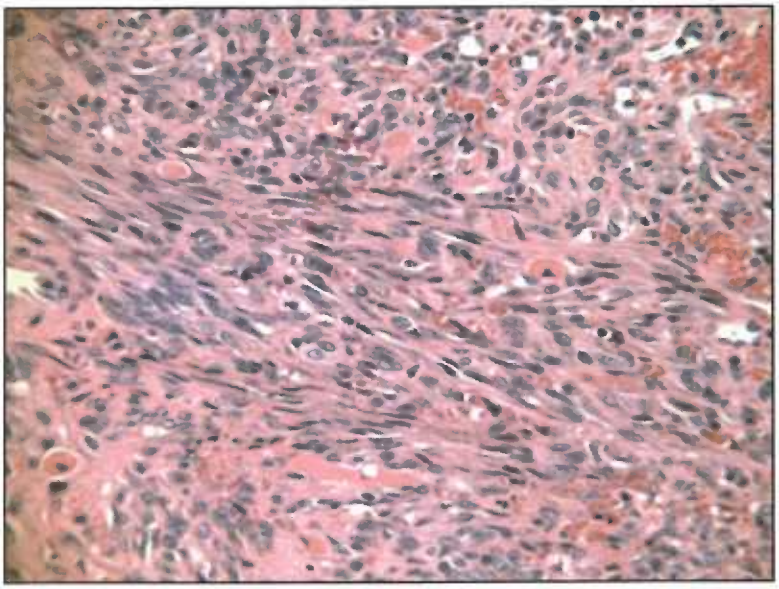


b

FIG. 2. Histological features of KS lesions. (A) Hematoxylin/Eosin stain of biopsy tissue from a dermal lesion and (B) graphic representation of the dominant histological features of KS (reproduced from appendix 1 of this dissertation). The dominant histological features of all epidemiological forms of KS include disorganized networks of abnormal microvascular spaces, tissue edema, and extravasated blood cells including erythrocytes and inflammatory cells. Spindle cells are the predominant cell type of KS lesions and represent the tumor cell component; infiltrating inflammatory cells drive lesion development by secretion of cytokines, chemokines and other metabolites. Data in Chapter 2 of this dissertation proposes a functional consequence of the extravasated erythrocytes and a phenotypic response of KS spindle cells.

Figure 2

a



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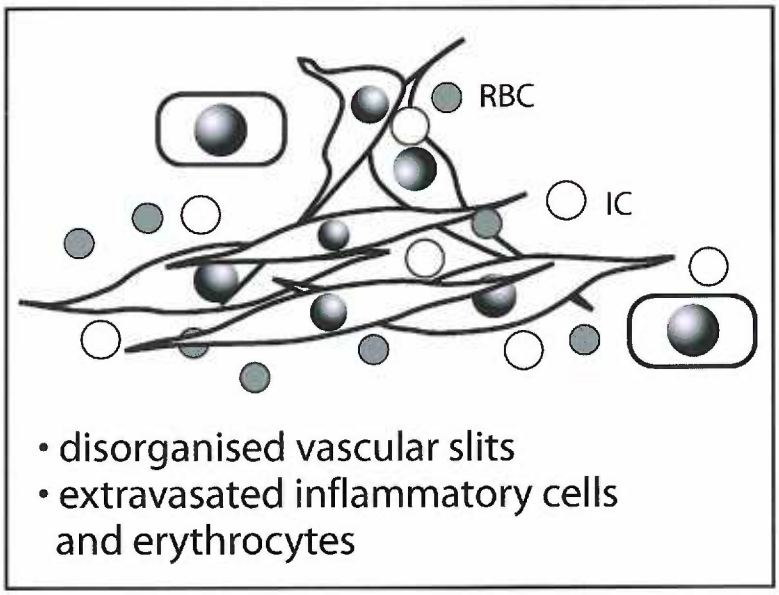

RBC
IC
• disorganised vascular slits
• extravasated inflammatory cells and erythrocytes

b. ***Standard and experimental KS therapy***

The extent and severity of KS, as well as comorbid factors (e.g. HIV serostatus, iatrogenic immune suppression), determine the clinical course of KS therapy. Patients with stable or slowly progressive AIDS-KS with a limited distribution (including dermal, lymph node, and oral lesions or non-symptomatic visceral disease) are routinely started on highly active antiretroviral therapy (HAART). AIDS-KS is known to be responsive to HAART, and control of HIV load is the primary goal in the treatment of AIDS-KS[17,18]. Pain, edema, and cosmetic concerns arising even from slowly progressive disease can be addressed with local therapies such as irradiation or excision [19,20], Alitretinoin gel (a retinoid approved for topical application) [21,22], interferon-alpha [23], or experimental therapies [24] such as novel angiogenesis inhibitors (reviewed in [17,25-27]) have been employed with varying degrees of success. Advanced or quickly progressing disease can be treated with systemic cytotoxic chemotherapy. Liposomal anthracyclines (daunorubicin, doxorubicin) are considered first line agents[28-30] while paclitaxel is approved as a second line agent[31-33]. However, bone marrow suppression associated with systemic chemotherapy limits the usefulness of these therapies in patients who are already immunosuppressed. Regression of iatrogenic KS has been noted following dose reduction of immunosuppressive therapy; treatment of KS in organ transplant patients requires a careful balance between preservation of grafted tissues and prevention of death due to disseminated KS[34,35]. Pathogenesis-targeted chemotherapeutic agents are in various stages of development, from preliminary observation to clinical trials[17,25-27] and two such agents will be discussed in more detail below.

c. *Development of KS therapies amenable to use in Africa*

Some of the severest manifestations of KS occur in parts of Africa (figure 1b). KS was common in Africa prior to the AIDS epidemic, but has increased over the last 10-15 years, presumably as a result of the spread of HIV[36-42]. Now 57,000 new cases of KS occur in Africa each year, an incidence rate similar to that of colon cancer in industrialized nations[43,44]. Effective KS therapy is prohibitively expensive for most of those afflicted; thus KS in Africa is associated with a 90% mortality rate[43]. A recent article by Dedicoat, *et al.* describes an attempt to identify effective KS therapeutics that could be used in resource-poor settings such as Africa[45]. The authors sought to identify studies examining drugs found on the World Health Organization (WHO) essential drug list, a minimal list of medications assumed to be normally available in developing countries. None of the over six hundred studies reviewed identified chemotherapies that would be affordable in resource-poor areas. To effectively address the problem of KS in Africa it will be necessary to develop significantly less expensive drugs. The study presented in Chapter Two of this dissertation identifies one such potential agent.

2. KS as an infectious disease

a. *Discovery of KSHV and establishment of causality*

Several agents have previously been hypothesized to induce KS. An association with AIDS led to the speculation that HIV was responsible for KS, but HIV DNA could

not be demonstrated in KS spindle cells. Furthermore, epidemiological studies of AIDS-KS revealed that considerable variation in KS risk existed between different populations of AIDS patients [46]. HIV-positive gay men were found to be 20-30 times more likely to develop KS than HIV-positive hemophiliacs or IV drug users. The occurrence of KS in children with vertically-acquired HIV was found to be even more rare. Thus an indirect role for HIV in KS pathogenesis was hypothesized and the search for other candidate agents continued.

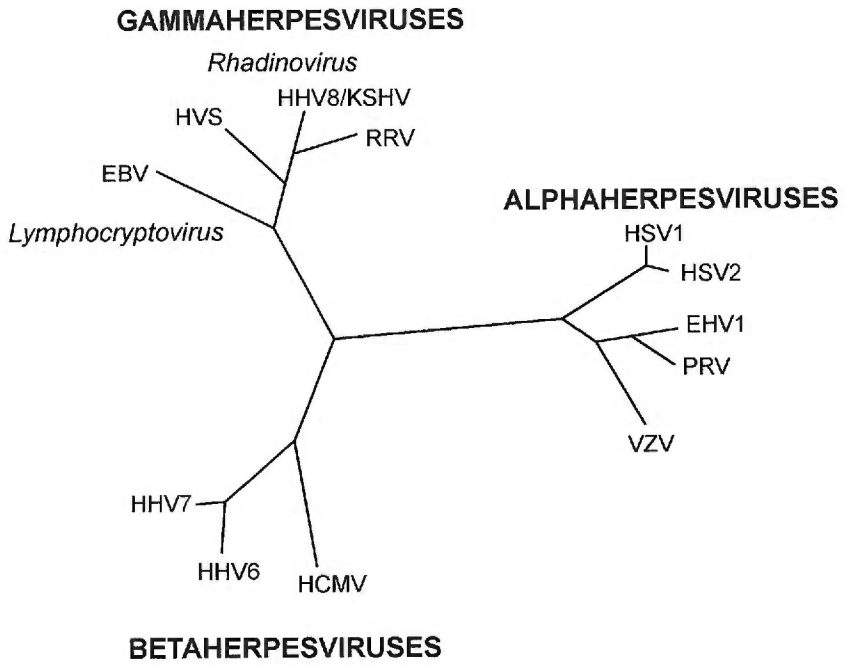
Representational difference analysis (RDA) is a technique used to identify minor differences between complex genomic samples. DNA from KS tissue and normal skin was compared by RDA, which led to the discovery of KSHV in 1994[47]. Sequences from this new human herpesvirus were detected in nearly all KS tumors but not in most other pathologic samples examined. Importantly, viral sequences were found in lymph node biopsies from gay men with AIDS, which suggested a link not only to patients with KS but also to groups known to be at increased risk of developing KS. Seroprevalence studies have since shown that KSHV infection is more common in populations known to be at greater risk for developing KS [48] and that KSHV viral load increases prior to the onset of disease (though the utility of viral load assessments as predictors of KS development is debated [49-51]). KSHV is now considered necessary for all clinical forms of KS [27,47,52] as well as two rare lymphoproliferative disorders, primary effusion lymphoma (PEL)[24,53,54] and multicentric Castleman's disease[55]. However, as the seroprevalence of KSHV far exceeds the incidence of these diseases (even in populations known to be at higher

risk), we can conclude that KSHV is necessary but not sufficient for the development of disease [56].

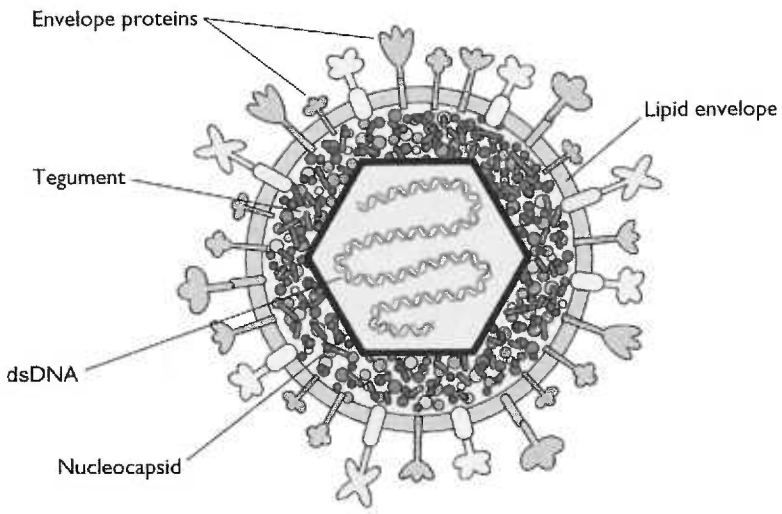
FIG. 3. KSHV. (A) A dendrogram showing relatedness of a selection of mammalian herpesviruses, including the eight known to date that infect humans. KSHV is classified as a gamma-2 herpesvirus, the first human herpesviral pathogen thus categorized. RRV and HVS, the closest relatives of KSHV, do not induce KS-like disease in their natural hosts; the lack of appropriate animal models for KSHV/KS pathogenesis studies is a significant limitation in this field. (B) A schematic illustrating general herpesvirus virion architecture. Salient features include a capsid-enclosed double-stranded DNA genome, tegument, and a glycoprotein-studded lipid envelope (adapted from "Principles of Virology" Flint, Enquist, Racaniello, and skalka (Eds) 2nd Edition, ASM Press, 2004).

Figure 3

a


GAMMAHERPESVIRUSES
Rhadinovirus
HHV8/KSHV
HVS
RRV
EBV
Lymphocryptovirus
ALPHAHERPESVIRUSES
HSV1
HSV2
EHV1
PRV
VZV
HHV7
HHV6
HCMV
BETAHERPESVIRUSES

b


Envelope proteins
Lipid envelope
Tegument
dsDNA
Nucleocapsid

b. *The viral family* Herpesviridae

The family *Herpesviridae* is a diverse family of large enveloped double-stranded DNA viruses known to be associated with most animal species. Mammalian-associated herpesviruses are divided into three subfamilies, *Alpha-, Beta-, and Gammaherpesvirinae* (figure 3a), that vary in genomic size, base composition, and organization but share five distinct characteristics: 1) a common virion architecture consisting of a genomic core within an icosahedral capsid (the nucleocapsid), surrounded by an amorphous tegument and phospholipid envelope (figure 3b); 2) a group of approximately 43 herpesvirus genes thought to have descended from a common ancestor of all contemporary mammalian herpesviruses; 3) synthesis and encapsidation of viral DNA within the host cell nucleus; 4) production and release of progeny virions that usually results in host cell death; and 5) establishment of a life-long, predominantly latent infection in the natural host.

Herpesvirus infection is mediated by interaction of viral glycoproteins in the virion envelope with cognate receptors in the plasma membrane of host cells. Entry is mediated by fusion of the particle and host cell membranes, resulting in release of tegument proteins and the viral nucleocapsid into the cytoplasm. The nucleocapsid is then transported along host-cell microtubules to the nuclear envelope where the viral DNA is delivered to the nucleoplasm. Both viral and cellular genes are required for completion of the viral life cycle (e.g. a viral DNA polymerase mediates replication of the viral genome while transcription requires cellular RNA polymerase II). Modulation of host cell gene expression by KSHV and the associated implications for the development of novel KS therapies will be discussed in detail in this dissertation.

Herpesvirus genes are expressed in two general patterns, latent and lytic, though the biological signals governing the switch from latency to lytic replication are not well understood. Although type-specific patterns exist, a generalized herpesvirus lifecycle can be described. The latancy program is characterized by expression of relatively few viral genes and results in maintenance of the viral genome as a circularized episome tethered to host-cell chromosomes. The lytic cycle involves a more complicated program characterized by coordinated expression of all viral lytic genes in three kinetic classes: immediate-early (IE), early (E), and late (L). IE gene products are detectable immediately following infection, and do not require *de novo* synthesis of viral DNA or proteins to be expressed. In contrast, E genes are involved in replication of viral DNA and can be blocked by inhibitors of protein synthesis such as cycloheximide (CHX). L genes are structural or packaging proteins and can be blocked by both CHX and inhibitors of DNA synthesis such as phosphonoacetic acid (PAA; foscarnet). Productive replication proceeds from expression of all necessary viral genes to packaging of viral genomic complements into preformed capsids within the nucleus; subsequent egress through the nuclear membrane and various components of the secretory pathway ensues whereby particles acquire a tegument layer and a lipid envelope studded with virally-encoded glycoproteins as well as some cellular proteins. Release of progeny virions occurs by budding through the plasma membrane or lysis of the infected cell, in either case inducing cell death. Herpesvirus particles are unstable once released from the host cell and can be quickly inactivated by the host immune system or degraded upon

shedding into the environment. Transmission to new hosts thus requires intimate contact, including saliva, breast-feeding, transplacental, blood, and sexual routes.

There are currently eight herpesviruses known to infect humans, differing in the severity of disease associated with infection and the anatomical sites where latency is established. Three alphaherpesviruses, herpes simplex virus 1 and 2 (HSV-1 and 2; HHV1 and 2, respectively; genus *Simplexvirus*) and varicella-zoster virus (VZV; HHV3; genus *Varicellovirus*), are associated with recurrent ulcerative diseases of skin and mucosae. These viruses initially infect and replicate in epithelial cells; retrograde axonal transport of virions to the sensory ganglia innervating the site(s) of infection leads to establishment of latency. Of the three betaherpesviruses, cytomegalovirus (CMV; HHV5; genus *Cytomegalovirus*) is the most studied. CMV infection is a common cause of infectious mononucleosis, and perinatal infection is a significant cause of birth defects. CMV latency is established in various tissues including mononuclear cells, secretory glands, and kidneys. HHV6 and HHV7, classified as betaherpesviruses, are not currently known to be associated with any specific disease. Two gammaherpesviruses, Epstein-Barr virus (EBV; HHV4; genus *Lymphocryptovirus*) and Kaposi's sarcoma-associated herpesvirus (KSHV; HHV8; genus *Rhadinovirus*), establish latency in lymphoid organs. These viruses have the notable capacity to induce proliferation and transformation of host cells and are accordingly associated with proliferative disorders including lymphomas and sarcomas. EBV is the prototypical gammaherpesvirus and will be discussed in more detail in a later section of this introduction. The severity of disease caused by any of these herpesviral pathogens is increased dramatically by immunosuppressive

comorbidities and are, therefore, associated with AIDS- and organ transplant-associated complications.

c. *Classification and genomic organization of KSHV*

Complete sequencing of the KSHV genome revealed relatedness to members of the *Gammaherpesvirinae* subfamily[57-59]. KSHV has been placed in the *Rhadinovirus* or γ2 group within this subfamily, becoming the first human herpesvirus thus categorized. The KSHV genome is ~ 165 kb with a central unique coding region of ~ 140 kb flanked on either end by variable numbers of noncoding, GC-rich repeat sequences [60,61]. The coding region of KSHV consists of seven blocks of conserved herpesvirus genes that align closely with those of the prototypical γ2 virus, herpesvirus saimiri (HVS), a virus which causes fatal T cell lymphomas in new world primates [62,63]. These conserved genes are labeled open reading frames (ORF) 1 through 75 and most encode proteins required for production of progeny virions (transcription factors, DNA synthesis-related enzymes, as well as capsid, tegument, and envelope proteins) or proteins involved in the establishment and maintenance of latency. Interspersed between the conserved blocks are genes that are unique to gammaherpesviruses or to KSHV, labeled K 1 through 15. Many of the K genes have homologs to cellular genes involved in intra- and inter-cellular signaling including cytokines, chemokines, chemokine receptors, and interferon response factors[64].

d. *KSHV latent cycle genes*

Both *in vitro* and *in vivo*, KSHV is maintained as a viral episome in a primarily latent state characterized by expression of few viral gene products [65-72]. At least three viral gene products, vFLIP, vCYC, and LANA-1, are believed to comprise the minimal latency expression program of KSHV and are consistently expressed in all virally infected cells in KS, PEL, and MCD [65-67,73]. The gene product of ORF 71, vFLIP (viral FLICE-inhibitory protein; K13) inhibits apoptosis [74,75] and activates NFκB[76]. ORF 72, the viral cyclin (v-Cyclin), is a homolog of cellular D-type cyclin that can drive quiescent cells into S phase in part by inhibiting retinoblastoma protein (Rb) activity[77-80]. In addition, v-Cyclin inactivates the cyclin-dependent kinase inhibitor p27[81] and promotes cytoplasmic export of human Orc1, a component of the host origin recognition complex[82]. The latency-associated nuclear antigen (LANA-1; ORF 73) targets the tumor suppressor proteins p53 and Rb[83,84], providing both anti-apoptotic and cell cycle-regulatory functions. LANA-1 is also a transcriptional modulator of various cellular and viral promoters [85], and tethers multiple copies of the viral episome to host cell chromosomes, a function indispensable for maintenance of the viral genome during cell division [86].

Two additional viral genes, K10.5 and K12 are transcribed during latency but have additional features deserving of mention. K10.5 is expressed during latency in the B cell disorders MCD and PEL, but not KS [87]. The gene product of K10.5, LANA-2, also known as viral interferon regulatory factor 3 (vIRF3), has partial homology to members of the cellular IRF family (particularly IRF4) and two other viral IRFs K9 (vIRF1) and K11.1 (vIRF2) [87,88]. A role for LANA-2 in inhibition of

p53-induced apoptosis has been proposed, both through direct interaction with p53 [87] and via inhibition of NFκB activity [89]; thus, LANA-2 could contribute to proliferative or neoplastic expansion of KSHV-infected B cells.

Kaposin (K12) was initially identified as an abundant latent transcript in KS tumor samples[59]; unlike other KSHV latency genes, however, K12 transcript levels increase following lytic cycle induction, suggesting a more complicated transcriptional regulation pattern. Questions awaiting elucidation include whether Kaposin utilizes different transcription units during latency and lytic replication, as well as how cell cycle and tumor type influence transcriptional regulation. The Kaposin locus encodes at least three proteins (Kaposin A, B and C) via a translational program that is similarly complex and incompletely understood [73,90-92]. Transforming functions have been ascribed to the Kaposin family, particularly to Kaposin A [91], and recently a role for Kaposin B in stabilization of cytokine transcripts via activation of the cellular kinase MK2 was proposed [93]. Overall, the latency program of KSHV enables expansion of a population of latently-infected spindle cells by inducing proliferation and preventing apoptosis of infected cells as well as maintaining the viral genome in daughter cells.

e. *KSHV lytic gene expression*

In vivo, the majority of KSHV-infected spindle cells and neoplastic B cells maintain the virus as a latent infection, with only a small percentage of cells expressing lytic cycle genes [59,94-97]. The KSHV gene ORF 50 , a homolog of Epstein-Barr virus Rta, encodes a replication and transcriptional activator that is necessary and sufficient to

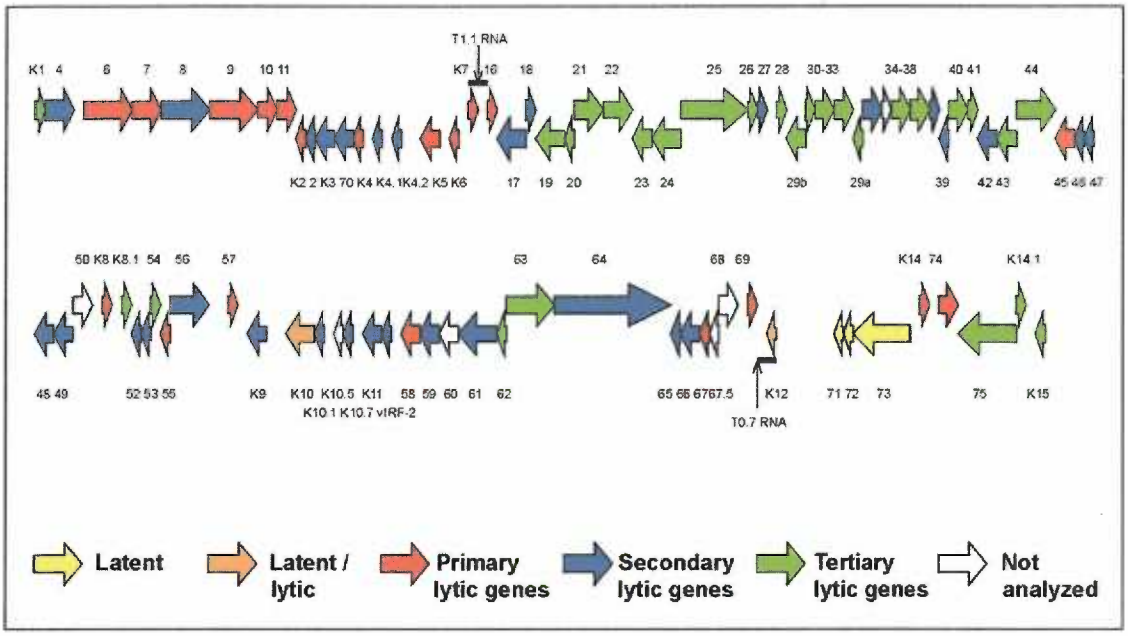
trigger lytic replication and production of viral progeny (reviewed by West and Wood[98]). ORF 50 is one of the earliest immediate early genes induced upon lytic reactivation in B cells and endothelial cells, [99-101] and is considered the master regulator of the switch from latency to lytic replication. *In vitro*, KSHV-infected cells can be induced to enter lytic cycle by ectopic expression of ORF 50 [100-102] or by treatment with phorbol esters [103] or sodium butyrate[104,105]. The biological signals that initiate the lytic cascade are not fully understood, though hypoxia[106,107], inflammatory cytokines (IC) [108-110], HIV-1 *tat*[109,111], and CMV coinfection [112], have all been implicated. A role for LANA-1 in controlling lytic induction via regulation of ORF 50 expression and function has also been proposed[113].

Upon reactivation, KSHV expresses a wide repertoire of gene products in a coordinated cascade, culminating in release of progeny virions from infected cells. Chemical induction significantly increases the percentage of cells that express lytic gene products and was used by two groups for initial classification of KSHV lytic genes into kinetic classes using microarray analysis[114,115]. Lytic replication is essential for viral dissemination, and the observation that treatment of KSHV-seropositive AIDS patients with ganciclovir decreases the incidence of KS development suggests that lytic replication plays an important role in viral pathogenesis[116]. A number of KSHV lytic genes code for regulatory proteins that could conceivably drive proliferation or transformation as well as genes that modulate apoptotic signals and recognition by the host immune system (see [117] and [118] for recent comprehensive reviews). These genes include homologs of cellular cytokines (vIL-6/K2), chemokines (vMIP-I/K6, vMIP-II/K4, vMIP-III/K4.1), chemokine

receptors (vGPCR/ORF74), interferon regulatory factors (vIRF-1/K9), anti-apoptotic factors (vIAP/K7), and modulators of immune recognition (K3/MIR1 and K5/MIR2). Thus, while lytic replication is incompatible with host cell survival, reservoirs of lytic infection are though to exert essential paracrine mechanisms that sustain tumorigenesis (see [118,119] for recent reviews). Additionally, some speculate that a limited subset of lytic genes could be expressed under certain conditions in the absence of full lytic replication[99,120-122]. Such lytic gene expression could contribute to pathogenesis without leading to host-cell death resulting from production and release of progeny virus. Of particular importance here may be expression of the immediate early gene K5, which can be expressed independent of ORF50 expression in PEL cells [123] and experimentally infected 293A cells (Jae Jung, personal communication). K5, along with another IE gene K3, perform a crucial immune evasion function via downregulation of MHC-1 on infected cells [124].

FIG. 4. Kinetic classes of KSHV genes (reproduced from reference 113). KSHV ORFs are color coded according to categorization into kinetic classes: latent, latent/lytic (expressed without chemical induction but with levels significantly increased following induction), primary lytic, secondary lytic, and tertiary lytic genes. Expression of primary lytic genes is required for DNA replication.

Figure 4


T1.1 RNA
K1 4 6 7 8 9 10 11 K7 16 18 21 22 25 26 27 28 30-33 34-38 40 41 44
K2 2 K3 70 K4 K4.1K4.2 K5 K6 17 19 20 23 24 29b 29a 39 42 43 45 46 47
50 K8 K8.1 54 56 57 63 64 68 69 K14 74 K14.1
45 49 52 53 55 K9 K10 K10.5 K11 58 59 60 61 62 65 66 6767.5 K12 71 72 73 75 K15
K10.1 K10.7 vIRF-2
T0.7 RNA
Latent
Latent / lytic
Primary lytic genes
Secondary lytic genes
Tertiary lytic genes
Not analyzed

3. KSHV-infected cell lines derived from primary effusion lymphomas

Primary effusion lymphomas (PEL; also body cavity-based lymphomas) are KSHV-related neoplasms that can present independently of HIV-infection[54] but are more frequent and severe in AIDS patients[53]. Because most PEL tumors are CD20-negative, PEL patients cannot be treated with B cell-targeted therapies; the prognosis of PEL, therefore, is poor. KSHV was first recovered by cultivation of cell lines derived from PEL [103]. Numerous clonal PEL cell lines have now been established [125-132], about half of which are coinfected with EBV [54,103,126,133-135]. Cell lines can be readily established from PEL tumors, and, unlike explanted KS spindle cells, maintain the KSHV genome in 100% of cells even following extensive tissue culture passage [66,87,114,115,136-140]. In PEL lines, the KSHV genome is maintained as 50-150 latent episomes per cell, which is approximately 10-fold higher than in KS [52,141]. While PEL infection is predominantly latent, lytic replication can be induced by chemical induction or ectopic expression of ORF50 [61,100]. Collectively, these characteristics have made PEL lines an extremely consistent and convenient model in which to study KSHV biology. PEL were used for the initial *in vitro* studies of KSHV biology, including those that yielded the viral genomic sequences, and have since been widely used to determine the kinetics of viral gene expression and mechanisms of viral latency and reactivation [57,58,114,115,142,143 100,101,104,144,145]. PEL systems have also been used to develop novel KSHV-specific therapeutic strategies that may find clinical application. Klass, *et al*[146], demonstrated that inducing replication in PEL cells with valproate (an anti-seizure medication with histone deacetylase inhibitor action), while simultaneously blocking herpesviral DNA replication with ganciclovir

and phosphonoformic acid, led to apoptosis in the tumor cells without increasing virus load. Godfrey, *et al*[147], tested RNA interference as a therapeutic strategy for PEL. Using lentiviral-delivered short hairpin RNA (shRNA) to target latency genes, inhibition of v-Cyclin and v-FLIP led to apoptosis in all 4 PEL lines (BC-3, BCP-1, JSC-1 and HBL-6) tested. Non-KSHV cell lines including the Burkitt's lymphoma line RAMOS were unaffected by this treatment. Interestingly, LANA-1 was not as reliable a target; stable cell lines maintaining the sh-LANA vector, and consequent lower KSHV copy numbers, could be generated. Whether this result reveals a capacity for KSHV to integrate into the host genome, thus becoming LANA-independent for segregation during cell division, remains to be established. Another interesting finding emerging from this study was the fact that LANA knock-down increased expression of ORF50, a result consistent with a recent report that LANA inhibits ORF50 expression and function[113]. In a murine model, injection of the sh-v-Cyclin vector prevented development of PEL-driven ascites and reduced established ascites. Other researchers have also used PEL lines to establish animal models for the study of KSHV biology and PEL tumorigenesis *in vivo* [126,132,148-150]. In the future, such models should greatly facilitate *in vivo* screening and validation of anti KSHV drugs that may have efficacy for KS as well as PEL.

Despite their considerable contribution to our current understanding of KSHV biology, PEL cell lines are not amenable to studying KSHV-induction of transformation because the transformation event precedes cell line establishment *in vitro*. Interestingly, primary B cells and some established B cell lines can be infected with KSHV *in vitro*, but infection does not lead to prolonged maintenance of the viral

genome nor to cellular transformation [122,151,152]. This is in marked contrast to B cell infection by two closely related gamma herpesviruses, EBV and RRV, both of which readily transform B cells and establish persistent infections. The reasons for this disparity are unknown and further studies are required to clarify these issues. Additionally, PEL are of limited utility in studying the cell-specific role of KSHV in KS, where the target cell is of endothelial lineage. Therefore, development of endothelial cell-based systems that support *de novo* KSHV-infection has been essential for studying KSHV infection and gene expression patterns in non-B-lineage cells, for identifying KSHV-induced changes in endothelial cell physiology that contribute to KS pathogenesis, and for developing effective KS-targeted therapies.

4. Experimental infection of endothelial cells with KSHV

Spindle cells harbor the KSHV genome *in vivo* but all described cell lines derived from KS lesions appear to rapidly lose the KSHV genome upon serial *in vitro* passage [10,52,94,129,153-155]. Genome loss could be explained by an insufficiency of the episomal maintenance mechanism in endothelial-lineage cells. Others have argued that this phenomenon is artifactual, reflecting the different selective pressures inherent in tissue culture conditions versus the KS lesional microenvironment. Because explanted KS cells lose the KSHV genome after serial passage, the development of *in vitro* models for KS using experimental *de novo* infection has proven invaluable for investigation of KSHV- endothelial cell (EC) interaction.

EC are the presumptive precursors of the characteristic spindle cells in KS lesions [6-10] although, as will be discussed in more detail later, whether KSHV

preferentially infects lymphatic, vascular, or precursor EC is a matter of debate. Spindle cells harbor KSHV DNA *in vivo* [52,94,153], but the loss of the genome from explanted tumor cells, as well as the need to evaluate the consequences of *de novo* infection, stimulated efforts to develop *in vitro* EC-based culture systems that supported KSHV infection [120,156-159]. All EC-based KS models aim to generate age- and passage-matched infected and uninfected cultures, illuminating an important advantage over PEL cell lines that lack uninfected counterparts for use as controls. The EC-based *in vitro* systems described to date differ to varying degrees as regards the protocol used and the observed consequences of infection, but key similarities have also been noted. Collectively, when evaluated in the context of the differences in experimental design, these models have yielded valuable information about KSHV biology and pathogenesis in EC. The degree to which any of these models reproduces the spindle component within the complex tumor microenvironment will be discussed further towards the end of this chapter.

Flore, *et al*, were the first to describe infection of primary EC *in vitro*, using adult bone marrow-derived EC as initial targets and HUVEC as secondary recipients to demonstrate both productive infection and paracrine influences[157]. Virus inoculum was prepared from concentrated TPA-induced supernatants from the BC-3 PEL line. The KSHV-exposed cultures acquired telomerase activity, exhibited extended lifespans, and formed colonies in soft agar, suggesting a KSHV-mediated transformation event. However, the frequency of initial infection was low and KSHV was maintained in only a minority of cells (<10%) following serial passage. Thus, the long-term survival of these cultures was attributed to indirect influences that could

also be reproduced by transferring supernatants from infected cultures to naive, uninfected HUVEC. Specifically, the type 2 VEGF receptor, KDR, was upregulated on all cells, regardless of infection status, via a KSHV-initiated paracrine effect, rendering the KDR+ cells responsive to VEGF in the culture medium. While more recent models have allowed a higher percentage of infected cells, the paradigm whereby a subset of infected cells influences the larger microenvironment remains extremely relevant to KS pathogenesis.

Ciufo, *et al* describe infection of primary adult and neonatal dermal microvascular EC (DMVEC) using infectious KSHV prepared from the supernatants of three different TPA-induced PEL lines, BCP-1, BC-3 and JSC1[156]. Virus was concentrated to generate high titer inoculae allowing for a high initial infection rate. Interestingly, inoculum prepared from the JSC1 line allowed the most efficient infection, due to the production of a higher number of progeny virions, and perhaps some qualitative differences between PEL lines as well. Upon infection, EC morphology changed from a cobblestone shape with a flat aspect to aggregates of cells with elongated spindloid morphology. Infection of the primary DMVEC appeared to be predominantly latent; almost all converted spindle cells were LANA-1-positive, with approximately 5-10% of cells expressing immediately early or early lytic proteins (e.g. K8, K5, ORF50 and ORF59) and 1-2% expressing the late lytic glycoprotein K8.1A. K8.1A-positive cells were typically a sub-fraction of rounded up cells exhibiting CPE associated with the final stages of productive replication. In some of the K8.1A-positive or ORF59-positive cells, intranuclear inclusions resembling herpesviral replication compartments were visible. In summary, infection

of primary DMVEC with JSC1-derived KSHV at high MOI led to the establishment of a predominantly latently infected cell pool that showed marked morphological changes reminiscent of KS spindle cells, while a subset of cells underwent spontaneous lytic replication with release of infectious progeny. These findings are reminiscent of KS tumor cells *in vivo*, both with respect to morphology and viral gene expression patterns. The KSHV-infected cultures could not be maintained indefinitely, however, due in part to a chronic loss of cells from lytic infection and failure of the latently infected cells to be maintained for more than a few cycles as a population of dividing cells that maintained the viral episome. Addition of uninfected cells to the cultures at a 1:10 ratio provided fresh targets for infection, providing a means to generate additional infected cultures without establishing new PEL-initiated infections. Thus, multiple rounds of *de novo* infection, as opposed to long-term maintenance of latently-infected cells, define this culture model.

Similar protocols have since been used to generate KSHV-infected EC cultures to examine patterns of viral [99] or host [122,160-162] gene expression by microarray analysis, RT-PCR and/or immunostaining following *de novo* infection with PEL-derived virus. Krishnan and colleagues [99] were the first to comprehensively examine viral gene expression patterns in primary endothelial cells following *de novo* infection. In this report, adult DMVEC were infected with concentrated BCBL-1-derived KSHV and the kinetics of latent and lytic gene expression was examined by whole genome array (Celonex HHV8 viruChip), RT-PCR, and immunostaining at time points from 30 minutes to 5 days PI. This report revealed a number of interesting findings. Expression of a limited set of immediate early (IE) and early (E) lytic genes

was initiated concurrently with latent genes in the majority of cells immediately following infection, with a sharp decline thereafter. Compared to TPA-induced BCBL cells, only a limited number of ORF50-activated genes were expressed, and the majority of genes involved in DNA replication and viral assembly were not expressed. Of particular interest, the IE gene K5 was expressed at early time points and continued to be well expressed after a decline in other IE and E genes. Another interesting finding was confirmation of the absence of LANA 2 (ORFK10.5) expression in primary EC. Expression of a limited set of IE and E genes with immune evasion and anti-apoptotic function, with a subsequent decline thereafter, may play a crucial role *in vivo* in allowing establishment of infection and tumor initiation/progression. Signals that determine which cells progress through a full lytic cycle and produce infectious progeny, and which establish latency, remain to be determined; indeed their elucidation may require the multi-factorial tumor environment to be more fully represented in *in vitro* KS models.

Host endothelial cell gene expression has been evaluated after only 2 and 4 hrs PI [122], after several days [161,162], or after 3 weeks [160]. Poole *et al* [160] also performed microarray analysis following reseeding of infected cultures with uninfected cells [160]. Naranatt and colleagues [122] examined gene expression in KSHV-infected primary DMVEC with Affymetrix HG-U133A gene arrays as early as 2 and 4 hrs PI, and discovered a significant early reprogramming of the host transcriptome that included alterations in genes involved in signaling, apoptosis, transcription, host defense, cell cycle, metabolism, inflammation, angiogenesis, and tumorigenesis. The authors also evaluated infected fibroblasts and the B cell line BJAB, to create a database that

illuminated both cell-type specific and common responses to infection. The study by Poole and colleagues [160] differed in that the authors' aim was to interrogate host reprogramming after establishment of a latent infection and following exposure of latently infected cells to TPA; hence, cells were harvested 3 weeks post infection (PI) as well as two weeks after reseeding infected cells with new uninfected targets. Clontech Human Atlas or Incyte Human UniGemV2.0 cDNA arrays were used, with RT-PCR analysis confirming changes in selected individual genes. This study revealed that even after establishment of latency, KSHV-infected cells exhibited a profound alteration in gene expression patterns; between 1.4 and 2.5% of genes represented were significantly upregulated or downregulated. Of particular interest was the induction of interferon-induced genes, genes involved in cell signaling and angiogenesis, and genes involved in cell cycle progression and apoptosis. The studies by Hong and colleagues and Wang and colleagues [161,162] were specifically designed to evaluate lymphatic reprogramming and are discussed later along with a similar study using immortalized EC [163].

