High Glucose Induces Reactivation of Latent Kaposi’s Sarcoma-Associated Herpesvirus

Fengchun Ye
Case Western Reserve University, fxy63@case.edu

Yan Zeng
University of Nebraska-Lincoln

Jingfeng Sha
Case Western Reserve University

Tiffany Jones
University of Texas Health Sciences Center at San Antonio

Kurt Kuhne
University of Texas Health Sciences Center at San Antonio

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/virologypub

Part of the Biological Phenomena, Cell Phenomena, and Immunity Commons, Cell and Developmental Biology Commons, Genetics and Genomics Commons, Infectious Disease Commons, Medical Immunology Commons, Medical Pathology Commons, and the Virology Commons


http://digitalcommons.unl.edu/virologypub/312

This Article is brought to you for free and open access by the Virology, Nebraska Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Virology Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
High Glucose Induces Reactivation of Latent Kaposi’s Sarcoma-Associated Herpesvirus

Fengchun Ye1,2,*, Yan Zeng3,5, Jingfeng Sha1, Tiffany Jones2, Kurt Kuhne2, Charles Wood3, and Shou-Jiang Gao2,4,*

1Department of Biological Sciences, School of Dental Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA;
2Department of Pediatrics, Greehey Children’s Cancer Research Institute, University of Texas Health Sciences Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229, USA;
3Nebraska Center for Virology and School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68583, USA;
4Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, California 90033, USA;
5Current address: Department of Biochemistry and Key Laboratory of Xinjiang Endemic and Ethic Diseases, Shihezi University School of Medicine, Xinjiang 83202, China.

*Correspondence to: Fengchun Ye, e-mail: fxy63@case.edu; Shou-Jiang Gao, email: shoujiang@usc.edu.

Key words: Diabetes, Kaposi’s sarcoma, Blood glucose, Hydrogen peroxide, and KSHV
High prevalence of Kaposi’s sarcoma (KS) is seen in diabetic patients. It is unknown if the physiological condition of diabetes contributes to KS development. We found elevated levels of viral lytic gene expression when Kaposi’s sarcoma-associated herpesvirus (KSHV) infected cells were cultured in high glucose medium. To demonstrate the association between high glucose and KSHV replication, we xenografted telomerase-immortalized human umbilical vein endothelial cells that are infected with KSHV (TIVE-KSHV) into hyperglycemic and normal nude mice. The injected cells expressed significantly higher levels of KSHV lytic genes in hyperglycemic mice than in normal mice. We further demonstrated that high glucose induced production of hydrogen peroxide (H$_2$O$_2$), which down regulated silent information regulator 1 (SIRT1), a class-III histone deacetylase (HDAC), resulting in epigenetic transactivation of KSHV lytic genes. These results suggest that high blood glucose in diabetic patients contributes to development of KS by promoting KSHV lytic replication and infection.

Multiple epidemiological studies have reported a higher prevalence of classic KS in diabetic patients. By using both in vitro and in vivo models, we demonstrated an association between high glucose and KSHV lytic replication. High glucose induces oxidative stress and production of H$_2$O$_2$, which mediates reactivation of latent KSHV through multiple mechanisms. Our results provide the first experimental evidence and mechanistic support for the association of classic KS with diabetes.
INTRODUCTION

Kaposi sarcoma (KS) is a vascular neoplasia etiologically associated with Kaposi’s sarcoma-associated herpesvirus (KSHV) infection (1). KSHV establishes a lifelong persistent latent infection following acute infection. Reactivation of the latent virus into productive lytic replication plays a pivotal role in the initiation and progression of KS as viral load positively correlates with KS progression. Indeed, treatment of KS patients with anti-herpesviral drugs effectively leads to regression of KS tumors (2-6).

Unlike iatrogenic or AIDS-associated KS, classic KS predominantly occurs in elderly men of Mediterranean or Jewish decent, with no apparent immune suppression (7). The exact cause for the development of classic KS remains undefined. Asthma, allergies in males, topical corticosteroid use, and infrequent bathing have been suggested as risk factors for classic KS (8-9). Multiple studies have also documented a high prevalence of classic KS in patients with diabetes mellitus (10-13), a metabolic syndrome that manifests with elevated levels of blood glucose and episodic ketoacidosis, either due to a lack of insulin (Type-1 diabetes) or cellular resistance to insulin (Type-2 diabetes). High levels of KSHV DNA and sero-positivity have been seen in diabetic patients (14-16). However, no study has ever determined if diabetes is the cause or effect of KS and whether high glucose level plays a role in the development of KS.

