Latent Herpesvirus 8 infection improves both insulin and glucose uptake in primary endothelial cells

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INTRODUCTION

Human Herpesvirus 8 (HHV8) has a characteristic tropism for B lymphocytes and is known as the causative agent of Kaposi sarcoma (KS), as well as of some B cell lymphoproliferative diseases, namely primary effusion lymphoma (PEL) and multicentric Castleman disease (reviewed by Ablashi et al., 2002; Hengge et al., 2002; Dourmishev et al., 2003; Ganem, 2010; Wen and Damania, 2010). KS lesions are characterized by neoangiogenesis and the production of typical spindle cells of endothelial origin. In vitro HHV8 infection of endothelial cells causes dramatic changes in the cellular phenotype resembling the spindle shape of KS lesion cells (Ablashi et al., 2002). On the other hand, the effects of lytic and latent HHV8 infection on endothelial cell functions and triggering of inflammatory processes are still largely unknown (Caselli et al., 2007; Gregory et al., 2012). HHV8 infection induces profound modifications in the behaviour of both primary and immortalized endothelial cells.

HHV8 causes an intense transcriptional reprogramming in HUVEC cells (Wang et al., 2004), stimulates the Warburg effect in latently infected TIME endothelial cells with an increase in glycolysis and glucose consumption (Delgado et al., 2010) and activates hypoxia-induced factors (Carroll et al., 2006). In a recent work, Rose et al. (2007) found that in HHV8-infected dermal microvascular cells (E-DMVEC) the expression of the insulin receptor (IR) was strongly induced in latently infected cells. Binding of ligands to the IR triggered a signal cascade that led to a similar activation of downstream effector molecules, which regulated cell growth and survival (Ottensmeyer et al., 2000; Raggo et al., 2005; McAllister & Moses, 2007). Over-expression of the IR in KS tissue compared to normal skin was reported by Wang et al. (2004)
and Rose et al. (2007). They independently showed that IR gene expression was induced in HHV8-infected primary DMVEC, AIDS-KS tissue and immortalized endothelial (TIME) cells. HHV8 is believed to establish persistent infection for the duration of the host’s existence, with a limited gene expression program, which maintains the circularized viral DNA genome during cellular replication, with only occasional switching to the lytic phase infection (Parravicini et al., 2000; Douglas et al., 2010). In addition, Watanabe et al. (2003) and Wang and Damania (2008) found that the HHV8 latency-associated nuclear antigen (LANA) prolonged the life span of primary HUVEC cells and augmented cell survival in the presence of apoptotic inducers and under conditions of serum deprivation. Many other works stress that HHV8 infection induces intense and durable changes in the physiological properties of HHV8-infected cells (Dourmishev et al., 2003; Carroll et al., 2006; Ganem, 2010; Guilluy et al., 2011), but very little is known about the metabolic modifications underlying these altered cell behaviours.

This work performed a series of experiments designed to verify whether overexpression of the IR could affect insulin and glucose uptake in HHV8-infected HUVEC cells. The results demonstrated that HHV8 induced a marked and significant enhancement of both insulin and glucose uptake in infected HUVEC cells during the latent phase of viral infection.

MATERIALS AND METHODS

Cells and viruses
HUVEC cells were purchased from Invitrogen (Life Technologies, UK) and grown in M200 medium (Gibco, Life Technologies, UK) enriched with low serum growth supplement (LSGS, Invitrogen). HUVEC cells were always kept in a semi-confluent state and were sub-cultured at least once a week. For all the experiments HUVEC cells from sub-cultures 2 to 5 were used. HHV8 permanently infected BC3 cells were kindly donated by Caselli et al. (2007) and were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (Invitrogen). BC3 cells were used to produce 100x concentrated stocks of HHV8, as described by Cerimele et al. (2001). The virus pellet was suspended in RPMI, filtered through a 0.22 micron filter and kept at -80°C until used. The quantitative analysis of virus genomes present in the stock preparation was obtained by a real-time polymerase chain reaction (rtPCR), with a Step-One Real Time System (Applied Biosystems, Life Technologies, UK) using primers to amplify the orf 26 gene. The standard HHV8 DNA curve was generated by a serial dilution of a plasmid (pBlueScript SK-ORF26) containing a cloned fragment of HHV8 DNA (ORF26, nucleotides 47261 to 47550). The purified cell-free inoculums contained an average number of $4.7 \times 10^5$ copies of viral DNA/mL. For cell infection, about $5 \times 10^4$/mL HUVEC were seeded in 12 multiwell plates.