Primary infection of EC has also been used to study KSHV binding and entry and the signaling pathways induced in the earliest phases of infection [164]. This study identified a role for integrin $\alpha 3\beta 1$-FAK dependent phosphatidylinositol 3- (PI 3-) kinase activation in KSHV entry and a role for the PKC-zeta-MEK-ERK signaling cascade during the earliest stages of KSHV infection. Activation of a mitogenic cascade during KSHV entry may have important implications for establishment of latency and the division of latently-infected cells, as well as creation of a host

microenvironment conducive to the expression of immediate early lytic cycle proteins.

A recent report by Krug, *et al*, is worthy of mention since infection was performed at low MOI to specifically explore the ability of latently infected EC to replicate, as well as the ability of infected EC to produce infectious virus for dissemination via *de novo* infection [159]. Pooled neonatal DMVEC were infected with KSHV derived from TPA-induced BCBL-1 cells. While less than 5% of cells were initially infected, infection spread primarily due to proliferation of latently infected cell with episome maintenance. A limited contribution by *de novo* infection from virions produced from the small percentage that entered lytic cycle was also noted. Spread of infection could not be blocked by treating cultures with compounds that block herpesviral lytic replication; therefore, proliferation of latently infected cells characterizes this culture system. A growth advantage for LANA-1-positive cells as compared to uninfected cells was noted, and cell spindling with loss of contact inhibition following post-confluent growth was observed. Thus, of all primary infection protocols, this is the most similar to the system described by Moses, *et al* that will be discussed in the following section. Of note, infection of the primary EC induced expression of cellular antiviral genes, specifically dsRNA-activated protein kinase (PKR) and 2'5'-oligoadenylate synthetase (2'5'-OAS), a phenotype that could be reproduced by IFNα treatment. IFNα treatment was also effective in preventing lytic activation and viral replication. However, because infection was initiated at low MOI and was maintained primarily by proliferation of latently infected cells, induction of antiviral genes did not eliminate viral infection in this system. Other

protocols for infection of primary cells that utilize high titer inoculae may induce higher levels of these host-defense genes, which may in turn interfere with establishment and maintenance of infection.

The life-span of primary EC *in vitro* is limited due to replicative senescence. To overcome this limitation, some investigators have employed life-extended EC prepared by ectopic expression of human telomerase or genes from other transforming viruses [120,158]. Expression of these genes does not induce laboratory evidence of transformation other than extending the length of time cells can be serially propagated in culture. A system described by Moses, *et al* was the first to use life-extended EC for KSHV infection and the first to describe cultures in which the majority of cells became infected and maintained the genome in a predominantly latent state [120]. In this model, adult primary DMVEC are immortalized by retroviral transduction of the E6 and E7 genes of human papillomavirus (HPV) type 16. The life-extended cells exhibit no overt signs of transformation (i.e. they become contact-inhibited at confluence and do not form colonies in soft agar) but with serial passage can be maintained significantly longer than their primary counterparts. HPV E6 and E7 are known to have significant effects on the cell cycle, most notably through targeting p53 and Rb. E6 participates in the ubiquitination and degradation of p53 [165], while E7 binds and sequesters Rb [166] and induces Rb phosphorylation (i.e. inactivation) [167]. These are two pathways that KSHV latent genes also inhibit; LANA-1 disrupts the function of both p53 [83] and Rb [84], and v-Cyclin induces Rb phosphorylation via activation of CDK-6 [79]. In addition, LANA and p53 co-localization is observed in KSHV-associated tumors *in vivo* where p53 is usually wild

type, suggesting that LANA-mediated p53 inactivation is important for tumorigenesis [168]. Therefore, immortalization of DMVEC by E6/E7 expression may augment some of the alterations in cellular physiology that are themselves induced by KSHV, thus creating a cellular microenvironment conducive to KSHV infection. It cannot be ruled out, however, that these HPV gene products do not complicate results obtained when using this culture system for studies of KSHV biology. Thus, key findings obtained using E6/E7-immortalized DMVEC have been verified in primary cells and KS tissue [120,169-171].

For studies performed with the E6/E7-DMVEC model to date, infectious KSHV has been derived from unconcentrated TPA-induced BCBL-1 cultures, yielding a relatively low titer inoculum and thus the expectation of a low percentage of initially infected cells. Indeed, evaluation of LANA-1 expression by IFA at early times (12 h) PI, revealed that not more than 10% of EC in a treated culture were KSHV infected [120]. LANA expression increased with time, such that by 14 days PI up to 80% of cells in a KSHV-exposed culture were infected. PCR for the KSHV genome at days 7 and 14 PI revealed increased intensity of the amplified product by day 14, supporting evidence of virus spread. A similar trend was seen with expression of the early lytic protein ORF 59: at 1 week PI <1% of cells were ORF 59-positive, but by 8 weeks PI up to 5 % of cells expressed this lytic marker. Expression of the late lytic glycoprotein K8.1A/B followed similar expression kinetics though to a consistently 5- to 10-fold lesser degree than ORF 59. Lytic replication could be induced in infected cells by treatment with TPA, sodium butyrate or corticosteroids, but never in more than 40% of LANA-positive cells.

Prior to exposure to KSHV, E6/E7-immortalized DMVEC retained a classical cobblestone appearance. KSHV-infection, however, induced marked changes in cellular morphology reminiscent of the spindle cells observed in KS lesions including elongated cells with oval cell bodies, uniformly narrow elongated cells, and extremely narrow light-refractile cells displaying scattering. A low percentage of rounded up cells that tended to detach from the monolayer were also observed; the nuclei of these cells as well as the nuclei of extremely spindled cells displayed intranuclear inclusions resembling typical herpesviral CPE. The extent of morphologic change within virus-exposed cultures increased with time PI and correlated strongly with the percentage of KSHV-infected cells, suggesting a direct effect of the virus on cell morphology. Evaluation of gene expression in concert with morphology revealed the following: LANA-1 expression was sufficient for spindling; ORF59-positive cells were spindle-shaped, with a proportion displaying intranuclear inclusions, and all rounded cells were strongly ORF59 positive; rare K8.1A/B positive cells displayed severe spindling but were more frequently rounded. The presence of infectious virions in conditioned supernatants that could transfer infection to naïve cultures, albeit at a low MOI, confirmed that DMVEC could support a fully permissive replication cycle. The relative ratios of ORF73 to ORF59 to K8.1A/B-positive cells at any one time suggested that the majority of cells harbored latent infection and that completion of the lytic cycle occurred in only a fraction of the lytic cell population. These observations are very similar to those reported by Ciufo and colleagues upon infection of primary DMVEC [156].

Evaluation of KSHV gene expression and cell morphology suggests that the attainment of completely infected cultures in the E6/E7-DMVEC model reflects a combination of the proliferation of latently infected cells and *de novo* infection by EC-generated virus. Once all cells are infected and naive viral targets are absent, latently infected cells continue to proliferate with only a minor fraction of cells lost due to productive lytic infection. Infected cultures can be expanded by passaging, but the genome is best maintained when low split ratios are used; presumably a delicate balance exists between cell division and episome replication and segregation to daughter cells. A key difference between the immortalized DMVEC and primary EC is the increased length of time for which latently-infected E6/E7-DMVEC can be passaged with genome maintenance. It is possible that the presence of E6/E7 allows for a degree of episome maintenance more akin to what is seen in PEL cells that, unlike KS tumor cells, are able to maintain the KSHV episome *in vitro* as well as *in vivo*. The latent gene ORFK10.5/LANA-2 that inhibits p53 function is well expressed in PEL cell but not in primary DMVEC [69,99]; possessing multiple ways to disable this tumor-suppressor protein may be a reason for efficient episome maintenance in PEL cells. Distinct from their role as oncoproteins, E6 and E7 are also known to be required for stable maintenance of HPV episomes in undifferentiated human keratinocytes[172]. The HPV oncogenes in the immortalized DMVEC may assist in episome maintenance by duplicating the function of LANA-2 or by acting as surrogates for other currently unappreciated mechanisms occurring in the lesional microenvironment. Interestingly, after several weeks in culture, there is a decrease in both spontaneous ORF 59-expression and inducibility of lytic cycle proteins by

chemical induction in KSHV-infected E6/E7-DMVEC. Thus, a predominantly latent infection is established, possibly by selection of clones that are resistant to lytic induction but that can propagate as continually-dividing, latently-infected cells. In such cultures, CD31 is strongly expressed on the LANA-positive cells, suggesting that K5 is not expressed and thus a true latency exists [Mandana Mansouri and Klaus Früh, unpublished observations].

As described above, the E6/E7-DMVEC model appears to represent two distinct stages: one in which the viral infection and genome spread occurs via both lytic and latent means, and a second where latently infected cells survive for multiple passages and maintain the genome in a latent state. When such cells are not passaged prior to achieving tissue culture confluence, a third state is generated, characterized by the continued post-confluent growth of the cells into 3-dimensional foci. Importantly, uninfected cells grow to confluence and enter a quiescent state under similar conditions. Post-confluent growth reflects loss of contact inhibition, one of the hallmarks of cellular transformation. Infected cells are also able to form colonies in soft agar, a measure of anchorage-independent growth, and form tumors in mice when injected into the tail base in a matrigel solution [Shane McAllister and Ashlee Moses; unpublished observations]. Krug and colleagues [159] similarly report that when primary DMVEC are infected at low MOI with subsequent spread of infection primarily through division of latently infected cells, loss of contact inhibition is observed. The piled up aggregates of infected cells reported by Ciufo and colleagues [156] are also reminiscent of 3-dimensional focus formation. Thus, acquisition of a

transformed phenotype is not unique to the E6/E7 DMVEC system, although the potential for additional contributions from the HPV proteins should be considered.

Latently infected E6/E7-DMVEC have been used in gene expression profiling studies to examine KSHV-induced cellular gene reprogramming and identify potential therapeutic targets for KS. These studies have employed cDNA arrays [173] as well as Affymetrix U95A and U133A and B GeneChips [170,171] and have interrogated several different KSHV-infected cultures relative to age- and passage-matched uninfected controls. For all of these studies, KSHV-infected DMVEC were infected with BCBL-1-derived KSHV at low MOI and passaged when confluent at low split ratios. Cells were harvested for microarray analysis when immunofluorescent staining of parallel cultures revealed that >90% of KSHV-infected cultures were LANA positive. This typically took 3-4 weeks and encompassed approximately 5-7 tissue culture passages. A minimum of two biological replicates were used for each comparison. The complete data sets from these different microarray experiments are available online (ohsu.edu/vgti/fruh.htm) and further details can be found in the specific papers referenced. To date, this database has been used to identify a handful of potential targets for KS chemotherapy[169,171,173]. Briefly, the receptor tyrosine kinase c-Kit was identified as a gene induced by KSHV using cDNA arrays [173], and confirmed using Affymetrix arrays, RT-PCR analysis and immunofluorescence, the latter being on primary DMVEC. An independent analysis of gene expression in primary DMVEC using U133A GeneChips also reported KSHV-induction of c-Kit [161]. A role for c-Kit in proliferation and post-confluent growth of KSHV-infected DMVEC was then demonstrated using both a pharmacological inhibitor of c-Kit

(Imatinib Mesylate, Gleevec; formerly STI 571 [173]) and gene knockdown approaches [170]. These data combined with evidence of c-Kit expression in KS tumors [174] contributed to a recent clinical trial that demonstrated the efficacy of Imatinib Mesylate as a therapeutic regimen for KS [175]. Because both c-Kit and PDGF-R are expressed in KS, the relative importance of these two targets remains unclear. However, the study is notable since it was the first to attempt to identify novel drug targets for KSHV using microarrays, and to test the efficacy of a pharmacologic agent in a disease model [176]. The same conceptual approach was used to identify two KSHV-induced cellular proteins, RDC-1 and Neuritin, with novel oncogenic properties [171]. RDC-1 was also identified as one of the most highly-induced genes in the microarray studies performed on primary DMVEC by Poole and colleagues [160]. E6/E7-DMVEC were recently used in a proteomics-based screen to identify KSHV induction of the enzyme heme oxygenase-1 (HO-1) [169]. HO-1 expression in KS tissue was confirmed by the authors and by a recent SAGE study [177]. Inhibition of HO-1-induced proliferation of infected cells by treatment with mesoporphyrin compounds may, upon further study, offer an additional treatment option for KS [169,178]

A second efficient culture system for studying KSHV biology based on the use of immortalized EC was described by Lagunoff and colleagues [158]. In this model, human neonatal DMVEC were immortalized by retroviral transduction of the telomerase reverse transcriptase subunit (hTERT) [179]. The telomerase-immortalized microvascular endothelial cells (TIME cells) retained a normal karyotype as well as many of the properties of the primary cells from which they were derived, including expression of CD31 and $\alpha V\beta 3$-integrin, LDL uptake and tubule formation in

matrigel. TIME cells grow well when serially passaged and become contact-inhibited at confluence, with the only morphologic change being a mild cell spindling.

TIME cells are infected at a high MOI with KSHV concentrated from TPA-induced BCBL-1 supernatants. Nearly all TIME cells are LANA-positive at 48 h following infection, and latency is the predominant outcome; only about 1% of infected cells express the early lytic protein ORF 59, and a smaller subset express the late structural glycoprotein K8.1. This system thus accurately reflects the state of the viral genome *in vivo* [94] and is reminiscent of what has been observed following *de novo* infection of primary and E6/E7-DMVEC [120,156]. In addition, due to the high frequency of initial infection, TIME cells provide a valuable system for studying early events in *de novo* infection. With increasing time PI, however, there is a rapid reduction in the infected TIME cell population, such that by tissue culture passage 7, less than 0.1% of cells are infected. Such loss of latently infected cells suggests inefficient maintenance of the viral episome and resembles what is seen with explanted KS spindle cells. However, because a percentage of TIME cells are lytically infected, infectious virus can be serially transferred or cultures can be maintained by addition of uninfected cells. An advantage of the TIME system is the ease with which TIME cells can be cultivated and infected with high efficiency. In addition, potential effects from oncoproteins such as E6 and E7 are not a concern in this model.

While TIME cells establish a predominantly latent infection, they can be induced to lytically reactivate by infection with an ORF50-expressing adenovirus vector [152]. Using this system, Glausinger and Ganem [180] demonstrated that lytic

KSHV infection strongly inhibits host gene expression by accelerating global mRNA turnover. Shut-off is mediated by the viral SOX (shutoff exonuclease) protein, the product of the HSV alkaline exonuclease homolog ORF37. The TIME cells were subsequently used to demonstrate that a subset of host transcripts, including IL-6 and the IL-1 type 1 receptor, escape host shut-off [180].

TIME cells express very little or no Prox-1 protein prior to KSHV infection, suggesting that the original immortalized clone was derived from blood vascular, as opposed to lymphatic, endothelium. Prox-1 is required for expression of two key markers that differentiate lymphatic from vascular endothelium, VEGFR3 and podoplanin [181]. TIME cells have thus also proved useful for investigating the hypothesis that KSHV infection drives EC to a more lymphatic phenotype, a hypothesis supported by the robust expression of lymphatic EC markers on KS spindle cells [182,183]. Carroll and colleagues [163] used RT-PCR and cDNA microarray techniques to investigate expression of genes specific to lymphatic EC following KSHV infection of TIME cells and reported significant induction of such genes including Prox-1, VEGFR3, podoplanin and LYVE-1. The microarray studies were performed after 24, 48 and 96 hrs of infection; 147 genes (about 1% of genes on the array) were significantly induced (>1.8 fold; $P < 0.001$) at all timepoints and 61 genes significantly repressed. This list comprises another valuable data set with which to examine KSHV reprogramming of the host transcriptome, particularly at early times PI and when considering the blood vascular phenotype of the uninfected controls. Array analysis performed with primary cells may reflect a mixed population of both blood and lymphatic EC, since both types can be present in primary cultures [184]. In

addition to the differential expression of lymphatic markers observed in KSHV-infected TIME cells, other interesting findings included significant upregulation of host IL-6 and significant downregulation of IL-8. Induction of IL-6 expression is consistent with the finding that IL-6 escapes host shutoff; loss of IL-8 protein expression in KSHV-infected E6/E7-DMVEC stimulated with IL-1β has also been observed [Ashlee Moses and Michael Jarvis, unpublished observations].

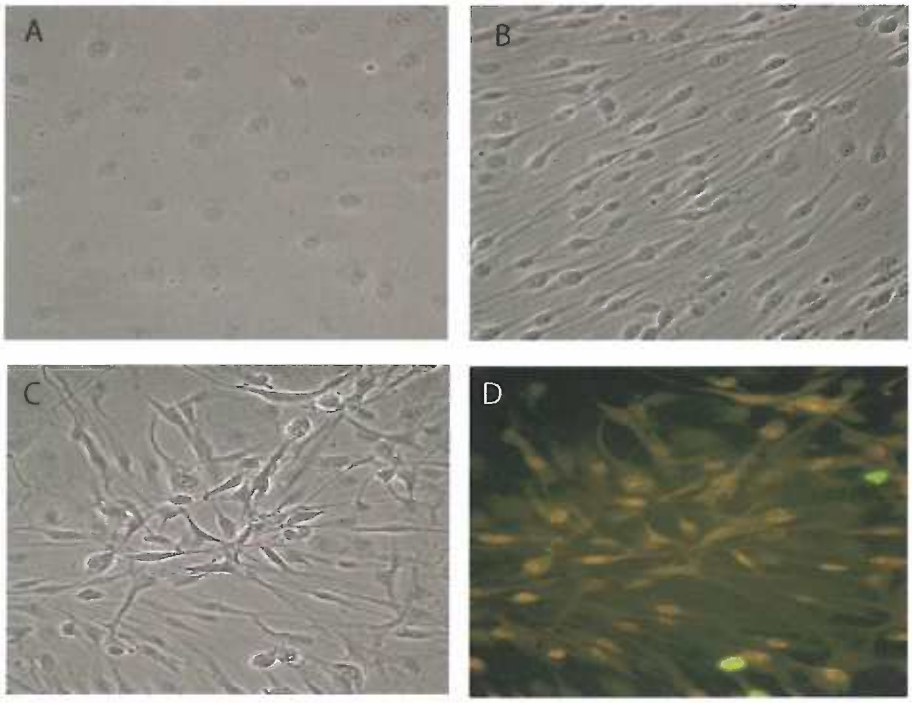
Telomerase-immortalized KSHV-infected DMVEC have also been described by Tomescu and colleagues [185]. The cells used in this study, TIME-T4 cells, were derived from a different parental cell than that of Lagunoff, *et al*, but were transduced with the same retroviral hTERT expression vector. TIME-T4 cells were infected with concentrated BCBL-1-derived virus or via co-culture with BCBL cells. The TIME-T4 cells respond differently to KSHV infection than the original TIME cells in that a dramatic spindling was observed as soon as 24 hrs PI. This may be due to an inherent difference in the immortalized cell clone or due to quantitative or qualitative differences in the inoculae used. The T4 cells demonstrated downregulation of major histocompatibility complex (MHC) class I, and the adhesion molecules ICAM-1 and CD31, within 48 hrs of infection, indicating that this system is extremely useful for the study of KSHV immune evasion mechanisms in endothelial cells.. The effective downregulation of MHC1 suggests that the T4 cells were expressing latent and IE lytic genes concurrently, as suggested by the studies of Krishnan and colleagues in primary cells [99]. TIME T4 cells should prove valuable for determining the kinetics of IE expression in EC, particularly for K3 and K5, as well as the window of time after

their down regulation for which MHC1 and adhesion molecule surface expression remains functionally compromised.

The above text contains multiple references to gene expression profiling experiments performed on KSHV-infected EC. Taking into account the differences in experimental design, including cells and virus origin, infection and culture conditions and the microarray platforms used, it is not surprising that the correlation between these data sets is modest. Genes that are commonly induced could reveal patterns that are general responses to virus infections, as well as genes so tightly linked to KSHV pathogenesis that their dysregulation transcends differences in experimental design. On the other hand, differences may reveal valid temporal changes in gene expression or culture-specific events. Analyses performed at early times PI would no doubt include host genes deregulated by those early lytic viral genes that may be transiently expressed, while those performed after many tissue culture passages (with episome maintenance) would more reflect the influences of latent gene expression, as well as the delayed effects of those early events PI. Collectively, these studies should be viewed as a valuable database from which to further analyze links between KSHV pathogenesis and cellular gene expression and function. Valuable progress has already been made in this regard.

FIG. 5. In vitro EC-based KS model used in the endothelial cell-based studies included in this dissertation. (A) Phase image of uninfected DMVEC showing the classical cobblestone appearance of normal endothelial cells. (B) Phase image of age- and passage-matched KSHV-infected DMVEC showing spindle cell morphology following viral infection. (C) Phase image of KSHV-infected DMVEC demonstrating loss of contact inhibition. Image shows approximately three layers of cells in a three dimensional focus. (D) Corresponding immunofluorescence microscopic image showing staining for the latent viral protein ORF 73/LANA (red) and the lytic viral protein ORF 59/PF-8 (green). Cells were used for experimentation at 3 weeks post-infection when >90% of cells stained positively for ORF 73. Spontaneous lytic reactivation of KSHV, as assessed by ORF 59 expression, was generally restricted to <2% of cells.

Figure 5



4. Parental lineage of spindle cells

Identification of the cellular origin of KS lesional spindle cells has been a matter of ongoing debate. Spindle cells express endothelial cell, smooth muscle cell, macrophage, fibroblast and dendritic cell markers [12,186], but are generally accepted to originate from an endothelial cell precursor. In addition to expressing markers of blood vascular endothelium, KS spindle cells express several markers specific for lymphatic endothelium, including VEGF-R3 and podoplanin [182,183,187]. These observations suggest either that KSHV preferentially infects lymphatic endothelium *in vivo*, or that KSHV infects precursor EC, lymphatic EC (LEC) or blood vascular EC (BEC) and drives the gene expression profile to a more convergent one, where lymphatic-lineage markers are induced or retained. As discussed in detail in this chapter, KSHV infects blood vascular EC *in vitro*; if however lymphatic EC were the preferred target *in vivo*, this may in part explain the inability of KSHV-infected EC to maintain the genome in tissue culture for extended periods. Wang, *et al* recently infected both LEC and BEC with KSHV and found using quantitative PCR that KSHV genomes were maintained at higher copy number in LEC [162]. This same group compared gene expression profiles of nodular KS samples and normal skin using Affymetrix U133A arrays and developed a KS expression signature of 1,482 genes by removing genes expressed at similar levels in both KS and normal dermis or epidermis. Using expression profiles similarly generated from purified LEC and BEC, the authors found that while both LEC and BEC markers were present in KS tissue, the KS expression signature was more like that of the LEC. Interestingly, infection of LEC and BEC led to a convergence of their profiles such that they were more like

each other than the uninfected counterparts. Hong and colleagues [161] compared gene profiles of primary DMVEC at day 7 PI and found significant upregulation of key lymphatic lineage-specific genes following KSHV infection including Prox1, LYVE-1, reelin, follistatin, desmplakin and leptin receptor. In addition, retroviral transduction of BEC with LANA led to induction of Prox1, the master gene responsible for lymphatic vessel development [181]. This study is in agreement with a study done in TIME cells, an immortalized BEC line, reporting KSHV induction of lymphatic-lineage markers [163].

Collectively, these studies suggest that *in vitro* KSHV induces a transcriptional drift in BEC and LEC towards a more convergent phenotype. Since commercial EC preparations contain both LEC and BEC, the relative ratios in cell preparations used in different laboratories may have some bearing on the observed outcome of infection. Regarding immortalized EC, the BEC lineage of TIME cells was recently established [163]. E6/E7-immortalized EC on the other hand appear to have a more LEC-like phenotype; genes forLYVE-1, podoplanin, VEGFR-3, leptin receptor, oncostatin M receptor, c-MAF, and reelin are all expressed [Patrick Rose and Ashlee Moses; unpublished observations]. Since KSHV genomes appear to be better maintained in LEC than BEC [162], these findings may contribute to the ability of E6/E7-DMVEC to maintain the KSHV episome for long periods of time. The relevance of the above studies to KS tumors is clearly the appreciation that lymphangiogenic molecules are involved in KS pathogenesis; if KSHV infection of EC results in a cell type with characteristics of both vascular and lymphatic EC,

understanding this unique tumor phenotype will be important for understanding the disease and developing effective clinical approaches.

6. A model of KS lesion progression

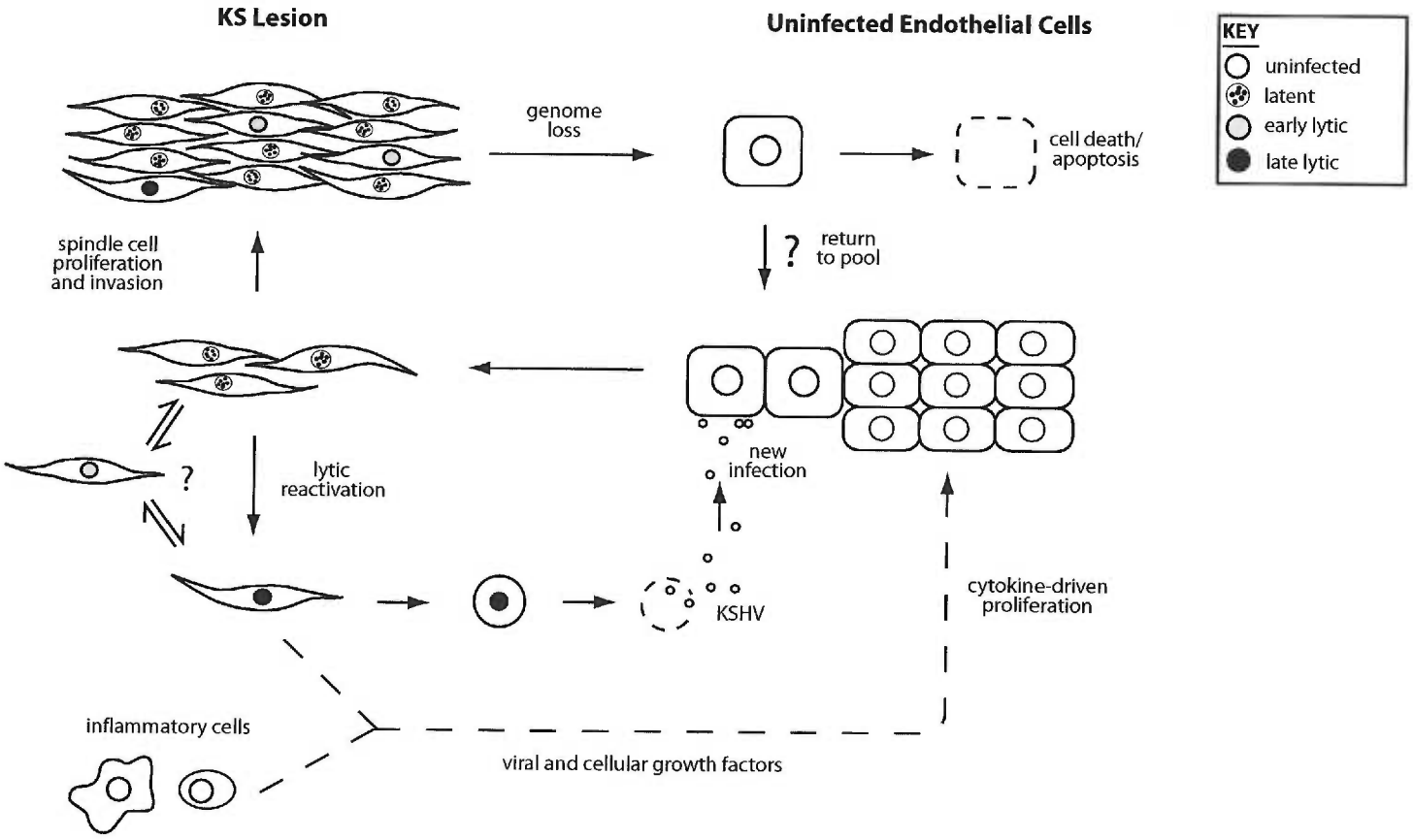
The observed frequency of immunoreactivity for KSHV markers in EC infected *in vitro* using a limited panel of antibodies is LANA >> ORF 59 >> K8.1 (i.e. latent >> early lytic >> late lytic). As described elsewhere in this chapter, these ratios have been observed in studies using both primary and immortalized EC and different viral infection protocols. These ratios also accurately reflect gene expression patterns found in KS lesions by immunohistochemistry and *in situ* hybridization [69]. Two theoretical outcomes of lytic reactivation could account for the relative excess of early lytic versus late lytic gene expression. First, if all cells activated to lytic cycle consistently support viral DNA replication with assembly and release of infectious progeny, then all early lytic-positive cells would, in time, progress to cell lysis and death. Alternately, if only a fraction of lytically activated cells produce infectious progeny, then a pool of cells must exist that express several IE and early genes without supporting productive viral replication. Evidence that anti-herpes agents such as ganciclovir and foscarnet, which inhibit lytic but not latent herpesviral infection, can improve the clinical outcome of KS strengthens the assertion that ongoing lytic gene expression is involved in KS pathogenesis [116,188,189]. If every lytic event leads to cell death, then a continual pool of new targets for *de novo* infection must be recruited; on the other hand, limited expression of IE and E genes would allow for immune evasion and anti-apoptotic mechanisms, as well for as the angiogenic and

chemoattractant properties of lytic genes, to contribute to tumor formation without a net loss of cells. The clinical responsiveness of KS to drugs that block proliferation of latently-infected cells [175] indicates that lytic reactivation is not the only mechanism driving KS lesion progression. Accumulation of latently-infected cells in KS lesions is due to proliferation and tissue invasion of latently-infected cells. The rapid loss of the KSHV genome upon culture of explanted spindle cells may reflect an insufficiency of the episomal maintenance machinery in endothelial lineage cells. It is unknown if this occurs *in vivo* when virally-infected spindle cells are stimulated to divide. Loss of the cytoprotective functions of some KSHV genes could lead to apoptotic cell death of spindle cells following cell division without episomal maintenance. Another possibility is that spindle cells that have lost the viral genome could become targets for re-infection; multiple rounds of re-infection could contribute to the accumulation of mutations leading eventually to the outgrowth of truly transformed clonal populations of spindle cells.

The lack of a well characterized animal model currently precludes the *in vivo* examination of KSHV deletion mutants, so the specific gene expression requirements for KS disease states must be inferred indirectly. The clinical responsiveness of KS to drugs that block herpesvirus lytic cycle, and to drugs that block proliferation of latently-infected cells, indicates that both of these mechanisms can drive KS lesion establishment and progression, perhaps cooperatively (figure 6).

FIG. 6. The partial clinical regression of KS lesions in response to drugs that block herpesvirus lytic cycle, as well as to drugs that block proliferation of latently-infected cells, indicates that both of these mechanisms cooperate to drive KS lesion establishment and progression. The model of KS lesion progression presented here is based on recent clinical advances in KS chemotherapy and draws together multiple theories of KS that by themselves cannot account for the complexities of KS lesions: the accumulation of spindle cells as KS lesions progress is driven by both continuous *de novo* infection and proliferation of latently infected cells with episome maintenance. Lytic reactivation results in both the production of infectious virus and also in elaboration of a paracrine signaling network that contributes to lesion progression by inducing proliferation of neighboring latently infected and uninfected cells. Loss of the KSHV genome upon multiple rounds of infected cell division may be followed by cell death or repeated rounds of reinfection; the latter may contribute to the outgrowth of truly transformed clones that have accumulated multiple mutations. There are many EC-based *in vitro* KS models used to dissect viral mechanisms of pathogenesis (see text for details). All models aim to generate age- and passage-matched infected and uninfected cultures, though the degree to which these models accurately represent the *in vivo* tumor microenvironment is a matter of rigorous debate. The model that I have used for studies in this dissertation is uniquely well-suited for studying mechanisms that contribute to proliferation of latently-infected cells.

Figure 6


KS Lesion
Uninfected Endothelial Cells
KEY
uninfected
latent
early lytic
late lytic
genome loss
cell death/ apoptosis
spindle cell proliferation and invasion
? return to pool
new infection
lytic reactivation
?
KSHV
cytokine-driven proliferation
inflammatory cells
viral and cellular growth factors

6. Control of the cell cycle

The development of cancer requires the acquisition of a set characteristics that together account for the dramatic physiological differences between normal and cancerous cells. These characteristics, which will be discussed in detail in the next section, arise from accumulating genomic changes within proliferating, pre-malignant cell populations. That cancer is rare in the average human lifespan reflects the vigor with which normal cells prevent and correct genomic changes during the course of cell division. Cell division is driven by the cell cycle, a temporally-organized sequence of molecular events that ensures fidelity of DNA replication and segregation[190]. The engines and safe-guards that produce this fidelity are discussed here; derangements of these normal processes leading to genetic mutations will be discussed in the next section.

Cyclin-dependent kinases (CDKs) drive progression through the stages of the cell cycle by controlling the onset and sequence of different cell cycle stages and coordinating these stages with cellular growth[190]. CDKs are composed of a catalytic protein kinase subunit and a cyclin subunit[191]; enzymatic activity of these heterodimers is controlled by differential phosphorylation of the kinase active site, the availability of cyclin subunits (which oscillates over the course of the cell cycle due to transcriptional and proteolytic controls governing the levels of specific cyclins), and by association with CKI inhibitors[192]. Expression of different CDKs as well as fluctuations in CDK activity initiates S phase and M phase[193]. In addition, CDK activity in early G1 leads to the phosphorylation of the retinoblastoma tumor suppressor (pRb). Unphosphorylated pRb binds and sequesters E2F transcription

factors that are necessary for S phase; phosphorylation inactivates pRb, releasing E2F transcription factors and allowing progression into S phase[194].

As stated above, the molecular events of the cell cycle ensure fidelity of DNA replication and segregation during cell division. Occasionally errors arise that, if left uncorrected, result in the propagation of genetic mutations. Under normal circumstances (i.e. in a non-cancerous cell) detection of such errors at various check-points leads to a halt in cell cycle progression to allow repair[195]. For example, signals induced by recognition of DNA structures or protein complexes indicative of DNA damage or active DNA repair prevent progression through mitosis in part by inhibiting CDK activation, thus preventing the formation of daughter cells with damage DNA sequences or inappropriately partitioned chromosomes. Another important checkpoint blocks progression through S phase when derangements in DNA replication, chromosome segregation, or cell division are sensed. This level of control is mediated by the tumor suppressor gene p53[196]. The activity of p53 blocks progression of the cell cycle, in part by feedback on pRb resulting in E2F transcription factor sequestration, to allow time for repair mechanisms to act. In the case of irreparable DNA damage, p53 induces apoptotic cell death.

7. Mechanisms of Tumorigenesis

Six characteristic alterations of cellular physiology, acquired during the proliferation of populations of pre-malignant cells, govern the transformation of normal human cells into malignant derivatives: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, angiogenesis, limitless replicative potential,

and tissue invasion and metastasis[197]. These characteristics, each representing failure of cellular anti-cancer mechanisms, result from either gain of function mutations of oncogenes or loss of function mutations of tumor suppressor genes, i.e., genomic alterations representing a failure of cell cycle fidelity.

To proliferate, normal cells require mitogenic signals arising from extracellular growth factors binding their cognate receptors on the plasma membrane and the subsequent elaboration of an intracellular signaling cascade. The greatly reduced dependence of cancer cells on exogenous growth signals can arise from alterations of the extracellular growth factor concentration, the growth factor receptor, or the intracellular signaling cascade induced by receptor ligandation. For example, platelet-derived growth factor (PDGF) upregulation in glioblastomas[198,199] and KS[175,200] establishes an autocrine signaling loop that reduces the requirements of these tumor cells for PDGF expression by other cell types. Upregulation of growth factor receptors in some cancers, such as HER2/*neu* in mammary tumors[201] or c-Kit in KS[173,175], can lead to an increase responsiveness to physiological levels of growth factor. Elevation of receptor expression can in some cases result in ligand-independent signaling, as can expression of constitutively-signaling receptor mutants[202]. The growth signals resulting from growth factor stimulation are in many cases similar to the signals resulting from the interaction of pro-growth integrins with specific components of the extracellular matrix (ECM); accordingly, alteration of the integrin expression profile is a mechanism used by some cancer cells to achieve autonomy from growth factor signaling[203,204]. Finally, structural mutations within the intracellular growth signaling circuitry can lead to mitogenic signaling in the absence

of input from upstream regulators. This common mechanism of growth factor autonomy is typified by Ras structural mutations, which are identified in approximately 25% of tumors[205].

In addition to growth stimulatory signals, normal cells within tissues are subject to anti-growth signals that induce a reversible resting state (quiescence) or irreversible terminal differentiation. Cancer cells must develop an insensitivity to such signals, which are transmitted by soluble growth inhibitors and growth inhibitors immobilized on ECM components or neighboring cells. Antiproliferative signals are funneled through pRb, which modulates the ability of a cell to progress through S phase. For example, TGFβ prevents the inactivating phosphorylation of pRb by inhibiting specific cyclin:CDK complexes[206,207]. Insensitivity to TGFβ activity can be achieved by cancer cells by downregulation of the TGFβ receptor, expression of mutant receptors, or by alterations of the intracellular signaling cascade elicited by receptor ligandation[208,209]. Interference with pRb directly, such as sequestration by viral oncogenes like HPV E7, can also reduce sensitivity to antigrowth signals[210].

The apoptotic pathway of programmed cell death is triggered by many of the alterations of physiology manifest in pre-malignant cells; insensitivity to apoptotic signals, therefore, is a hallmark of cancer development. Apoptosis can be induced or prevented by the exposure of cells to exogenous death or survival factors, respectively. TNFα and FAS activate the pro-apoptotic "gatekeeper" caspase 8[211], whereas IGF-1/2 and IL-3 signal through the pro-survival PI3 kinase-AKT/PKB pathway[212,213]. The intracellular environment is also monitored and signals such as those resulting from DNA damage or oncogenic overexpression can elicit an

apoptotic response. Irreparable DNA damage activates the p53 pro-apototic pathway, resulting in cytochrome C release by cellular mitochondria and subsequent activation of caspase 9. Unrestricted signaling through the PI3 kinase-AKT/PKB pathway or loss of p53 represent the most common mechanisms of apoptosis resistance that develops in cancer. Mutations of the p53 locus is seen in more than 50% of human cancers[214].