In the present study, we found increased levels of viral lytic gene expression when KSHV–infected primary effusion lymphoma cells were cultured in medium containing high levels of glucose. To further examine the association between high blood glucose and KSHV replication, we generated hyperglycemic nude mice with streptozotocin (STZ), which damages pancreatic β cells to result in hypoinsulinemia and hyperglycemia.
(17). We then xeno-grafted telomerase-immortalized human umbilical vein endothelial cells (18) that are re-infected with a recombinant Kaposi’s sarcoma-associated herpesvirus [TIVE-KSHV (BAC16)] (19), into the hyperglycemic and control healthy nude mice. The original TIVE-KSHV cells were malignantly transformed and grow “KS-like” tumors in nude mice (18). Although hyperglycemia did not seem to enhance tumor growth, the injected TIVE-KSHV (BAC16) cells expressed significantly higher levels of KSHV lytic genes in hyperglycemic mice than in normal mice. Results from cells cultured in vitro demonstrate that high glucose induces production of H$_2$O$_2$, which has been previously shown to trigger reactivation of latent KSHV through activation of the MAKP pathways (20-21). Interestingly, H$_2$O$_2$ also mediates down regulation of the class-III HDAC SIRT1 (22) to induce histone hyperacetylation of viral chromatins, resulting in active transcription of KSHV lytic genes. Our results suggest that H$_2$O$_2$ mediates high glucose induction of KSHV lytic gene expression and replication through multiple mechanisms.

To our knowledge, this study provides the first experimental evidence to support an association of diabetes with development of KS that has been suggested by previous epidemiological studies.

MATERIALS and METHODS

Cell culture, media, and reagents

TIVE-KSHV cells, originally infected with native KSHV (18), were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium plus 10% fetal bovine serum (FBS). We re-infected these cells with the recombinant KSHV BAC16 to obtain TIVE-
KSHV (BAC16) cells that stably express green fluorescence protein (GFP). RPMI 1640 medium without glucose was purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). BCBL1 cells were grown in RPMI 1640 medium with 1, 3, or 6 g/L D-glucose plus 10% FBS. Primary human umbilical vein endothelial cells (HUVECs) were grown in EBM-2 medium with growth factor supplements (Lonza, Allendale, New Jersey, USA).

A mouse monoclonal antibody to KSHV lytic protein RTA was a gift from the Pasteur Research Institute in Shanghai, China. A mouse monoclonal antibody to KSHV lytic protein K8α was purchased from MyBiosource, Inc. (San Diego, California, USA). A rat antibody to KSHV latent nuclear antigen (LANA) was purchased from Advanced Biotechnologies, Inc. (Columbia, Maryland, USA). A mouse monoclonal antibody to SIRT1 was purchased from EMD Millpore (Temecula, California, USA). D-glucose and L-glucose were purchased from Sigma-Aldrich.

Generation of hyperglycemic mice and xeno-grafting of TIVE-KSHV (BAC16) cells

A total number of 32 athymic nude mice (4 week old, female) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The blood glucose level of each mouse was measured before any treatment by using a glucose meter. The mice were then randomly separated into two groups, one being treated with intra-peritoneal (IP) injection of Streptozotocin (STZ, Sigma-Aldrich) at a dose of 200 mg/kg body mass, twice a week for 2 weeks. The other group of untreated mice was used as a control. Two weeks after the last STZ treatment, the blood glucose level of each mouse from both groups was measured again to confirm development of hyperglycemia in the treated mice. Equal
numbers of TIVE-KSHV (BAC16) cells at 5 x 10^6 cells per injection site, 2 sites per mouse, were then subcutaneously injected into each mouse at the abdominal region. Tumor volumes (length x width x height) were measured once a week with a caliper. At the end of experiments, all tumors were surgically removed from the mice. All procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and following a protocol (2011-0802) that was approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University.

**Immuno-chemical staining and imaging**

Fresh frozen sections were prepared from the surgically removed tumors. A standard procedure for preparation and staining of acetone-fixed frozen tissue sections was followed, using primary antibodies to KSHV small capsid protein (ORF65), latent protein LANA, and control IgG. After multiples washes with PBS, the primary antibody-antigen signals were revealed with a biotinylated secondary antibody and streptavidin-horseradish peroxidase, and DAB (3,3’-diaminobenzidine) detection system (Biolegend, San Diego, California, USA). DAPI was used for nuclear staining. Images were captured under a microscope (Carl Zeiss, Inc., Thornwood, NY).

**Isolation of Total RNAs and Quantification of mRNA by qRT-PCR**

Total RNAs were isolated using a RNA purification kit from QIAGEN, which includes a step to remove residual genomic DNA prior to RNA purification. Reverse transcription (RT) of total RNA was performed by using Superscript Transcriptase II.
qRT-PCR was conducted to quantify different viral transcripts using primers described previously (23). The mRNA level of the housekeeping gene β-actin was used as a reference for normalization, using the primers

5’ATTGCCGACAGGATGCAGA3’ (forward) and

5’GAGTACTTGCCTCAGGAGGA3’(reverse). All qRT-PCR reactions were carried out in triplicates.

