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

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ABSTRACT

A high prevalence of Kaposi's sarcoma (KS) is seen in diabetic patients. It is unknown if the physiological conditions of diabetes contribute 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 levels and KSHV replication, we xenografted telomerase-immortalized human umbilical vein endothelial cells that are infected with KSHV (TIVE-KSHV cells) 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 levels induced the production of hydrogen peroxide (H₂O₂), which downregulated silent information regulator 1 (SIRT1), a class III histone deacetylase (HDAC), resulting in the epigenetic transactivation of KSHV lytic genes. These results suggest that high blood glucose levels in diabetic patients contribute to the development of KS by promoting KSHV lytic replication and infection.

IMPORTANCE

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 levels and KSHV lytic replication. High glucose levels induce oxidative stress and the production of H₂O₂, which mediates the 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.

Kaposi's 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 antiherpesvirus drugs effectively leads to regression of KS tumors (2–6).

Unlike iatrogenic or AIDS-associated KS, classic KS occurs predominantly in elderly men of Mediterranean or Jewish descent who have no apparent immune suppression (7). The exact cause of the development of classic KS remains undefined. Asthma, allergies in males, topical corticosteroid use, and infrequent bathing have been suggested to be 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, due to either a lack of insulin (type 1 diabetes) or cellular resistance to insulin (type 2 diabetes). High levels of KSHV DNA and seropositivity have been seen in diabetic patients (14–16). However, no study has ever determined if diabetes is the cause or an effect of KS and whether high glucose levels play 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 levels and KSHV replication, we generated hyperglycemic nude mice with streptozotocin (STZ), which damages pancreatic

β cells, resulting in hypoinsulinemia and hyperglycemia (17). We then xenografted telomerase-immortalized human umbilical vein endothelial cells (18) that are reinfected with recombinant Kaposi's sarcoma-associated herpesvirus [TIVE-KSHV (BAC16) cells] (19) into hyperglycemic and control healthy nude mice. The original TIVE-KSHV cells are 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 levels induce the production of H₂O₂, which was previously shown to trigger the reactivation of latent KSHV through the activation of mitogen-activated protein kinase (MAPK) pathways (20, 21). Interestingly, H₂O₂ also mediates the downregulation of the class III his-

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tone deacetylase (HDAC) silent information regulator 1 (SIRT1) (22) to induce histone hyperacetylation of viral chromatin, resulting in active transcription of KSHV lytic genes. Our results suggest that H₂O₂ 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 the development of KS, which was 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 reinfected these cells with recombinant KSHV BAC16 to obtain TIVE-KSHV (BAC16) cells that stably express green fluorescent protein (GFP). RPMI 1640 medium without glucose was purchased from Thermo Fisher Scientific (Waltham, MA, USA). BCBL1 cells were grown in RPMI 1640 medium with 1, 3, or 6 g/liter D-glucose plus 10% FBS. Primary human umbilical vein endothelial cells (HUVECs) were grown in EBM-2 medium with growth factor supplements (Lonza, Allendale, NJ, USA).

A mouse monoclonal antibody to the KSHV lytic protein replication and transcription activator (RTA) was a gift from the Pasteur Research Institute in Shanghai, China. A mouse monoclonal antibody to the KSHV lytic protein K8 α was purchased from MyBiosource, Inc. (San Diego, CA, USA). A rat antibody to KSHV latent nuclear antigen (LANA) was purchased from Advanced Biotechnologies, Inc. (Columbia, MD, USA). A mouse monoclonal antibody to SIRT1 was purchased from EMD Millipore (Temecula, CA, USA). D-Glucose and L-glucose were purchased from Sigma-Aldrich.

Generation of hyperglycemic mice and xenografting of TIVE-KSHV (BAC16) cells. A total of 32 athymic nude mice (4 weeks old and female) were purchased from the 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, with one being treated with an intraperitoneal (i.p.) injection of 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 the development of hyperglycemia in the treated mice. Equal numbers of TIVE-KSHV (BAC16) cells at 5×10^6 cells per injection site, with 2 sites per mouse, were then subcutaneously injected into each mouse in the abdominal region. Tumor volumes (length by width by height) were measured once a week with a caliper. At the end of the experiments, all tumors were surgically removed from the mice. All procedures were carried out in strict accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (23) and according to a protocol (2011-0802) that was approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University.