The acquisition of mutations that favor proliferation as well as mutations that impart resistance to apoptosis would seem to be sufficient for cancer cells to develop macroscopic tumors; however, human cells are restricted by an upper limit of replicative potential. In culture, many types of primary human cells can be passed 50 to 70 times before reaching senescence[215]. Senescence can be overcome by inactivation of both pRb and p53, whereafter cells can be passed additional times. However, eventually a second state is reached called crisis in which massive cell death and karyotypic disarray occurs. From crisis may emerge a rare (1 in 10^7) cell clone that is truly immortalized[216]. Immortalization represents a clone's ability to maintain the length of telomeric DNA above a critical threshold. In 85-90% of cancers this is accomplished by overexpression of the human telomerase gene, which maintains telomeric length by the addition of hexanucleotide repeats onto the ends of telomeric DNA[217].

A further hindrance to rigorous expansion of pre-malignant cells is the ongoing nutrient and soluble gas requirements. During organogenesis, the coordinated growth of vascular structures and parenchyma is controlled such that cell in a fully-formed tissue are within 100 μm of a capillary blood supply. Once

organogenesis is complete, however, angiogenesis is a tightly controlled and transient event due to the intrinsic limitation of cells to stimulate angiogenesis. Therefore, a tumor must overcome this barrier and stimulate angiogenesis so that proliferating tumor cells can form macroscopic tumors. Induction of pro-angiogenic soluble factors, notably VEGF and FGF-1/2[218], and downregulation of the anti-angiogenic CD36 (thrombospondin-1 receptor) [219] are two mechanisms by which tumor cells have been shown to induce angiogenesis. Additionally, alteration of the integrin expression profile has angiogenic consequences in addition to the effects on proliferation, and pro-angiogenic integrins are expressed by a number of tumors[203].

Cancer morbidity is attributable to metastasis in approximately 90% of cases[220]. The molecular processes underlying tissue invasion and metastasis involve the enzymatic activity of extracellular proteases and alterations in the coupling of cells to components of the microenvironment. Modulation of extracellular protease activity, necessary for the angiogenic activities as well as invasion, produces degraded stromal components, thus altering the binding requirements of cells within a developing tumor. Alterations in the integrin expression profile accommodate changing binding specificities as invading cells are in transit as well as at final sites of colonization[203]. Another mechanisms increasing the invasiveness of cancer cells is the alteration of cellular adhesion molecules such as E cadheren. E cadheren forms homotypic intercellular dimers that induce antigrowth signals via βcatenin; oblation of this signaling pathway is associated with the majority of epithelial cancers[221]. The extracellular protease activity and altered binding characteristics of cancer cells are essential for invasion and metastasis; however, the molecular machinery and

regulatory mechanisms remain the least well understood of the acquired oncogenic characteristics.

8. Epstein-Barr virus: the prototypical γ-herpesvirus

EBV (HHV4), the prototypical gammaherpesvirus, was discovered in 1964 [222] and sequenced in 1982 [223]. EBV establishes latency in lymphoid organs. EBV has the notable capacity to induce proliferation and transformation of host cells and is accordingly associated with proliferative disorders, including Burkitt's lymphoma (BL) and Hodgkin's disease as well as the epithelial tumor nasopharyngeal carcinoma (NPC)[224]. EBV infection is ubiquitous around the world but the distribution of EBV-associated neoplasms exhibit geographic restriction. EBV-associated BL is most frequently encountered in Africa, whereas NPC is common in Asia. Europe and the United states, in contrast, have relatively low levels of EBV-associated BL and NPC; rather, infectious mononucleosis is the predominant manifestation of EBV infection in industrialized nations. Numerous contributory factors have been proposed including variations in the age of EBV infection. EBV is acquired very early in childhood in Africa, slightly later childhood in Asia, and during adolescence in industrialized countries.

As in KSHV infection, where latency predominates in tumor tissues, EBV-induced tumors are primarily latently-infected. EBV is maintained as a circular episome anchored to the host cell nuclear membrane and is replicated during S phase of the cell cycle. KSHV latency in lymphocyte and endothelial lineage cells exhibit distinctions in viral gene expression (i.e. differential LANA2 expression). EBV

exhibits similar variation in latency gene expression profiles. The first of the three EBV latency programs is the program that dominates in EBV-mediated BL. Type I latency is the most restricted expression program and is characterized by the expression of EBV nuclear antigen-1 (EBNA-1) and the abundant small nuclear RNAs (EBERs). EBNA-1 is a sequence-specific DNA binding protein that binds the origin of replication (*oriP*) and is required for replication by host DNA polymerase. Transgenic mice expressing EBNA-1 have increased incidence of lymphomas, suggesting that EBNA-1 contributes to EBV-oncogenesis [225]. EBNA-1 is also expressed during productive infection. Interestingly, EBNA-1 does not induce an effective CTL response; this is due to alterations of proteosomal processing, preventing presentation of EBNA-1 by MHC molecules[226]. EBERs, transcribed by RNA polymerase III, are the most abundant viral RNA expressed in infected cells (approximately 10^6 copies/cell)[227]. EBERs are expressed in all EBV-associated neoplasms but are not necessary for transformation. EBERs may have a role in maintenance of latency as the expression decreases dramatically during productive infection[228].

In addition to EBNA-1 and EBERs, type II latency is associated with expression of the latent membrane protein-1 (LMP-1) and LMP-2. LMP-1 is a transmembrane protein that is essential for transformation of B cells *in vitro*. LMP-1 has been shown to contribute to transformation at many levels, including induction of anti-apoptotic proteins, p53 interference, increased invasiveness associated with E-cadherin downregulation, and to decreasing the dependency on exogenous EGF by upregulation of the EGF-receptor[229]. LMP-1 was recently shown to upregulate MMP-

1, a possible mechanism contributing to NPC pathogenesis[230]. LMP-2 is expressed as two splice variants, LMP-2A and B. LMP-2 is also an integral membrane protein and interferes with signaling via the immunoglobulin receptor, thus blocking B cell activation and EBV reactivation [231]. Type II latency is the program that predominates in NPC where the predominant lesional cell type is epithelial in origin. Transformation of epithelial cells by EBV was initially difficult to demonstrate. Transfection of epithelial cells with CD21, the EBV receptor, facilitated infection but the established infection was unstable and quickly lost[232]. Stably-infected clones could be established that retained the viral genome and expressed EBNA-1 and LMP-1; these cells appeared to be impaired in differentiation and were unable to reactivate a productive infection suggesting that alterations of cellular differentiation influence EBV latency and transformation.

Type III latency is the least restricted latency program, which includes expression of type II genes as well as EBNA-2, EBNA-3, and LP, which are involved in establishment of latency. In addition, EBNA-2 activates the promoter of LMP-1, thereby participating indirectly in the transformative functions of EBV. Type III is associated with infectious mononucleosis. Interestingly, lymphocytic cell lines exhibiting type III latency are readily established from biopsies of EBV-induced neoplasms.

Like KSHV, some of the lytic proteins of EBV have been shown to exhibit proliferative or anti-apoptotic functions. The immediate early protein ZEBRA, for example, is a homolog of the KSHV gene K8 and inhibits p53-mediated transcription. The early gene *BHRF1* is a homolog of the anti-apoptotic *bcl2* (also a homolog of

KSHV ORF 16), can block apoptosis induced by various agents [233]. *BARF1*, another early gene, has been shown to transform rodent fibroblasts [234]. Like KSHV lytic genes, the EBV genes associated with productive infection are expressed in a minority of cells within virus-induced tumors; therefore, the contribution of these genes to EBV-induced oncogenesis remains unclear.

9. Overview of work presented in this dissertation

Like the closely related herpesvirus EBV, the seroprevalence of KSHV greatly exceeds the incidence of KSHV-related diseases, even in populations known to be at increased risk for these diseases. This may be reflected by findings using *in vitro* systems that have shown that KSHV does not readily transform endothelial cells, the likely cell of origin of the KS spindle cell. However, clonal KS tumors have been described, indicating that transformation, though rare, can occur as part of KS progression. The multistep model of tumorigenesis describes six characteristics of tumor cells that must be attained in order for a tumor to develop beyond a small, localized collection of pre-malignant, non-clonal cells. That KSHV is not sufficient to cause KS indicates that other, non-KSHV-driven (but possibly KSHV-exacerbated) processes are required for disease development; however, KS has not been described in KSHV-negative individuals. Clearly, elucidation of KSHV-mediated changes in host cell physiology that lead to KS development is essential for our understanding of this disease process and for development of better therapeutic regimens.

My central hypothesis in this dissertation is that KSHV infection of endothelial cells (the precursors of KS spindle cells) contributes to KS development

by inducing or accelerating acquisition of key tumor cell characteristics, as well as by making the tumor cell uniquely suited to the KS microenvironment, and that these changes come about in part through viral reprogramming of the infected cell. To test this hypothesis, I started with large-scale gene expression profiling studies, using both microarray and proteomics methods, to evaluate changes in DMVEC gene expression following KSHV infection. While microarray procedures had been previously employed by collaborators and colleagues, I was the first to evaluate KSHV-associated changes in DMVEC protein profiles. Functional clustering of genes identified in these screens revealed that numerous cellular pathways were modified following KSHV infection, including those that regulate apoptosis, proteolysis, and proliferation. I next used this information to develop and test several sub-hypothesis to help elucidate the link between KSHV infection and KS lesion development.

It is well known that endothelial cells retain the ability to proliferate and invade the extracellular matrix in response to growth or injury stimuli, but these processes are typically tightly regulated. Because spindle cells within KS lesions are known to proliferate and invade surrounding tissues, I hypothesized that KSHV contributes to KS lesion progression by deregulating the expression of genes involved in cell proliferation and invasion. In this dissertation, I have specifically shown that KSHV-infection upregulates HO-1 in DMVEC resulting in a proliferative advantage over uninfected cells when cultures are exposed to free heme, which is known to be present within the KS lesional microenvironment. In this way, the infected cell is able to thrive in a high-heme environment that could otherwise be toxic. Further, I have shown that the degradative enzyme MMP-1 is upregulated in DMVEC following

KSHV-infection, which may provide infected cells with a proliferative advantage over uninfected cells by reducing growth-restricting cell-cell and cell-matrix contacts, as well as increasing the invasive potential of infected cells. These two cellular genes may, therefore, represent possible targets for KS chemotherapy. Further, I hypothesized that KSHV lytic replication is closely associated with the cell cycle, and went on to shown that KSHV lytic reactivation was most efficient during S phase of the cell cycle. This novel finding contributes to our understanding of the control of KSHV lytic cycle induction and should help to further elucidate the contributions of KSHV lytic genes to KS pathophysiology.

Chapter Two

KSHV Induces Heme Oxygenase-1 in Infected Endothelial Cells

Citation:

Shane C. McAllister, Scott G. Hansen, Rebecca A. Ruhl, Camilo M. Raggo, Victor R. DeFilippis, Deborah Greenspan, Klaus Früh, and Ashlee V. Moses. 2004. Kaposi sarcoma-associated herpesvirus (KSHV) induces heme oxygenase-1 expression and activity in KSVH-infected endothelial cells. Blood 103 (9):3465-3473.

Coauthor contributions:

I developed the hypothesis regarding elevated levels of HO-1 and KS pathophysiology, designed and carried out of the experimental approaches, and prepared the original and revised versions of manuscript, tables, and figures. Coauthors contributed the following: S. G. Hansen assisted with Western blotting, R. A. Ruhl with tissue culture, C. M. Raggo and V. R. DeFilippis with quantitative RT-PCR (taqman); D. Greenspan provided oral AIDS-KS biopsy material that was used to demonstrate elevated HO-1 protein in lesional tissue; K. Fruh and my graduate mentor A. V. Moses acted as advisors to me during the development and presentation of this project.

ABSTRACT

Kaposi's sarcoma (KS) is the most common AIDS-associated malignancy and is characterized by angiogenesis and the presence of spindle cells. Kaposi's sarcoma-associated herpesvirus (KSHV) is consistently associated with all clinical forms of KS and *in vitro* infection of dermal microvascular endothelial cells (DMVEC) with KSHV recapitulates many of the features of KS including transformation, spindle cell proliferation and angiogenesis. To study the molecular mechanisms of KSHV pathogenesis, we compared the protein expression profiles of KSHV-infected and uninfected DMVEC. This comparison revealed that Heme Oxygenase-1 (HO-1), the inducible enzyme responsible for the rate-limiting step in heme catabolism, was upregulated in infected endothelial cells. Recent evidence suggests that the products of heme catabolism have important roles in endothelial cell biology including apoptosis and angiogenesis. Here we show that HO-1 mRNA and protein are upregulated in KSHV-infected cultures. Comparison of oral and cutaneous AIDS-KS tissues with normal tissues revealed that HO-1 mRNA and protein were also upregulated *in vivo*. Increased HO-1 enzymatic activity *in vitro* enhanced proliferation of KSHV-infected DMVEC in the presence of free heme. Treatment with the HO-1 inhibitor chromium mesoporphyrin IX abolished heme-induced proliferation. These data suggest that HO-1 is a potential therapeutic target for KS that warrants further study.

1. Introduction

Kaposi's Sarcoma-associated herpesvirus (KSHV; also human herpesvirus-8) is implicated in all clinical forms of KS [47,52] as well as the lymphoproliferative disorders primary effusion lymphoma (PEL) [53] and multicentric Castleman's disease (MCD) [55]. Endothelial cells harbor the KSHV genome *in vivo* [52,94,153], are permissive for virus infection *in vitro* [120,156-158], and are thought to be the precursors of spindle cells [6-9]. We and others have shown that *in vitro* infection of human dermal microvascular endothelial cells (DMVEC) with KSHV induces a spindle cell morphology and characteristics of a transformed phenotype including loss of contact inhibition and growth in soft agar [120,156-158]. Because explanted KS cells fail to maintain the KSHV genome following serial passage [10,155], *in vitro* infection of DMVEC has proved an invaluable tool for the study of KSHV pathogenic mechanisms [170,173].

KSHV, like other herpesviruses, has a substantial coding capacity which includes viral genes unique to this human pathogen, genes which share homology with other members of the *herpesviridae*, as well as genes which appear to have originated in the host genome [58,235]. Of note, at least three of these genes products, vFLIP, vCYC, and LANA are believed to comprise the viral latency expression program and are consistently expressed in all virally infected cells in KS, PEL, and MCD [65-67]. The gene product of ORF 71, vFLIP (viral FLICE-inhibitory protein) is thought to play an important role in prevention of apoptotic cell death [74]. The activity of a homolog of cellular cyclin D, vCYC (ORF 72) [77], together with the modulation of cellular transcription wrought by the latency-associated nuclear antigen (LANA, ORF 73) [85], is thought to be involved in KSHV-induced host cell transformation.

Additionally, LANA is indispensable for maintenance of the viral genome [86]. Other gene products, such as vGCR [236,237] and vIL-6 [238], are classified as lytic gene products and are expressed in only a minority of cells *in vivo* [95-97]. Although reactivation of KSHV from the latent state and subsequent completion of the viral lytic expression cascade is incompatible with survival of a host cell, lytic gene products are thought to have paracrine influences on both latently-infected and uninfected cells and are thus considered vital for lesion development [239].

We have used our previously described *in vitro* KS model to dissect viral mechanisms of pathogenesis [170,173]. The salient features of our model include recapitulation of KS cell physiology including spindle cell formation, loss of contact inhibition and anchorage-dependent growth restriction; long term propagation of predominantly latently-infected cells; and the ability to generate age- and passage-matched KSHV-infected and uninfected cultures [120]. This system has been amenable to gene expression profiling by cDNA microarrays and has provided valuable information in this regard [170,173]. In the present study, we employed a proteomics expression profiling approach to study alterations in host cell gene expression following *in vitro* infection of DMVEC with PEL-derived KSHV. Out of approximately 850 gene products screened by western blotting, 52 genes exhibited significant up- or down-regulation in infected cells compared with uninfected controls. These altered genes have been implicated in diverse cellular processes including apoptosis, cell division, metabolism, morphology, transcription and tumorigenesis. One metabolic gene which showed significant upregulation in KSHV-infected cells was heme oxygenase-1 (HO-1). Given recent evidence implicating HO-

1 enzymatic activity in anti-apoptotic responses [240,241], cell proliferation [242,243], and angiogenesis [242-245], we reasoned that upregulation of this gene by KSHV-infection may have important consequences for endothelial cell biology and KS pathophysiology.

Heme Oxygenases (HO) are responsible for the oxidative cleavage of the heme ring, the rate limiting step in heme catabolism [246]. Enzymatic degradation of heme releases carbon monoxide (CO), free iron, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase [247]. To date, three isoforms have been identified: the stress-inducible HO-1 (HSP32) and the constitutive HO-2 and HO-3. These isozymes, products of distinct genes, differ in specific activity, inducibility, and tissue distribution. HO-2, an essentially uninducible isozyme, is present in high concentrations in tissues such as brain and testis [248]. HO-3, also uninducible, is distinguished by a lesser degree of homology to the other isoforms and a markedly reduced specific activity; this isozyme is thought to function in heme transport within cells rather than heme catabolism [249,250]. HO-1 is ubiquitously distributed in mammalian tissues and is strongly and rapidly upregulated by noxious stimuli leading to oxidative stress such as transitional metals, glutathione-depleting agents, UV light and heat shock [248]. Heme, the natural substrate of HO-1, is itself a potent inducer of HO-1 [248].

In the present study we show that HO-1 mRNA and protein are upregulated by KSHV infection of DMVEC *in vitro* as well as in KS biopsy tissue. Upregulation of HO-1 enzymatic activity *in vitro* conferred a proliferative advantage to infected cells suggesting that this enzyme may have an important role in KS pathophysiology.

2. Materials and Methods:

a. *Reagents*

Ethanolamine, pefabloc SC, leupeptin, glucose-6-dehydrogenase (type XV from baker's yeast), glucose-6-phosphate, and $NADP^+$ were from Sigma (St. Louis, MO); pooled rat liver cytosol was from Cedra Corporation (Austin, TX); human skin total RNA was from Stratagene (La Jolla, CA); Cu(II) mesoporphyrin IX (CuMP), Cr(III) mesoporphyrin IX (CrMP), and heme were from Porphyrin Products, Inc. (Logan, UT). Stock solutions of methemalbumin (1.5 mM heme and 0.15 mM BSA), CuMP, and CrMP (1.0 mM) were prepared as described previously [251]. Briefly, heme, CuMP, or CrMP was dissolved in 0.5 ml 10% (w/v) ethanolamine in deionized water. BSA was added to the heme solution in 2 ml deionized water. The volume was raised to 7 ml and adjusted to pH 7.4 with 1 N HCl and rapid stirring. The final volume was adjusted to 10 ml with deionized water. Heme and mesoporphyrin stock solutions were prepared in the dark and stored at –20°C for up to one month.

b. *Derivation of KSHV-infected DMVEC*

In vitro infection of DMVEC with KSHV was previously described in detail [120]. Briefly, primary DMVEC (BioWhittaker, Walkersville, MD) were immortalized by retroviral-mediated expression of HPV type 16 E6 and E7 genes and were subsequently infected with KSHV derived from tetradecanoyl phorbol acetate-treated BCBL-1 cell culture supernatants. DMVEC were cultured in endothelial-SFM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% human male AB serum

(HS; Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), endothelial cell growth supplement (50 μg/ml; Becton Dickinson, Bedford, MA), and G418 (200 μg/ml; GIBCO BRL). The BCBL-1 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health (contributed by Michael McGrath and Don Ganem) and was cultured in RPMI supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 2-mercaptoethanol (5×10^{-5} M). KSHV infection of DMVEC was verified by PCR amplification of the KS330 *Bam*HI fragment of the ORF 26 gene, reverse transcription (RT)-PCR amplification of the spliced mRNA from the ORF 29 gene and immunostaining for KSHV proteins. DMVEC were used for experimentation when >90% of cells expressed ORF 73 (LANA). Typically, 2% of infected cells in such LANA-positive cultures expressed the early lytic protein ORF 59 and <1% of ORF 59-positive cells expressed the late lytic glycoprotein ORF K8.1. Viral antigen expression was detected by immunofluorescent staining as previously described using antibodies generated and generously provided by Bala Chandran (The University of Kansas Medical Center, Kansas City, KA). For infection of primary DMVEC the recombinant EGFP-expressing clone rKSHV.152 [112] was used.

c. *BD PowerBlot*

The western array screening service offered by BD Transduction Laboratories (Franklin Lakes, NJ) measures changes in protein expression by simultaneously probing control and experimental cell protein extracts with a panel of more than 850 monoclonal antibodies. KSHV-infected and uninfected DMVEC cultures were harvested according to BD Transduction Laboratories specifications. Monolayers in T75 flasks were washed with PBS and lysed in 1 ml boiling lysis solution per flask (10 mM Tris, pH 7.4, 1 mM sodium ortho-vanadate, and 1% SDS). Lysates were scraped and transferred to 50 ml conical polypropylene tubes and subsequently heated in a microwave for 10 s uncapped. Cellular DNA was then sheared by passing lysates ten times through a 25-gauge blunt needle. A small volume was removed and diluted 1:10 to reduce the SDS concentration to 0.1% and protein concentration was determined using the Bradford's reagent according to manufacturer's instructions (Bio-Rad, Hercules, CA) with BSA as a standard. Samples were then shipped to BD Transduction Laboratories on dry ice where PowerBlot screening was conducted. Gradient SDS-PAGE (4-15%, 0.5 mm thick [Bio-Rad Criterion IPG well comb]) gels were loaded with 200 μg of protein in one continuous well across the entire width of the get. This yields the equivalent of 10 μg protein per lane on a standard 10 well mini-gel. Gels were run 1.5 h at 150 V. The gels were transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 2 h at 200 mAmp using a wet electrophoretic transfer TE Series apparatus (Hoefer Scientific Instruments, San Francisco, CA). Following transfer, membranes were air dried and subsequently rewet in methanol and blocked for 1 h in blocking buffer (LI-COR, Lincoln, NE).

Blocked membranes were then clamped into western blotting manifolds that isolate 40 channels across each membrane. Each channel was then loaded with a complex antibody cocktail and incubated for 1 h at 37°C. Blots were removed from manifolds, washed and hybridized for 30 minutes at 37°C with secondary goat anti-mouse conjugated to Alexa680 fluorescent dye (Molecular Probes, Eugene, OR). Membranes were washed, dried, and scanned using the Odyssey Infrared Imaging System (LI-COR). Samples were run in triplicate and fold change was reported following analysis of blots using a 3x3 comparison method.

d. ***Western blotting***

Uninfected and KSHV-infected cells were harvested by trypsanization and pelleted by centrifugation at 15,000 rpm for 30 min at 4°C and then resuspended in 100 μl of 1 x phosphate buffered saline. The resuspended pellet was frozen and then thawed three times to break open the cells. The total cell extract was then centrifuged at 2000 rpm to spin out excess cellular debris. Total protein was measured with a Bradford assay kit (Biorad). Samples for immunoblotting were prepared by taking 20 μg of total protein and combining with 3 x reducing sample buffer to make 30 μl total volume. The samples were boiled for 3 min and then centrifuged at 15,000 rpm for 1 min to pellet insoluble material. Each sample (15 μl) was loaded on two identical 12% polyacrylamide gels and resolved by discontinuous electrophoresis (SDS-PAGE) as previously described [252]. After electrophoresis, the gels were transferred to nitrocellulose and then subjected to immunoblot analysis using anti-heme oxygenase-1 (Transduction Laboratories, catalogue #610712) and anti-SAPK/JNK (Cell

Signaling Technologies) antibodies as per the manufacturers' recommendations. The antigen-antibody complex was then incubated with anti-mouse Ig horseradish peroxidase and anti-rabbit Ig horseradish peroxidase (Amersham Life Sciences). The blot was developed by incubation with chemiluminescent substrate (Pierce) and exposed to Kodak BioMAX MR film.

e. ***Immunofluorescent staining***

For detection of HO-1 protein, E6/E7 transformed DMVEC monolayers were rinsed two times with phosphate-buffered saline containing 1% normal goat serum and 0.02% sodium azide (staining buffer), fixed in 2% paraformaldehyde, permeabilized with 0.05% triton and stained with an anti-HO-1 monoclonal antibody (clone 23, BD Transduction Laboratories) and an anti-LANA rabbit polyclonal antibody followed by a goat anti-mouse FITC-labeled (Biosource International, Camarillo, CA) and goat anti-rabbit Alexa-labeled (Molecular Probes, Eugene, OR) secondary antibodies. For detection of HO-1 in non-transformed cell, primary DMVEC were infected at a low multiplicity of infection with the EGFP expressing KSHV clone. Monolayers were fixed, permeabilized and stained for HO-1 as above. All antibodies were used at a 1:100 dilution in staining buffer and incubated with cell monolayers for 60 min at 37°C. Primary antibodies were omitted from duplicate monolayers to control for nonspecific binding of secondary antibody. Stained cells were mounted and examined on a Zeiss fluorescent microscope.

f. ***Quantitative RT-PCR***

RNA was isolated using an RNeasy Total RNA kit (QIAGEN, Inc.). RNA samples were treated with RNase-free DNase I to remove any residual genomic DNA contamination (Ambion, Austin, TX). Quantification of RNA was performed by a two-step method. First, cDNA was synthesized using superscript II (Invitrogen). Synthesized cDNA was diluted in H_2O to a final concentration of 100 ng per reaction in TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed on an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) under standard reaction conditions. Relative quantitation of gene expression, as outlined in the Applied Biosystems User Bulletin 2, was conducted to compare uninfected and KSHV-infected samples. Following primer efficiency validation on RNA from KSHV-infected DMVEC, the comparative cycle threshold (C_T) method was performed as outlined in user bulletin 2. The comparative C_T method compares differences in the threshold cycles between samples after normalization to an endogenous control, in this case GAPDH. C_T values vary with target RNA concentration such that a higher target concentration yields an earlier threshold signal over background. Differences between samples during the exponential phase of PCR amplification were calculated by the following equation: $\Delta\Delta C_T = (C_{T\,(HO\text{-}1)} - C_{T\,(GAPDH)})\text{infected} - (C_{T\,(HO\text{-}1)} - C_{T\,(GAPDH)})\text{mock}$ and converted to *n*-fold change unites by the equation $2^{-\Delta\Delta CT}$. The following primers were generated using primer express v1.1 (ABI): HO-1 forward, 5'-GCCCTTCAGCATCCTCAGTTC-3', and reverse, 5'-GGTTTGAGACAGCTGCCACA-3', GAPDH forward, 5'-

GAAGGTGAAGGTCGGAGT-3', and reverse, 5'-GAAGATGGTGATGGGATTTC-3'.

g. ***Immunohistochemistry***

Paraffin embedded biopsy tissue from oral AIDS-KS lesions were sectioned and mounted on charged slides. The slides were deparaffinized, treated in citrate buffer (30 min, steamed), transferred to TBS/Tween Buffer, and placed on an automatic staining machine programmed for staining using the Vectastain Elite ABC kit with diaminobenzidine as the substrate (Vector Laboratories, Burlingame, CA). Polyclonal anti-HO-1 (Affinity Bioreagents, Golden, CO) was used at 1:40. Slides were counterstained with hematoxylin/eosin.

h. ***HO enzymatic activity***

Crude endothelial cell protein extracts were prepared as previously described [253]. Briefly, following overnight incubation in complete media alone or with 10 μM CuMP or CrMP, monolayers were rinsed with PBS and scraped directly into 300 μl sonication buffer (0.25 M sucrose, 20 mM Tris-HCl, 50 μg/ml Pefabloc SC, 4 μg/ml leupeptin; pH 7.4) sonicated on ice two times for 30 s, and centrifuged for 20 min at 18,000 g. The protein concentration of the resultant supernatant was determined using Bradford's reagent as described above. HO activity was measured by the spectrophotometric determination of bilirubin production as described [253]. Final reaction concentrations were 1 mg endothelial cell protein extract, 50 μM heme, 2 mg/ml pooled rat liver cytosol, 1 mM $MgCl_2$, 3 units glucose-6-dehydrogenase, 1 mM

glucose-6-phosphate, and 2 mM $NADP^+$ in 0.5 ml 0.1 M potassium phosphate buffer, pH 7.4, for 30 min at 37°C. The reaction was stopped by the addition of two volumes of chloroform. Bilirubin concentration in the chloroform extracts was determined using an Ultrospec 2100 Pro spectrophotometer (Amersham Parmacia Biotech, Piscataway, NJ) to measure the difference in optical density at 464 and 530 nm and the equation $A = c\ L\ \varepsilon$ assuming an extinction coefficient (ε) of 40 $mM^{-1}cm^{-1}$ for bilirubin in chloroform with a path length (L) of 1.0 cm. HO activity was reported as picomoles of bilirubin produced per milligram endothelial cell protein extract per hour.

i. ***Assessment of heme-induced proliferation***

The effect of low-dose heme treatment on proliferation of KSHV-infected and uninfected DMVEC was assessed by direct cell counting. Cells were plated in 96-well plates at 1.25×10^4 cells per well and incubated overnight in complete medium. The next morning cells were refed with serum-free medium (SFM) in the presence or absence of CuMP or CrMP and incubated for 24 h. The next morning (t = 0) cells were refed with SFM containing CuMP or CrMP in the presence or absence of 5 μM heme and incubated for an additional 48 h (t = 48). Quadruplicate wells were trypsanized and counted by hemocytometer at t = 0 and 48. Trypan blue exclusion showed that cell viability was >95% for all treatment conditions. To ensure that the ability of these cells to proliferate in this experimental setting was not non-specifically limited, a separate experiment was conducted whereby the proliferative response of serum starved cells was assessed following a 48 h incubation in SFM

containing 10% human serum in the presence or absence of endothelial cell growth supplement (50 μg/ml), a complex mixture containing known endothelial cell mitogens including VEGF and bFGF.

3. Results

a. *Western blot screening of KSHV-infected DMVEC*

To screen changes in gene expression following experimental infection of DMVEC with KSHV, we raised age- and passage-matched cultures of KSHV-infected and uninfected cells (figure 1) as previously described [120]. Uninfected DMVEC retained the classical cobblestone morphology of normal endothelial cells and remained contact inhibited after serial passage (figure 1a). KSHV-infected cultures, however, assumed a spindle cell morphology reminiscent of the neoplastic endothelial cell-derived spindle cells in KS lesions (figure 1b). Cultures were harvested for expression profiling once infected cultures had become >90% spindled and ORF 73 positive (figure 1c and d). Alterations in gene expression resulting from KSHV infection are most likely due to latent infection as staining for the lytic marker ORF 59 revealed <2% spontaneous lytic reactivation. We note, however, that a paracrine influence conferred by even sporadic reactivation could also effect gene expression [239]. Total protein extracts were prepared according to BD Transduction Laboratories specifications and analyzed by the PowerBlot method as described in the Methods section.

Out of approximately 850 gene products screened by PowerBlot western array screening, 52 genes exhibited significant up- or down-regulation in infected cells as compared with uninfected controls. Functional clustering of the data revealed several potentially important alterations in host cell physiology caused by KSHV infection (table 1). Genes involved in cellular processes including apoptosis, cell adhesion, morphology, proliferation, DNA damage repair, metabolism, protein sorting, transcription, and tumorigenesis all showed significant changes in expression levels. We chose to focus further efforts on HO-1, a gene with potentially interesting and important implications in KS pathophysiology.

FIG. 1. Derivation of uninfected and KSHV-infected DMVEC. (A) Phase image of uninfected DMVEC showing the classical cobblestone appearance of normal endothelial cells. (B) Phase image of age- and passage-matched KSHV-infected DMVEC showing spindle cell morphology following viral infection. (C) Phase image of KSHV-infected DMVEC demonstrating loss of contact inhibition. Image shows approximately three layers of cells in a three dimensional focus. (D) Corresponding immunofluorescence microscopic image showing staining for the latent viral protein ORF 73/LANA (red) and the lytic viral protein ORF 59/PF-8 (green). Cells were used for experimentation at 3 weeks post-infection when >90% of cells stained positively for ORF 73. Spontaneous lytic reactivation of KSHV, as assessed by ORF 59 expression, was generally restricted to <2% of cells.

Figure 1

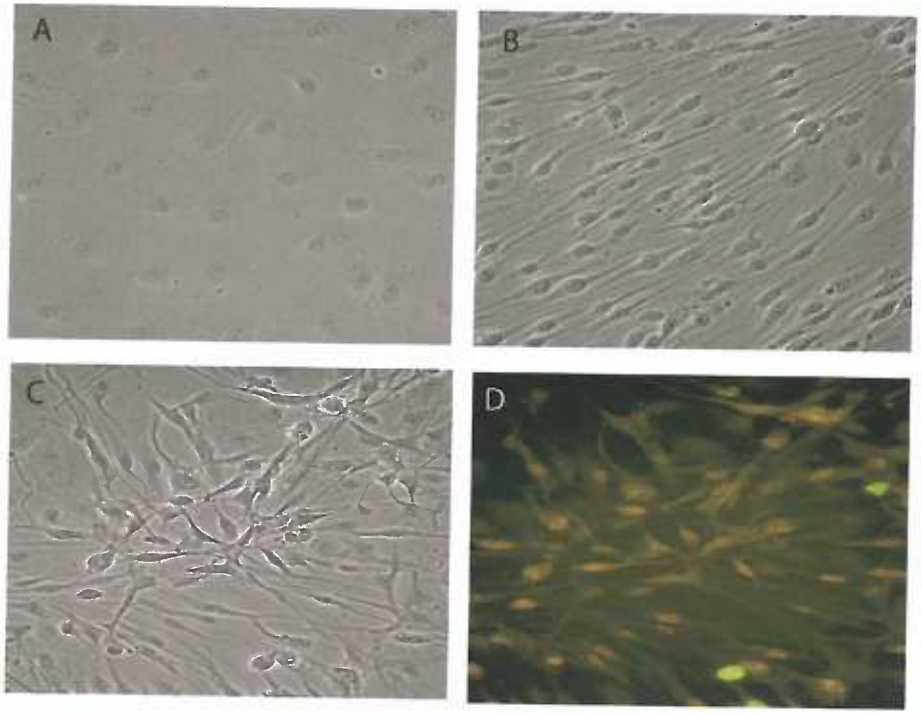


TABLE 1. Genes modulated in DMVEC after KSHV-infection[a]

UG Cluster	Gene ID	Symbol	PowerBlot Value	Name	Function	Group[b]
Hs.82395	A40036	Fas	8.8	CD 95	Apoptosis	A
Hs.89862	Q15628	TRADD	**-1.5**	TNFR1-associated death domain protein	Apoptosis	A
Hs.381231	NP_001219	CASP8	**absent**[c]	Caspase 8	Apoptosis	A
Hs.287797	P05556	ITGB1	1.8	Integrin beta 1	Cell adhesion	CA
Hs.216381	A45973	Nexilin	**-4.2**	Nexilin	Cell adhesion	CA
Hs.119537	A38219	KHDRBS1	3.0	Src-associated in mitosis, 68 kDa	Mitosis	CD
Hs.3849	AB045981	FKBP10	2.4	FK506 binding protein 10, 65 kDa	Cell proliferation	CD
Hs.433496	Q9UN86	G3BP2	2.3	Ras-GAP	Mitogenesis	CD
Hs.104741	NP_060962	TOPK	**-6.3**	T-LAK cell-originated protein kinase	MAPKK	CD
Hs.317432	P54687	BCAT1	**-3.5**	Branched chain aminotransferase 1	Cell proliferation	CD
Hs.22868	JN0805	PTPN11	**-2.9**	Protein tyrosine phosphatase, non-receptor type II	Protein-tyrosine phosphatase	CD
Hs.181046	P51452	DUSP3	**-2.8**	Dual specificity phosphatase 3	Regulator of CDKs	CD
Hs.211593	A45416	PRKCQ	**-2.5**	Protein Kinase C theta	Cell proliferation	CD
Hs.75770	NP_000312	RB1	**-1.9**	Retinoblastoma protein	Cell proliferation	CD
Hs.169449	P17252	PRKCA	**absent**	Protein Kinase C alpha	Cell proliferation	CD
Hs.93183	S51797	VASP	3.0	Vasodilator-stimulated phosphoprotein	Cytoskeleton	CS
Hs.13405	Q9NQX3	GPHN	2.9	Gephyrin	Cytoskeleton	CS
Hs.74034	Q03135	CAV1	2.6	Caveolin 1	Structural role in caveolae	CS
Hs.171374	NP_055785	KIFAP3	2.5	Kinesin-associated protein 3	Mictrotubule organization	CS
Hs.325474	NP_149129	CALD1	**-2.6**	L-Caldesmon	Cytoskeleton	CS
Hs.146409	A36382	CDC42	**-2.4**	Cell division cycle 42	Regulation of actin cytoskeleton	CS
Hs.377973	NP_005563	LMNA	**-1.9**	Lamin A/C	Nuclear envelope formation	CS
Hs.180141	NP_068733	CFL2	**-1.6**	Cofilin 2	Actin-binding protein	CS
Hs.84981	A32626	Ku80	1.8	Ku antigen 80K chain	Double-stranded break repair	DR
Hs.192803	P23025	XPA	**absent**	Xeroderma pigmentosum, complementation group A	Nucleotide excision repair	DR
Hs.202833	P09601	HMOX1	4.2	Heme oxygenase (decycling) 1	Heme catabolism	M
Hs.171280	Q99714	HADH2	2.6	Hydroxyacyl-coenzyme A dehydrogenase, type II	Short-chain alcohol dehydrogenase	M
Hs.170222	A31311	SLC9A1	2.3	Solute carrier family 9, isoform 1	pH homeostasis	M
Hs.111039	JC1343	NMT1	2.1	N-myristoyltransferase, type 1	Myristylation	M
Hs.6906	NP_005393	RALA	1.6	RAS-like protein A	GTP-binding protein	M
Hs.1239	A30325	ANPEP	**-3.5**	Aminopeptidase M	Enkephalin processing	M
Hs.122647	NP_004799	NMT-2	**-2.2**	N-myristoyltransferase, type 2	Myristylation	M
Hs.9302	Q13371	PHLP	2.4	Phosducin-like protein	G-protein regulation	O
Hs.153924	P53355	DAP Kinase	1.9	Death-associated protein kinase 1	IFN-gamma-associated cell death	O
Hs.11866	O14925	TIMM23	2.7	Translocase of inner mitochondrial membrane 23	Mitochondrial protein translocation	PS
Hs.16258	NP_570137	RAB24	2.1	RAB24	Vesicle sorting	PS
Hs.84153	Q13561	DCTN2	1.9	Dynactin 2	Microtubule-based organelle transport	PS
Hs.300631	NP_057610	GS15	1.8	Golgi SNARE, 15 kDa	Vesicle sorting	PS
Hs.75410	P11021	HSPA5	1.6	Heat shock 70 kDa protein 5	Prevents aggregation	PS
Hs.12797	O60231	DDX16	3.0	DEAH-box protein 16	Helicase	T
Hs.96055	Q01094	E2F1	1.8	E2F transcription factor 1	Transcription repressor	T
Hs.408442	O14901	TIEG2	**-5.7**	TGFB inducible early growth response 2	Transcription repressor	T
Hs.21486	P42224	STAT1	**-2.6**	Signal transducer and activator of transcription 1	Interferon-induced transcripion	T
Hs.146355	NP_009297	ABL1	2.6	Abelson murine leukemia viral oncogene homolog 1	Oncogenesis	TG
Hs.41714	NP_004314	BAG-1	2.4	Bcl-2-associated athanogene-1	Oncogenesis	TG
Hs.1345	A33166	MCC	2	Mutated in Colorectal Cancer	Tumor suppressor	TG
Hs.343475	1LYW_F	CTSD	1.7	Cathepsin D	Endopeptidase; estrogen inducible	TG
Hs.226795	14GS_B	GSTP1	1.5	Glutathione S-transferase pi	Oncogenesis	TG
Hs.58169	NP_006092	HEC	**-3.0**	Highly expressed in cancer	Oncogenesis	TG
Hs.82483	Q15796	Smad 2/3	**-2.1**	Mothers against decapentaplegic homolog 2	Tumor suppressor	TG
Hs.77183	P10398	ARAF1	**absent**	A-Raf proto-oncogene	Oncogenesis	TG
Hs.118796	NP_001146	ANXA6	**-4.9**	Annexin A6	Unknown	U

[a]Boldface type indicates down-regulated genes

[b]Genes are organized into the following groups: A = apoptosis, CA = cell adhesion, CD = cell division, CS = cell shape, DR = DNA repair, M = metabolism O = other, PS = protein sorting, T = transcription, TG = tumorigenesis, U = unknown

[c]absent = potein present in mock-infecfted cells but not in KSHV-infected cells: fold change cannot be called

b. ***Experimentally-infected DMVEC express increased levels of HO-1 mRNA and protein***

KSHV induction of HO-1 was of particular interest because virus upregulation of this gene might confer a proliferative advantage to infected cells in the KS lesional environment. The PowerBlot data showing upregulation of HO-1 in KSHV-infected DMVEC (table 1) were independently confirmed in our laboratory by quantitative RT-PCR, Western blotting and indirect immunofluorescence. KSHV-infected DMVEC expressed HO-1 mRNA at a level 3.48-fold higher than uninfected cells (table 2). PowerBlot analysis revealed upregulation of HO-1 by a factor of 4.24 and Western blot analysis comparing uninfected and KSHV-infected DMVEC confirmed significant viral induction of HO-1 (figure 2a). As expected, modulation of HO-1 enzymatic activity by treatment with the heme analogs CrMP (inhibitory of HO-1 activity) and CuMP (non-inhibitory) did not significantly alter the expression of HO-1 in KSHV-infected cells (figure 2a).