**KSHV Virion Production and Titration**

The culture supernatants of BCBL1-BAC36 cells were collected 5 days after culturing in RPMI 1640 medium plus 10% FBS and various concentrations of D-glucose, followed by low-speed centrifugation (4,000 g, 15 min) to remove cellular debris. To determine the relative viral titers in the supernatants, 1 ml of the supernatant was used to infect HUVECs in 6-well plates. At 72 h post-infection, cells were harvested and counted with a hemocytometer under a fluorescent microscope. The numbers of KSHV infected GFP-positive cells and the numbers of total cells from 8 independent readings were used to calculate the average percentage of GFP-positive cells, which was used as the relative viral titer of the supernatant in question.

**Chromatin Immuno-Precipitation (ChIP) Assay**

Equal numbers of BCBL1-BAC36 cells (8 x 10^6 cells) were cultured in RPMI 1640 medium with various concentrations of D-glucose with and without catalase (400 unites/ml) for 24 h, followed by fixation with 0.5% formaldehyde for 15 min. Chromatin suspensions were prepared, and ChIP assays were performed using a ChIP assay kit.
(Invitrogen) with antibodies to RNA polymerase II (RN Pol II), acetylated histone-4 (H4K12-Ac), histone-3 (H3K9-Ac), histone-3 (H3), LANA, and rabbit IgG, all from Millipore, as well as a rat monoclonal antibody to LANA and a rat IgG (as control) as described above. DNA from input and the end ChIP products were isolated by using a DNA purification kit (QIAGEN). The purified DNA was re-suspended in 200 μl sterile water, and used for qPCR quantification for specific viral chromatin with the following primers: 5’CTCATCGTGGAGCTGTCACACG3’ (RTA promoter-forward) and 5’TCTCCCGATGGCGACGTGCACTAC3’ (RTA promoter-reverse) from RTA (ORF50) promoter region.

**Measurement of intracellular H₂O₂**

BCBL1-BAC36 cells were cultured in RPMI 1640 medium plus 10% FBS with 1, 3, and 6 g/L D-glucose for 24 hours. The cells were collected and washed twice with ice-cold PBS, and re-suspended in 1 x assay buffer of the OxiSelect™ hydrogen peroxide/peroxidase assay kit from Cell Biolabs, Inc. (San Diego, California, USA) at a concentration of 2 x 10⁶/ml. The cells were homogenized by sonication, followed by high speed centrifugation (10,000 g, 15 minutes at 4°C). The supernatants and 1 x assay buffer (as background) were loaded into 96-well plate for measurement of the relative levels of H₂O₂, with 6 repeats per sample. In parallel, a series of different concentrations (0 to 10 μM) of H₂O₂ were loaded into the same plate. The florescence H₂O₂ detection reaction was read under a fluorescence microplate reader (BIO-TEK, Winooski, Vermont, USA) at 560 nm (excitation) and 600 nm (emission). Upon subtraction of each reading with that of the background, a standard curve was established with relative fluorescence...
units (RFU) from the different concentrations of H₂O₂, and the concentrations of H₂O₂ in the samples were determined by comparing RFU of the samples with the standard curve.

RESULTS

Cells cultured in high concentrations of D-glucose display increased KSHV lytic gene expression

To test the effect of high concentration of glucose on KSHV gene expression, we conducted independent experiments in three different laboratories. The Wood laboratory cultured the original KSHV-infected BCBL1 cells in RPMI 1640 medium containing 1, 3, and 6 g/L D-glucose, which are equivalent to 100, 300, and 600 mg/dL as measured by clinic glucose meters, respectively. Clearly, BCBL1 cells expressed significantly higher levels of RTA and K8.1 mRNA (Fig. 1A), as well as RTA and K8α proteins when cultured in medium containing 3 and 6 g/L D-glucose (Fig. 1B and C).

Adding more D-glucose also changes the osmolality of the medium. To rule out possible effect of osmolality on KSHV gene expression, the Gao and Ye laboratories cultured BCBL1 cells carrying the recombinant KSHV, BAC36 (24), in RPMI 1640 medium containing 1, 3, and 6 g/L of D-glucose and 5, 3, and 0 g/L L-glucose, respectively. L-glucose, which cannot be metabolized by the cells, was used to balance the osmolality in medium with lower levels of D-glucose, in order for all cells to be compared with the same osmolality. As shown in Fig. 1E, F, and G, under these conditions, higher concentrations of D-glucose increased expression of RTA and ORF65 in BCBL1-BAC36 cells. Similar effects were also seen in TIVE-KSHV (BAC16) cells (Fig. 1D). In addition, BCBL1-BAC36 cells cultured in high levels of D-glucose
produced higher titers of virions (Fig. 1H). Collectively, these results indicate that high glucose enhances KSHV lytic gene expression and replication in different types of cells.