Immunochemical staining and imaging. Fresh frozen sections were prepared from the surgically removed tumors. A standard procedure for the preparation and staining of acetone-fixed frozen tissue sections was performed, using primary antibodies to KSHV small capsid protein (open reading frame 65 [ORF65]), the latent protein LANA, and control IgG. After multiple washes with phosphate-buffered saline (PBS), primary antibody-antigen signals were revealed with a biotinylated secondary antibody, streptavidin-horseradish peroxidase, and a DAB (3,3'-diaminobenzidine) detection system (BioLegend, San Diego, CA, USA). DAPI (4',6-diamidino-2-phenylindole) 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 by using an 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 (Invitrogen, Carlsbad, CA). RT-quantitative PCR (qPCR) (qRT-PCR) was conducted to quantify the levels of different viral transcripts by using primers described previously (24). The mRNA level of the housekeeping gene β -actin was used as a reference for normalization, using the primers 5'-ATTGCCGACAGGATGCAGA-3' (forward) and 5'-GAGTACTTGCCTCAGGAGGA-3' (reverse). All qRT-PCRs 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 ($4,000 \times g$ for 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 postinfection, cells were harvested and counted with a hemocytometer under a fluorescence 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 immunoprecipitation assay. Equal numbers of BCBL1-BAC36 cells (8×10^6 cells) were cultured in RPMI 1640 medium with various concentrations of D-glucose with and without catalase (400 U/ml) for 24 h, followed by fixation with 0.5% formaldehyde for 15 min. Chromatin suspensions were prepared, and chromatin immunoprecipitation (ChIP) assays were performed by using a ChIP assay kit (Invitrogen) with antibodies to RNA polymerase II (Pol II), acetylated histone H4 (H4K12-Ac), acetylated histone H3 (H3K9-Ac), histone H3 (H3), LANA, and rabbit IgG, all from Millipore, as well as a rat monoclonal antibody to LANA and rat IgG (as a control), as described above. DNAs from the input and the end ChIP products were isolated by using a DNA purification kit (Qiagen). The purified DNA was resuspended in 200 μ l sterile water and used for qPCR quantification of specific viral chromatin with the primers 5'-CTCATCGTCGGAGCTGTCACACG-3' (RTA promoter forward) and 5'-TCTCCCGATGGCGACGTGCACTAC-3' (RTA promoter reverse) from the 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/liter D-glucose for 24 h. The cells were collected, washed twice with ice-cold PBS, and resuspended in $1 \times$ assay buffer from the OxiSelect hydrogen peroxide-peroxidase assay kit from Cell Biolabs, Inc. (San Diego, CA, USA), at a concentration of 2×10^6 cells/ml. The cells were homogenized by sonication, followed by high-speed centrifugation ($10,000 \times g$ for 15 min at 4°C). The supernatants and $1 \times$ assay buffer (as the background) were loaded into a 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 μ M) of H₂O₂ were loaded into the same plate. The fluorescent H₂O₂ detection reaction was read under a fluorescence microplate reader (Bio-Tek, Winooski, VT, USA) at 560 nm (excitation) and 600 nm (emission). Upon the subtraction of each reading from 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 the RFU of the samples with the standard curve.

RESULTS

Cells cultured with high concentrations of D-glucose display increased KSHV lytic gene expression. To test the effect of high concentrations 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/liter D-glucose, which are equivalent to 100, 300, and 600 mg/dl, respectively, as measured by clinic glucose meters. Clearly, BCBL1 cells expressed significantly higher levels of RTA and K8.1 mRNAs

(Fig. 1A) as well as RTA and K8 α proteins when cultured in medium containing 3 and 6 g/liter D-glucose (Fig. 1B and C).