Double staining for KSHV LANA and HO-1 by indirect immunofluorescence (IF) showed that HO-1 was upregulated in KSHV-infected, LANA-positive cells but not uninfected neighboring cells (figure 2b). A range of HO-1 staining intensities is apparent by IF in latently-infected cells suggesting that in addition to viral components, cellular factors may also influence HO-1 expression in these cells. Parallel monolayers stained for the lytic marker ORF 59 showed that <1% of cells had undergone spontaneous lytic reactivation (data not shown), thus suggesting that latent infection with KSHV was sufficient for upregulation of HO-1. HO-1 upregulation by KSHV infection was confirmed in primary DMVEC infected with

the recombinant EGFP-expressing clone rKSHV.152 [112] at a low multiplicity of infection (figure 2c). IF after one passage post-infection revealed HO-1 expression in EGFP-positive, spindle-shaped primary DMVEC but not neighboring, EGFP-negative cells suggesting a direct rather than paracrine effect of KSHV infection on upregulation of HO-1.

TABLE 2. Quantitative RT-PCR Analysis of HO-1 Expression

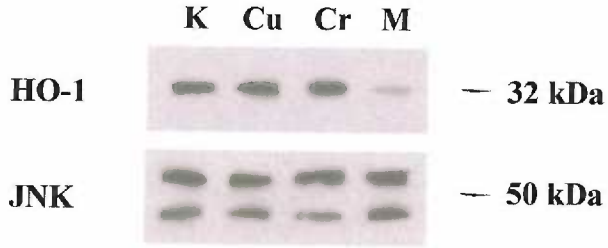
tissue culture[a]	3.48
KS tissue[b]	3.81

[a]compared to mock-infected cells
[b]compared to normal skin

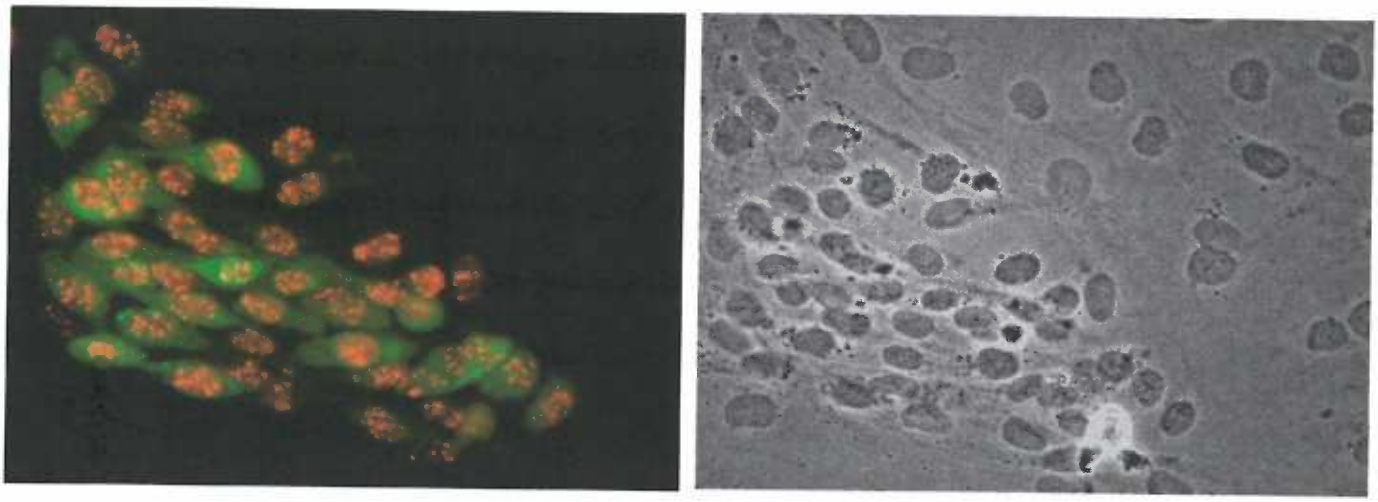
FIG. 2. Upregulation of HO-1 in KSHV-infected DMVEC. (A) Age- and passage-matched uninfected and KSHV-infected DMVEC were harvested and lysed with 3 freeze/thaw cycles. Clarified supernatants from whole cell extracts were resolved on SDS-PAGE, transferred to nitrocellulose membranes and probed with a monoclonal antibody (MAb) raised against the 32 kDa protein HO-1. HO-1 expression was increased in KSHV-infected extracts (lane K) relative to uninfected extract (lane M). Treatment of KSHV-infected cells with the heme analogs CuMP and CrMP (lanes Cu and Cr, respectively) did not significantly alter HO-1 expression. SAPK/JNK was used as a loading control. (B) Monolayers of KSHV-infected DMVEC were fixed with 2% paraformaldehyde, permeabilized with 0.05% Triton X, and stained with an anti-HO-1 MAb and an anti-ORF 73 polyclonal antibody followed by FITC-labeled goat-anti-mouse and Texas Red-labeled goat-anti-rabbit secondary antibodies. HO-1 staining (green) was found exclusively in cells expressing ORF 73 (red), but not in uninfected neighboring cells, visible in the phase image in right panel. (C) The recombinant EGFP-expressing clone rKSHV.152 was used to infect primary DMVEC at a low multiplicity of infection. Monolayers were fixed and permeabilized as above and stained for HO-1 (red). EGFP-KSHV-positive, spindle-shaped DMVEC stained positive for HO-1 (red) while uninfected EGFP-negative cells, visible in the phase image in right panel, did not.

Figure 2

A



B



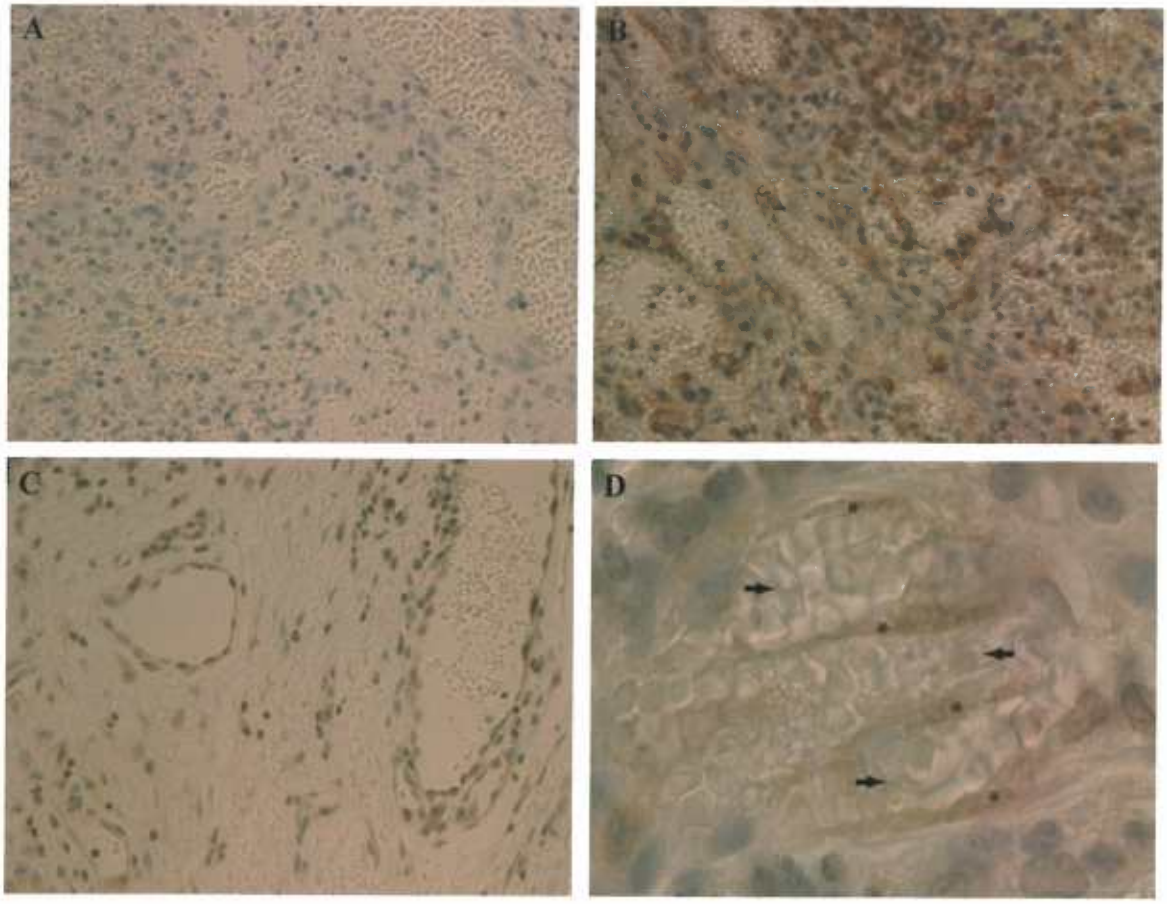
C

c. *HO-1 is upregulated in KS tissue*

Quantitative RT-PCR on RNA extracted from a biopsy of cutaneous AIDS-KS revealed upregulation of HO-1 message by a factor of 3.8 relative to normal skin (table 2). HO-1 levels from both *in vitro* and *in vivo* sample sets were normalized to an internal control, GAPDH. HO-1 protein levels were assessed in biopsy tissue of oral AIDS-KS lesions by immunohistochemistry (IHC, figure 3). Examination of normal tissue at the tumor margin showed HO-1 expression within the intact endothelial lining of normal vascular spaces as well as sporadic expression in non-vascular regions (figure 3c). KS lesional tissue showed a higher degree of cellularity, numerous abnormal vascular spaces with extravasated erythrocytes, and substantially higher HO-1 reactivity compared with normal tissue (figure 3b). Examination of the KS lesional tissue at higher magnification revealed numerous HO-1 expressing spindle-shaped cells traversing the abnormal vascular spaces (figure 3d). The strong HO-1 reactivity observed in spindle-shaped cells within KS lesional tissue suggests that this protein may play an important role in the pathophysiology of KS.

FIG. 3. HO-1 expression in oral AIDS-KS tissue. Paraffin embedded biopsy tissues from oral AIDS-KS lesions were sectioned and mounted on charged slides. The slides were deparaffinized and stained with a polyclonal anti-HO-1 and counterstained with hematoxylin/eosin. (A) 40x. Rabbit Ig was used as a control for secondary specificity in sections taken from identical tissue blocks. Panel A shows low background staining in an area of tumor involvement . (B) 40x. A similar area of tumor involvement from a section derived from the same tissue block shows a high degree of cellularity, numerous abnormal vascular spaces with extravasated erythrocytes and a marked level of HO-1 expression (brown). (C) 40x. HO-1 expression in normal tissue at the tumor margin of the same tissue section shown in panel B. HO-1 was found within the endothelial lining of normal vascular spaces and sporadically in non-vascular regions. (D) 63x. Higher magnification of an area of tumor involvement from the same tissue section shown in panel B showing HO-1 staining in spindle-shaped cells (asterisks) traversing abnormal, erythrocyte-congested vascular spaces (arrows).

Figure 3

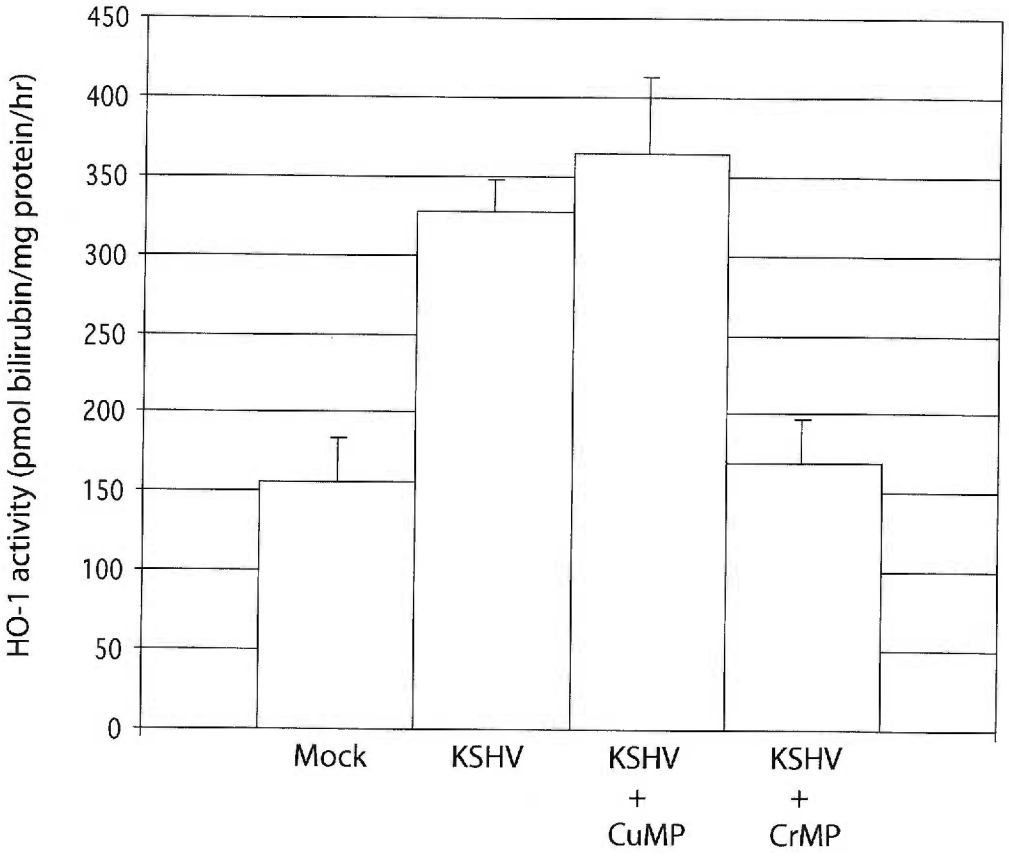


d. ***HO-1 enzymatic activity is upregulated in KSHV-infected cells compared to uninfected cells***

We verified that HO-1 induced by KSHV infection of DMVEC was enzymatically active by measuring the production of bilirubin by crude whole cell extracts. HO-1 activity was increased 2.1-fold in KSHV-infected DMVEC over uninfected controls (figure 4). Treatment of KSHV-infected cultures with an inhibitor of HO-1 activity, the heme analog CrMP (10 μM overnight), resulted in reduction of HO-1 activity to baseline levels while treatment with the structurally related but non-inhibitory CuMP had no net effect on HO-1 activity. Note that these analogs do not influence HO-1 expression levels (figure 2a). Therefore, the HO-1 expressed in KSHV-infected DMVEC is enzymatically active and susceptible to pharmacological inhibition.

FIG. 4. Measurement of HO activity in DMVEC cell extracts. HO activity in uninfected and KSHV-infected DMVEC cell extracts was determined by spectrophotometric measurement of bilirubin production. Cell extracts were incubated with 50 μM heme for 30 minutes at 37oC. The reaction was stopped by the addition of two volumes of chloroform, and bilirubin concentration was determined by comparing the difference in optical density at 464 and 530 nm. HO activity was reported as picomoles of bilirubin produced per milligram endothelial cell protein extract per hour. KSHV-infected cells (KSHV) had 2.1-fold greater HO activity relative to uninfected cells (Mock). Treatment with the HO inhibitor CrMP reduced HO activity in infected cells to the level of uninfected cells (KSHV + CrMP) while treatment with the non-inhibitory heme analog CuMP did not reduce HO activity (KSHV + CuMP). Results were determined in triplicate (+/- SD [error bars]).

Figure 4


HO-1 activity (pmol bilirubin/mg protein/hr)
450
400
350
300
250
200
150
100
50
0
Mock
KSHV
KSHV + CuMP
KSHV + CrMP

e. ***HO-1 upregulation increases proliferation of KSHV-infected DMVEC in the presence of free heme***

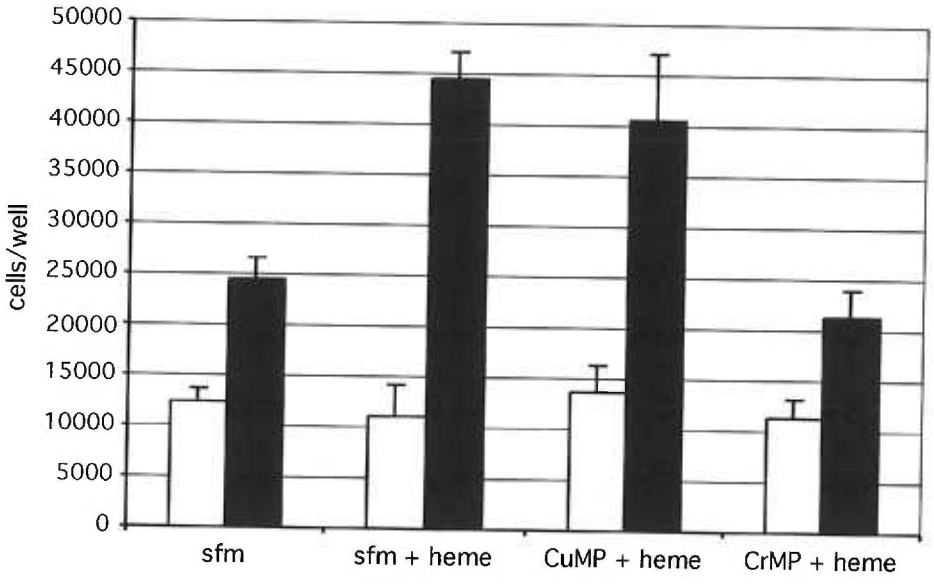
Because of the potential for increased exposure of KS cells to free heme and heme-containing proteins in KS tissue [254-258], we investigated the possibility that HO-1 upregulation might affect the response of KSHV-infected cells to heme. Overexpression of HO-1 in endothelial cells by viral vector transduction has been shown to increase cell proliferation [242,243]. To assess the effect of upregulation of HO-1 on proliferation of DMVEC, uninfected and KSHV-infected cells were plated in 96-well plates and cultured for 24 h in serum-free media followed by the addition of a non-toxic dose of heme (5 μM) for 48 h. Selected cultures were additionally treated with the heme analogs CrMP (an inhibitor of HO-1 activity) and CuMP (non-inhibitory analog). Proliferation of DMVEC in response to treatment was assessed by performing direct cell counts at 0 and 48 h post heme treatment as described in the methods section. Importantly, treatment with heme had no effect on the proliferation of uninfected DMVEC, but significantly stimulated the growth of KSHV-infected cells (figure 5a). This increase in proliferation was due to HO-1 enzymatic activity since inhibition of HO-1 activity by treatment with CrMP abolished this increase in cell division whereas treatment with the non-inhibitory heme analog CuMP did not. Uninfected cells did exhibit a robust proliferative response when serum-starved cells were incubated with 10% human serum in the presence or absence of endothelial cell growth supplement, a complex mixture containing known endothelial cell mitogens, indicating that the lack of proliferation of these cells in the presence of heme was due to an unresponsiveness to heme rather than a non-specific limitation resulting from

the experimental procedure employed (figure 5b). Cell viability was >95% by trypan blue exclusion for all treatment conditions (data not shown) indicating that the steady state of uninfected cell numbers under all treatment conditions was due to minimal proliferation in serum-free conditions rather than a cytotoxic effect of heme on these cells. Given the increased concentration of heme within the KS lesional microenvironment, upregulation of HO-1 in KSHV-infected cells and the subsequent increase in cellular proliferation in the presence of the enzyme's substrate may provide a survival and growth advantage for spindle cells compared with uninfected cells. Thus, KSHV induction of HO-1 may represent a novel strategy for viral tumorigenesis.

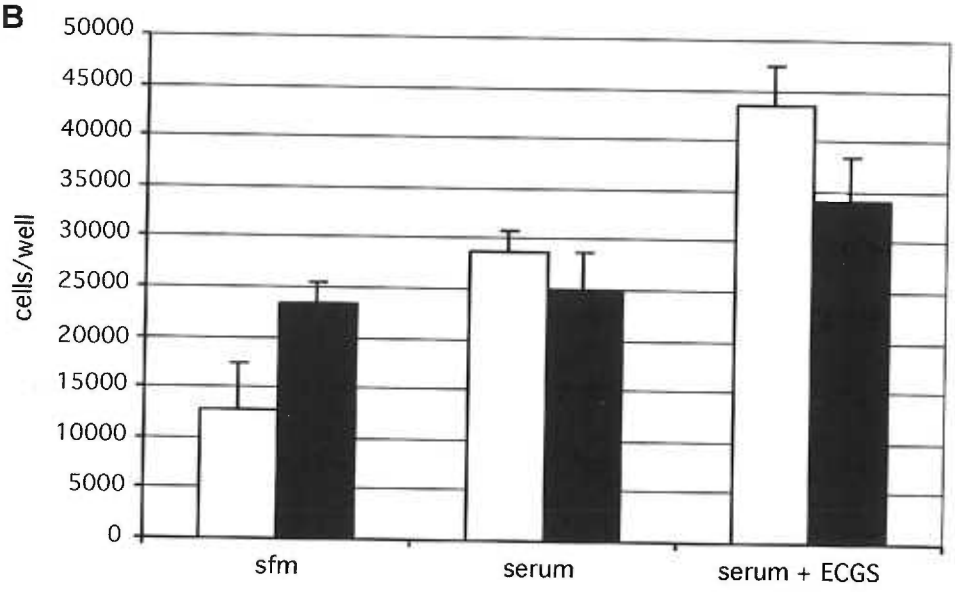
FIG. 5. Proliferation of DMVEC in response to free heme. The proliferative response of uninfected and KSHV-infected cells to heme was determined by direct cell counts. (A) Cells were plated in complete media overnight followed by culture in serum-free media (SFM) for 24 h in the presence or absence of the heme analogs CrMP (to inhibit HO-1 activity) and CuMP (non-inhibitory). Cells were then incubated with and without 5 μM heme in SFM and proliferation measured 48 h later. Uninfected cells (white bars) did not proliferate in response to incubation with heme while KSHV-infected cells (black bars) proliferated to a greater extent following exposure to heme. Enhanced proliferation of KSHV-infected cells in the presence of heme was HO-dependent since treatment with CrMP abolished the proliferative response. (B) The lack of proliferation of uninfected cells was due to an unresponsiveness to heme rather than a non-specific limitation resulting from the experimental procedure employed. These cells did exhibit a robust proliferative response when serum-starved cells were incubated with 10% human serum in the presence or absence of endothelial cell growth supplement (50 μg/ml), a complex mixture containing known endothelial cell mitogens. Results were determined in quadruplicate (+/- SD [error bars]).

Figure 5

A


50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
cells/well
sfm
sfm + heme
CuMP + heme
CrMP + heme

B


50000
45000
40000
35000
30000
25000
20000
15000
10000
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0
cells/well
sfm
serum
serum + ECGS

4. Discussion

We report here the results of a proteomics screening approach undertaken to identify alterations in gene expression in DMVEC experimentally infected with KSHV. Of the 850 genes examined by western blot array, 52 showed significant positive or negative regulation compared to uninfected DMVEC. In the present study we chose to focus our efforts on HO-1 because of the potential role this gene has in angiogenesis. Given the vascular nature of KS lesions, host genes involved in angiogenesis which are induced by KSHV infection may provide targets for pharmacological intervention. Additionally, the availability of specific HO-1 inhibitors in clinical use for control of hyperbilirubinemia in neonates [259,260] aided our initial dissection of the role of HO-1 upregulation in KSHV-induced pathogenesis.

HO-1 is a heme-degrading enzyme that cleaves the porphyrin ring releasing equimolar quantities of carbon monoxide (CO), free iron, and biliverdin. Free iron then stimulates the production of the iron-scavenging protein ferritin, while biliverdin is rapidly reduced to bilirubin by biliverdin reductase [246]. The effects of these HO-1 metabolites on endothelial physiology suggest that HO-1 might play an important and interesting role in KS pathogenesis. For example, CO produced by HO-1 activity was shown to protect endothelial cells from both CD95/Fas- and TNFα-mediated apoptosis [240,241]. In addition, generation of the cytoprotectant bilirubin, along with ferritin upregulation following release of free iron, has been shown to protect endothelial cells from oxidative damage resultant from myriad noxious stimuli [261-263].

In the present study we show that KSHV infection of DMVEC results in upregulation of HO-1 at both the message and protein levels. *In vitro*,

immunofluorescent analysis revealed upregulation of HO-1 in infected DMVEC but not uninfected neighboring cells in both primary and E6/E7 transformed cultures. Recently it was shown by serial analysis of gene expression (SAGE) that HO-1 expression in AIDS-KS biopsy tissue is significantly greater than in control tissue [177]. Here we independently confirm this finding with quantitative RT-PCR results on cutaneous AIDS-KS tissue which show elevated HO-1 mRNA compared to normal skin. Furthermore, immunohistochemistry on oral AIDS-KS lesional tissue revealed increased expression of HO-1 protein compared with normal tissue at the tumor margin. *In vitro*, upregulated HO-1 was enzymatically active and susceptible to chemical inhibition with a heme analog, CrMP.

We show here that increased HO-1 activity in KSHV-infected endothelial cells results in increased cell proliferation following exposure to free heme, whereas uninfected cells grown under the same conditions did not exhibit a proliferative response. Because of the importance of angiogenesis in the pathophysiology of KS, heme induced proliferation of KSHV-infected cells may be an important factor contributing to lesion development. There is evidence that KS lesions have locally elevated concentrations of hemoglobin and free heme and therefore contain ample substrate for HO-1. Intracellular junctions between neoplastic endothelial cells are frequently defective allowing the extravasation of large numbers of erythrocytes from vessel lumens into the surrounding tissue [254-257]. These renegade cells become more numerous as the disease progresses and abnormal networks of slit-like vascular spaces expand. Erythrophagocytosis by KS cells is also a common feature [258]. Many spindle cells contain membrane-bound intracytoplasmic erythrocytes

(erythrophagosomes) surrounded by lysosomal granules. These erythrophagosomes show various stages of disintegration leading ultimately to extruded hemoglobin and empty ghost cells (dead RBCs). Others have described the presence of hyaline globules in plaque- and nodular-stage KS and histologic staining has revealed that these are the remnants of degenerated erythrocytes [255]. In light of these histologic findings, it is conceivable that upregulation of HO-1 by KSHV infection could confer a selective advantage to KS cells within the high heme lesional microenvironment compared with neighboring uninfected cells. The availability of potent inhibitors of HO-1 in clinical use elsewhere [259,260] makes this enzyme a potentially important treatment target in KS.

Upregulation of HO-1 has been recognized in other experimental pathogenesis systems. Infections by influenza virus [246], encephalomyocarditis virus [264], *Listeria monocytogenes*[265], and *Rickettsia rickettsii* [266] all show significant upregulation of HO-1, suggesting that this enzyme may participate in cellular responses to some intracellular pathogens. We have assessed the expression of HO-1 in our DMVEC system following infection with the KSHV-related rhesus rhadinovirus (RRV). RRV is associated with the development of lymphomas and retroperitoneal fibromatosis (RF) in rhesus macaques coinfected with simian immunodeficiency virus, an animal model similar to HIV and KSHV coinfection in humans (S. Wong, manuscript in preparation). The expression profile of DMVEC infected with a recombinant EGFP-expressing clone of RRV was compared with that of uninfected cells by cDNA microarray (manuscript in preperation). HO-1 was found not to be induced in these cells by RRV infection (S. Wong, personal

communication). We verified this finding by taqman in our laboratory (data not shown). Alternately, another gene which we have reported as upregulated by KSHV infection, cKit, was found to be upregulated by RRV infection (data not shown), suggesting that there are similar yet distinct subsets of cellular genes induced by these closely related, endothelial-tropic viruses. It is interesting that RRV does not induce HO-1, a phenomenon which might be explained by the histological differences that exist between RF and KS. One of the dominant histopathological features of KS, the disorganized network of abnormal vascular spaces filled with extravasated erythrocytes, is notably absent in RF [267,268]. It is conceivable that in the absence of a high heme microenvironment within RF tissue, RRV may not have experienced the selective pressure to upregulate HO-1 because no proliferative advantage is to be derived by that enzyme's expression in the absence of substrate. Therefore, the specificity of HO-1 upregulation in endothelial cells by KSHV infection may be a modulation of cellular gene expression resulting from the unique erythrocyte-rich microenvironment characteristic of KS.

Recent reports indicate that HO-1 expression in endothelial cells results in upregulation of VEGF [244,269]. Current studies in our laboratory are focused on defining the mechanism(s) by which KSHV induces HO-1 in DMVEC as well as determining the cellular pathways activated by HO-1-derived metabolites which result in enhanced proliferation of infected cells following exposure to heme.

5. Acknowledgments: This work was supported in part by a sponsored research agreement from Virogenomics (AVM and KF), NOT-HL-02-013 (AVM), NIH-NIDCR PO1 DE-07496. The UCSF AIDS Specimen Bank is supported by P30 A127763-12. We acknowledge Dr. Molly Kulesz-Martin and Dr. Jon Hanifin, OHSU Department of Dermatology, for cutaneous AIDS-KS tissue from the Molecular Profiling Resource in Dermatology, partially supported by NIH CA31101 and OHSU Cancer Center grant CA69533; Dr. Bala Chandran, University of Kansas Medical Center, for KSHV-specific antibodies; Dr. Jeff Vieira, Department of Laboratory Medicine, University of Washington, and Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, for the recombinant EGFP-expressing clone rKSHV.152; and Linda Jauron-Mills, Oregon Cancer Center, for help with IHC.

Chapter Three

KSHV Induces Matrix Metalloprotease-1 in Infected Endothelial Cells

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Status of manuscript: will be submitted following defense.

AUTHORS

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I developed the hypothesis regarding elevated levels of MMP-1 and KS pathophysiology, designed and carried out the majority of the experimental approaches, and prepared the manuscript, tables, and figures. Coauthors contributed the following: S. G. Hansen assisted with Western blotting and adenovirus construction; M. Luppi contributed KS biopsy materials; S. W. Wong and A. V. Moses acted as advisors to me during the development and presentation of this project.

ABSTRACT

Matrix metalloproteases (MMPs) are a family of enzymes that degrade extracellular matrix components. MMP activity is important in many normal processes, such as angiogenesis and wound repair, but is also involved in various pathophysiological processes, including cancer. Analysis of Kaposi's sarcoma (KS) biopsy material revealed abundant expression of MMP-1 and decreased expression of the naturally-occurring MMP inhibitors, the tissue inhibitors of matrix metalloproteases (TIMPs). Expression of MMP-1 is induced by numerous stimuli that are also known to be involved in KS lesion progression, including inflammatory cytokines and VEGF. KS spindle cell development and lesion progression is the consequence of infection of endothelial cells with KSHV. We confirmed that MMP-1 overexpression in KS spindle cells could be directly mediated by KSHV infection using our previously described *in vitro* KS model of KSHV-infected endothelial cells. The KSHV latency genes ORF 72 and ORF 73 were sufficient to mediate MMP-1 upregulation. This study is the first to propose a role for MMP-1 in KS pathophysiology and contributes to a body of work linking the MMP family of enzymes to KS progression. The manipulation of the expression of MMP-1 and the enzyme's endogenous inhibitors may potentiate KS tumor cell proliferation and invasion, thus contributing to KS lesion progression; targeted inhibition of MMP-1 may, therefore, be a useful treatement option for KS.

INTRODUCTION

Angiogenesis, the development and proliferation of new blood vessels, is essential for normal development and dynamic processes such as would healing, and is also critical for the vascularization and growth of tumors[270-273]. Early in the multistep process of angiogenesis, resting vascular endothelial cells become activated, overcome contact inhibitory signals, degrade components of the extracellular matrix (ECM), and invade their basement membrane; invading cells then migrate into tissue spaces, proliferate, and finally organize themselves into nascent vessels. Angiogenesisis is a hallmark feature of Kaposi's sarcoma (KS)[4,274,275]. However, significant derangements of the angiogenic process within KS lesions result in the formation of disorganized networks of abnormal vascular slits, rather than well-formed microvessels. These vascular slits are composed of spindle-shaped neoplastic endothelial cells that form defective intracellular junctions, accounting for lesional edema and abundant extravasation of inflammatory cells and erythrocytes [254-257]. KS lesional spindle cells express chemotactic and angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukins (IL)-6 and IL-8, which drive proliferation and invasiveness of both spindle cells and neighboring endothelial cells [276-281]. Because spindle cells are rare in early patch-stage KS lesions but become the predominant cell type in later plaque- and nodular-stage lesions, alterations in spindle cell physiology that induce proliferation and invasion are thought to be important in disease progression.

KS-associated herpesvirus (KSHV; also human herpesvirus 8) was discovered in 1994 and is now considered necessary for all clinical forms of KS, as well as two B

cell lymphoproliferative disorders, multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL). Both *in vitro* and *in vivo*, KSHV infection in tumor cells is maintained in a primarily latent state characterized by expression of few viral gene products [65-72]. At least three gene products, vFLIP, vCYC, LANA, are believed to comprise the viral latency expression program of KSHV and are consistently expressed in all virally infected cells in KS, PEL, and MCD [65-67,73]. The gene product of open reading frame (ORF) 71 viral FLICE-inhibitory protein (vFLIP; K13) inhibits FAS (CD95)-mediated apoptosis and is thought to act as a tumor progression factor by interfering with apoptotic signals induced by cytotoxic T lymphocytes[74,75]. ORF 72, the viral cyclin (vCYC) is a homolog of cellular D-type cyclin and can drive quiescent cells into S phase in part by inhibiting retinoblastoma protein (Rb) activity [77-80]. The latency-associated nuclear antigen (LANA; ORF 73) targets both p53 and Rb, modulating cellular transcription by blocking both activation of p53-dependent promoters and inducing activation of E2F-dependent genes [83,84]. Because products of the Kaposin (ORF K12) transcript are expressed at low levels during latency but are increased after induction of the lytic cycle, kinetic classification of Kaposin has been controversial. The Kaposin locus encodes at least 3 proteins, A, B and C, via a complex translational program that is as yet incompletely understood[73,90-92]. Transforming functions have been ascribed to the Kaposin family, particularly to Kaposin A[91], and recently, a role for Kaposin B in stabilization of cytokine transcripts via activation of the kinase MK2 was proposed (McCormick C and Ganem D: The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs. *Science* 307, 739-741 (2005).). Thus, the latency

program of KSHV can drive the proliferation of host cells and prevent their apoptosis, two functions considered vital for tumor development.