**Generation of hyperglycemic nude mice and xeno-grafting of TIVE-KSHV cells**

To further examine the association between high blood glucose in diabetic KS patients and KSHV replication, we next generated hyperglycemic nude mice by using the commonly used antibiotic STZ. Before treatment, the blood glucose level of each mouse was measured using a glucose meter. All 32 mice had a blood glucose level within the normal range (100 to 140 mg/dL) (Fig. 2A). The mice were then randomly divided into two groups. One group of mice were injected with STZ at a dose of 200 mg/kg body mass, twice weekly for 2 weeks, and the second group were injected with a placebo (PBS). Two weeks after the treatment, we measured the blood glucose levels of all mice again. All STZ-treated mice displayed permanent diabetic levels of blood glucose (Fig. 2A) and symptoms of diabetes such as excessive thirst and loss of weight (Fig. 2B).

We then subcutaneously injected TIVE-KSHV (BAC16) cells at the abdominal region at a dose of $5 \times 10^6$ cells per injection site, two sites per mouse, into the two groups of mice for tumor development. Eight weeks after inoculation, we surgically collected the tumors. As shown in Fig. 2C and D, no significant difference in tumor volume was seen between the two groups, except two of the STZ-treated mice developed a secondary tumor at the neck region. These secondary tumors had fewer cells that expressed KSHV latent protein LANA and contained large numbers of mouse inflammatory cells expressing the mouse macrophage marker F4/80 (data not shown).


TIVE-KSHV (BAC16) cells express higher levels of KSHV lytic genes in hyperglycemic mice

To examine how blood glucose level impacts viral gene expression, we isolated total RNA from 8 tumors from each group of mice and measured the mRNA levels of KSHV replication and transcription activator (RTA, ORF50) by qRT-PCR. All tumors from STZ-treated mice express higher levels of RTA mRNA (Fig. 3A). We then extracted total proteins from 8 tumors of each group and conducted Western blot analysis to measure viral proteins. As shown in Fig. 3B, all 8 tumors from STZ-treated mice expressed much higher levels of RTA protein than the control mice. To further confirm increased expression of KSHV lytic genes in tumors from STZ-treated mice, we performed immune-histochemical staining on sections of the other 8 tumors from each group with a monoclonal antibody to KSHV small capsid protein (ORF65). As shown in Fig. 3C and D, the numbers of cells expressing ORF65 are 4.8 times higher in tumors from STZ-treated mice than in tumors from the control mice.

Since we waited two weeks after the last STZ treatment before injecting TIVE-KSHV (BAC16) cells into the mice, it is unlikely that the increased expression of RTA and ORF65 resulted from STZ treatment itself. To rule out that possibility, we cultured TIVE-KSHV (BAC16) cells in the absence or presence of STZ, followed by Western blot detection of RTA and LANA proteins. As shown in Fig. 3E, STZ treatment had little effect on expression of RTA and LANA. Hence, the elevated levels of blood glucose in STZ-treated mice are responsible for the increased expression of KSHV lytic genes.
High concentrations of glucose enhances KSHV lytic gene expression by inducing H$_2$O$_2$

Metabolic syndromes including diabetes are well known for the production of excessive amounts of reactive oxygen species (ROS) such as H$_2$O$_2$ (25-30), which has previously been shown to trigger reactivation of latent KSHV into lytic replication (20-21, 31). To monitor changes in the level of intracellular H$_2$O$_2$, we used a previously established BCBL1 cell line that stably expresses the H$_2$O$_2$ sensor protein Hyper-cyto (21). The Hyper-cyto protein exhibits two excitation peaks at 420 and 500 nm and one emission peak at 516 nm. Upon exposure to H$_2$O$_2$, the excitation peak at 420 nm decreases in proportion to the increase in the peak at 500 nm, and cells become yellow fluorescent when intracellular H$_2$O$_2$ surpasses the threshold level (32). We cultured these cells in RPMI 1640 medium containing 1, 3, and 6 g/L D-glucose for 24 hours respectively. As shown in Fig. 4A and B, the intracellular level of H$_2$O$_2$ increased significantly when cells were cultured in higher concentrations of D-glucose. To further confirm that high glucose induces H$_2$O$_2$ production, we cultured BCBL1-BAC36 cells in RPMI 1640 medium containing 1, 3, and 6 g/L D-glucose for 24 hours respectively, prepared cell lysates from equal numbers (2 x 10$^6$) of cells in 1 ml assay buffer, and measured their relative intracellular H$_2$O$_2$ concentrations by using a hydrogen peroxide/peroxidase assay kit from Cell Biolabs, Inc. Cells cultured in medium containing 3 and 6 g/L D-glucose definitely produced higher levels of H$_2$O$_2$ (Fig. 4C).