The addition of more D-glucose also changes the osmolality of the medium. To rule out possible effects of osmolality on KSHV gene expression, the Gao and Ye laboratories cultured BCBL1 cells carrying the recombinant KSHV BAC36 (25) in RPMI 1640 medium containing 1, 3, and 6 g/liter of D-glucose and 5, 3, and 0 g/liter 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 to G, under these conditions, higher concentrations of D-glucose increased the 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 with high levels of D-glucose produced higher titers of virions (Fig. 1H). Collectively, these results indicate that high glucose levels enhance KSHV lytic gene expression and replication in different types of cells.

Generation of hyperglycemic nude mice and xenografting of TIVE-KSHV cells. To further examine the association between high blood glucose levels 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 by 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 was injected with STZ at a dose of 200 mg/kg body mass twice weekly for 2 weeks, and the second group was injected with a placebo (PBS). Two weeks after 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 into the abdominal region at a dose of 5×10^6 cells per injection site, with two sites per mouse, in 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 that two of the STZ-treated mice developed a secondary tumor at the neck region. These secondary tumors had fewer cells that expressed the 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 levels impact viral gene expression, we isolated total RNA from 8 tumors from each group of mice and measured the mRNA levels of KSHV RTA (ORF50) by qRT-PCR. All tumors from STZ-treated mice expressed higher levels of RTA mRNA (Fig. 3A). We then extracted total proteins from 8 tumors from each group and conducted Western blot analysis to measure viral protein levels. As shown in Fig. 3B, all 8 tumors from STZ-treated mice expressed much higher levels of RTA protein than did those of the control mice. To further confirm the increased expression levels of KSHV lytic genes in tumors from STZ-treated mice, we performed immunohistochemical staining on sections of the other 8 tumors from each group with a monoclonal antibody to the KSHV small capsid protein (ORF65). As shown in Fig. 3C and D, the numbers

FIG 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/liter D-glucose (D-Glu) for 24 h. (B) Western blot detection of RTA and K8 α proteins from BCBL1 cells treated as described above for panel A. (C) Relative levels of RTA and K8 α proteins in Western blots shown in panel B. The intensity of the RTA or K8 α band from each sample was first normalized to that of the β -tubulin band from the same sample. The levels of RTA and K8 α proteins in cells cultured in medium containing 1 g/liter 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 containing 10% FBS and 1 or 6 g/liter D-glucose plus 5 or 0 g/liter 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/liter D-glucose plus 5, 3, or 0 g/liter L-glucose for 24 h (for RTA mRNA) and 72 h (for ORF65 mRNA), respectively. (F) Western blot detection of RTA and ORF65 proteins from BCBL1-BAC36 cells treated as described above for panel E. (G) Relative levels of RTA and ORF65 proteins in the Western blots shown in panel F, which were calculated as described above for panel C. (H) Percentages of GFP-positive HUVECs at 48 h postinfection with supernatants from equal numbers (8×10^6) of BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/liter D-glucose plus 5, 3, or 0 g/liter L-glucose for 5 days, respectively. All qRT-PCRs 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/liter D-glucose and those in cells cultured in medium containing 3 or 6 g/liter D-glucose were all significant, with P values of <0.005 .

FIG 2 Generation of hyperglycemic nude mice and xenografting of TIVE-KSHV (BAC16) cells for tumor development. A total of 32 athymic nude mice (4 weeks old and female) were randomly separated into two groups, with one group of mice being treated with STZ (200 mg/kg body mass, with 2 i.p. injections per week for 2 weeks) to develop hyperglycemia and the other group of mice being injected with PBS (placebo) as a control. Equal numbers (5×10^6 cells/injection site, at 2 sites/mouse) of TIVE-KSHV (BAC16) cells were subcutaneously injected into mice for tumor development 2 weeks after the last STZ treatment. (A) Average blood glucose levels of STZ-treated mice and untreated mice (control) measured 4 and 12 weeks after the first STZ treatment. (B) Average weights of the two groups of mice 2 and 12 weeks after the first STZ treatment. (C) Representative tumors from the two groups of mice collected at the end of the experiment. (D) Average volumes (length by width by height) of tumors from the two groups of mice at different weeks after inoculation of TIVE-KSHV (BAC16) cells.

of cells expressing ORF65 are 4.8 times higher in tumors from STZ-treated mice than in tumors from control mice.