Upon reactivation, KSHV expresses a wider repertoire of lytic gene products in a coordinated cascade, which culminates in release of progeny virions from the infected cell. Many of these lytic genes have homology to human oncogenes and growth factors or transforming genes of other herpesviruses. These KSHV-encoded regulatory proteins include homologs of cellular cytokines (viral interleukin-6 [vIL-6]) and chemokines (viral macrophage inflammatory protein I [vMIP-I]; K4, vMIP-II; K6, and vMIP-III; K4.1); the anti-apoptotic protein viral B-cell lymphoma 2 (vBCL-2; ORF 16); an inhibitor of interferon signaling (viral interferon regulatory factor [vIRF]; K9); and a viral G-protein-coupled receptor with homology to the human IL-8 receptor (vGCR; ORF 74) that has been directly implicated in angiogenesis[121,274,282]. While completion of the viral lytic cascade may be incompatible with host cell survival and therefore transformation, lytic gene products are thought to elicit important paracrine effects on adjacent latently-infected and uninfected cells and are therefore considered vital for lesion development. Interestingly, it has also been proposed that a population of KSHV-infected cells can support a restricted lytic infection with expression of certain lytic genes but without completion of the viral life cycle and concomitant cell lysis. The extent to which this program operates in vivo is unclear, but it would allow the effects of a wider repertoire of viral genes to be exerted.

The MMPs are a family of functionally and structurally related calcium-dependent, zinc-containing endopeptidases that degrade components of the

extracellular matrix (ECM)[283]. The 23 MMPs found in humans are divided into subfamilies based on sequence homology, substrate specificity, and domain organization. MMPs are secreted as inactive zymogens and are activated extracellularly by cleavage of a pro-domain [284,285], resulting in an 8-10 kDa reduction in molecular mass of the active protein [286]. MMPs can also be activated *in vivo* by other proteases, including plasmin[287]. The biological activities of MMPs are inhibited by the action of a family of four tissue inhibitors of MMPs (TIMP-1 through 4)[288]. The TIMPs are closely related proteins that are broadly active against members of the MMP family. TIMPs bind both the zymogen and activated forms of MMPs, inhibiting enzyme activation and enzyme activity, respectively. Regulation of the interactions between cells and ECM components by controlled expression of MMPs and TIMPs is essential for many normal physiological processes such as embryogenesis, angiogenesis, and wound repair[289-291]. Dysruption of the balance between proteolysis and stasis, however, is associated with numerous pathophysiological processes including arthritis, cardiovascular disease, tissue ulceration, and cancer[270-273].

Individual members of the MMP family have previously been implicated in KS pathogenesis. This work has focused mainly on MMP-2 and MMP-9, which degrade collagen type IV, the main component of vascular basement membranes[275]. Cells explanted from KS lesions were found to constitutively express several MMPs[292] and supernatants conditioned by explanted KS induce expression of MMPs by cultured endothelial cells[293]. Invasion through experimental basement membrane preparations could be blocked by treatment with TIMP-2, suggesting that invasiveness was mediated, at least in part, by MMP-2. Others have demonstrated

that KS cell invasion can be inhibited *in vitro* by inhibiting pro-MMP-2 activation with *N*-acetylcysteine[294]. *In vivo*, HIV protease inhibitors have been found to induce regression of KS lesions. These drugs do not inhibit MMP-2 directly, but they are able to indirectly block activation of pro-MMP-2, possibly accounting for their anti-KS activity[295,296]. An inhibitor of MMP-2 and MMP-9, COL-3 (Metastat; Collagenex Pharmaceuticals, Newton, PA), was recently studied in a Phase I trial for treatment of AIDS-associated KS. In 18 COL-3-treated AIDS-KS patients, 1 full and 7 partial responses were scored for an overall response rate of 44%. COL-3 can inhibit the activity of MT1-MMP, which is known to be important for pro-MMP-2 activation.

For in vitro studies described below, we have used our previously characterized *in vitro* KS model based on KSHV infection of dermal microvascular endothelial cells (DMVEC). This model recapitulates several aspects of KS cell physiology including spindle cell formation; loss of contact inhibition and anchorage-dependent growth restriction; long term propagation of predominantly latently-infected cells; and the ability to generate age- and passage-matched KSHV-infected and uninfected cultures[120]. Expression profiling of KSHV-infected DMVEC using Affymetrix GeneChips revealed that MMP-1 was consistently and significantly upregulated in 4 of 4 infected cultures relative to uninfected DMVEC. In the present study we confirm this finding by complementary methods, and describe a possible role for MMP-1 in KS pathogenesis. MMP-1 (also known as interstial collagenase) is noted for specificity for the interstitial collagens, collagen types I, II, and III and is produced by invading cells for breakage through interstitial barriers. MMP-1 has also been shown to mediate endothelial cell migration during angiogenesis[297]. Further,

MMP-1 can activate cell proliferation by activating insulin-like growth factors[298]. Epidermal hyperplasia and an increased susceptibility to tumor formation has also been noted in a transgenic mouse model[299]. To our knowledge, the study presented here is the first to demonstrate KSHV-induced MMP-1 expression in endothelial cells. Demonstration of strong MMP-1 expression in KSHV-infected cells contributes to a growing body of work that links the MMP family of enzymes to KS progression. As described here, manipulation of the expression of MMP-1 and it's endogenous inhibitors by viral infection may potentiate KS tumor cell proliferation and invasion, thus contributing to KS lesion progression. Thus, MMP-1 represents an attractive target for KS chemotherapy that may not be efficiently blocked by current KS therapeutic efforts.

RESULTS

a. *MMP-1 is abundantly expressed in KS*

MMPs have been implicated in KS but studies have so far focused almost exclusively on MMP-2 and -9. Analysis of microarray data sets generated in our laboratory from comparative profiling of DMVEC following *in vitro* infection and transformation with KSHV (Moses et al, 2002 NYAS paper; Raggo et al, in press, 2005) indicated that MMP-1 was significantly upregulated following viral infection. Interestingly, MMP-2 was found to be downregulated by KSHV infection. The level of MMP-9 was unchanged by viral infection. The observed elevation of MMP-1 by KSHV infection was of interest since MMP-1 expression is known to be low in normal

skin[300]. Therefore, overexpression of MMP-1 could contribute to the significant architectural alterations in dermal, and perhaps visceral, KS lesions by increasing the invasive potential of the tumor component, the EC-derived spindle cell. To confirm the biological relevance of the *in vitro* finding, we assessed MMP-1 mRNA expression in biopsy material taken from an AIDS-KS lesion and from normal skin by quantitative RT-PCR (qPCR; table 1). MMP-1 message was approximately 25-fold more abundant in KS tissue compared with normal skin. To confirm that MMP-1 protein was also strongly expressed in KS lesions, MMP-1 levels were assessed by immunohistochemistry (IHC) in cutaneous biopsy material taken from five classical KS patients. These KS tumors exhibited the hallmark features of KS histology, including numerous abnormal vascular spaces and extravasated erythrocytes. Importantly, MMP-1 expression was strong in nearly all lesional spindle cells surrounding and traversing abnormal vascular spaces (figure 1a). Omission of the primary antibody from the IHC protocol revealed almost undetectable background reactivity, confirming that the strong MMP-1 signal found in KS lesions was due to increased expression of MMP-1 protein rather than nonspecific binding of secondary antibody. Therefore, MMP-1 message and protein is significantly overexpressed in KS tissue, as well as in experimental models, and may represent a novel KS lesion progression factor.

TABLE 1. Quantitative RT-PCR of MMP-1 and TIMP expression in AIDS-KS tissue[a]

MMP-1	25.3
TIMP-1	NC
TIMP-2	-4.2
TIMP-3	-2.9
TIMP-4	-22.2

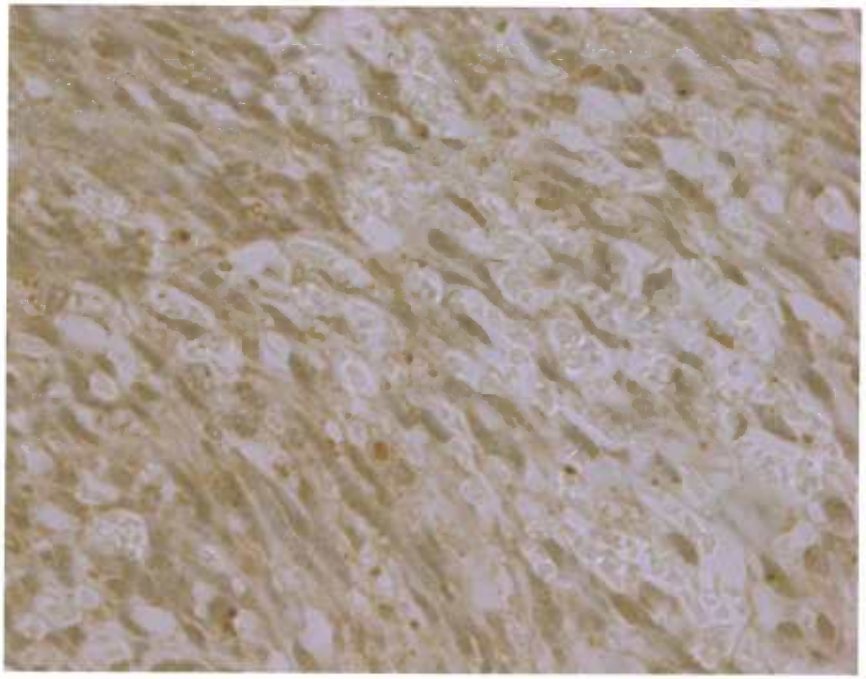
[a]compared to normal skin

FIG.1. MMP-1 expression in tissue from dermal classic KS. Five paraffin-embedded biopsies from dermal classic KS lesions were sectioned and mounted on charged slides. The slides were deparaffinized and stained with a monoclonal anti-MMP-1 anti-mouse and counterstained with hematoxylin/eosin. (A) 40x. Omission of primary antibody from the staining protocol revealed low non-specific background staining in an area of tumor involvement. (B) 40x. A similar section shows a high degree of cellularity, numerous abnormal vascular spaces with extravasated erythrocytes and a marked level of MMP-1 expression (brown) in spindle-shaped cells traversing abnormal, erythrocyte-congested vascular spaces.

Figure 3

a

b



b. *KS tissue expresses lower TIMP levels than normal skin*

TIMPs competitively inhibit both the zymogen (inactive) and activated forms of MMPs and are important biological mediators of MMP activity; dysregulation of TIMP expression has been described in many neoplastic disorders[283,288,289]. Here, expression of mRNA encoding the four isotypic TIMPs was determined by qPCR using biopsy material from AIDS-associated KS (table 1). Expression of TIMP-2, 3, and 4 was decreased in KS biopsy material compared to normal skin. TIMPs can inhibit different classes of MMPs with little selectivity. The finding that several TIMPs are downregulated in KS tissue suggests that MMP-1 expressed by KS spindle cells could be subject to decreased TIMP-mediated inhibition, resulting in a net increase of the enzymatically active pool of MMP-1 within KS lesions.

c. *KSHV-infected endothelial cells upregulate MMP-1* in vitro

Expression of MMP-1 is induced by numerous stimuli that are also known to be involved in KS lesion progression, including inflammatory cytokines and VEGF. Thus, to verify that MMP-1 overexpression in KS tissue could be directly mediated by KSHV infection of lesional spindle cells, we used our previously described experimental model of KS development that is based on in vitro infection of dermal microvascular endothelial cells (DMVEC) with KSHV [120]. KSHV infection of DMVEC in this system recapitulates many features of KS cell physiology including spindle cell formation, anchorage-independent growth, and loss of contact inhibition[120]. Because infection is maintained in the majority of cells and there is evidence of transformation, our culture system is likely to be reflective of the pattern

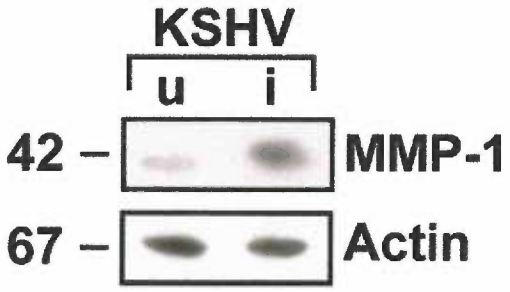
and consequences of KSHV-infection in established lesions. Importantly, long term propagation of predominantly latently-infected cells with parallel age- and passage-matched KSHV-infected and uninfected controls makes this system well-suited for studying modulation of endothelial cell gene expression mediated by KSHV infection. This system has been amenable to genomic and proteomic expression profiling and has provided valuable information in this regard [170,173,301].

As mentioned above, interrogation of previously generated microarray data revealed induction og thr MMP-1 transcript after KSHV infection. To confirm that these increased transcript levels correlated with an increase in MMP-1 protein expression, we assessed expression of MMP-1 by age- and passage-matched KSHV-infected and uninfected control DMVEC by Western blot (figure 2a). A 42 kDa band corresponding to the uncleaved, pro-enzyme form of MMP-1 (pro-MMP-1) was detected, with a stronger signal produced by samples derived from virally-infected cells. Interestingly, the absence of a lower molecular weight band indicated that pro-MMP-1 was not activated in this tissue culture system. This data was confirmed using an MMP-1 activity assay that distinguishes between activated- and pro-MMP-1, which indicated that pro-MMP-1 was expressed by KSHV-infected DMVEC in > 30-fold excess over uninfected cells (figure 2b). Pro-MMP-1 is activated *in vivo* by proteases including plasmin. KS cell express urokinase type plasminogen activator. Inflammatory cells that infiltrate or marginate KS lesions are also rich sources of ptoteases and protease activators. Thus, KS lesions likely have locally elevated levels of enzymatically active plasmin and other proteases available to activate the pro-MMP-1 induced by KSHV infection [292].

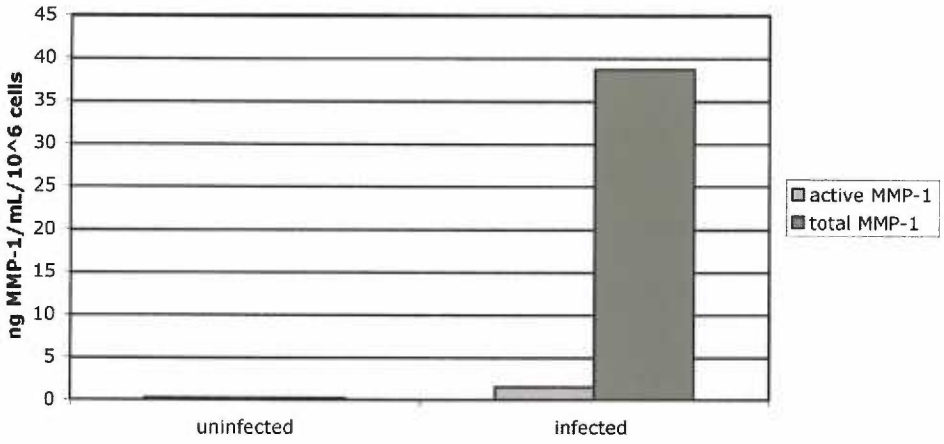
FIG. 2. MMP-1 expression in experimentally-infected DMVEC *in vitro*. (A) Expression of MMP-1 by KSHV-infected and uninfected control DMVEC was assessed by Western blot. A 42 kDa band corresponding to the uncleaved (i.e. unactivated zymogen) form of MMP-1 was noted. KSHV-infected DMVEC expressed more MMP-1 protein than uninfected controls (lane 'i' versus 'u'), consistent with the increased expression of MMP-1 message by infected cells (table 1). (B) MMP-1 enzymatic activity was determined using the Amersham Biosciences Biotrak Assay. This assay distinguishes between zymogen and activated forms of MMP-1. Uninfected DMVEC expressed almost undetectable levels of MMP-1 protein. KSHV-infected cells, however, expressed > 30 fold more MMP-1 protein. Of note, the MMP-1 expressed by infected DMVEC was secreted as inactive zymogen, consistent with the Western blot finding.

Figure 2

a


KSHV
u
i
42 –
MMP-1
67 –
Actin

b


45
40
35
30
25
20
15
10
5
0
ng MMP-1/mL/10^6 cells
uninfected
infected
active MMP-1
total MMP-1

d. ***KSHV latency genes upregulate MMP-1 in DMVEC***

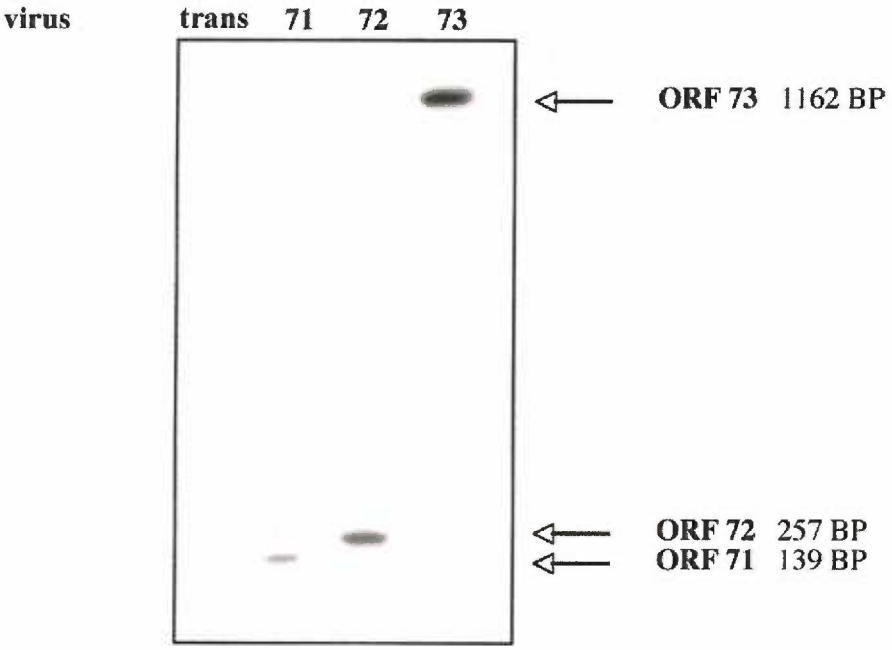
Because the majority of spindle cells, both in our *in vitro* system and within KS lesions, are latently infected with KSHV, we reasoned that one or more of the KSHV latency-associated genes could be responsible for the upregulation of MMP-1 in infected EC and spindle cells. To test this hypothesis, the open reading frames (ORFs) for the three latency-associated genes expressed in all KS lesional spindle cells, ORF 71, 72 and 73, were cloned with HA tags into adenoviral expression vectors and recombinant viruses were used to transduce uninfected DMVEC along with an adenovirus transactivator (Ad-trans) necessary for optimal expression of the desired gene[302]. Expression of each KSHV latency gene in DMVEC cultures was confirmed by Western blot (figure 3a). Transduction with Adenovirus-expressing (Ad)-ORF71, Ad-ORF72, or Ad-ORF73 at an multiplicity of infection (MOI) of 1000 plus Ad-trans at an MOI of 200 resulted in discrete bands with apparent molecular weights corresponding to the predicted sizes of the KSHV latency genes. RNA extracted from cells expressing KSHV latency genes was analyzed by qPCR four days following transduction. Only the viral cyclin analog, ORF 72 (vCYC) affected the expression of MMP-1 mRNA relative to Ad-trans alone, upregulating the enzyme by a factor of 3.2 (table 2). However, when cells expressing KSHV latency genes were assessed for MMP-1 activity as described in figure 2b, ORF 72- *and* ORF 73-expressing cells revealed significantly increased MMP-1 levels compared with Ad-trans only (figure 3b). These data suggest that KSHV genes can regulate MMP-1 expression at both transcriptional and post-transcriptional levels, creating multiple pathways for MMP-1 dysregulation. In KSHV-infected cells, where ORF72 and

ORF73 are co-expressed, this dual influence could have profound effects on the physiology of the tumor cells.

FIG. 3. Expression of KSHV ORFs 72 and 73 is sufficient to induce MMP-1 activity in DMVEC. (A) The KSHV latency genes ORFs 71, 72, and 73 were individually cloned into replication-deficient adenoviral (Ad) expression vectors and used to transduce DMVEC. HA-tagging of the latency genes allowed detection by Western blot. At an MOI of 1000 for Ad/Orfs, and 200 for the Ad transactivator (Ad/trans)contsruct (MOI that were used for MMP-1 assays), expression was sufficient for detection by standard Western blot analysis; bands correspond to the predicted molecular weights of ORFs 71, 72, and 73 (B) Expression of both ORFs 72 and ORF 73, but not ORF 71, induced MMP-1 protein expression over background induced by infection with Ad/trans alone. Consistent with the activity status of MMP-1 expressed by KSHV infected cells, MMP-1 secreted by ORF 72- and ORF 73-transduced cells was in the unactive zymogen form.

Figure 3

a


virus
trans
71
72
73
ORF 73 1162 BP
ORF 72 257 BP
ORF 71 139 BP

b

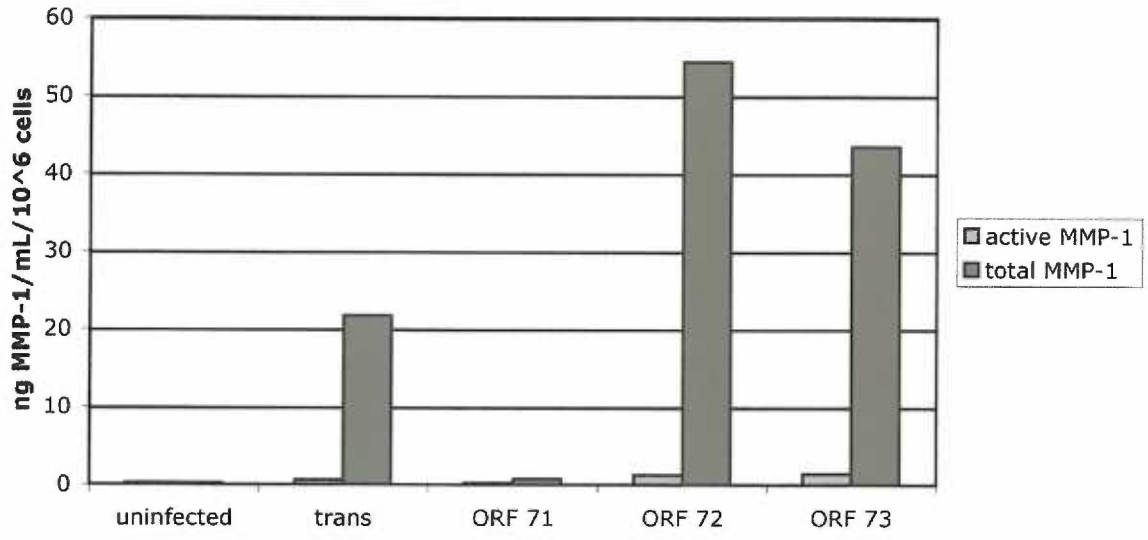

ng MMP-1/mL/10^6 cells
60
50
40
30
20
10
0
uninfected
trans
ORF 71
ORF 72
ORF 73
active MMP-1
total MMP-1

TABLE 2. Quantitative RT-PCR of MMP-1 expression in DMVEC

ORF 71[a]	NC
ORF 72[a]	3.2
ORF 73[a]	NC
KSHV[b]	3.4

[a]compared to trans only
[b]compared to uninfected

DISCUSSION

The ECM is a dynamic network of macromolecules, including collagens, fibronectin, laminin, and proteoglycans, that provides adhesion and survival signals to associated cells[283,303]. Many normal physiological processes involve some degree of ECM remodeling. For example, angiogenesis, the formation of new blood vessels branching from existing vessels, requires that vascular endothelial cells become locally-invasive, degrading ECM components of the basement membrane allowing migration into tissue spaces and subsequent proliferation and organization into nascent vessels[270-273].. The normal process of angiogenesis is exploited by tumors that must stimulate ingrowth of new blood vessels to provide sufficient nutrient and oxygen delivery to support proliferating cells within the tumor mass. In addition, tumor metastasis requires degradation and penetration of connective tissues. Tumor cell invasion requires the activity of the gelatinases, MMP-2 and MMP-9, for degradation of basement membrane components. Interstitial collagenases, such as MMP-1, are then required to degrade the exposed interstitial connective tissues[304].

KS is a vascular tumor in which dysregulated angiogenesis drives lesion progression. Studies by us and others have indicated that KSHV induces numerous cellular enzymes involved in endothelial cell activation and proliferation[169,170,173,275]. In addition, angiogenic cofactors such as vascular endothelial cell growth factor (VEGF) and basis fibroblast growth factor (bFGF), that are abundant in KS lesions due to the extensive inflammatory infiltrate are thought to be essential for lesion progression. KS spindle cells also have alterations in the expression of adhesion molecules that mediate cell-cell and cell-matrix interactions. Thus, proliferating

spindle cells do not form intact, anastamosing vascular spaces, but rather disorganized networks of leaky slit-like vascular spaces that allow abundant extravasation of blood cells. Extensive degradation of the extracellular matrix could thus contribute to the disorganization of KS lesions since migration requires specific interactions with matrix components[303]. The dependence of cell-ECM interactions during migration is highlighted by the finding that soluble endostatin, a potent angiostatic agent, blocks cellular attachment to the ECM by binding integrins, thus inhibiting migration[305]. The increase in the ratio of MMP-1 and TIMP expression demonstrated here, resulting in a tip of the balance towards proteolysis, may further contribute to the disorganized KS lesional architecture by increased turnover of ECM components during local invasion of spindle cells.

In addition to increasing the invasiveness of cancer cells, MMP enzymatic activity can contribute to cancer progression by releasing ECM-bound growth factors, thus elevating local concentrations of soluble growth factors. MMPs also display proteolytic activity against non-ECM components; activity against molecules that mediate cell-cell and cell-matrix associations are implicated in reducing intracellular cell contacts. This process may increase the mobility KSHV-infected cells, contributing to invasion, but also reduce contact-inhibitory signals allowing for increased proliferation. Normal cells require survival signals induced by cell-matrix interactions. The loss of these signals can result in anoikis, a form of apoptosis induced by loss of cell contacts. KSHV-infected cells are resistant to apoptotic cell deathbut uninfected cells would remain susceptible[74,83,99,306,307]; therefore, reduction of cell adhesion signals and the differing effects on KSHV-infected versus uninfected

cells could contribute to KS lesion progression by increased proliferation of infected cells and increased apoptotic-cell death of uninfected cells.

A recent study demonstrated that EBV, a closely-related gammaherpesvirus, upregulates MMP-1. Specifically, MMP-1 protein was also found to be abundantly expressed in nasopharyngeal carcinoma, an EBV-mediated neoplasm[230]. *In vitro*, EBV-induced MMP-1 was enzymatically activated, possibly due to co-expression of MMP-2, as MMP-2 is known to activate MMP-1[308]. In our system, however, KSHV was associated with a decrease in MMP-2 expression, a finding corroborated by others. The reduction of MMP-2 expression in our system may explain the lack of pro-MMP-1 activation in *in vitro* culture where only EC are present. Invasion of type I collagen by melanoma and breast cancer cell lines involves expression of pro-MMP-1; however, interaction with other cell types, such as stromal cells, is thought to be required for activation the enzyme *in vivo*, thus contributing to matrix degradation and tumor cell invasion[309,310].

A role for MMP-1 has been described for other invasive pathogeneic processes, including breast cancer, melanoma, and rheumatoid arthritis. Given the important role of MMP-1 in various invasive processes, including possibly KS, development of MMP inhibitors specific for MMP-1 may yield significant clinical benefit. The majority of compounds in clinical development for the inhibition of MMP function act by preventing zymogen activation or blocking the activity of activated enzymes. These compounds, however, are reletively non-selective[311,312]. Because MMPs are involved in numerous physiological processes, the development of highly-specific MMP-inhibitors may facilitate the development of tailored anti-

MMP therapies. This could increase treatment efficacy and decrease toxicity due to the broad inhibition of multiple members of this enzyme family. Specifically targeting MMP-1 by retroviral transduction of synovial fibroblasts with a ribozyme that degrades MMP-1 mRNA, but not that of other MMPs, significantly reduced cellular invasiveness in an *in vivo* model of rheumatoid arthritis [313]. Further, an inhibitor that selectively inhibits expression of MMP-1 has been described but has yet to be extensively tested in vivo[314]. These methods of MMP-1 inhibition may have application in anti-KS therapy.

EXPERIMENTAL PROCEDURES

a. *Immunohistochemistry*

Paraffin embedded tissue was sectioned and mounted on charged slides. The slides were deparaffinized, treated in citrate buffer (30 min, steamed), transferred to TBS/Tween Buffer, and placed on an automatic staining machine programmed for staining using the Vectastain Elite ABC kit with diaminobenzidine as the substrate (Vector Laboratories, Burlingame, CA). Monoclonal anti-MMP-1 (Oncogene Research Products, Boston, MA). Slides were counterstained with hematoxylin/eosin.

b. *Derivation of KSHV-infected DMVECs*

Our *in vitro* KS model has previously been described in detail[120]. Briefly, adult primary DMVEC are immortalized by retroviral transduction of the E6 and E7 genes of human papillomavirus HPV type 16 and maintained in endothelial-SFM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% human male AB serum (HS; Sigma, St. Louis, MO), penicillin (100 U/ml), streptomycin (100 mg/ml), glutamine (2 mM), endothelial cell growth supplement (ECGS; 50 mg/ml; Becton Dickinson, Bedford, MA), and G418 (200 mg/ml; GIBCO BRL). These life-extended cells exhibit no overt signs of transformation (i.e. they become contact-inhibited at confluency and do not form colonies in soft agar) but with serial passage can be maintained significantly longer than primary cells. Infectious KSHV is derived from phorbol-ester-treated PEL cells; KSHV-infected DMVEC develop a spindle cell morphology and form colonies in soft agar and foci when monolayers are cultured

post-confluence. Importantly, infected cells maintain the KSHV genome even after extensive passaging.

c. *Western blotting*

Uninfected and KSHV-infected cells were harvested by trypsanization and pelleted by centrifugation at 16,000 g for 30 min at 4°C, then resuspended in 100 ml of 1 x phosphate buffered saline and subjected to three freeze/thaw cycles. The total cell extract was then centrifuged at 16,000 g to spin out excess cellular debris. Total protein was measured with a Bradford assay kit (Biorad). Samples for immunoblotting were prepared by taking 20 mg of total protein and combining with 3 x reducing sample buffer to make 30 ml total volume. The samples were boiled for 3 min and then centrifuged at 16,000 g for 1 min to pellet insoluble material. Each sample (15 ml) was loaded on two identical 12% polyacrylamide gels and resolved by discontinuous electrophoresis (SDS-PAGE) as previously described[252]. After electrophoresis, the gels were transferred to nitrocellulose and then subjected to immunoblot analysis using anti-matrix metalloprotease-1 (Oncogene Research Products) and anti-actin antibodies as per the manufacturers' recommendations. The antigen-antibody complex was then incubated with anti-mouse Ig horseradish peroxidase and anti-rabbit Ig horseradish peroxidase (Amersham Life Sciences). The blot was developed by incubation with chemiluminescent substrate (Pierce) and exposed to Kodak BioMAX MR film.

d. *Quantitative RT-PCR (taqman)*

RNA was isolated using an RNeasy Total RNA kit (QIAGEN, Inc.). RNA samples were treated with RNase-free DNase I to remove any residual genomic DNA contamination (Ambion, Austin, TX). Quantification of RNA was performed by a two-step method. First, cDNA was synthesized using superscript II (Invitrogen). Synthesized cDNA was diluted in H_2O to a final concentration of 5 ng per reaction in TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed on an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) under standard reaction conditions. Relative quantitation of gene expression, as outlined in the Applied Biosystems User Bulletin 2, was conducted to compare uninfected and KSHV-infected samples. Following primer efficiency validation on RNA from KSHV-infected DMVEC, the comparative cycle threshold (C_T) method was performed as outlined in user bulletin 2. The comparative C_T method compares differences in the threshold cycles of target genes between samples after normalization to an endogenous control or calibrator, in this case GAPDH. C_T values vary with target RNA concentration such that a higher target concentration yields an earlier threshold signal over background. Differences between samples during the exponential phase of PCR amplification were calculated by the following equation: $DDC_T = (C_{T\,(target)} - C_{T\,(calabrator)})^{infected} - (C_{T\,(target)} - C_{T\,(calabrator)})^{uninfected}$ and converted to *n*-fold change units by the equation 2^{-DDCT}. The following primers were generated using primer express v1.1 (ABI): MMP-1 *forward*, 5'-GCCCTTCAGCATCCTCAGTTC-3', and *reverse*, 5'-GGTTTGAGACAGCTGCCACA-3'; TIMP-1 *forward*, 5'-

GCCCTTCAGCATCCTCAGTTC-3', and *reverse*, 5'-GGTTTGAGACAGCTGCCACA-3'; TIMP-2 *forward*, 5'-GCCCTTCAGCATCCTCAGTTC-3', and *reverse*, 5'-GGTTTGAGACAGCTGCCACA-3'; TIMP-3 *forward*, 5'-GCCCTTCAGCATCCTCAGTTC-3', and *reverse*, 5'-GGTTTGAGACAGCTGCCACA-3'; TIMP-4 *forward*, 5'-GCCCTTCAGCATCCTCAGTTC-3', and *reverse*, 5'-GGTTTGAGACAGCTGCCACA-3'; GAPDH *forward*, 5'-GAAGGTGAAGGTCGGAGT-3', and *reverse*, 5'-GAAGATGGTGATGGGATTTC-3'.

e. *Construction of adenoviral vectors containing KSHV latency genes*

To examine the role of individual viral genes in the absence of KSHV infection, full-length ORF 71, 72, and 73 transcripts were cloned into an adenoviral expression vector as previously described[302] to create recombinant adenoviruses expressing individual latency-associated genes (Ad/KSHV ORF-73, Ad/KSHV ORF-73, and Ad/KSHV ORF-73). This places each construct under the control of a *tet*-regulated promoter-enhancer element, and protein expression driven by coinfection with an adenovirus expressing the requisite transactivator (Ad/trans). To allow detection of expression of these genes, an N-terminal HA epitope was added to each construct. Recombinant viruses were screened by PCR, and protein expression was confirmed by western immunoblot of infected cell lysates using a mouse monoclonal HA-specific antibody (Sigma, Someplace). Recombinant adenoviruses were plaque

purified, and viral stocks were grown and titered on 293 cells. For DMVEC infections, monolayers at ~80% confluency were incubated with Ad/KSHV ORF-73, Ad/KSHV ORF-73, or Ad/KSHV ORF-73 at a multiplicity of infection (MOI) of 1000 and Ad/trans at an MOI of 200 for 2 h in minimal medium supplemented with 2 % human serum and polybrene (2 μg/ml; Sigma), after which complete culture media was used to bring each culture up to appropriate culture volume. The following morning, cultures were refed with complete medium and incubated an additional 3 days. As a control for infection efficiency and nonspecific effects of adenovirus infection, duplicate monolayers were infected with Ad/trans at an MOI of 1200 under identical infection conditions.

f. ***MMP-1 enzymatic activity***

The MMP-1 Biotrak Assay (Amersham biosciences) was used according to manufacterers's instructions to compare MMP-1 levels in conditioned tissue culture suppernatants from KSHV-infected and uninfected DMVEC, as well as DMVEC transduced by adenovirus constructs encoding the KSHV latency genes (ORFs 71-73). Use of a chemical activator of MMPs, p-aminophenylmercuric acetate (AMPA), allowed us to distinguish between levels of pro-MMP-1 and activated MMP-1 in conditioned supernatants.

Chapter Four

Increased efficiency of phorbol ester-induced lytic reactivation of Kaposi's sarcoma-associated herpesvirus during S-phase

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Citation:

Shane C. McAllister, Scott G. Hansen, Ilhem Messaoudi, Janko Nikolich-Zugich, and Ashlee V. Moses. 2005. Increased Efficiency of Phorbol Ester-Induced Lytic Reactivation of Kaposi's Sarcoma-Associated Herpesvirus during S phase. Journal of Virology 79 (4): 2626-2630.

Coauthor contributions: I developed the hypothesis of this study, designed and carried out of the majority of the experimental approaches, and prepared the original and revised versions of manuscript, tables, and figures. Coauthors contributed the following: S. G. Hansen assisted with qPCR and titering; I. Messaoudi assisted with BrdU staining; J. Nikolich-Zugich and A. V. Moses acted as advisors to me during the development and presentation of this project.

ABSTRACT

Expression of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic genes is thought to be essential for the establishment and progression of KSHV-induced diseases. The inefficiency of lytic reactivation in various *in vitro* systems hampers the study of lytic genes in the context of whole virus. We report here increased expression of KSHV lytic genes and increased release of progeny virus when synchronized cultures of body cavity-based lymphoma-1 cells are treated with a phorbol ester during S-phase of the cell cycle.

Kaposi's sarcoma-associated herpesvirus (KSHV; also human herpesvirus 8) is a gamma-2 herpesvirus and is widely accepted to be the causative agent of Kaposi's sarcoma (KS) as well as the lymphoproliferative disorders primary effusion lymphoma (PEL)[53,54] and multicentric Castleman's disease[55]. KSHV DNA is consistently found associated with almost all clinical forms of KS[47], localizing specifically to endothelial and spindle cells[52] as well as infiltrating monocytes[315]. *In vivo*, the majority of infected cells maintain the virus as a latent infection with only a small percentage of cells spontaneously entering the lytic replication cycle[59,94]. The biological signals that promote lytic induction are not fully understood, though hypoxia[106], coinfection with other viruses[111,112], and expression of KSHV ORF 50 [316], a homolog of Epstein-Barr virus Rta , have been implicated. *In vitro*, KSHV-infected cells can be induced to enter lytic cycle by treatment with phorbol esters or sodium butyrate or by overexpression of ORF 50 [102]. Chemical induction significantly increases the percentage of cells that express lytic gene products above the typical background of 0.5 to 5%, but in most cases the maximum level of lytic induction does not exceed 20%[109,317]. Efforts to study lytic cycle genes *in vitro* within the context of whole virus are thus complicated by the inefficiency of chemical induction.

Fig 1. Schematic of cell synchronization protocol. Asynchronously dividing BCBL-1 cells were arrested in G0 by 24 h incubation in serum free medium. Following the reintroduction of serum, TPA was added to induce lytic replication at 0, 6, 16, and 24 h after release from G0-block (asterisks). Parallel unsynchronized BCBL-1 cells, TPA-treated or untreated, were prepared as controls.