To demonstrate that H$_2$O$_2$ was responsible for the increased KSHV lytic gene expression, we next cultured BCBL1-BAC36 cells in RPMI 1640 medium containing 1 and 6 g/L D-glucose respectively, in the absence or presence of catalase or the
antioxidants N-acetyl-cysteine (NAC) and glutathione. As shown in Fig. 4D, catalase abolished high glucose-induced transcription of RTA and ORF65. The three different antioxidants inhibited high glucose-induced expression of ORF65 protein in a dose-dependent manner (Fig. 4E). Collectively, these results suggest that induction of KSHV lytic gene expression by high glucose is mediated by \( \text{H}_2\text{O}_2 \).

High glucose down regulates class-III HDAC SIRT1 to increase histone acetylation and transactivate viral chromatin

We previously showed that \( \text{H}_2\text{O}_2 \) activated the MAP kinases ERK-1/2, JNK, and p38 to induce expression of KSHV lytic genes (21). Consistent with our previous finding, BCBL1-BAC36 cells cultured in medium containing high concentration of D-glucose displayed increased phosphorylation of ERK1/2, JNK, and p38 and expression of KSHV lytic protein RTA, which can be inhibited by catalase (Fig. 5A). In addition, inhibitors of ERK1/2, JNK, and p38 significantly inhibited high glucose induction of RTA transcription (Fig. 5B), thus confirming a critical role of MAPK activation in high glucose induction of RTA expression.

To investigate other mechanisms that might be involved in high glucose induction of KSHV lytic replication, we examined the expression of SIRT1, which is a member of class-III HDAC and a key factor involved in the development of diabetes (33-39). In addition, several studies have demonstrated the involvement of SIRT1 in the regulation of KSHV lytic gene expression through epigenetic remodeling (40-42). Immuno-fluorescence antibody (IFA) staining showed that SIRT1 expression was substantially reduced in BCBL1-BAC36 cells cultured in medium containing 6 g/L D-
glucose compared to cells cultured in medium containing 1 g/L D-glucose (Fig. 6A and 
B). Consistent with the IFA results, data from Western blot analysis showed that the 
protein level of SIRT1 was reduced by D-glucose in a dose-dependent manner (Fig. 7A). 
To examine if H2O2 plays a role in SIRT1 down regulation, we cultured BCBL1-BAC36 
cells in medium containing low and high glucose in the presence of various doses of 
catalase. As shown in Fig. 7B, catalase dose-dependently blocked SIRT1 down regulation 
in cells that were cultured in medium containing 6 g/L D-glucose. In a parallel 
experiment, we found that treating BCBL1-BAC36 cells with H2O2 also resulted in 
SIRT1 down regulation, which could be blocked by catalase as well (Fig. 7C). Together, 
these results suggest that H2O2 mediates SIRT1 down regulation in cells that are cultured 
in medium containing high concentration of glucose.

As a consequence of SIRT1 down regulation, BCBL1-BAC36 cells cultured in 
medium containing high concentrations of D-glucose displayed increased levels of 
acetylated histones, which could be reduced by adding catalase to the culture medium 
(Fig. 7D). To demonstrate that these epigenetic changes indeed occur in viral 
chromatins, we next conducted ChIP assays. By performing qPCR using primers specific 
for the promoter region of KSHV lytic gene RTA, we detected significantly higher levels 
of acetylated histones and RNA polymerase II in this region of viral chromatin when cells 
were cultured in medium containing high concentrations of glucose (Fig. 5E and F). 
These results suggest that, in addition to activation of MAKP pathways, high glucose also 
transactivate KSHV lytic gene expression via epigenetic modifications of the RTA 
promoter region.
DISCUSSION

Multiple studies have reported high prevalence of classic KS in patients with diabetes mellitus (10-13), and KSHV DNA was detected in more than 50% of type-2 diabetic patients (14-16). These clinical studies seem to suggest that diabetes patients are more prone to KSHV infection and that diabetes is a risk factor for development of classic KS. However, whether this metabolic syndrome really contributes to KS tumor development has never been experimentally tested.

Type-1 diabetes results from insulin deficiency due to the lack of insulin-producing β cells in the pancreas. In contrast, type-2 diabetes occurs in adults as a consequence of the development of cellular resistance to insulin. Despite the different mechanisms, a common outcome of both types of diabetes is high glucose levels in the plasma. In the present study, we found increased levels of KSHV lytic gene expression when KSHV infected BCBL1 and TIVE-KSHV cells were cultured in media containing diabetic levels of glucose. In full support of data from the \textit{in vitro} study, TIVE-KSHV cells also displayed substantially higher expression of KSHV lytic genes in hyperglycemic mice than in mice with normal level of blood glucose. These results strongly suggest that high levels of blood glucose promote development of KS by inducing productive KSHV lytic replication.