Since we waited 2 weeks after the last STZ treatment before injecting TIVE-KSHV (BAC16) cells into mice, it is unlikely that the increased expression of RTA and ORF65 resulted from the STZ treatment itself. To rule out this possibility, we cultured TIVE-KSHV (BAC16) cells in the absence or presence of STZ, followed by Western blot detection of the RTA and LANA proteins. As shown in Fig. 3E, STZ treatment had little effect on the 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 enhance KSHV lytic gene expression by inducing H_2O_2 . Metabolic syndromes, including diabetes, are well known for the production of excessive amounts of reactive oxygen species (ROS) such as H_2O_2 (26–31), which was previously shown to trigger the reactivation of latent KSHV into lytic replication (20, 21, 32). To monitor changes in the levels of intracellular H_2O_2 , we used a previously established BCBL1 cell line that stably expresses the H_2O_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_2O_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 the level of intracellular H_2O_2 surpasses the threshold level (33). We cultured these cells in RPMI 1640 medium containing 1, 3, and 6 g/liter D-glucose for 24 h. As shown in Fig. 4A and B, the intracellular level of H_2O_2 increased significantly when cells were cultured with higher concentrations of D-glucose. To further confirm that high glucose levels induce H_2O_2 production, we cultured BCBL1-BAC36 cells in RPMI 1640 medium containing 1, 3,

and 6 g/liter D-glucose for 24 h; prepared cell lysates from equal numbers of cells (2×10^6) in 1 ml assay buffer; and measured their relative intracellular H_2O_2 concentrations by using a hydrogen peroxide-peroxidase assay kit from Cell Biolabs, Inc. Cells cultured in medium containing 3 and 6 g/liter D-glucose definitely produced higher levels of H_2O_2 (Fig. 4C).

To demonstrate that H_2O_2 was responsible for the increased KSHV lytic gene expression levels, we next cultured BCBL1-BAC36 cells in RPMI 1640 medium containing 1 and 6 g/liter D-glucose in the absence or presence of catalase or the antioxidants N-acetyl-cysteine (NAC) and glutathione. As shown in Fig. 4D, catalase abolished the high-glucose-induced transcription of RTA and ORF65. The three different antioxidants inhibited high-glucose-induced expression of the ORF65 protein in a dose-dependent manner (Fig. 4E). Collectively, these results suggest that the induction of KSHV lytic gene expression by high glucose levels is mediated by H_2O_2 .

High glucose levels downregulate the class III HDAC SIRT1 to increase histone acetylation and transactivate viral chromatin. We previously showed that H_2O_2 activated the MAPKs extracellular signal-regulated kinase 1/2 (ERK1/2), Jun N-terminal kinase (JNK), and p38 to induce the expression of KSHV lytic genes (21). Consistent with our previously reported findings, BCBL1-BAC36 cells cultured in medium containing high concentrations of D-glucose displayed increased phosphorylation of ERK1/2, JNK, and p38 and expression of the 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.

FIG 3 Tumors from hyperglycemic mice express significantly higher KSHV lytic gene expression levels. (A) Average mRNA levels of the KSHV lytic gene ORF50 (RTA) from 8 tumors of STZ-treated and untreated (control) mice. (B) Western blot detection of the lytic protein RTA and the latent protein LANA from 8 tumors of each group. β -Tubulin was used as a loading control. (C) Immunohistochemical staining of the 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 μ M) and tetradecanoyl phorbol acetate (TPA) (25 ng/ml) for 24 h, respectively.

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 the class III HDACs and a key factor involved in the development of diabetes (34–40). In addition, several previous studies demonstrated the involvement of SIRT1 in the regulation of KSHV lytic gene expression through epigenetic remodeling (41–43).