Figure 1

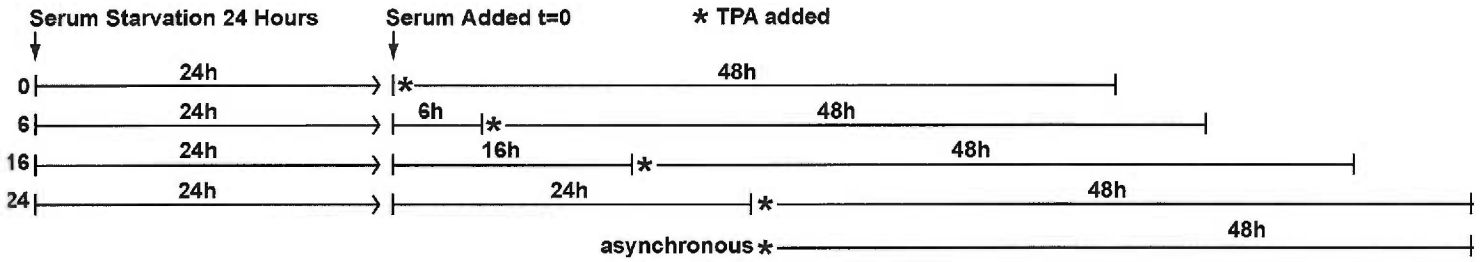
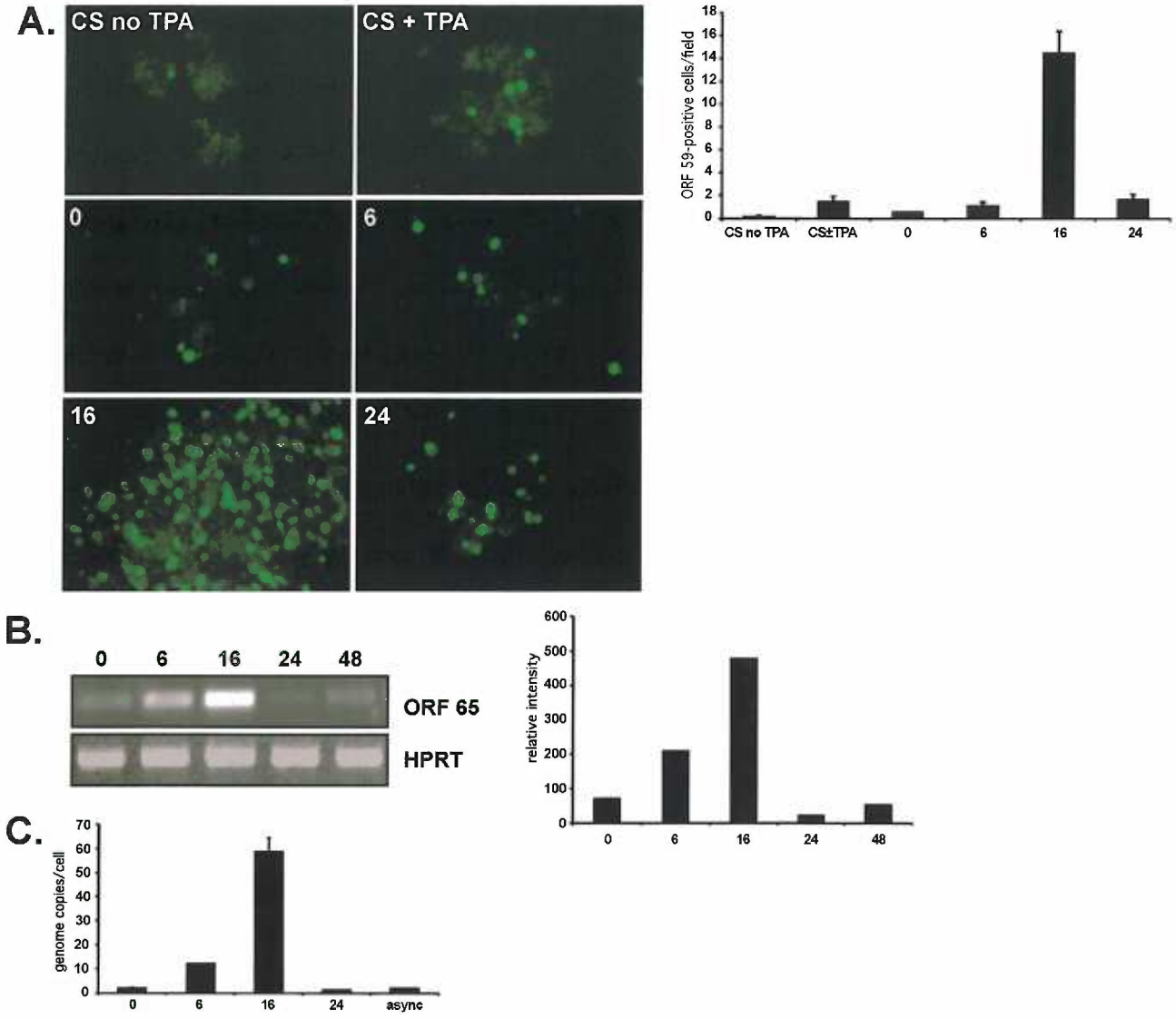

Serum Starvation 24 Hours
Serum Added t=0
* TPA added
0
24h
*
48h
6
24h
6h
*
48h
16
24h
16h
*
48h
24
24h
24h
*
48h
asynchronous
*
48h

Fig 2. Staining BCBL-1 cells for an early lytic KSHV protein (ORF 59, green) revealed that cells were most susceptible to lytic induction by TPA-treatment when synchronized cells were induced 16 h following release from G0 block (A, panel 16). The average number of ORF 59-positive cells counted over nine random fields was 10-fold higher in synchronized cells 16 h following the reintroduction of serum compared with cells incubated in constant serum (CS) treated with TPA (A, bar graph, CS + TPA vs. 16). Expression of a late lytic gene (ORF 65, determined by RT-PCR analysis) was also greatest at this time point (B, lane 16 of agarose-ethidium bromide gel). Densitometry analysis of PCR-band intensity showed an approximately seven-fold elevation of ORF 65 expression at 16 h compared with the 0 h sample (B, bar graph,16 vs. 0). Increased expression of lytic genes at 16 h was accompanied by a thirty-fold increase in release of viral progeny as determined quantitative SYBR-green PCR (D).

Figure 2


A.
CS no TPA
CS + TPA
0
6
16
24
ORF 59-positive cells/field
CS no TPA
CS±TPA
0
6
16
24
B.
0
6
16
24
48
ORF 65
HPRT
relative intensity
C.
genome copies/cell
0
6
16
24
async

DNA viruses, including herpesviruses, are known to influence cell cycle progression in host cells to optimize the cellular environment for viral replication[318,319]. We hypothesized, therefore, that inducing lytic reactivation in synchronized cultures of KSHV-infected cells at different stages of the cell cycle might reveal a point of maximum inducibility. To test this hypothesis, asynchronous cultures of BCBL-1 cells, a PEL cell line persistently infected with KSHV but not Epstein-Barr virus [obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health, contributed by Michael McGrath and Don Ganem], were synchronized at G_0 by 24 h incubation in serum free RPMI-1640 medium (SFM) supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine. Asynchronously dividing cells (1.2×10^7) were pelleted, resuspended in 20 ml SFM, and then distributed evenly between 4 T25 flasks (3×10^6 cells in 5 ml SFM/culture). After 24 h, 500 µl fetal calf serum (FCS; 10% final concentration) was added to induce reentry into the cell cycle. At 0, 6, 16, and 24 h following the addition of serum, lytic replication was induced by the addition of tetradecanoyl phorbol acetate (TPA; 20 ng/ml) and cultures were incubated for an additional 48 h (figure 1). Parallel unsynchronized cultures, TPA-treated and untreated, were prepared as controls. Trypan blue exclusion verified that cell viability was not appreciably effected by any of the experimental conditions employed (data not shown). Following TPA induction, cultures were spotted onto glass slides and air dried, fixed with 2% paraformaldehyde, permeabilized with 0.05% triton, stained for a KSHV early lytic marker (ORF 59) with a monoclonal antibody generously

provided by Bala Chandran (The University of Kansas Medical Center, Kansas City, KA) followed by goat anti-mouse FITC-labeled secondary antibody (Biosource International, Camarillo, CA), and examined on a Zeiss fluorescent microscope. Figure 2a shows representative images for each treatment. Calculation of the average number of ORF 59-positive cells from nine random fields shows a ten-fold increase in ORF 59 reactivity in the synchronized BCBL-1 culture that was induced with TPA 16 h after release from G_0-block compared with the unsynchronized TPA-treated culture (figure 2b, 16h vs. constant serum [CS] + TPA; error bars = standard error of the mean [SEM] from quadruplicate determinations). This observation suggests that the cellular environment 16 h into the cell cycle is better able to support lytic replication following chemical induction.

We next compared expression levels of a late lytic gene, ORF 65, by reverse transcriptase-polymerase chain reaction (RT-PCR) in synchronized cultures prepared as described in figure 1. Total cellular RNA was harvested using the RNeasy RNA isolation kit (QIAGEN Inc., Valencia, CA). Five ng DNase-treated RNA was used as template for RT-PCR for ORF 65 using the Titan one-tube RT-PCR kit (Roche, Indianapolis, IN) per manufacturer's instructions (ORF 65 forward primer: 5'-GGCGTTAATTAAGCTAGCAT GTCCAACTTTAAGGT GAGA-3', ORF 65 reverse primer: 5'-AAACCTATTTCT TT TTGCCAGAGG-3'; cycle conditions: 50°C for 30 min, 94°C for 5 min [1 cycle] followed by 94°C for 30 sec, 56°C for 1 min, 68°C for 1 min [40 cycles], followed by 72°C for 10 min [1 cycle]). The cellular hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was amplified from each sample as a control for cDNA synthesis and yielded consistent amplification

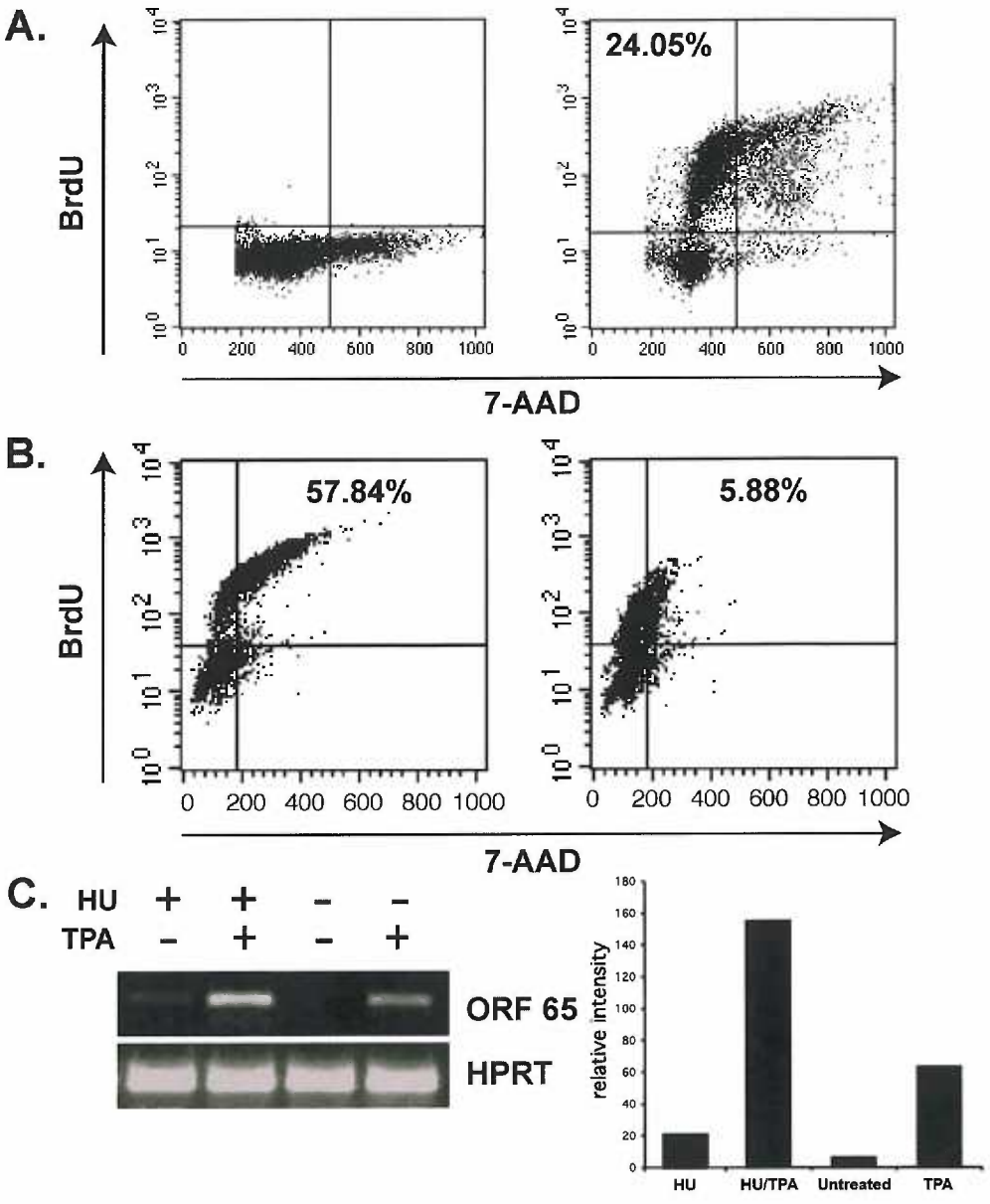
products from sample to sample. Parallel reactions using Vent polymerase (NEB, Beverly, MA) showed that there was no DNA contamination in template RNAs (data not shown). Cultures without TPA induction had ORF 65 levels below the level of detection, as did TPA-induced cultures treated with gancyclovir, a drug that blocks viral replication[320] (data not shown). Figure 2c shows the levels of ORF 65 expression when RT-PCR products were visualized by electrophoresis through agarose-ethidium bromide gels. Densitometry analysis of band intensity revealed that ORF 65 expression was highest in the synchronized BCBL-1 culture that was induced with TPA 16 h after release from G_0-block (figure 2c, bar graph), again indicating that the intracellular environment of BCBL-1 cells is most conducive to KSHV lytic reactivation by chemical induction 16 h following release from G_0-block.

To determine if the increase in lytic gene expression seen in the previous experiments represents abortive reactivation or if there is a concomitant increase in release of viral progeny, KSHV genome copies in culture supernatants were determined by quantitative SYBR-green PCR. Culture supernatants from synchronized and unsynchronized cultures prepared as in figure 1 were centrifuged for 1 h at 22,000 rpm over 5 ml 20% sorbitol. DNA from the resultant pellets was harvested using the Purgene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to manufacturer's instructions and resuspended in 10 μl dH_2O; 1 μl was used as template per reaction using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Quantitative PCR was performed on an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems; Foster City, CA) under standard reaction conditions. Full length ORF 65 was cloned into pGEM-

TEasy (pGEM-TEasy Vector System; Promega, Madison, WI; ORF 65 forward primer: 5'-CAGGAGCGACTGGATCATGA-3', ORF 65 reverse primer: 5'-TTTCCCTGATCCAGGGTATTCA-3') and serially diluted for construction of a standard curve. Culture supernatant from synchronized BCBL-1 cells chemically induced 16 h following release from G_0-block yielded an average of approximately 60 KSHV genomes/cell, a 30-fold increase in virus production compared with the unsynchronized TPA-induced culture (figure 2d), indicating that the increased expression of lytic genes under these culture conditions is reflective of increased productive rather than abortive lytic replication.

Fig 3. Quantification of DNA content showed that after 24 h incubation in serum free medium synchronized BCBL-1 cells were arrested in G_0, as shown by the majority of cells having a 2N DNA content and no BrdU-incorporation (A, left panel). However, 16 h after release from G_0-block, cells reach S-phase of the cell cycle as shown by an increase in the percentage of cells that have incorporated BrdU into newly synthesized DNA (A, right panel). Blockade at early S phase following HU treatment was verified by the same method (B). HU-treated cultures had significantly reduced numbers of cells progressing through S phase (6%; B, upper right quadrant of right panel) compared with untreated cultures (63%; upper right quadrant of left panel). Expression of the late lytic gene ORF 65 (determined by RT-PCR analysis) was greater in TPA-induced cultures of BCBL-1 cells that had been previously synchronized at early S phase of the cell cycle by incubation with HU (C).

Figure 3


A.
BrdU
24.05%
7-AAD
B.
BrdU
57.84%
5.88%
7-AAD
C.
HU + + - -
TPA - + - +
ORF 65
HPRT
relative intensity
HU
HU/TPA
Untreated
TPA

The S phase of the cell cycle is reached between 12 and 16 h after the addition of serum to cultures synchronized by serum starvation[321]. To determine whether synchronized BCBL-1 cells were in S phase 16 h following release from G_0-block, we determined the DNA content of these cells using the BrdU Flow kit (BD Pharmingen, San Diego, CA) per manufacturer's instructions. As expected, BCBL-1 cells synchronized by 24 h serum starvation were predominantly stalled in G_0, indicated by a 2N DNA content (7-AAD staining of total DNA content; figure 3a, lower left quadrant of left panel) and the absence of DNA replication (BrdU staining of actively replicating DNA; figure 3a, upper left and right quadrants of left panel). In contrast, 16 h following the reintroduction of serum, 70% of cells began DNA synthesis (figure 3a, upper left and right quadrants of middle panel) with 24% of the total achieving a 4N DNA content (upper right quadrant only). TPA-treatment did not alter the progression through the cell cycle past S-phase (data not shown). Therefore, G_0-synchronized BCBL-1 cells are predominantly in S-phase 16 h after reentering the cell cycle.

We next employed hydroxyurea (HU; 1.5 mM; Fisher, Pittsburgh, PA) to halt cell cycle progression in S phase; HU inhibits ribonucleotide reductase, thereby limiting the ribonucleotide pool and blocking DNA synthesis[322]. We verified that HU treatment blocked BCBL-1 cells in S-phase by measuring the DNA content of HU-treated and untreated cells by BrdU and 7-AAD staining as above. BCBL-1 cultures were first synchronized by serum starvation for 24 h; 10% serum was then added and cultures were allowed to progress through the cell cycle for 20 h in the presence or

absence of 1.5 mM HU plus 10 μM BrdU. Figure 3b demonstrates that approximately 58% of gated cells from untreated cultures were able to achieve a 4N DNA content (upper right quadrant of left panel), whereas 10-fold less HU-treated cells (upper right quadrant of right panel) were able to proceed past early S-phase, thus verifying that HU-treatment synchronized BCBL-1 cells at S-phase. To corroborate our evidence that BCBL-1 cells are most responsive to lytic induction during S phase, BCBL-1 cultures with and without HU were divided into TPA-treated and untreated pools. ORF 65 expression, determined by RT-PCR as described in figure 2c, was approximately 2.5-fold greater in TPA-induced BCBL-1 cultures that had been previously synchronized at early S-phase with HU compared with asynchronous cells treated with TPA (3c, bar graph). This finding is consistent with other data presented here and indicates that the intracellular environment of BCBL-1 cells is most able to support KSHV lytic replication following chemical induction during S-phase of the cell cycle.

This simple method may prove useful in the study of KSHV lytic cycle genes in the context of whole virus as well as increasing the efficiency of high-titer KSHV stock preparation. More broadly, determination of specific S-phase cellular products which account for the increased inducibility of infected cells as demonstrated here may shed further light on the molecular mechanisms governing induction of KSHV lytic replication.

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Chapter Five

Summary of findings

KS begins as hyperproliferative lesions driven in part by the alterations of endothelial cell physiology resulting from KSHV infection. Development of truly transformed sarcomas is associated with advanced disease, though the essential mechanisms governing the outgrowth of clonal spindle cell populations is poorly understood. The direct action of KSHV genes leads to many of the alterations of endothelial cells physiology seen in KS, but these changes are only part of the story. The main aim of my graduate studies was to identify cellular participants in KSHV-mediated transformation of endothelial cells that represent targets for novel therapeutics. Among the six characteristic alterations of cellular physiology governing the transformation of normal human cells into malignant derivatives, I have described two mechanisms by which KSHV contributes to the transformation of endothelial cells.

The initial derivation of KSHV-infected B cell lines from patients with primary effusion lymphoma, another rare KSHV-associated tumor, facilitated the characterization of the KSHV genome and gene expression patterns. Later, the development of endothelial cell-based *in vitro* KS models (reviewed in chapter 1 and appendix 2 of this dissertation) contributed to our understanding of KS pathogenesis by allowing the identification of specific changes in host-cell gene expression

induced by *de novo* and long-term KSHV infection. The work presented in this dissertation describes my specific contributions to KSHV research and has utilized both of the above-mentioned systems to do so. The overall model of KS pathogenesis that I propose in this dissertation (figure 6 of chapter 1) incorporates the experimental work of myself and others, takes into account recent clinical advances in KS chemotherapy and draws together multiple theories of KS that by themselves cannot account for the complexities of KSHV pathogenesis. Here I present my experimental findings in the context of this model.

1. KSHV upregulation HO-1: induction of KS spindle cell proliferation and protection from heme-mediated toxicity

Comparison of the protein expression profiles of KSHV-infected and uninfected DMVEC by a Western blot-based proteomics method revealed that the enzyme HO-1, a known mediator of angiogenesis, was among the cellular genes upregulated by KSHV infection (Chapter 2). Immunohistochemical studies on KS lesional tissue revealed strong HO-1 reactivity, thus confirming the *in vivo* relevance of this finding. Given the vascular nature of KS lesions, host genes involved in angiogenesis that are induced by KSHV infection may provide pathogenesis-specific targets for pharmacological intervention. I have demonstrated that upregulation of HO-1 in DMVEC *in vitro* leads to an increase in cellular proliferation upon exposure to free heme, a possible mechanism contributing to KS lesion progression where the presence of extravasated and degenerating erythrocytes creates a heme-rich microenvironment. Importantly, characterized HO-1 inhibitors are in clinical use for

other disorders. I propose for the first time that these inhibitors are worth evaluating as potential chemotherapy for KS, either as systemic or topical agents. This work is unique in that it proposes a functional consequence of a prominent histological feature of KS lesions (extravasation of erythrocytes) and is also the first study to examine changes in EC gene expression following KSHV-infection using a proteomics method. No other herpesvirus has been demonstrated to upregulate HO-1. This is likely a reflection of the unique feature of KS lesions, i.e. extravasated erythrocytes. No other lesions known to be caused by herpesviral pathogens exhibit this unique histological feature. The upregulation of HO-1 in such a setting would not be expected to yield any growth or survival advantage (figure 1).

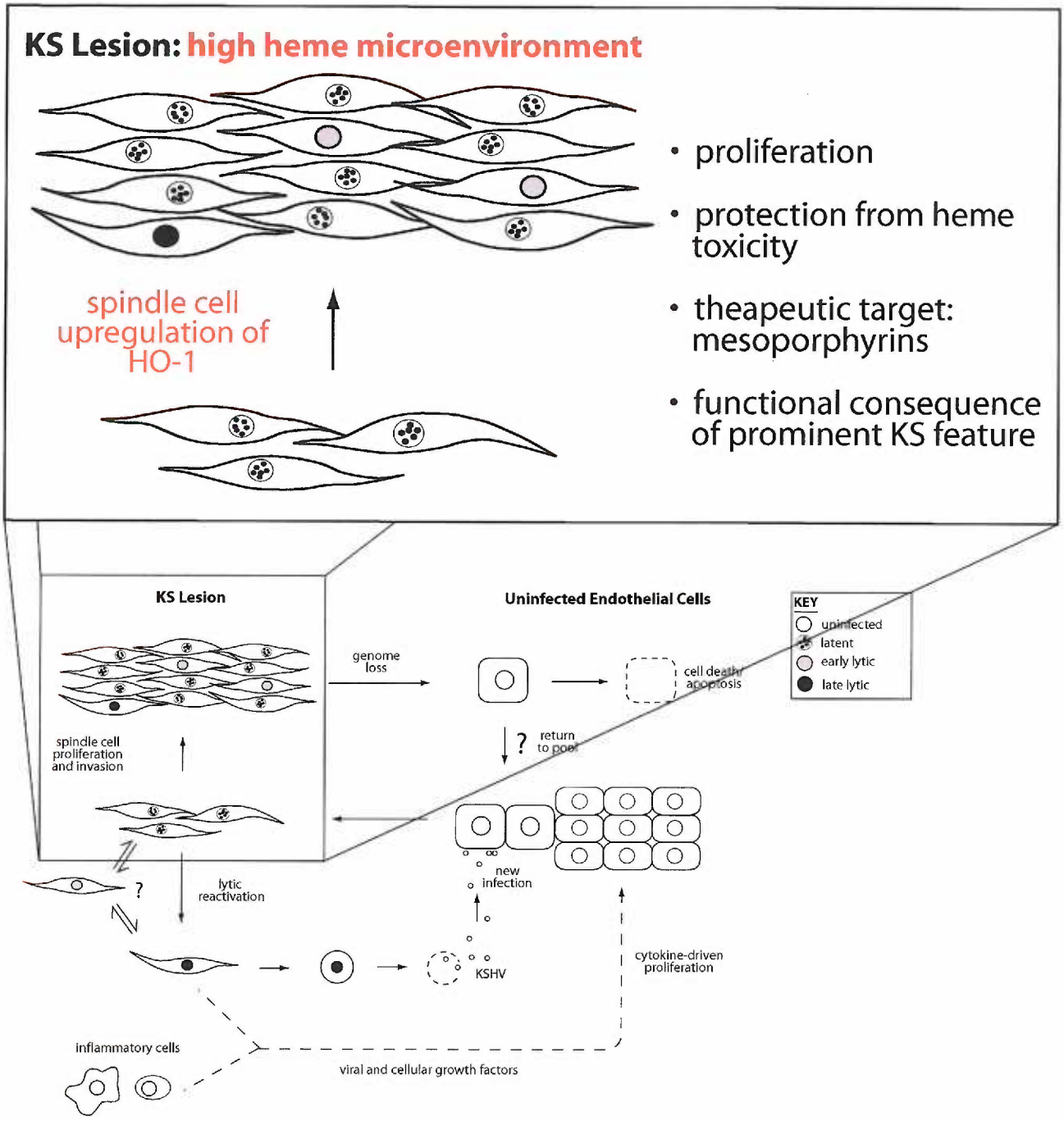
In addition to stimulating proliferation, the products of HO-1 enzymatic activity have been shown by others to act as cytoprotectants through antioxident and anti-apoptitic activities. HO-1 upregulation by KS spindle cells may therefore represent a mechanism by which these cells achieve autonomy from growth factor signaling and prevent apoptosis, two key features that pre-malignant cells must acquire. In this light, the physiological changes in endothelial cell physiology wrought by KSHV infection that lead to the formation of defective cellular junctions and subsequently RBC extravasation can be viewed as a progression factors for KS. Alternately, RBC extravasation could be a byproduct of KSHV-induced alterations in gene expression rather than an evolutionarily selected phenomenon. In this case, upregulation of HO-1 would be an adaptive response necessary for spindle cells to cope with the potentially toxic levels of heme within the KS microenvironment. Future work should clarify which of the heme metabolites is responsible for the

proliferative advantage induced by HO-1 overexpression. In addition, screening of a wider panel of KS biopsy material for HO-1 expression should be conducted to confirm that other epidemiological forms of KS display a similar trend. Provided that HO-1 expression is found to be a common feature of KS, evaluation of HO-1 inhibitors should be evaluated as therapeutic agents for KS chemotherapy.

FIG. 1. The model of KS lesion progression presented in this dissertation (chapter 1) is based on recent clinical advances in KS chemotherapy and draws together multiple theories of KS that by themselves cannot account for the complexities of KS lesions. My experimental findings contribute to our understanding of mechanisms governing the accumulation of spindle cells in progressing KS lesions; these findings are reproduced in the three figures of this chapter.

No functional significance has previously been ascribed to the abundant extravasated red blood cells observed in KS lesions. However, my work demonstrating upregulation of HO-1 by endothelial cells following KSHV infection is suggests a possible consequence of this curious histological feature: the heme-rich lesional microenvironment may drive KS spindle cells to proliferate. In addition, heme exposure can induce severe oxidative damage; upregulation of HO-1 may, therefore, also represent a mechanism by which spindle cells protect themselves from the toxic properties of heme within KS lesions. I propose for the first time that inhibitors of HO-1, the mesoporphyrins, are worth evaluating as potential chemotherapy for KS, either as systemic or topical agents.

Figure 1


KS Lesion: high heme microenvironment
spindle cell upregulation of HO-1
• proliferation
• protection from heme toxicity
• theapeutic target: mesoporphyrins
• functional consequence of prominent KS feature
KS Lesion
spindle cell proliferation and invasion
genome loss
Uninfected Endothelial Cells
cell death/ apoptosis
? return to pool
KEY
uninfected
latent
early lytic
late lytic
new infection
lytic reactivation
KSHV
cytokine-driven proliferation
inflammatory cells
viral and cellular growth factors

2. KSHV upregulation MMP-1: induction of KS spindle cell invasion

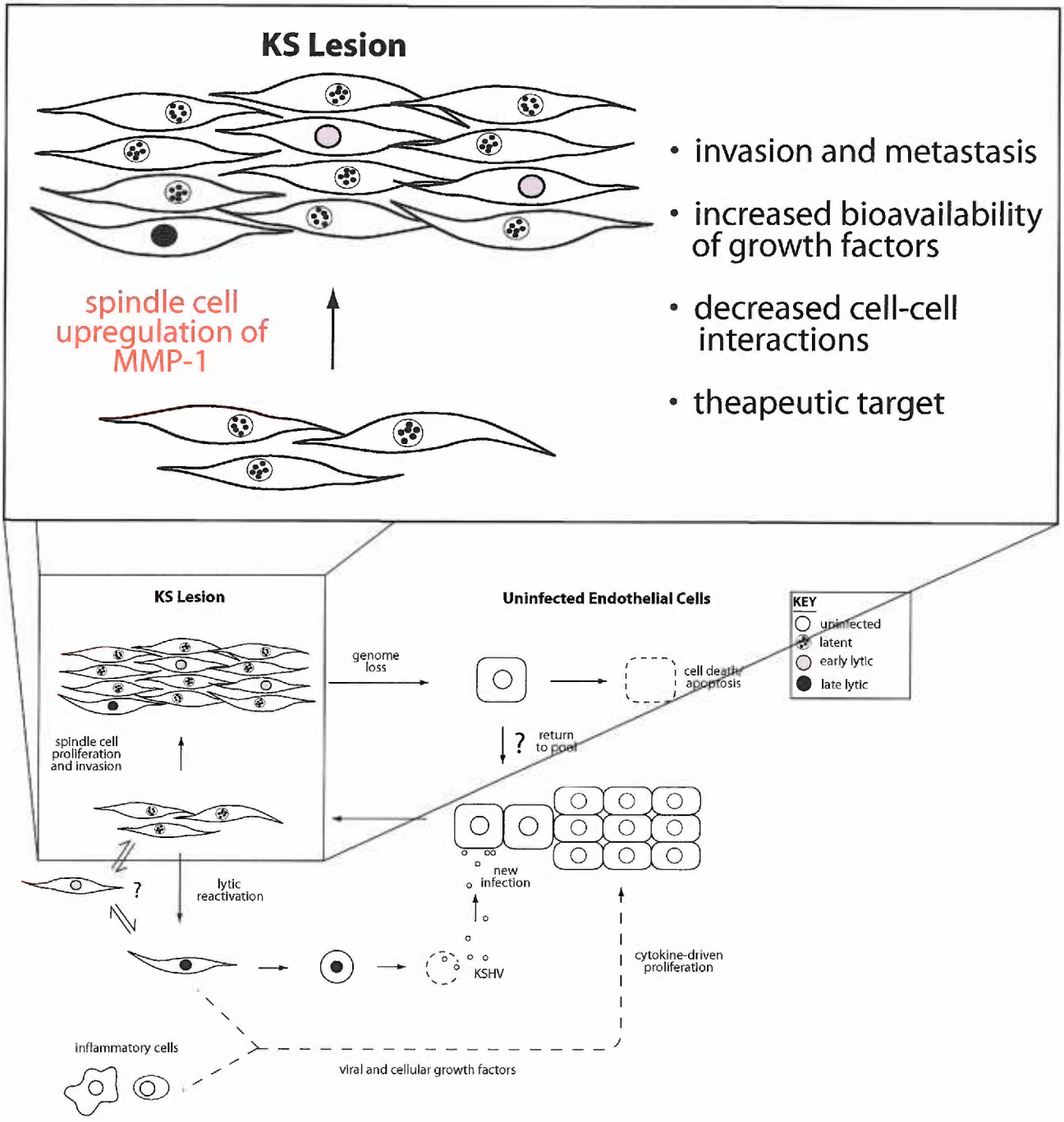
Chapter 3 describes my studies demonstrating a KSHV-induced imbalance in expression of the cellular enzyme MMP-1 and its endogenous protease inhibitors. The tissue remodeling machinery of higher organisms is tightly regulated under normal physiological conditions, and dysregulation is a hallmark of many diseases, including cancers. In KS, manipulation of the balance between stasis and proteolysis may potentiate KSHV-infected spindle cell invasion of the extracellular matrix, thus contributing to lesion progression and metastasis via circulating spindle cells. A role for MMP-1 has been described for other invasive pathogenic processes, including breast cancer, melanoma, and rheumatoid arthritis. Given the important role of MMP-1 in various invasive processes, potentially including KS, development of inhibitors specific for MMP-1 may yield significant clinical benefit. The majority of compounds in clinical development for the inhibition of MMP function act by preventing zymogen activation or blocking the function of activated enzymes. These compounds, however, are relatively non-selective. Because MMPs are involved in numerous physiological processes, the development of highly-specific MMP-inhibitors may facilitate the development of tailored anti-MMP therapies. This could increase treatment efficacy and decrease toxicity due to the broad inhibition of multiple members of this enzyme family. For example, specifically targeting MMP-1 by retroviral transduction of synovial fibroblasts with a ribozyme that degrades MMP-1 mRNA, but not that of other MMPs, significantly reduced cellular invasiveness in an *in vivo* model of rheumatoid arthritis. Of note, an inhibitor that selectively inhibits expression of MMP-1 has been described, but has yet to be extensively tested *in vivo*

for different pathological conditions. Such methods of MMP-1 inhibition may have application in anti-KS therapy (figure 2).

MMP-1 protein is abundantly expressed in nasopharyngeal carcinoma, an EBV-mediated neoplasm[230]. Thus MMP-1 may represent a common mechanism underlying gammaherpesvirus-mediated oncogenesis. Expression of MMP-1 by KS spindle cells may represent an important mechanism by which these cells invade local tissue, one of the key attributes of transformed cells. It is unknown currently whether KS metastasizes. Treatment of cancer with MMP inhibitors has been relatively disappointing so far, in part perhaps because of the differences in specificity of compounds tested as well as conspicuous gaps in our knowledge of the regulation of and interplay between different members of the large MMP family. In time we may have a more complete understanding of the role specific MMP family members play in KS progression. It may be that MMP-1 expression in KS lesions contributes to lesion progression by increasing the bioavailability of extracellular growth factors. Disruption of the extracellular matrix by MMPs produces ECM fragments with different binding and signaling characteristics: it is possible that MMP-1 contributes in this fashion and may be acting cooperatively with changes in the integrin expression profile of spindle cells. Similar mechanisms are at work in other tumors, driving proliferation of tumor cells as well as angiogenesis.

FIG. 2. Upregulation of MMP-1 by KS spindle cells may facilitate local tissue invasion and metastasis by increasing the ability of lesional spindle cells to degrade components of the extracellular matrix. MMP expression can also contribute to tumor development by increasing the bioavailability of ECM-bound growth factors and by decreasing growth-inhibitory cell-to-cell contacts. MMP inhibitors have been tested in clinical trials against numerous cancers including KS. However, the majority of MMP inhibitors are not active against MMP-1. Therefore, inhibitors with activity against MMP-1 should be considered possible therapeutic agents for the treatment of KS.

Figure 2


KS Lesion
spindle cell upregulation of MMP-1
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KS Lesion
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KEY
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genome loss
cell death/ apoptosis
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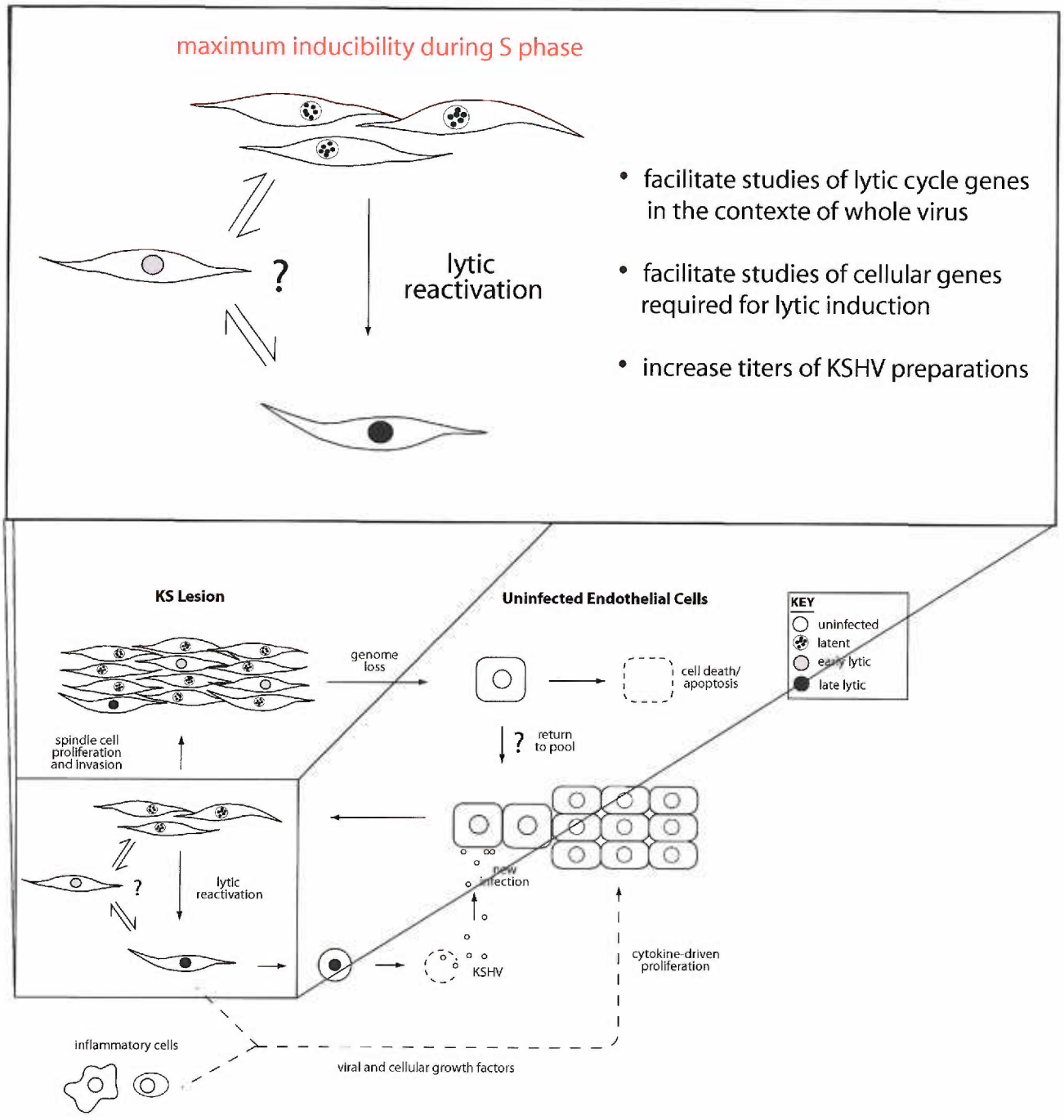
KSHV
cytokine-driven proliferation
inflammatory cells
viral and cellular growth factors

3. Increased reactivation of KSHV during S phase of the cell cycle

KSHV pathogenesis results from the action of both latent and lytic viral genes. The tendency of established EC and B cell-based *in vitro* culture systems to favor latency reflects the *in vivo* state, but hampers efficient study of lytic gene function. In chapter 4, I describe the development of experimental methods that should facilitate study of the induction and consequences of lytic gene expression. I report increased expression of KSHV lytic genes and increased release of progeny virus when synchronized cultures of body cavity-based lymphoma-1 cells (BCBL-1 cells) are treated with a phorbol ester during S-phase of the cell cycle indicating that the intracellular environment of BCBL-1 cells is most able to support KSHV lytic replication during S-phase. This simple method may prove useful in the study of KSHV lytic cycle genes in the context of whole virus as well as increasing the efficiency of high-titer KSHV stock preparation. More broadly, determination of specific S-phase cellular products which account for the increased inducibility of latently-infected cells may shed further light on the molecular mechanisms governing induction of KSHV lytic replication.