One of the manifestations by metabolic syndromes such as obesity and diabetes is production of excessive levels of ROS (25-30). By using a previously established BCBL1 cell line that stably expressing the H$_2$O$_2$ sensor protein pHyper-cyto (21), we demonstrated that cells cultured in high concentrations of glucose produce increased levels of intracellular H$_2$O$_2$. This result was further confirmed by another intracellular
H$_2$O$_2$ measurement assay. Notably, addition of catalase, which converts H$_2$O$_2$ into H$_2$O and O$_2$, and the anti-oxidants NAC and glutathione, effectively blocked high glucose induction of KSHV lytic gene expression in a dose-dependent manner. Therefore, H$_2$O$_2$ mediates high glucose induction of KSHV lytic gene expression, which further supports previous reports that H$_2$O$_2$ is an important physiological factor involved in reactivation of latent KSHV (20-21, 31). Interestingly, H$_2$O$_2$ has also been shown to enhance viral entry (43-45). It is therefore highly possible that the hyperglycemic environment in diabetic KS patients contributes to development of KS by promoting both productive KSHV replication and recurrent de novo infection.

Similar to stimulation with H$_2$O$_2$ (21), culturing cells in medium containing high concentrations of D-glucose also activates ERK1/2, JNK, and p38, and inhibitors of these MAPK pathways inhibit high glucose induction of KSHV gene expression. Interestingly, we found that high glucose and H$_2$O$_2$ also cause down regulation of the class-III HDAC SIRT1, leading to increased levels of histone acetylation in the promoter region of KSHV key lytic gene RTA. Thus, high glucose also engages this epigenetic mechanism to promote KSHV lytic gene expression. SIRT1 is well known for its anti-aging, anti-oxidative stress, and anti-inflammation properties (22, 46-47), and down regulation of SIRT1 has been linked to development of diabetes (48). Suppression of SIRT1 has been shown to trigger reactivation of latent KSHV (42, 49). SIRT1 is a member of the Sirtuin protein family that couples histone lysine deacetylation to NAD hydrolysis (50-52). The dependence of SIRT1 on NAD links its enzymatic activity directly to the energy status of cells via the cellular NAD to NADH ratio, the absolute levels of NAD, NADH or nicotinamide, or a combination of these variables.
The development of KS is a complex process. KSHV infection resulting from productive lytic replication plays an essential role in the initiation and progression of KS. However, in already formed KS tumors, KSHV-infected tumor cells are predominantly latent (53). Inflammatory cytokines, stress, and ROS are known to stimulate KSHV reactivation from latency (54). Under highly inflammatory and stressful conditions such as diabetes, it is expected that the latent virus undergoes reactivation. In this study, we xeno-grafted the KS tumor model cell line TIVE-KSHV (BAC16) into normal and hyperglycemic nude mice. While no obvious difference in tumor growth was seen between the two groups of mice, we did find significantly higher numbers of TIVE-KSHV (BAC16) cells undergoing lytic replication in hyperglycemic mice than in normal mice. Nevertheless, the majority of TIVE-KSHV cells in tumors from hyperglycemic mice remain latently infected, suggesting that the virus might have evolved unique mechanisms to overcome the highly inflammatory and stressful conditions to maintain latency. One possible mechanism might be through modulation of the cellular metabolic status. In support to this hypothesis, our recent study demonstrated that KSHV inhibits cellular aerobic glycolysis and oxidative phosphorylation by inhibiting expression of GLUT1 and GLUT3, thus preventing overflow of the metabolic pathways and maintaining the homeostasis of the latently infected and malignantly transformed cells (55).

In summary, our study provides the first evidence for a link between diabetes and higher levels of KSHV replication, which may lead to development of classic KS. Our results highlight H$_2$O$_2$ as the mediator for high glucose induction of KSHV lytic
replication through multiple mechanisms, which may shed lights on development of new strategies to prevent KSHV infection and KS development in diabetic patients.

ACKNOWLEDGEMENT

The present work was supported by the Immunology Alliance Fund from Case Western Reserve University to Fengchun Ye. This work was also supported by grants from NIH to Shou-Jiang Gao (CA096512, CA124332, CA132637, DE025465 and CA197153); and to Charles Wood (CA65903, P30 GM103509 and Fogarty D43 TW01492). We thank Dr. Ke Lan from the Pasteur Research Institute in Shanghai, China for providing antibodies to KSHV lytic protein RTA. We are grateful to Dr. Jae U. Jung from University of Southern California, USA for providing the recombinant KSHV BAC16. We declare no conflict of interest.

FIGURE LEGENDS

Figure-1. Cells cultured in medium containing higher concentrations of D-glucose express increased levels of KSHV lytic genes. A, relative levels of RTA and K8.1 mRNAs in BCBL1 cells that were cultured in RPMI 1640 medium containing 1, 3, and 6 g/L D-glucose (D-Glu) for 24 hours respectively. B, Western blot detection of RTA and K8α proteins from BCBL1 cells treated as described in A. C, relative levels of RTA and K8α protein in Western blots shown in B. The intensity of RTA or K8α band from each sample was first normalized to that of the β-tubulin band from the same sample. The levels of RTA and K8α proteins in cells cultured in medium containing 1 g/L D-glucose
were then set as the reference with a value of 1.0, and the relative levels of these proteins in other cells were the ratios between their band intensities and that of the reference. 