Immunofluorescent-antibody (IFA) staining showed that SIRT1 expression was substantially reduced in BCBL1-BAC36 cells cultured in medium containing 6 g/liter D-glucose compared to that in cells cultured in medium containing 1 g/liter 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 H_2O_2 plays a role in SIRT1 downregulation, we cultured BCBL1-BAC36 cells in medium containing low and high glucose concentrations in the presence of various doses of catalase. As shown in Fig. 7B, catalase dose-dependently blocked SIRT1 downregulation in cells that were cultured in medium containing 6 g/liter D-glucose. In a parallel experiment, we found that treatment of BCBL1-BAC36 cells with H_2O_2 also resulted in SIRT1 downregulation, which could be blocked by catalase as well (Fig. 7C). Together,

FIG 4 High glucose levels induce H_2O_2 to induce KSHV lytic gene expression. (A and B) BCBL1 cells stably expressing the H_2O_2 sensor protein pHyper-cyto were used to measure the relative levels of intracellular H_2O_2 . The number of circularly permuted yellow fluorescent protein (cpYFP)-positive cells (A) and fluorescence intensities (B) were quantified by flow cytometry analysis, following 24 h of culture in RPMI 1640 medium containing 10% FBS and 1 g/liter (red), 3 g/liter (green), or 6 g/liter (purple) D-glucose. Regular BCBL1 cells cultured in RPMI 1640 medium containing 10% FBS and 2 g/liter 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_2O_2 concentrations from equal numbers (2×10^6) of BCBL1-BAC36 cells that were cultured as described above for panels A and B. Measurement of H_2O_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/liter 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/liter and cells cultured in medium containing 6 g/liter D-glucose or between cultures with and without catalase were all significant, with P values of <0.005 . (E) Western blot detection of the KSHV small capsid protein (ORF65) in BCBL1-BAC36 cells cultured in RPMI 1640 medium containing 1 or 6 g/liter D-glucose in the presence of different doses of catalase and the antioxidants NAC and glutathione for 72 h, respectively.

FIG 5 High glucose levels activate MAPK pathways to induce KSHV gene expression. (A) Western blot detection of ERK1/2, 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/liter D-glucose (D-Glu) or stimulated with 400 μM H_2O_2 in the presence or absence of 200 U/ml catalase (Cat) for 24 h. (B) Levels of RTA mRNA in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium with 1 or 6 g/liter D-glucose in the presence or absence of different MAPK inhibitors for 24 h. Concentrations of the inhibitors were described previously (21).

these results suggest that H_2O_2 mediates SIRT1 downregulation in cells that are cultured in medium containing a high concentration of glucose.

As a consequence of SIRT1 downregulation, BCBL1-BAC36 cells cultured in medium containing high concentrations of D-glucose displayed increased levels of acetylated histones, which could be reduced by the addition of catalase to the culture medium (Fig. 7D). To demonstrate that these epigenetic changes indeed occur in viral chromatin, we next conducted ChIP assays. By performing qPCR using primers specific for the promoter region of the 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 the activation of MAPK pathways, high glucose concentrations also transactivate KSHV lytic gene expression via epigenetic modifications of the RTA promoter region.

DISCUSSION

Multiple studies have reported a high prevalence of classic KS in patients with diabetes mellitus (10–13), and KSHV DNA was de-

FIG 6 High glucose levels downregulate the expression of the class III 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/liter D-glucose for 24 h, using a mouse monoclonal antibody to SIRT1 and 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 40 $\times$ oil objective. (B) Average numbers of SIRT-1 foci (red dots) per cell when cells were cultured with different concentrations of D-glucose.

tected in >50% of type 2 diabetes 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 the development of classic KS. However, whether this metabolic syndrome truly 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 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 medium containing diabetic levels of glucose. In full support of data from the *in vitro* study, TIVE-KSHV cells also displayed substantially higher expression levels of KSHV lytic genes in hyperglycemic mice than in mice with normal levels of blood glucose. These results strongly suggest that high levels of blood glucose promote the development of KS by inducing productive KSHV lytic replication.