FIG. 3. Demonstration of increased induction of lytic replication during S-phase of the cell cycle is an important finding that will surely aid studies of the signals governing induction and the consequences of lytic replication. More pragmatically, this simple method should be useful for the preparation of high titer KSHV stocks.

Figure 3


maximum inducibility during S phase
?
lytic reactivation
• facilitate studies of lytic cycle genes in the contexte of whole virus
• facilitate studies of cellular genes required for lytic induction
• increase titers of KSHV preparations
KS Lesion
Uninfected Endothelial Cells
KEY
uninfected
latent
early lytic
late lytic
genome loss
cell death/ apoptosis
? return to pool
spindle cell proliferation and invasion
?
lytic reactivation
new infection
KSHV
cytokine-driven proliferation
inflammatory cells
viral and cellular growth factors

4. Summary of findings

I have identified two novel therapeutic targets for KS, described a role for one of the dominant histological features of KS lesions, described a simple method that may help other scientists in their studies of KSHV lytic genes and lytic reactivation, and developed a unifying model describing our current understanding of KS pathogenesis (figure 1). In addition to my experimental contributions to the field of KSHV pathogenesis, I have written two reviews that critically evaluate the work of myself and others, and look to provide a model for KS pathogenesis that encompasses contributions from multiple sources. Appendix 1 describes the identification of novel therapeutic targets for KS therapy using experimental models, genomic and proteomic profiling and clinical studies. Appendix 2 is a review of *in vitro* cell culture systems used in KSHV pathogenesis studies that acknowledges the strengths and weaknesses in these models and puts into perspective how each has contributed to our understanding of the complex KS lesional environment. These reviews are unique in the scope undertaken and should provide both a useful chronology of studies to date and a valuable perspective.

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Appendix One

Functional Genomics and the Development of Pathogenesis-Targeted Therapies for Kaposi's Sarcoma

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Citation: Shane C. McAllister, Klaus Frueh, and Ashlee V. Moses.

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ABSTRACT

Kaposi's sarcoma (KS) is a multifocal angioproliferative disorder affecting the skin, mucosa, and viscera of individuals infected with human herpesvirus-8 (HHV-8; also Kaposi's sarcoma-associated herpesvirus, KSHV). KS is the most common neoplasm in AIDS patients; the clinical outcome of AIDS-KS is significantly improved by highly active anti-retroviral therapy (HAART). However, in Africa, where the severest manifestations of KS occur, there is limited access to these and other effective but expensive drugs. Here we present a review of current efforts to identify novel therapeutic targets for treatment of KS using functional genomics, with recommendations regarding the development of economically-feasible treatments for use in Africa.

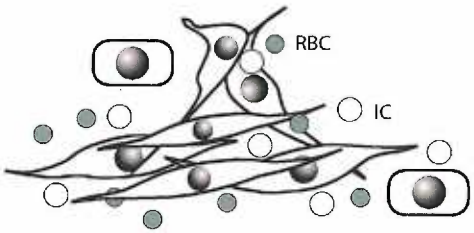
1. Kaposi's sarcoma

Kaposi's sarcoma (KS) was first described in 1872 by the Hungarian dermatologist Moriz Kaposi [323] but the disease did not benefit from substantial investigation until an aggressive, AIDS-associated variant was described over a hundred years later [2]. Four distinct epidemiological forms of KS are now recognized [4] and are distinguished by relative severity, HIV serostatus, and geographic distribution: classical, the indolent form first described in elderly Mediterranean men; AIDS-KS, severe and often fatal, the most common AIDS-associated neoplasm; endemic, an aggressive but non-HIV-associated variant found in regions of Africa; and iatrogenic, a form occasionally complicating organ transplantation in HIV seronegative patients. The four classes of KS are histologically indistinguishable, characterized principally by disorganized networks of abnormal microvasculature with numerous extravasated inflammatory cells and erythrocytes [5] (figure 1a).

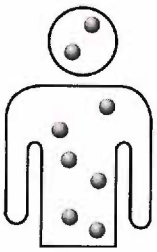
Fig 1. (A) KS is a multifocal angioproliferative disorder affecting the skin, mucosa, and viscera. Lesions are characterized by disorganized networks of abnormal microvasculature with numerous extravasated inflammatory cells (IC) and red blood cells (RBC). Chemotherapy for KS involves control of HIV if applicable and several cytotoxic agents. Pathogenesis-specific chemotherapies for KS are in development. (B) *De novo* infection of EC with KSHV induces a spindle cell phenotype with evidence of transformation, including loss of contact inhibition and colony formation in soft agar assays. There are many EC-based *in vitro* KS models used to dissect viral mechanisms of pathogenesis (see text for details). All models aim to generate age- and passage-matched infected and uninfected cultures, though the degree to which these models accurately represent the *in vivo* tumor microenvironment is a matter of rigorous debate.

Figure 1

A.

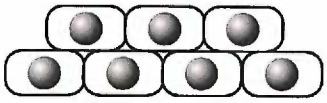


progression
- disorganised vascular slits
- extravasated inflammatory cells and erythrocytes



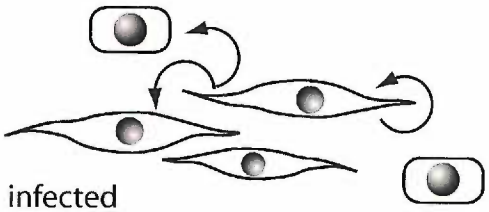
treatment
- dermal, mucosal, & visceral lesions
- HAART for AIDS-KS
- alitretinoin gel, liposomal daunorubicin, liposomal doxorubicin, paclitaxel, IFN-alpha

B.



uninfected
- contact inhibited
- anchorage-dependent

KSHV



infected
- anchorage-independent growth
- loss of contact inhibition
- paracrine & autocrine influences

2. Kaposi's sarcoma-associated herpesvirus

A putative etiological agent for KS, Kaposi's sarcoma-associated herpesvirus (KSHV; also human herpesvirus-8) was first described in 1994 [47] and is now widely acknowledged as the etiologic agent of all forms of KS [47,52] as well as the lymphoproliferative disorders multicentric Castleman's disease (MCD) [55] and primary effusion lymphoma (PEL) [53,54]. KSHV is a gamma-2 lymphotropic herpesvirus; like all herpesviruses, KSHV is a large enveloped virus with a double-stranded DNA genome of substantial coding capacity. The KSHV genome includes viral genes unique to this human pathogen, genes that share homology with other members of the *herpesviridae*, as well as genes that appear to have originated in the host genome [58,324].

Both *in vitro* and *in vivo*, KSHV is maintained as a viral episome in a primarily latent state characterized by expression of few viral gene products [65-67]. At least four gene products, vFLIP, vCYC, LANA, and Kaposin, are believed to comprise the viral latency expression program of KSHV and are consistently expressed in all virally infected cells in KS, PEL, and MCD [65-67,73]. An additional gene, LANA 2 [87], is expressed during latency in MCD and PEL but not KS. The gene product of ORF 71, vFLIP (viral FLICE-inhibitory protein/K13) can block FAS-mediated apoptosis and is thought to act as a tumor progression factor by interfering with apoptotic signals induced by cytotoxic T lymphocytes [74,75]. There is evidence that vFLIP is responsible for constitutive NF-kB activation [76,325], which is thought to mediate some of the anti-apoptotic function of vFLIP[307] and also aide the maintenance of latency by blocking lytic reactivation [326]. vCYC (ORF 72) is a homolog of cellular D-type cyclin and can drive quiescent cells into S phase in part

by inhibiting retinoblastoma protein (Rb) activity [77-80]. The latency-associated nuclear antigen (LANA, ORF 73) targets both p53 and Rb, enabling modulation of cellular transcription by blocking both activation of p53-dependent promoters [83] and inducing activation of E2F-dependent genes [84] . Additionally, LANA can tether multiple copies of the viral episome to host cell chromosomes and is thus indispensable for maintenance of the viral genome during host cell division [86]. Kaposin (K12) codes for three proteins, A, B, and C, which are translated from each reading frame in a common message [73,90], some of which have been shown to induce transformation [91,92,327]. Thus the latency program of KSHV appears to result in maintenance of the KSHV episome by inducing proliferation of host cells and prevention of apoptosis.

Upon reactivation, KSHV expresses a wider repertoire of gene products in a coordinated cascade, which culminates in release of progeny virions from the infected cell; these genes, some encoding homologs of cellular angiogenesis- and proliferation-modifying genes such as vGCR [236,237] and vIL-6 [238], are classified as lytic gene products and are expressed in only a minority of cells *in vivo* [95-97]. A number of KSHV lytic genes code for interesting regulatory proteins that could conceivably drive proliferation or transformation; however, reactivation of a latent herpesvirus infection and subsequent completion of the viral lytic expression cascade is normally considered incompatible with survival of a host cell. Thus there is a diversity of opinion concerning the timing and consequences of lytic gene expression. For example, lytic gene products could have paracrine influences on both latently-infected and uninfected cells [239]. Additionally, the dogma of cytotoxicity upon lytic replication has been challenged and some speculate that a limited subset of lytic

genes could be expressed under certain conditions resulting in paracrine and autocrine signaling [121,122]. Evidence that anti-herpes agents such as ganciclovir and foscarnet, which inhibit lytic but not latent infection, can improve the clinical outcome of KS strengthens the assertion that ongoing lytic gene expression is involved in KS pathogenesis [116,188,189] and further studies are sure to clarify the specific consequences of lytic gene expression in KS pathogenesis.

3. KS therapy

The extent and severity of KS, as well as comorbid factors (e.g. HIV serostatus, iatrogenic immune suppression), determines the clinical course of KS therapy. Patients with stable or slowly progressive AIDS-KS with a limited distribution (including dermal, lymph node, and oral lesions or non-symptomatic visceral disease) are routinely started on HAART. AIDS-KS is known to be responsive to HAART, and control of HIV load is the primary goal in the treatment of AIDS-KS [17]. Pain, edema, and cosmetic concerns arising even from slowly progressive disease can be addressed with local therapies such as irradiation [19,20] or excision, Alitretinoin gel (a retinoid approved for topical application) [21], interferon-alpha [23], or experimental therapies [24] such as novel angiogenesis inhibitors (reviewed in [17,25-27]). Advanced or quickly progressing disease can be treated with systemic cytotoxic chemotherapy. Liposomal anthracyclines (daunorubicin, doxorubicin) are considered first line agents [28-30] while paclitaxel is approved as a second line agent [31-33]. Regression of iatrogenic KS has been noted following dose reduction of immunosuppressive therapy; treatment of KS in organ transplant patients requires a careful balance between

preservation of grafted tissues and prevention of death due to disseminated KS [34,35]. Pathogenesis-targeted chemotherapeutic agents are in various stages of development, from preliminary observation to clinical trials [17,25-27]. Because of the recurrent nature of KS and the development of disease that is refractory to standard treatments, novel therapeutics for the treatment of KS are needed. Because the prevalence of advanced KS is highest in the developing world where health care resources are limited [45], development of less expensive treatment options should be a priority.

4. KSHV and endothelial cells

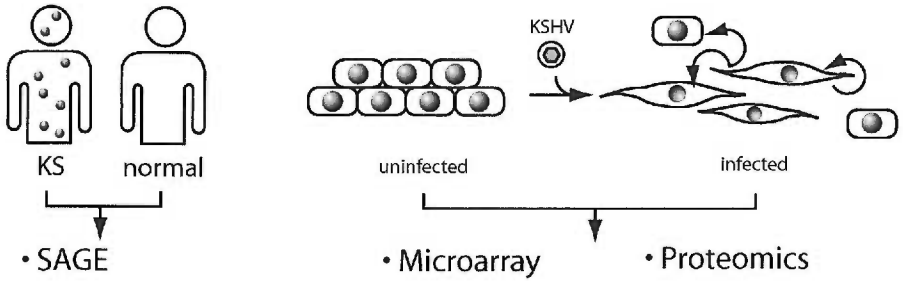
Endothelial cells are the presumptive precursors of the characteristic spindle cells in KS lesions [6-10]. Spindle cells harbor the KSHV genome *in vivo* [52,94,153], an association recapitulated by us and others in *in vitro* endothelial cell- (EC-) based models [120,156-158]. *De novo* infection of DMVEC with KSHV induces a spindle cell phenotype with evidence of transformation, including loss of contact inhibition and colony formation in soft agar assays (figure 1b). Because explanted KS cells lose the KSHV genome after serial passage [10,155], the development of *in vitro* models for KS using experimental infections has proven invaluable for investigation of KSHV biology and KS pathophysiology. Additionally, all EC-based KS models aim to generate age- and passage-matched infected and uninfected cultures. This is an important advantage of these systems over previous KSHV studies done in PEL cell lines that lack appropriate uninfected controls.

Several groups have employed functional genomics methods to identify cellular genes that are important for KS pathogenesis (figure 2). Cornelissen, et al [177],

compared gene expression in tissue from two AIDS-KS lesions with normal skin by serial analysis of gene expression (SAGE). Others have used experimentally-infected endothelial cells *in vitro* to screen for changes in cellular gene expression by cDNA microarray or a proteomics method [122,160,169,173]. Functional patterning of changes in cellular gene expression following KSHV infection has revealed general pathways by which KSHV induces a proliferative, pro-survival, and oncogenic shift in endothelial cell physiology, though there was considerable variation in the specific genes found in each study. This variation may result from the diversity of screening methods employed (i.e. Western blot array, cDNA microarray, SAGE) as well as the dissimilarity between source material. Also, the *in vitro* KS systems described differ notably in cell types employed and consequences of infection observed. For example, in our culture system there is evidence of spindling and transformation of dermal microvascular EC (DMVEC) and maintenance of a predominately latent infection even with extensive passaging [120]. As observed in tumor biopsy material, KSHV lytic gene expression is demonstrable in a small percentage of infected cells [120]. Other culture systems show varying degrees of spindle formation, lytic reactivation, and transformation, but infection is maintained in only a minority of cells following serial passage [156-158]. Because infection is maintained in the majority of cells and there is evidence of transformation, our culture system is likely to be reflective of KSHV-infection in an established lesion and the consequences thereof. The two novel therapeutic targets reviewed below were identified in our DMVEC system and may potentially be involved in proliferation of KSHV-infected lesional spindle cells.

Fig 2. References for functional genomic studies conducted using KS biopsy material (A) or samples derived from *in vitro* KS models (B).

Figure 2



Cornelissen, et al. 2003 (29)

Poole, et al. 2002 (83)
Moses, et al. 2002 (74)
Naranatt, et al. 2004 (77)
Hong, et al. 2004 (53)

McAllister, et al. 2004 (66)

5. c-Kit

Analysis of endothelial cell gene expression using microarrays has independently revealed that numerous cellular enzymes, including the receptor tyrosine kinase c-Kit, are overexpressed following experimental infection with KSHV[161,173]. We confirmed upregulation of c-Kit transcript and protein *in vitro* by quantitative real-time PCR analysis and immunofluorescent staining [173], while we and others have demonstrated c-Kit protein in KS biopsy samples (our unpublished observation, and [174]). Overexpression of c-Kit was also shown to be necessary and sufficient for induction of the transformed phenotype of KSHV-infected cells observed in our *in vitro* culture system. Importantly, infected DMVEC displayed a ligand-dependent growth advantage (figure 3a) that could be abolished by coexpression of a dominant negative c-Kit mutant or knock-down of c-Kit expression by gene silencing antisense molecules [170,173].

Imatinib mesylate (Gleevec; Novartis) has been used to successfully treat gastrointestinal stromal tumors (where the target was c-Kit [328]) as well as dermatofibrosarcoma protuberans and hypereosinophilic syndrome (targeting the PDGF pathway [329,330]). PDGF and c-Kit have both been implicated in KS progression [161,170,173,174,200,331-333]. Accordingly, the anti-tumor activity of Gleevec was recently evaluated in patients with AIDS-related cutaneous KS [175]. In this pilot trial, ten male patients with AIDS-KS refractory to standard therapy were administered 300 mg Gleevec orally, twice daily for four weeks. Serial tumor measurements showed a partial response in five subjects; the other five subjects, who had been developing new lesions at the beginning of the study, had stable disease after four weeks of

therapy. Tumors biopsies from four of six subjects showed histological regression, while immunohistochemistry showed a decrease in activated (phosphorylated) PDGFR. The activation state of c-Kit could not be directly measured in these biopsies because of antibody cross-reactivity; however, decreased activation of the known c–Kit downstream effecter ERK suggests that c-Kit signaling was inhibition by Gleevec. The most common adverse event was diarrhea, which required dose-reduction in six patients. A phase II trial is currently underway to further evaluate Gleevec as a single-agent in patients with KS that is refractory to standard therapies.

6. HO-1

Comparison of the protein expression profiles of KSHV-infected and uninfected endothelial cells by a Western blot-based proteomics method revealed that heme oxygenase-1 (HO-1), the inducible enzyme responsible for the rate-limiting step in heme catabolism [246], is among the metabolic cellular genes upregulated in infected endothelial cells [169]. HO-1 was also found upregulated in KS tissue by SAGE analysis [177]. Recent evidence implicates HO-1 enzymatic activity in anti-apoptotic responses [240,241], cell proliferation [242,243], and angiogenesis [242-245,334]. Given the vascular nature of KS lesions, host genes involved in angiogenesis which are induced by KSHV infection may provide pathogenesis-specific targets for pharmacological intervention.

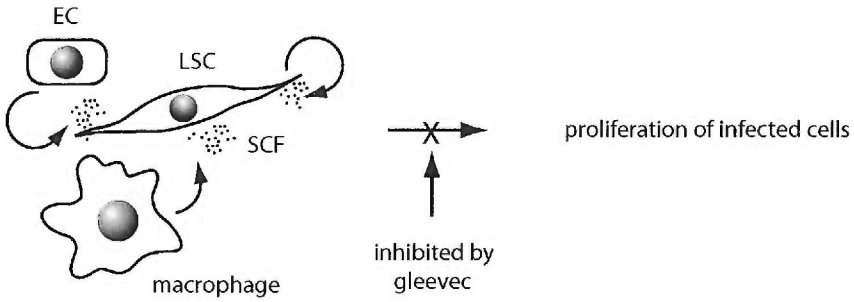
We confirmed upregulation of HO-1 mRNA and protein in KSHV-infected cells *in vitro*. Comparison of oral and cutaneous AIDS-KS tissues with normal tissues revealed that HO-1 mRNA and protein were also upregulated *in vivo*. Increased HO-1 activity in KSHV-infected endothelial cells resulted in increased cell proliferation

following exposure to free heme, whereas uninfected cells grown under the same conditions did not exhibit a proliferative response. Treatment with a HO-1 inhibitory heme analog (chromium mesoporphyrin IX) abolished heme-induced proliferation. There is evidence that KS lesions have locally elevated concentrations of hemoglobin and free heme and therefore contain ample substrate for HO-1 (figure 3b). Because of the importance of angiogenesis in the pathophysiology of KS, heme-induced proliferation of KSHV-infected cells may be an important factor contributing to lesion development. The availability of specific HO-1 inhibitors, such as chromium mesoporphyrin IX, in clinical use elsewhere [259,260] aided the initial investigation of the role of HO-1 upregulation in KSHV-induced pathogenesis; these HO-1 inhibitors are worth evaluating as potential chemotherapy for KS, either as systemic or topical agents.

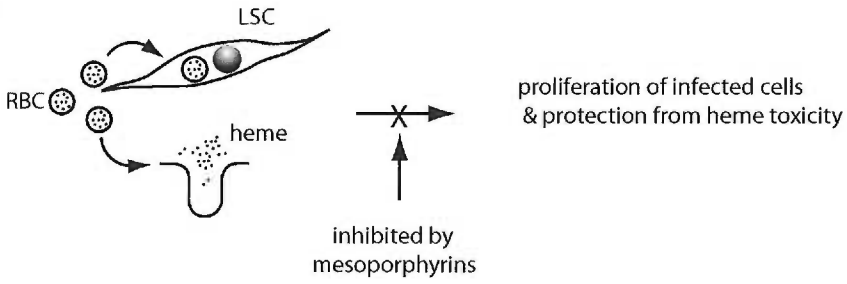
Fig 3. (A) Expression of the c-Kit ligand stem cell factor (SCF) by lesional spindle cells (LSC), uninfected endothelial cells (EC), and infiltrating macrophages, is expected to stimulate proliferation of KSHV-infected spindle cells that express elevated levels of c-Kit. This growth advantage has been demonstrated *in vitro* by blocking SCF-mediated proliferation of infected cells by treatment with Gleevec. Gleevec was subsequently shown to be effective chemotherapy for AIDS-KS, where c-Kit and PDGFR are potential targets. (B) KS lesions are known to have elevated levels of free heme due to the extravasation of numerous red blood cells (RBC) from the abnormal microvasculature. These extravasated RBCs then either disintegrate in the extracellular space, thereby releasing their heme (hemoglobin) into the local microenvironment, or are phagocytosed (erythrophagocytosis) by spindle cells. Erythrophagocytosis would be expected to elevate the intracellular concentration of heme. The growth advantage of KSHV-infected endothelial cells *in vitro* in the presence of free heme is blocked by treatment with the HO-1 inhibitor chromium mesoporphyrin IX; this and similar compounds represent potential new chemotherapeutic agents for KS.

Figure 3

A.


EC
LSC
SCF
macrophage
inhibited by
gleevec
proliferation of infected cells

B.


LSC
RBC
heme
inhibited by
mesoporphyrins
proliferation of infected cells
& protection from heme toxicity

7. Other candidate targets

The initial investigation of HO-1 and c-Kit was expedited by the availability of specific chemical inhibitors approved for clinical use in other disorders as well as the bias towards maintenance of latent infection inherent in our culture system. However, many promising candidate targets identified by the functional genomics methods described here suffer from the lack of specific chemical inhibitors; analyses of functional significance are subsequently hindered. The use of antisense molecules to inhibit c-Kit signaling demonstrated the utility of this approach in knocking down specific gene expression without using specific pharmacological agents [170,173]. We have applied this principle to other molecules identified by microarray, including neuritin and the orphan G-protein-coupled receptor RDC-1, and demonstrated a possible role for these genes in KS (manuscript submitted).

Anti-herpes agents such as ganciclovir and foscarnet have been shown to improve the clinical outcome of KS [116,188,189]. Other groups are investigating RNA interference techniques as possible pathogenesis-specific therapy for KS [145,147,307,335,336]. Gaps in our understanding of KS pathogenesis, however, further hinder rapid screening of possible treatment targets using functional genomics, for though a number of cytokines and growth factors are thought to be important in KS establishment and progression, the critical factors *in vivo* are not yet widely agreed upon [200,274,277,279,331,333,337]. An *in vivo* system amenable to investigation of lesion development using recombinant KSHV strains and targeted gene silencing would be a valuable tool for dissecting key molecular events in KS pathogenesis and evaluation of novel therapeutic targets. Unfortunately no such system for KS is currently available. Until one is available, however, further efforts akin

to the study by Cornelissen, et al [177] could help in clarifying some of the uncertainty inherent in this field by aiding in the further molecular characterization of the KS lesional microenvironment.

8. Expert opinion and outlook

A recent article published in *The Cochrane Database of Systematic Reviews* aimed to identify effective KS therapeutics that could be used in resource-poor settings such as Africa [45]. The authors sought to identify studies examining drugs found on the WHO essential drug list, a minimal list of medications assumed to be normally available in developing countries. None of the over six hundred studies reviewed identified chemotherapies that would be affordable in resource-poor areas. The seroprevelance of both KSHV and HIV is greater in Africa than most of the rest of the world and is expected to continue rising, and the incidence of KS will rise accordingly. Already 57,000 new cases of KS occur in Africa each year, and due to poor prognosis of AIDS-associated disease, result in 52,000 deaths [43]. To effectively address the problem of KS in the developing world, then, it will be necessary to develop significantly less expensive drugs. The functional genomics methods described here have been used to identify numerous possible therapeutic targets; some of these, such as HO-1 and maybe others, have chemical inhibitors already in clinical use that with further development could find application in resource-poor settings such as Africa.

9. Highlights

- Functional genomic studies have identified numerous potential therapeutic targets for KS, including c-Kit and HO-1.
- An open-label pilot trial has shown that Gleevec is an effective monotherapy for KS refractory to standard therapy; a phase II trial for Gleevec monotherapy is underway.
- African KS is a large and growing problem; development of cheaper therapies is necessary to meet the needs of poor populations.
- With further development, HO-1-inhibitory heme analogs (mesoporhpyrins) may be appropriate for use in resource-poor settings.
- Development of an *in vivo* system amenable to the study of recombinant KSHV strains and targeted gene deletion with antisense could accelerate identification of possibly important treatment targets for which no chemical agents are currently available.
- Numerous pathogenesis-related agents, such as angiogenesis inhibitors, are in various stages of pre-clinical and clinical development.

9. Acknowledgments: The authors wish to thank Andrew Townsend for his help with the preparation of this manuscript.

Appendix 2

In vitro systems used for KSHV pathogenesis studies

This appendix will appear in similar form in a chapter entitled "Endothelial cell- and lymphocyte-based *in vitro* systems for understanding KSHV pathogenesis" to be published in a forthcoming volume on KSHV in the Current Topics in Microbiology and Immunology book series.

1. Description and classification of Kaposi's sarcoma

Moritz Kaposi, a dermatologist at the University of Vienna, described the case histories of five middle aged and elderly men who presented with "multiple idiopathic pigmented sarcomas of the skin" in 1872 [1]; this disease was named Kaposi's sarcoma (KS) a few years later, but remained a medical rarity in the developed world for the next hundred or so years. In 1981 an aggressive variant of KS became one of the sentinel diseases of the AIDS pandemic[2]. The poor prognosis of AIDS-associated KS led one playwright to describe the disease as "the wine-dark kiss of the angel of death."[3].

Four distinct epidemiological forms of KS are now recognized [4] and are distinguished by relative severity, HIV serostatus, and geographic distribution: classical, the variant first described in elderly Mediterranean men; AIDS-KS, severe and often fatal, the most common AIDS-associated neoplasm; endemic, an aggressive but non-HIV-associated variant common in sub-Saharan Africa; and iatrogenic, a form occasionally complicating organ transplantation. In all forms, KS usually presents as multiple reddish-purple dermal or mucosal lesions that may be flat or raised. Lesions may coalesce as disease progresses, becoming nodular and even ulcerated. Dissemination to visceral organs, including the lungs, liver, lymph nodes, and gut may also occur and is associated with poor prognosis. The four epidemiological forms of KS are histologically indistinguishable, characterized by disorganized networks of abnormal microvasculature composed of spindle-shaped cells[5]; these cells do not maintain the integrity of microvascular channels, accounting for lesional edema and abundant extravasation of inflammatory cells and erythrocytes. Spindle cells, thought to be of endothelial lineage, are present in early patch-stage lesions but become the predominant cell type in later

plaque- and nodular-stage lesions [6-13]. Patch-stage lesions are typically polyclonal, but oligoclonal and monoclonal later-stage lesions have been described[14-16]. Thus KS exhibits characteristics of both a reactive hyperproliferative lesion and a truly transformed sarcoma.

2. Kaposi's sarcoma-associated herpesvirus

Representational difference analysis (RDA) is a technique used to identify minor differences between complex genomic samples. DNA from KS tissue and normal skin was compared by RDA, leading to the discovery of KSHV in 1994 [47]. Sequences from this new human herpesvirus were detected in nearly all KS tumors but not in most other pathologic samples examined. Importantly, viral sequences were found in lymph node biopsies from gay men with AIDS, which suggested a link not only to patients with KS but also to groups known to be at increased risk of developing KS. Seroprevalence studies have since shown that KSHV infection is more common in populations known to be at greater risk for developing KS [48] and that KSHV viral load increases prior to the onset of disease (although the utility of viral load as a predictor of KS development is debated [49-51]). KSHV is now considered necessary for all clinical forms of KS [27,47,52] as well as two rare lymphoproliferative disorders, primary effusion lymphoma (PEL)[24,53,54] and multicentric Castleman's disease [55]. However, as the seroprevalence of KSHV far exceeds the incidence of these diseases (even in populations known to be at higher risk), we can conclude that KSHV is necessary but not sufficient for the development of disease [56].

Complete sequencing of the KSHV genome revealed relatedness to members of the *Gammaherpesvirinae* subfamily[57-59]. KSHV has been placed in the *Rhadinovirus* or γ2 group within this subfamily, becoming the first human herpesvirus thus categorized. The KSHV genome is ~ 165 kb with a central unique coding region of ~ 140 kb flanked on either end by variable numbers of noncoding, GC-rich repeat sequences [60,61]. The coding region of KSHV consists of seven blocks of conserved herpesvirus genes that align closely with those of the prototypical γ2 virus, herpesvirus saimiri (HVS), a virus which causes fatal T cell lymphomas in new world primates [62,63]. These conserved genes are labeled open reading frames (ORF) 1 through 75 and most encode proteins required for production of progeny virions (transcription factors, DNA synthesis-related enzymes, as well as capsid, tegument, and envelope proteins) or proteins involved in the establishment and maintenance of latency. Interspersed between the conserved blocks are genes that are unique to gammaherpesviruses or to KSHV, labeled K 1 through 15. Many of the K genes have homologs to cellular genes involved in intra- and inter-cellular signaling including cytokines, chemokines, chemokine receptors, and interferon response factors[64].

Both *in vitro* and *in vivo*, KSHV is maintained as a viral episome in a primarily latent state characterized by expression of few viral gene products [65-72]. At least three viral gene products, vFLIP, vCYC, and LANA-1, are believed to comprise the minimal latency expression program of KSHV and are consistently expressed in all virally infected cells in KS, PEL, and MCD [65-67,73]. The gene product of ORF 71, vFLIP (viral FLICE-inhibitory protein; K13) inhibits apoptosis [74,75] and activates NFκB[76]. ORF 72, the viral cyclin (v-Cyclin), is a homolog of cellular D-type cyclin that can drive quiescent

cells into S phase in part by inhibiting retinoblastoma protein (Rb) activity[77-80]. In addition, v-Cyclin inactivates the cyclin-dependent kinase inhibitor p27[81] and promotes cytoplasmic export of human Orc1, a component of the host origin recognition complex[82]. The latency-associated nuclear antigen (LANA-1; ORF 73) targets the tumor suppressor proteins p53 and Rb[83,84], providing both anti-apoptotic and cell cycle-regulatory functions. LANA-1 is also a transcriptional modulator of various cellular and viral promoters [85], and tethers multiple copies of the viral episome to host cell chromosomes, a function indispensable for maintenance of the viral genome during cell division [86].

Two additional viral genes, K10.5 and K12 are transcribed during latency but have additional features deserving of mention. K10.5 is expressed during latency in the B cell disorders MCD and PEL, but not KS [87]. The gene product of K10.5, LANA-2, also known as viral interferon regulatory factor 3 (vIRF3), has partial homology to members of the cellular IRF family (particularly IRF4) and two other viral IRFs K9 (vIRF1) and K11.1 (vIRF2) [87,88]. A role for LANA-2 in inhibition of p53-induced apoptosis has been proposed, both through direct interaction with p53 [87] and via inhibition of NFκB activity [89]; thus, LANA-2 could contribute to proliferative or neoplastic expansion of KSHV-infected B cells.

Kaposin (K12) was initially identified as an abundant latent transcript in KS tumor samples[59]; unlike other KSHV latency genes, however, K12 transcript levels increase following lytic cycle induction, suggesting a more complicated transcriptional regulation pattern. Questions awaiting elucidation include whether Kaposin utilizes different transcription units during latency and lytic replication, as well as how cell cycle

and tumor type influence transcriptional regulation. The Kaposin locus encodes at least three proteins (Kaposin A, B and C) via a translational program that is similarly complex and incompletely understood [73,90-92]. Transforming functions have been ascribed to the Kaposin family, particularly to Kaposin A [91], and recently a role for Kaposin B in stabilization of cytokine transcripts via activation of the cellular kinase MK2 was proposed [93]. Overall, the latency program of KSHV enables expansion of a population of latently-infected spindle cells by inducing proliferation and preventing apoptosis of infected cells as well as maintaining the viral genome in daughter cells.

In vivo, the majority of KSHV-infected spindle cells and neoplastic B cells maintain the virus as a latent infection, with only a small percentage of cells expressing lytic cycle genes [59,94-97]. The KSHV gene ORF 50 , a homolog of Epstein-Barr virus Rta, encodes a replication and transcriptional activator that is necessary and sufficient to trigger lytic replication and production of viral progeny (reviewed by West and Wood[98]). ORF 50 is one of the earliest immediate early genes induced upon lytic reactivation in B cells and endothelial cells, [99-101] and is considered the master regulator of the switch from latency to lytic replication. *In vitro*, KSHV-infected cells can be induced to enter lytic cycle by ectopic expression of ORF 50 [100-102] or by treatment with phorbol esters [103] or sodium butyrate[104,105]. The biological signals that initiate the lytic cascade are not fully understood, though hypoxia[106,107], inflammatory cytokines (IC) [108-110], HIV-1 *tat*[109,111], and CMV coinfection [112], have all been implicated. A role for LANA-1 in controlling lytic induction via regulation of ORF 50 expression and function has also been proposed[113].

Upon reactivation, KSHV expresses a wide repertoire of gene products in a

coordinated cascade, culminating in release of progeny virions from infected cells. Chemical induction significantly increases the percentage of cells that express lytic gene products and was used by two groups for initial classification of KSHV lytic genes into kinetic classes using microarray analysis[114,115]. Lytic replication is essential for viral dissemination, and the observation that treatment of KSHV-seropositive AIDS patients with ganciclovir decreases the incidence of KS development suggests that lytic replication plays an important role in viral pathogenesis[116]. A number of KSHV lytic genes code for regulatory proteins that could conceivably drive proliferation or transformation as well as genes that modulate apoptotic signals and recognition by the host immune system (see [117] and [118] for recent comprehensive reviews). These genes include homologs of cellular cytokines (vIL-6/K2), chemokines (vMIP-I/K6, vMIP-II/K4, vMIP-III/K4.1), chemokine receptors (vGPCR/ORF74), interferon regulatory factors (vIRF-1/K9), anti-apoptotic factors (vIAP/K7), and modulators of immune recognition (K3/MIR1 and K5/MIR2). Thus, while lytic replication is incompatible with host cell survival, reservoirs of lytic infection are though to exert essential paracrine mechanisms that sustain tumorigenesis (see [118,119] for recent reviews). Additionally, some speculate that a limited subset of lytic genes could be expressed under certain conditions in the absence of full lytic replication[99,120-122]. Such lytic gene expression could contribute to pathogenesis without leading to host-cell death resulting from production and release of progeny virus. Of particular importance here may be expression of the immediate early gene K5, which can be expressed independent of ORF50 expression in PEL cells [123] and experimentally infected 293A cells (Jae Jung, personal communication). K5, along with another IE gene K3, perform a crucial immune evasion function via downregulation of

MHC-1 on infected cells [124].