**D,** relative levels of RTA and ORF57 mRNAs in TIVE-KSHV cells cultured in DMEM medium containing 10% FBS and 1 or 6 g/L D-glucose (D-Glu) plus 5 or 0 g/L L-glucose (L-Glu) respectively. 

**E,** relative levels of ORF50 (RTA) and ORF65 mRNAs in BCBL1-BAC36 that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/L D-glucose (D-Glu) plus 5, 3, or 0 g/L L-glucose (L-Glu) for 24 hours (for RTA mRNA) and 72 hours (for ORF65 mRNA), respectively. 

**F,** Western blot detection of RTA and ORF65 proteins from BCBL1-BAC36 cells treated as described in **E; G,** relative levels of RTA and ORF65 proteins in Western blots shown in **F,** which were calculated as described in **C.** 

**H,** percentages (%) of GFP-positive HUVECs at 48 hours post infection (hpi) with supernatant from equal numbers (8 x 10⁶) of BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS, 1, 3, or 6 g/L D-glucose (D-Glu) plus 5, 3, or 0 g/L L-glucose (L-Glu) for 5 days respectively. All qRT-PCR reactions consisted of triplicates, and the differences in relative mRNA levels (fold) of RTA, ORF57, K8.1, and ORF65 between cells cultured in medium containing 1 g/L D-glucose and those cultured in medium containing 3 or 6 g/L D-glucose were all significant with P values < 0.005.

**Figure-2.** Generation of hyperglycemic nude mice and xeno-grafting TIVE-KSHV (BAC16) cells for tumor development. A total of 32 athymic nude mice (4 weeks old, female) were randomly separated into two groups, with one group of mice being treated with STZ (200 mg/kg body mass, 2 IP injections per week, for 2 weeks) to develop...
hyperglycemia and the other group of mice injected with PBS (placebo) as a control. Equal numbers (5x10^6/injection site, 2 sites/mouse) of TIVE-KSHV (BAC16) cells were subcutaneously injected into the mice for tumor development two weeks after the last STZ treatment. A, average blood glucose levels of STZ-treated mice and un-treated mice (control) measured at 4 and 12 weeks after the first STZ treatment. B, average weights of the two groups of mice at 2 and 12 weeks after the first STZ treatment. C, representative tumors from the two groups of mice collected at the end of experiment. D, average volumes (length x width x height) of tumors from the two groups of mice at different week post inoculation of TIVE-KSHV (BAC16) cells.

Figure-3. Tumors from hyperglycemic mice express significantly higher levels of KSHV lytic gene expression. A, average mRNA levels of KSHV lytic gene ORF50 (RTA) from 8 tumors of STZ-treated and untreated (control) mice respectively. B, Western blot detection of lytic protein RTA and latent protein LANA from 8 tumors of each group. β-tubulin was used as loading control. C, immuno-chemical staining of KSHV small capsid protein (ORF65) and LANA on tumors from three STZ-treated and three control mice (M1, M2, and M3). Staining with mouse or rat IgG was done in parallel as a negative control. D, average numbers of ORF65-positive cells per microscopic field in tumor sections from the two groups of mice. E, Western blot detection of RTA and LANA proteins from TIVE-KSHV (BAC16) cells that were cultured with and without STZ (1 μM) and TPA (25 ng/ml) for 24 hours respectively.
Figure-4. High glucose induces H$_2$O$_2$ to induce KSHV lytic gene expression. A and B, BCBL1 cells stably expressing the H$_2$O$_2$ sensor protein pHyper-cyto were used to measure the relative levels of intracellular H$_2$O$_2$. The number of cpYFP-positive cells (A) and fluorescence intensity (B) were quantified by flow cytometry analysis, following a 24 hours culture in RPMI 1640 medium containing 10% FBS and 1 (red), 3 (green), or 6 (purple) g/L D-glucose respectively. Regular BCBL1 cells cultured in RPMI 1640 medium containing 10% FBS and 2 g/L D-glucose were used as a background control for flow cytometry analysis (black). Culture with each glucose concentration consisted of 6 replicates. C, relative intracellular H$_2$O$_2$ concentrations from equal numbers ($2 \times 10^6$) of BCBL1-BAC36 cells that were cultured as described in A and B. Measurement of H$_2$O$_2$ was carried out by using the OxiSelect™ hydrogen peroxide/peroxidase assay kit from Cell Biolabs, Inc. D, ORF50 (RTA) and ORF65 mRNA levels in BCBL1-BAC36 cells cultured in RPMI 1640 medium containing 1 or 6 g/L D-glucose (D-Glu) in the presence or absence of 400 U/ml of catalase (Cat) for 24 h (for RTA mRNA) and 72 h (for ORF65 mRNA) respectively. Differences in mRNA levels between cells cultured in medium containing 1 g/L and those cultured in medium containing 6/L D-glucose or between culture with and without catalase were all significant with P values <0.005. E, Western blot detection of KSHV small capsid protein (ORF65) in BCBL1-BAC36 cells cultured in RPMI 1640 medium containing 1 or 6 g/L D-glucose (D-Glu) in the presence of different doses of catalase and antioxidants NAC and glutathione for 72 hours respectively.
Figure-5. High glucose activates MAPK pathways to induce KSHV gene expression. A, Western blot detection of ERK-1/2, JNK, p38, and their phosphorylated counterparts, as well as RTA and β-tubulin in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 1 or 6 g/L D-glucose (D-Glu), or stimulated with 400 µM H₂O₂, in the presence or absence of 200 U/ml catalase (Cat) for 24 hours. B, levels of RTA mRNA in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium with 1 or 6 g/L D-glucose (D-Glu) in the presence or absence of the different MAPK inhibitors for 24 hours. Concentrations of the inhibitors were as described previously (21).