One of the manifestations of metabolic syndromes such as obesity and diabetes is the production of excessive levels of ROS (26–31). By using a previously established BCBL1 cell line that stably expresses the H_2O_2 sensor protein pH_{Hyper}-cyto (21), we demonstrated that cells cultured with high concentrations of glucose produce increased levels of intracellular H_2O_2 . This result was further confirmed by another intracellular H_2O_2 measurement assay. Notably, the addition of catalase, which converts H_2O_2 into H_2O and O_2 , and the antioxidants NAC and glutathione effectively blocked high-glucose induction of KSHV lytic gene expression in a dose-dependent manner. Therefore, H_2O_2 mediates high-glucose in-

duction of KSHV lytic gene expression, which further supports data from previous reports that H_2O_2 is an important physiological factor involved in the reactivation of latent KSHV (20, 21, 32). Interestingly, H_2O_2 has also been shown to enhance viral entry (44–46). It is therefore highly possible that the hyperglycemic environment in diabetic KS patients contributes to the development of KS by promoting both productive KSHV replication and recurrent *de novo* infection.

Similarly to stimulation with H_2O_2 (21), culture of 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 levels and H_2O_2 also cause the downregulation of the class III HDAC SIRT1, leading to increased levels of histone acetylation in the promoter region of the KSHV key lytic gene RTA. Thus, high glucose concentrations also engage this epigenetic mechanism to promote KSHV lytic gene expression. SIRT1 is well known for its antiaging, anti-oxidative-stress, and anti-inflammation properties (22, 47, 48), and downregulation of SIRT1 has been linked to the development of diabetes (49). Suppression of SIRT1 has been shown to trigger the reactivation of latent KSHV (42, 43). 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 xenografted 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 found 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 remained latently infected, suggesting that the virus might have evolved unique mechanisms to overcome highly inflammatory and stressful conditions to maintain latency. One possible mechanism might be through modulation of the cellular metabolic status. In support of this hypothesis, our recent study demonstrated that KSHV inhibits cellular aerobic glycolysis and oxidative phosphorylation by inhibiting the expression of GLUT1 and GLUT3, thus preventing overflow of the metabolic pathways and maintaining the homeostasis of 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 the development of classic KS. Our results highlight H_2O_2 as the mediator of high-glucose induction of KSHV lytic replication through multiple mechanisms, which may shed light on the development of new strategies to prevent KSHV infection and KS development in diabetic patients.

FIG 7 H_2O_2 mediates high-glucose downregulation of SIRT1 to epigenetically activate the expression of the KSHV lytic gene RTA. (A and B) Western blot detection of the SIRT1 protein in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/liter 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 the SIRT1 protein in BCBL1-BAC36 cells treated with different doses of H_2O_2 and catalase. (D) Western blot detection of the SIRT1 protein in BCBL1-BAC36 cells that were cultured in RPMI 1640 medium containing 10% FBS and 1, 3, or 6 g/liter D-glucose in the presence or absence of 400 U/ml catalase for 24 h. (E and F) ChIP assay detection of acetylated histones (H3K9-Ac and H4K12-Ac) and RNA polymerase II in the RTA promoter in BCBL1-BAC36 cells that were cultured with different concentrations of D-glucose with and without 400 U/ml catalase for 24 h. The relative amount of DNA in the RTA promoter (RTA) from each ChIP reaction was determined by qPCR and calculated as the average ratio between the level of the ChIP product and that of the input DNA from three repeats (F). Real-time PCR products from inputs and ChIP assay mixtures were also analyzed on a 1.5% agarose gel (E). Differences in the levels of H3K9-Ac, H4K12-Ac, and RNA Pol in the RTA promoter between cells cultured in medium containing 1 g/liter D-glucose and those cultured in medium containing 3 or 6 g/liter D-glucose or between cells cultured with and those cultured without catalase were all significant, with P values of <0.005 .

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