3. KSHV-infected cell lines derived from primary effusion lymphomas

Primary effusion lymphomas (PEL; also body cavity-based lymphomas) are KSHV-related neoplasms that can present independently of HIV-infection[54] but are more frequent and severe in AIDS patients[53]. Because most PEL tumors are CD20-negative, PEL patients cannot be treated with B cell-targeted therapies; the prognosis of PEL, therefore, is poor. KSHV was first recovered by cultivation of cell lines derived from PEL [103]. Numerous clonal PEL cell lines have now been established [125-132], about half of which are coinfected with EBV [54,103,126,133-135]. Cell lines can be readily established from PEL tumors, and, unlike explanted KS spindle cells, maintain the KSHV genome in 100% of cells even following extensive tissue culture passage [66,87,114,115,136-140]. In PEL lines, the KSHV genome is maintained as 50-150 latent episomes per cell, which is approximately 10-fold higher than in KS [52,141]. While PEL infection is predominantly latent, lytic replication can be induced by chemical induction or ectopic expression of ORF50 [61,100]. Collectively, these characteristics have made PEL lines an extremely consistent and convenient model in which to study KSHV biology. PEL were used for the initial *in vitro* studies of KSHV biology, including those that yielded the viral genomic sequences, and have since been widely used to determine the kinetics of viral gene expression and mechanisms of viral latency and reactivation [57,58,114,115,142,143 100,101,104,144,145]. PEL systems have also been used to develop novel KSHV-specific therapeutic strategies that may find clinical application. Klass, *et al*[146], demonstrated that inducing replication in PEL cells with valproate (an anti-seizure medication with histone

deacetylase inhibitor action), while simultaneously blocking herpesviral DNA replication with ganciclovir and phosphonoformic acid, led to apoptosis in the tumor cells without increasing virus load. Godfrey, *et al*[147], tested RNA interference as a therapeutic strategy for PEL. Using lentiviral-delivered short hairpin RNA (shRNA) to target latency genes, inhibition of v-Cyclin and v-FLIP led to apoptosis in all 4 PEL lines (BC-3, BCP-1, JSC-1 and HBL-6) tested. Non-KSHV cell lines including the Burkitt's lymphoma line RAMOS were unaffected by this treatment. Interestingly, LANA-1 was not as reliable a target; stable cell lines maintaining the sh-LANA vector, and consequent lower KSHV copy numbers, could be generated. Whether this result reveals a capacity for KSHV to integrate into the host genome, thus becoming LANA-independent for segregation during cell division, remains to be established. Another interesting finding emerging from this study was the fact that LANA knock-down increased expression of ORF50, a result consistent with a recent report that LANA inhibits ORF50 expression and function[113]. In a murine model, injection of the sh-v-Cyclin vector prevented development of PEL-driven ascites and reduced established ascites. Other researchers have also used PEL lines to establish animal models for the study of KSHV biology and PEL tumorigenesis *in vivo* [126,132,148-150]. In the future, such models should greatly facilitate *in vivo* screening and validation of anti KSHV drugs that may have efficacy for KS as well as PEL.

Despite their considerable contribution to our current understanding of KSHV biology, PEL cell lines are not amenable to studying KSHV-induction of transformation because the transformation event precedes cell line establishment *in vitro*. Interestingly, primary B cells and some established B cell lines can be infected with KSHV *in vitro*, but infection does not lead to prolonged maintenance of the viral genome nor to cellular

transformation [122,151,152]. This is in marked contrast to B cell infection by two closely related gamma herpesviruses, EBV and RRV, both of which readily transform B cells and establish persistent infections. The reasons for this disparity are unknown and further studies are required to clarify these issues. Additionally, PEL are of limited utility in studying the cell-specific role of KSHV in KS, where the target cell is of endothelial lineage. Therefore, development of endothelial cell-based systems that support *de novo* KSHV-infection has been essential for studying KSHV infection and gene expression patterns in non-B-lineage cells, for identifying KSHV-induced changes in endothelial cell physiology that contribute to KS pathogenesis, and for developing effective KS-targeted therapies.

4. Experimental infection of endothelial cells with KSHV

Spindle cells harbor the KSHV genome *in vivo* but all described cell lines derived from KS lesions appear to rapidly lose the KSHV genome upon serial *in vitro* passage [10,52,94,129,153-155]. Genome loss could be explained by an insufficiency of the episomal maintenance mechanism in endothelial-lineage cells. Others have argued that this phenomenon is artifactual, reflecting the different selective pressures inherent in tissue culture conditions versus the KS lesional microenvironment. Because explanted KS cells lose the KSHV genome after serial passage, the development of *in vitro* models for KS using experimental *de novo* infection has proven invaluable for investigation of KSHV-endothelial cell (EC) interaction.

EC are the presumptive precursors of the characteristic spindle cells in KS lesions [6-10] although, as will be discussed in more detail later, whether KSHV preferentially

infects lymphatic, vascular, or precursor EC is a matter of debate. Spindle cells harbor KSHV DNA *in vivo* [52,94,153], but the loss of the genome from explanted tumor cells, as well as the need to evaluate the consequences of *de novo* infection, stimulated efforts to develop *in vitro* EC-based culture systems that supported KSHV infection [120,156-159]. All EC-based KS models aim to generate age- and passage-matched infected and uninfected cultures, illuminating an important advantage over PEL cell lines that lack uninfected counterparts for use as controls. The EC-based *in vitro* systems described to date differ to varying degrees as regards the protocol used and the observed consequences of infection, but key similarities have also been noted. Collectively, when evaluated in the context of the differences in experimental design, these models have yielded valuable information about KSHV biology and pathogenesis in EC. The degree to which any of these models reproduces the spindle component within the complex tumor microenvironment will be discussed further towards the end of this chapter.

Flore, *et al*, were the first to describe infection of primary EC *in vitro*, using adult bone marrow-derived EC as initial targets and HUVEC as secondary recipients to demonstrate both productive infection and paracrine influences[157]. Virus inoculum was prepared from concentrated TPA-induced supernatants from the BC-3 PEL line. The KSHV-exposed cultures acquired telomerase activity, exhibited extended lifespans, and formed colonies in soft agar, suggesting a KSHV-mediated transformation event. However, the frequency of initial infection was low and KSHV was maintained in only a minority of cells (<10%) following serial passage. Thus, the long-term survival of these cultures was attributed to indirect influences that could also be reproduced by transferring supernatants from infected cultures to naive, uninfected HUVEC. Specifically, the type 2

VEGF receptor, KDR, was upregulated on all cells, regardless of infection status, via a KSHV-initiated paracrine effect, rendering the KDR+ cells responsive to VEGF in the culture medium. While more recent models have allowed a higher percentage of infected cells, the paradigm whereby a subset of infected cells influences the larger microenvironment remains extremely relevant to KS pathogenesis.

Ciufo, *et al* describe infection of primary adult and neonatal dermal microvascular EC (DMVEC) using infectious KSHV prepared from the supernatants of three different TPA-induced PEL lines, BCP-1, BC-3 and JSC1[156]. Virus was concentrated to generate high titer inoculae allowing for a high initial infection rate. Interestingly, inoculum prepared from the JSC1 line allowed the most efficient infection, due to the production of a higher number of progeny virions, and perhaps some qualitative differences between PEL lines as well. Upon infection, EC morphology changed from a cobblestone shape with a flat aspect to aggregates of cells with elongated spindloid morphology. Infection of the primary DMVEC appeared to be predominantly latent; almost all converted spindle cells were LANA-1-positive, with approximately 5-10% of cells expressing immediately early or early lytic proteins (e.g. K8, K5, ORF50 and ORF59) and 1-2% expressing the late lytic glycoprotein K8.1A. K8.1A-positive cells were typically a sub-fraction of rounded up cells exhibiting CPE associated with the final stages of productive replication. In some of the K8.1A-positive or ORF59-positive cells, intranuclear inclusions resembling herpesviral replication compartments were visible. In summary, infection of primary DMVEC with JSC1-derived KSHV at high MOI led to the establishment of a predominantly latently infected cell pool that showed marked morphological changes reminiscent of KS spindle cells, while a subset of cells underwent

spontaneous lytic replication with release of infectious progeny. These findings are reminiscent of KS tumor cells *in vivo*, both with respect to morphology and viral gene expression patterns. The KSHV-infected cultures could not be maintained indefinitely, however, due in part to a chronic loss of cells from lytic infection and failure of the latently infected cells to be maintained for more than a few cycles as a population of dividing cells that maintained the viral episome. Addition of uninfected cells to the cultures at a 1:10 ratio provided fresh targets for infection, providing a means to generate additional infected cultures without establishing new PEL-initiated infections. Thus, multiple rounds of *de novo* infection, as opposed to long-term maintenance of latently-infected cells, define this culture model.

Similar protocols have since been used to generate KSHV-infected EC cultures to examine patterns of viral [99] or host [122,160-162] gene expression by microarray analysis, RT-PCR and/or immunostaining following *de novo* infection with PEL-derived virus. Krishnan and colleagues [99] were the first to comprehensively examine viral gene expression patterns in primary endothelial cells following *de novo* infection. In this report, adult DMVEC were infected with concentrated BCBL-1-derived KSHV and the kinetics of latent and lytic gene expression was examined by whole genome array (Celonex HHV8 viruChip), RT-PCR, and immunostaining at time points from 30 minutes to 5 days PI. This report revealed a number of interesting findings. Expression of a limited set of immediate early (IE) and early (E) lytic genes was initiated concurrently with latent genes in the majority of cells immediately following infection, with a sharp decline thereafter. Compared to TPA-induced BCBL cells, only a limited number of ORF50-activated genes were expressed, and the majority of genes involved in DNA

replication and viral assembly were not expressed. Of particular interest, the IE gene K5 was expressed at early time points and continued to be well expressed after a decline in other IE and E genes. Another interesting finding was confirmation of the absence of LANA 2 (ORFK10.5) expression in primary EC. Expression of a limited set of IE and E genes with immune evasion and anti-apoptotic function, with a subsequent decline thereafter, may play a crucial role *in vivo* in allowing establishment of infection and tumor initiation/progression. Signals that determine which cells progress through a full lytic cycle and produce infectious progeny, and which establish latency, remain to be determined; indeed their elucidation may require the multi-factorial tumor environment to be more fully represented in *in vitro* KS models.

Host endothelial cell gene expression has been evaluated after only 2 and 4 hrs PI [122], after several days [161,162], or after 3 weeks [160]. Poole *et al* [160] also performed microarray analysis following reseeding of infected cultures with uninfected cells [160]. Naranatt and colleagues [122] examined gene expression in KSHV-infected primary DMVEC with Affymetrix HG-U133A gene arrays as early as 2 and 4 hrs PI, and discovered a significant early reprogramming of the host transcriptome that included alterations in genes involved in signaling, apoptosis, transcription, host defense, cell cycle, metabolism, inflammation, angiogenesis, and tumorigenesis. The authors also evaluated infected fibroblasts and the B cell line BJAB, to create a database that illuminated both cell-type specific and common responses to infection. The study by Poole and colleagues [160] differed in that the authors' aim was to interrogate host reprogramming after establishment of a latent infection and following exposure of latently infected cells to TPA; hence, cells were harvested 3 weeks post infection (PI) as

well as two weeks after reseeding infected cells with new uninfected targets. Clontech Human Atlas or Incyte Human UniGemV2.0 cDNA arrays were used, with RT-PCR analysis confirming changes in selected individual genes. This study revealed that even after establishment of latency, KSHV-infected cells exhibited a profound alteration in gene expression patterns; between 1.4 and 2.5% of genes represented were significantly upregulated or downregulated. Of particular interest was the induction of interferon-induced genes, genes involved in cell signaling and angiogenesis, and genes involved in cell cycle progression and apoptosis. The studies by Hong and colleagues and Wang and colleagues [161,162] were specifically designed to evaluate lymphatic reprogramming and are discussed later along with a similar study using immortalized EC [163].

Primary infection of EC has also been used to study KSHV binding and entry and the signaling pathways induced in the earliest phases of infection [164]. This study identified a role for integrin α3β1-FAK dependent phosphatidylinositol 3- (PI 3-) kinase activation in KSHV entry and a role for the PKC-zeta-MEK-ERK signaling cascade during the earliest stages of KSHV infection. Activation of a mitogenic cascade during KSHV entry may have important implications for establishment of latency and the division of latently-infected cells, as well as creation of a host microenvironment conducive to the expression of immediate early lytic cycle proteins.

A recent report by Krug, *et al*, is worthy of mention since infection was performed at low MOI to specifically explore the ability of latently infected EC to replicate, as well as the ability of infected EC to produce infectious virus for dissemination via *de novo* infection [159]. Pooled neonatal DMVEC were infected with KSHV derived from TPA-induced BCBL-1 cells. While less than 5% of cells were

initially infected, infection spread primarily due to proliferation of latently infected cell with episome maintenance. A limited contribution by *de novo* infection from virions produced from the small percentage that entered lytic cycle was also noted. Spread of infection could not be blocked by treating cultures with compounds that block herpesviral lytic replication; therefore, proliferation of latently infected cells characterizes this culture system. A growth advantage for LANA-1-positive cells as compared to uninfected cells was noted, and cell spindling with loss of contact inhibition following post-confluent growth was observed. Thus, of all primary infection protocols, this is the most similar to the system described by Moses, *et al* that will be discussed in the following section. Of note, infection of the primary EC induced expression of cellular antiviral genes, specifically dsRNA-activated protein kinase (PKR) and 2'5'-oligoadenylate synthetase (2'5'-OAS), a phenotype that could be reproduced by IFNα treatment. IFNα treatment was also effective in preventing lytic activation and viral replication. However, because infection was initiated at low MOI and was maintained primarily by proliferation of latently infected cells, induction of antiviral genes did not eliminate viral infection in this system. Other protocols for infection of primary cells that utilize high titer inoculae may induce higher levels of these host-defense genes, which may in turn interfere with establishment and maintenance of infection.

The life-span of primary EC *in vitro* is limited due to replicative senescence. To overcome this limitation, some investigators have employed life-extended EC prepared by ectopic expression of human telomerase or genes from other transforming viruses [120,158]. Expression of these genes does not induce laboratory evidence of transformation other than extending the length of time cells can be serially propagated in culture. A

system described by Moses, *et al* was the first to use life-extended EC for KSHV infection and the first to describe cultures in which the majority of cells became infected and maintained the genome in a predominantly latent state [120]. In this model, adult primary DMVEC are immortalized by retroviral transduction of the E6 and E7 genes of human papillomavirus (HPV) type 16. The life-extended cells exhibit no overt signs of transformation (i.e. they become contact-inhibited at confluence and do not form colonies in soft agar) but with serial passage can be maintained significantly longer than their primary counterparts. HPV E6 and E7 are known to have significant effects on the cell cycle, most notably through targeting p53 and Rb. E6 participates in the ubiquitination and degradation of p53 [165], while E7 binds and sequesters Rb [166] and induces Rb phosphorylation (i.e. inactivation) [167]. These are two pathways that KSHV latent genes also inhibit; LANA-1 disrupts the function of both p53 [83] and Rb [84], and v-Cyclin induces Rb phosphorylation via activation of CDK-6 [79]. In addition, LANA and p53 co-localization is observed in KSHV-associated tumors *in vivo* where p53 is usually wild type, suggesting that LANA-mediated p53 inactivation is important for tumorigenesis [168]. Therefore, immortalization of DMVEC by E6/E7 expression may augment some of the alterations in cellular physiology that are themselves induced by KSHV, thus creating a cellular microenvironment conducive to KSHV infection. It cannot be ruled out, however, that these HPV gene products do not complicate results obtained when using this culture system for studies of KSHV biology. Thus, key findings obtained using E6/E7-immortalized DMVEC have been verified in primary cells and KS tissue [120,169-171].

For studies performed with the E6/E7-DMVEC model to date, infectious KSHV has been derived from unconcentrated TPA-induced BCBL-1 cultures, yielding a

relatively low titer inoculum and thus the expectation of a low percentage of initially infected cells. Indeed, evaluation of LANA-1 expression by IFA at early times (12 h) PI, revealed that not more than 10% of EC in a treated culture were KSHV infected [120]. LANA expression increased with time, such that by 14 days PI up to 80% of cells in a KSHV-exposed culture were infected. PCR for the KSHV genome at days 7 and 14 PI revealed increased intensity of the amplified product by day 14, supporting evidence of virus spread. A similar trend was seen with expression of the early lytic protein ORF 59: at 1 week PI <1% of cells were ORF 59-positive, but by 8 weeks PI up to 5 % of cells expressed this lytic marker. Expression of the late lytic glycoprotein K8.1A/B followed similar expression kinetics though to a consistently 5- to 10-fold lesser degree than ORF 59. Lytic replication could be induced in infected cells by treatment with TPA, sodium butyrate or corticosteroids, but never in more than 40% of LANA-positive cells.

Prior to exposure to KSHV, E6/E7-immortalized DMVEC retained a classical cobblestone appearance. KSHV-infection, however, induced marked changes in cellular morphology reminiscent of the spindle cells observed in KS lesions including elongated cells with oval cell bodies, uniformly narrow elongated cells, and extremely narrow light-refractile cells displaying scattering. A low percentage of rounded up cells that tended to detach from the monolayer were also observed; the nuclei of these cells as well as the nuclei of extremely spindled cells displayed intranuclear inclusions resembling typical herpesviral CPE. The extent of morphologic change within virus-exposed cultures increased with time PI and correlated strongly with the percentage of KSHV-infected cells, suggesting a direct effect of the virus on cell morphology. Evaluation of gene expression in concert with morphology revealed the following: LANA-1 expression was

sufficient for spindling; ORF59-positive cells were spindle-shaped, with a proportion displaying intranuclear inclusions, and all rounded cells were strongly ORF59 positive; rare K8.1A/B positive cells displayed severe spindling but were more frequently rounded. The presence of infectious virions in conditioned supernatants that could transfer infection to naïve cultures, albeit at a low MOI, confirmed that DMVEC could support a fully permissive replication cycle. The relative ratios of ORF73 to ORF59 to K8.1A/B-positive cells at any one time suggested that the majority of cells harbored latent infection and that completion of the lytic cycle occurred in only a fraction of the lytic cell population. These observations are very similar to those reported by Ciufo and colleagues upon infection of primary DMVEC [156].

Evaluation of KSHV gene expression and cell morphology suggests that the attainment of completely infected cultures in the E6/E7-DMVEC model reflects a combination of the proliferation of latently infected cells and *de novo* infection by EC-generated virus. Once all cells are infected and naive viral targets are absent, latently infected cells continue to proliferate with only a minor fraction of cells lost due to productive lytic infection. Infected cultures can be expanded by passaging, but the genome is best maintained when low split ratios are used; presumably a delicate balance exists between cell division and episome replication and segregation to daughter cells. A key difference between the immortalized DMVEC and primary EC is the increased length of time for which latently-infected E6/E7-DMVEC can be passaged with genome maintenance. It is possible that the presence of E6/E7 allows for a degree of episome maintenance more akin to what is seen in PEL cells that, unlike KS tumor cells, are able to maintain the KSHV episome *in vitro* as well as *in vivo*. The latent gene

ORFK10.5/LANA-2 that inhibits p53 function is well expressed in PEL cell but not in primary DMVEC [69,99]; possessing multiple ways to disable this tumor-suppressor protein may be a reason for efficient episome maintenance in PEL cells. Distinct from their role as oncoproteins, E6 and E7 are also known to be required for stable maintenance of HPV episomes in undifferentiated human keratinocytes[172]. The HPV oncogenes in the immortalized DMVEC may assist in episome maintenance by duplicating the function of LANA-2 or by acting as surrogates for other currently unappreciated mechanisms occurring in the lesional microenvironment.

Interestingly, after several weeks in culture, there is a decrease in both spontaneous ORF 59-expression and inducibility of lytic cycle proteins by chemical induction in KSHV-infected E6/E7-DMVEC. Thus, a predominantly latent infection is established, possibly by selection of clones that are resistant to lytic induction but that can propagate as continually-dividing, latently-infected cells. In such cultures, CD31 is strongly expressed on the LANA-positive cells, suggesting that K5 is not expressed and thus a true latency exists [Mandana Mansouri and Klaus Früh, unpublished observations].

As described above, the E6/E7-DMVEC model appears to represent two distinct stages: one in which the viral infection and genome spread occurs via both lytic and latent means, and a second where latently infected cells survive for multiple passages and maintain the genome in a latent state. When such cells are not passaged prior to achieving tissue culture confluence, a third state is generated, characterized by the continued post-confluent growth of the cells into 3-dimensional foci. Importantly, uninfected cells grow to confluence and enter a quiescent state under similar conditions. Post-confluent growth reflects loss of contact inhibition, one of the hallmarks of cellular transformation.

Infected cells are also able to form colonies in soft agar, a measure of anchorage-independent growth, and form tumors in mice when injected into the tail base in a matrigel solution [Shane McAllister and Ashlee Moses; unpublished observations]. Krug and colleagues [159] similarly report that when primary DMVEC are infected at low MOI with subsequent spread of infection primarily through division of latently infected cells, loss of contact inhibition is observed. The piled up aggregates of infected cells reported by Ciufo and colleagues [156] are also reminiscent of 3-dimensional focus formation. Thus, acquisition of a transformed phenotype is not unique to the E6/E7 DMVEC system, although the potential for additional contributions from the HPV proteins should be considered.

Latently infected E6/E7-DMVEC have been used in gene expression profiling studies to examine KSHV-induced cellular gene reprogramming and identify potential therapeutic targets for KS. These studies have employed cDNA arrays [173] as well as Affymetrix U95A and U133A and B GeneChips [170,171] and have interrogated several different KSHV-infected cultures relative to age- and passage-matched uninfected controls. For all of these studies, KSHV-infected DMVEC were infected with BCBL-1-derived KSHV at low MOI and passaged when confluent at low split ratios. Cells were harvested for microarray analysis when immunofluorescent staining of parallel cultures revealed that >90% of KSHV-infected cultures were LANA positive. This typically took 3-4 weeks and encompassed approximately 5-7 tissue culture passages. A minimum of two biological replicates were used for each comparison. The complete data sets from these different microarray experiments are available online (ohsu.edu/vgti/fruh.htm) and further details can be found in the specific papers referenced. To date, this database has

been used to identify a handful of potential targets for KS chemotherapy[169,171,173]. Briefly, the receptor tyrosine kinase c-Kit was identified as a gene induced by KSHV using cDNA arrays [173], and confirmed using Affymetrix arrays, RT-PCR analysis and immunofluorescence, the latter being on primary DMVEC. An independent analysis of gene expression in primary DMVEC using U133A GeneChips also reported KSHV-induction of c-Kit [161]. A role for c-Kit in proliferation and post-confluent growth of KSHV-infected DMVEC was then demonstrated using both a pharmacological inhibitor of c-Kit (Imatinib Mesylate, Gleevec; formerly STI 571 [173]) and gene knockdown approaches [170]. These data combined with evidence of c-Kit expression in KS tumors [174] contributed to a recent clinical trial that demonstrated the efficacy of Imatinib Mesylate as a therapeutic regimen for KS [175]. Because both c-Kit and PDGF-R are expressed in KS, the relative importance of these two targets remains unclear. However, the study is notable since it was the first to attempt to identify novel drug targets for KSHV using microarrays, and to test the efficacy of a pharmacologic agent in a disease model [176]. The same conceptual approach was used to identify two KSHV-induced cellular proteins, RDC-1 and Neuritin, with novel oncogenic properties [171]. RDC-1 was also identified as one of the most highly-induced genes in the microarray studies performed on primary DMVEC by Poole and colleagues [160]. E6/E7-DMVEC were recently used in a proteomics-based screen to identify KSHV induction of the enzyme heme oxygenase-1 (HO-1) [169]. HO-1 expression in KS tissue was confirmed by the authors and by a recent SAGE study [177]. Inhibition of HO-1-induced proliferation of infected cells by treatment with mesoporphyrin compounds may, upon further study, offer an additional treatment option for KS [169,178]

A second efficient culture system for studying KSHV biology based on the use of immortalized EC was described by Lagunoff and colleagues [158]. In this model, human neonatal DMVEC were immortalized by retroviral transduction of the telomerase reverse transcriptase subunit (hTERT) [179]. The telomerase-immortalized microvascular endothelial cells (TIME cells) retained a normal karyotype as well as many of the properties of the primary cells from which they were derived, including expression of CD31 and αVβ3-integrin, LDL uptake and tubule formation in matrigel. TIME cells grow well when serially passaged and become contact-inhibited at confluence, with the only morphologic change being a mild cell spindling.

TIME cells are infected at a high MOI with KSHV concentrated from TPA-induced BCBL-1 supernatants. Nearly all TIME cells are LANA-positive at 48 h following infection, and latency is the predominant outcome; only about 1% of infected cells express the early lytic protein ORF 59, and a smaller subset express the late structural glycoprotein K8.1. This system thus accurately reflects the state of the viral genome *in vivo* [94] and is reminiscent of what has been observed following *de novo* infection of primary and E6/E7-DMVEC [120,156]. In addition, due to the high frequency of initial infection, TIME cells provide a valuable system for studying early events in *de novo* infection. With increasing time PI, however, there is a rapid reduction in the infected TIME cell population, such that by tissue culture passage 7, less than 0.1% of cells are infected. Such loss of latently infected cells suggests inefficient maintenance of the viral episome and resembles what is seen with explanted KS spindle cells. However, because a percentage of TIME cells are lytically infected, infectious virus can be serially transferred or cultures can be maintained by addition of uninfected cells. An advantage of

the TIME system is the ease with which TIME cells can be cultivated and infected with high efficiency. In addition, potential effects from oncoproteins such as E6 and E7 are not a concern in this model.

While TIME cells establish a predominantly latent infection, they can be induced to lytically reactivate by infection with an ORF50-expressing adenovirus vector [152]. Using this system, Glausinger and Ganem [180] demonstrated that lytic KSHV infection strongly inhibits host gene expression by accelerating global mRNA turnover. Shut-off is mediated by the viral SOX (shutoff exonuclease) protein, the product of the HSV alkaline exonuclease homolog ORF37. The TIME cells were subsequently used to demonstrate that a subset of host transcripts, including IL-6 and the IL-1 type 1 receptor, escape host shut-off [180].

TIME cells express very little or no Prox-1 protein prior to KSHV infection, suggesting that the original immortalized clone was derived from blood vascular, as opposed to lymphatic, endothelium. Prox-1 is required for expression of two key markers that differentiate lymphatic from vascular endothelium, VEGFR3 and podoplanin [181]. TIME cells have thus also proved useful for investigating the hypothesis that KSHV infection drives EC to a more lymphatic phenotype, a hypothesis supported by the robust expression of lymphatic EC markers on KS spindle cells [182,183]. Carroll and colleagues [163] used RT-PCR and cDNA microarray techniques to investigate expression of genes specific to lymphatic EC following KSHV infection of TIME cells and reported significant induction of such genes including Prox-1, VEGFR3, podoplanin and LYVE-1. The microarray studies were performed after 24, 48 and 96 hrs of infection; 147 genes (about 1% of genes on the array) were significantly induced (>1.8 fold; $P < 0.001$) at all

timepoints and 61 genes significantly repressed. This list comprises another valuable data set with which to examine KSHV reprogramming of the host transcriptome, particularly at early times PI and when considering the blood vascular phenotype of the uninfected controls. Array analysis performed with primary cells may reflect a mixed population of both blood and lymphatic EC, since both types can be present in primary cultures [184]. In addition to the differential expression of lymphatic markers observed in KSHV-infected TIME cells, other interesting findings included significant upregulation of host IL-6 and significant downregulation of IL-8. Induction of IL-6 expression is consistent with the finding that IL-6 escapes host shutoff; loss of IL-8 protein expression in KSHV-infected E6/E7-DMVEC stimulated with IL-1β has also been observed [Ashlee Moses and Michael Jarvis, unpublished observations].

Telomerase-immortalized KSHV-infected DMVEC have also been described by Tomescu and colleagues [185]. The cells used in this study, TIME-T4 cells, were derived from a different parental cell than that of Lagunoff, *et al*, but were transduced with the same retroviral hTERT expression vector. TIME-T4 cells were infected with concentrated BCBL-1-derived virus or via co-culture with BCBL cells. The TIME-T4 cells respond differently to KSHV infection than the original TIME cells in that a dramatic spindling was observed as soon as 24 hrs PI. This may be due to an inherent difference in the immortalized cell clone or due to quantitative or qualitative differences in the inoculae used. The T4 cells demonstrated downregulation of major histocompatibility complex (MHC) class I, and the adhesion molecules ICAM-1 and CD31, within 48 hrs of infection, indicating that this system is extremely useful for the study of KSHV immune evasion mechanisms in endothelial cells.. The effective

downregulation of MHC1 suggests that the T4 cells were expressing latent and IE lytic genes concurrently, as suggested by the studies of Krishnan and colleagues in primary cells [99]. TIME T4 cells should prove valuable for determining the kinetics of IE expression in EC, particularly for K3 and K5, as well as the window of time after their down regulation for which MHC1 and adhesion molecule surface expression remains functionally compromised.

The above text contains multiple references to gene expression profiling experiments performed on KSHV-infected EC. Taking into account the differences in experimental design, including cells and virus origin, infection and culture conditions and the microarray platforms used, it is not surprising that the correlation between these data sets is modest. Genes that are commonly induced could reveal patterns that are general responses to virus infections, as well as genes so tightly linked to KSHV pathogenesis that their dysregulation transcends differences in experimental design. On the other hand, differences may reveal valid temporal changes in gene expression or culture-specific events. Analyses performed at early times PI would no doubt include host genes deregulated by those early lytic viral genes that may be transiently expressed, while those performed after many tissue culture passages (with episome maintenance) would more reflect the influences of latent gene expression, as well as the delayed effects of those early events PI. Collectively, these studies should be viewed as a valuable database from which to further analyze links between KSHV pathogenesis and cellular gene expression and function. Valuable progress has already been made in this regard.

5. Parental lineage of spindle cells

Identification of the cellular origin of KS lesional spindle cells has been a matter of ongoing debate. Spindle cells express endothelial cell, smooth muscle cell, macrophage, fibroblast and dendritic cell markers [12,186], but are generally accepted to originate from an endothelial cell precursor. In addition to expressing markers of blood vascular endothelium, KS spindle cells express several markers specific for lymphatic endothelium, including VEGF-R3 and podoplanin [182,183,187]. These observations suggest either that KSHV preferentially infects lymphatic endothelium *in vivo*, or that KSHV infects precursor EC, lymphatic EC (LEC) or blood vascular EC (BEC) and drives the gene expression profile to a more convergent one, where lymphatic-lineage markers are induced or retained. As discussed in detail in this chapter, KSHV infects blood vascular EC *in vitro*; if however lymphatic EC were the preferred target *in vivo*, this may in part explain the inability of KSHV-infected EC to maintain the genome in tissue culture for extended periods. Wang, *et al* recently infected both LEC and BEC with KSHV and found using quantitative PCR that KSHV genomes were maintained at higher copy number in LEC [162]. This same group compared gene expression profiles of nodular KS samples and normal skin using Affymetrix U133A arrays and developed a KS expression signature of 1,482 genes by removing genes expressed at similar levels in both KS and normal dermis or epidermis. Using expression profiles similarly generated from purified LEC and BEC, the authors found that while both LEC and BEC markers were present in KS tissue, the KS expression signature was more like that of the LEC. Interestingly, infection of LEC and BEC led to a convergence of their profiles such that they were more like each other than the uninfected counterparts. Hong and colleagues [161] compared gene

profiles of primary DMVEC at day 7 PI and found significant upregulation of key lymphatic lineage-specific genes following KSHV infection including Prox1, LYVE-1, reelin, follistatin, desmplakin and leptin receptor. In addition, retroviral transduction of BEC with LANA led to induction of Prox1, the master gene responsible for lymphatic vessel development [181]. This study is in agreement with a study done in TIME cells, an immortalized BEC line, reporting KSHV induction of lymphatic-lineage markers [163].

Collectively, these studies suggest that *in vitro* KSHV induces a transcriptional drift in BEC and LEC towards a more convergent phenotype. Since commercial EC preparations contain both LEC and BEC, the relative ratios in cell preparations used in different laboratories may have some bearing on the observed outcome of infection. Regarding immortalized EC, the BEC lineage of TIME cells was recently established [163]. E6/E7-immortalized EC on the other hand appear to have a more LEC-like phenotype; genes forLYVE-1, podoplanin, VEGFR-3, leptin receptor, oncostatin M receptor, c-MAF, and reelin are all expressed [Patrick Rose and Ashlee Moses; unpublished observations]. Since KSHV genomes appear to be better maintained in LEC than BEC [162], these findings may contribute to the ability of E6/E7-DMVEC to maintain the KSHV episome for long periods of time. The relevance of the above studies to KS tumors is clearly the appreciation that lymphangiogenic molecules are involved in KS pathogenesis; if KSHV infection of EC results in a cell type with characteristics of both vascular and lymphatic EC, understanding this unique tumor phenotype will be important for understanding the disease and developing effective clinical approaches.

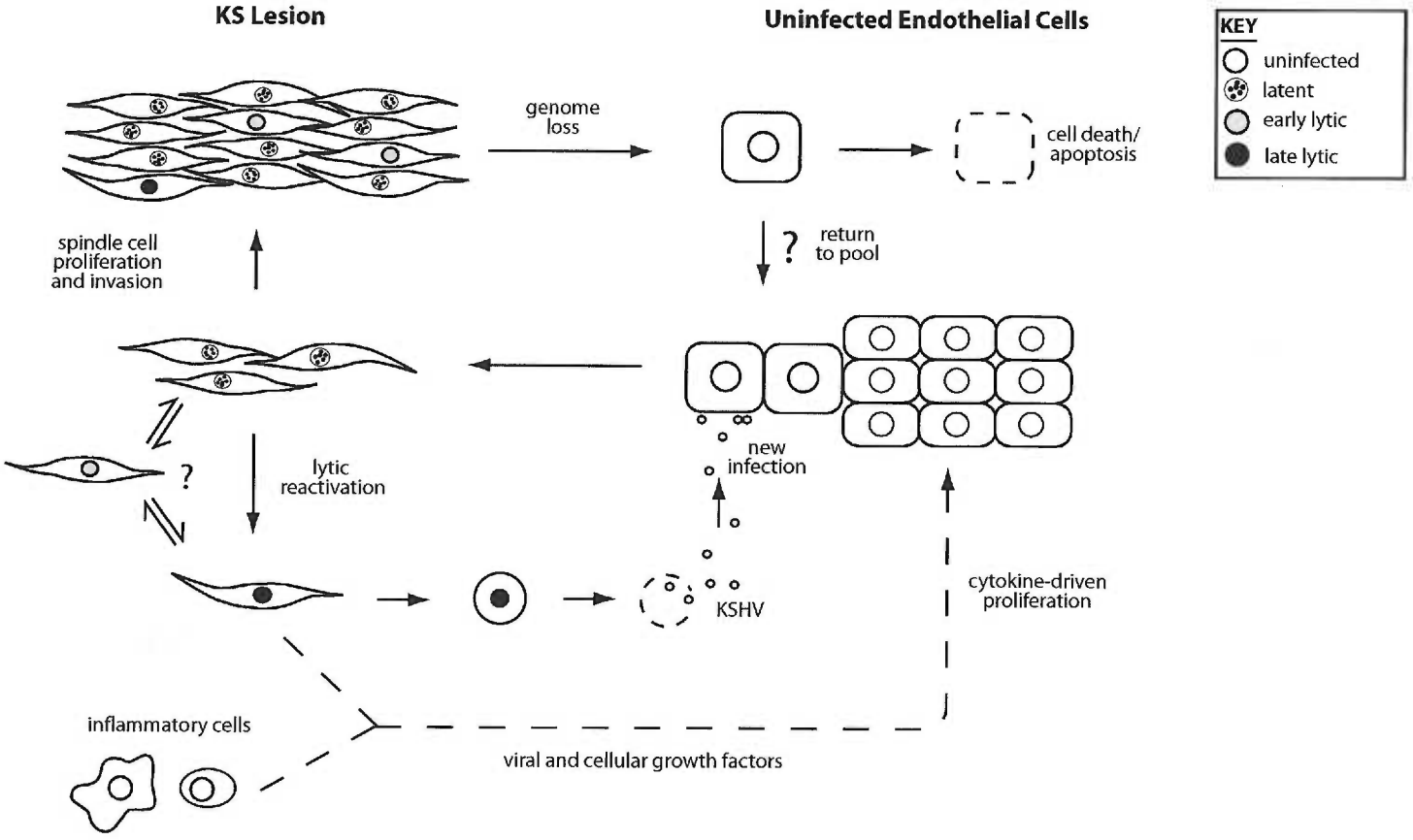
6. A model of KS lesion progression

The observed frequency of immunoreactivity for KSHV markers in EC infected *in vitro* using a limited panel of antibodies is LANA >> ORF 59 >> K8.1 (i.e. latent >> early lytic >> late lytic). As described elsewhere in this chapter, these ratios have been observed in studies using both primary and immortalized EC and different viral infection protocols. These ratios also accurately reflect gene expression patterns found in KS lesions by immunohistochemistry and *in situ* hybridization [69]. Two theoretical outcomes of lytic reactivation could account for the relative excess of early lytic versus late lytic gene expression. First, if all cells activated to lytic cycle consistently support viral DNA replication with assembly and release of infectious progeny, then all early lytic-positive cells would, in time, progress to cell lysis and death. Alternately, if only a fraction of lytically activated cells produce infectious progeny, then a pool of cells must exist that express several IE and early genes without supporting productive viral replication. Evidence that anti-herpes agents such as ganciclovir and foscarnet, which inhibit lytic but not latent herpesviral infection, can improve the clinical outcome of KS strengthens the assertion that ongoing lytic gene expression is involved in KS pathogenesis [116,188,189]. If every lytic event leads to cell death, then a continual pool of new targets for *de novo* infection must be recruited; on the other hand, limited expression of IE and E genes would allow for immune evasion and anti-apoptotic mechanisms, as well for as the angiogenic and chemoattractant properties of lytic genes, to contribute to tumor formation without a net loss of cells. The clinical responsiveness of KS to drugs that block proliferation of latently-infected cells [175] indicates that lytic reactivation is not the only mechanism driving KS lesion progression. Accumulation of latently-infected cells in

KS lesions is due to proliferation and tissue invasion of latently-infected cells. The rapid loss of the KSHV genome upon culture of explanted spindle cells may reflect an insufficiency of the episomal maintenance machinery in endothelial lineage cells. It is unknown if this occurs *in vivo* when virally-infected spindle cells are stimulated to divide. Loss of the cytoprotective functions of some KSHV genes could lead to apoptotic cell death of spindle cells following cell division without episomal maintenance. Another possibility is that spindle cells that have lost the viral genome could become targets for re-infection; multiple rounds of re-infection could contribute to the accumulation of mutations leading eventually to the outgrowth of truly transformed clonal populations of spindle cells.

The lack of a well characterized animal model currently precludes the *in vivo* examination of KSHV deletion mutants, so the specific gene expression requirements for KS disease states must be inferred indirectly. The clinical responsiveness of KS to drugs that block herpesvirus lytic cycle, and to drugs that block proliferation of latently-infected cells, indicates that both of these mechanisms can drive KS lesion establishment and progression, perhaps cooperatively (figure 1).

Figure 1


KS Lesion
Uninfected Endothelial Cells
KEY
uninfected
latent
early lytic
late lytic
genome loss
cell death/ apoptosis
spindle cell proliferation and invasion
?
return to pool
?
lytic reactivation
new infection
KSHV
cytokine-driven proliferation
inflammatory cells
viral and cellular growth factors