Figure-6. High glucose downregulates expression of class-3 HDAC SIRT1. A, IFA staining of SIRT1 (red) in BCBL1-BAC36 cells cultured in RPMI 1640 medium containing 10% FBS and 1 or 6 g/L D-glucose for 24 h, using a mouse monoclonal antibody to SIRT1 and a rabbit anti-mouse IgG conjugated to Alexa Fluor®-594. DAPI was used for nuclear staining. The cells were imaged and analyzed under a fluorescence microscope with a 40x oil objective. B, average numbers of SIRT-1 foci (red dot) per cell when cells were cultured at different concentrations of D-glucose.

Figure-7. H₂O₂ mediates high glucose downregulation of SIRT1 to epigenetically activate expression of KSHV lytic gene RTA. A and B, Western blot detection of SIRT1 protein in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/L D-glucose (D-Glu) for 24 h, in the absence (A) or presence (B) of various doses of catalase (Cat). C, Western blot detection of SIRT1 protein in BCBL1-BAC36 cells treated with different doses of H₂O₂ and catalase (Cat). D, Western
blot detection of acetylated histone-3 (H3K9-Ac) and histone-4 (H4K12-Ac), total histone-3 (H3) and histone-4 (H4), RTA and β-tubulin in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/L D-glucose (D-Glu) in the presence or absence of 400 U/ml catalase (Cat) for 24 h. E and F, ChIP assay detection of acetylated histones (H3K9-Ac and H4K12-Ac) and RNA polymerase II (RN Pol) in RTA promoter in BCBL1-BAC36 cells that were cultured with different concentrations of D-glucose (D-Glu) with and without 400 U/ml catalase (Cat) for 24 h. The relative amount of DNA in RTA promoter (RTA) from each ChIP reaction was determined by qPCR and calculated as the average ratio between the level of ChIP product and that of the input DNA from three repeats (F). Real-time PCR products from inputs and ChIP assays were also analyzed in a 1.5% agarose gel (E). Differences in the levels of H3K9-Ac, H4K12-Ac, and RN Pol in RTA promoter between cells cultured in medium containing 1 g/L and 3 or 6 g/L D-glucose, or between cells cultured with and without catalase, were all significant with P values <0.005.

REFERENCES


2. Broccolo, F., C. Tassan Din, M. G. Vigano, T. Rutigliano, S. Esposito, P. Lusso, G. Tambussi, and M. S. Malnati. 2016. HHV-8 DNA replication correlates with


for classical Kaposi's sarcoma. Journal of the National Cancer Institute 94:1712-1718.


A

\[
\begin{array}{c|cccc}
\text{H}_2\text{O}_2 (\text{uM}) & 0 & 0 & 0 & 200 \\
\text{Cat (U/ml)} & 0 & 0 & 200 & 0 \\
\text{D-Glu (g/L)} & 1 & 6 & 6 & 1 \\
p-\text{ERK1/2} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{ERK1/2} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{p-p38} & \text{---} & \text{---} & \text{---} & \text{---} \\
p-\text{p38} & \text{---} & \text{---} & \text{---} & \text{---} \\
p-\text{JNK} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{JNK} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{RTA} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{Tubulin} & \text{---} & \text{---} & \text{---} & \text{---}
\end{array}
\]

B

![Bar chart showing relative level of RTA mRNA with D-Glu (g/L) at 1, 6, 6, 6, 6, 6.](chart)

- ERK inh: - - + - -
- JNK inh: - - - + -
- P38 inh: - - + - -