The cells were infected with concentrated HHV8 at a multiplicity of at least 10-20 genomes per cell in M200 medium containing 2 μg/mL of polybrene for 2 h at 37°C. After 24-48 h the infected cells were observed with a light microscope to detect the typical spindle cell morphology. Cell infection was examined by a cytofluorometric method. Only cell monolayers with at least 60% of HHV8 infected cells were used for the experiments.

PCR and RT-PCR
The presence and level of transcription of HHV8 in HUVEC cells were analyzed by PCR and RT-PCR for the amplification of the orf 26 and orf 73 genes (LANA). Genomic DNA was isolated using the Easy-DNA kit (Invitrogen, UK) following the manufacturer’s instructions. Total RNA was extracted with a PureLink RNA Mini kit (Life Technologies) and treated with TURBO DNase (Applied Biosystems) before the synthesis of cDNA by random hexamers and Superscript II (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using, respectively, 100 ng of total DNA or 200 ng of total RNA extracted from the infected cells. More specifically, primers and conditions for PCR amplification were as follows: orf 26 took place with the primers orf26 fw 5’-GCCGAAAGGATTCACCATGCTGCT-3’ and orf26 rev 5’-GGGGCGCCGGAGGATTTTGG-3’ for 40 cycles (15” at 95°C, 1’ at 60°C and 15” at 72°C) plus 10’ at 72°C of extension; orf 73 amplification took place with primers orf73 fw 5’-ATCGGGAGAAGGATCCACCATGCTGCT-3’ and orf73 rev 5’-TTCAGCGTTTCACTGTCC-3’ for 40 cycles.
(15’ at 95°C, 1’ at 60°C and 15’ at 72°C) plus 10’ at 72°C of extension. The amplification of the housekeeping β-actin gene (ACTB) was used as a control. PCR products were run in 2% agarose gels and visualized by ethidium bromide staining. Acquisition and processing of images were performed by PhotoDoc-It Imaging System Digital (UVP, Cambridge, MA, USA) and PhotoPaint expression (Corel, Ottawa, Canada).

**qPCR and ELISA test for insulin receptor**

RT-PCR was done on a Step-One rtPCR System (Applied Biosystems). To normalize gene expression between the control and HHV8-infected HUVEC, β-actin was assessed. The total RNA isolated by PureLink RNA Mini kit (Life Technologies) was treated with Turbo DNase (Applied Biosystems); 2 μg of total RNA were used for the synthesis of cDNA using a Superscript VILO cDNA Synthesis kit (Invitrogen). The following primers were selected by using Primer Express software (Applied Biosystems): act fw 5’-CAC-CATTGGCAATGAGCGGTTC-3’, act rev 5’-AG-GTCTTTGCGGATGTCCACGT-3’ for the expression of the β-actin gene, and ins fw 5’-GAG-GCGAGGCTGCCAAT-3’, ins rev 5’-TC-CTGGCCAGACGTCAACGA-3’ for the expression of the IR gene (IRα). The reactions were run using cDNA 20-fold diluted with Power Sybr Green PCR Master mix (Applied Biosystems) following the manufacturer’s instructions. The relative expression values between the control and HHV8-infected HUVEC were calculated by the comparative cycle threshold (ct) method using Relative Quantitation Study software (Applied Biosystems). The specificity of the amplicons was monitored by analysing the dissociation curve of the amplified product. For the ELISA assay, the HUVEC cells were seeded in 24 multiwell plates at a multiplicity of 3x10^4 cells per well and infected or mock infected with HHV8 after 24-48 h, as described previously. Cells were washed twice with PBS at the scheduled times and kept in a serum-free medium for 2 h at 37°C. They were then fixed in iced methanol for 4 min at 4°C. After washing, the plates were treated for 2 h with 1% BSA to neutralize any aspecific uptake of antibodies. The wells were washed again and a primary anti-insulin receptor antibody, diluted 1:200 in PBS, was added for 1 h at 37°C (rabbit anti-IRβ, Santa Cruz Biotechnology CA, USA). The cells were washed and the secondary diluted 1:300 antibody was added for 45 min at 37°C (goat anti-rabbit IgG, Sigma-Aldrich, Milan, It). Finally, the cells were washed and the peroxidase substrate was added for 15 min. Sample absorbance was read at a WL of 370 in a Pharmacia ultruspec III spectrophotometer.

**Direct insulin-peroxidase assay**

The direct insulin uptake to HUVEC cells was analyzed with peroxidase-conjugated bovine insulin used at a dilution of 1:1000 in PBS, according to the manufacturer’s instructions (SIGMA, 200-300 IU/mg of protein). The assay was run as described in the previous paragraph for the ELISA test. The insulin-peroxidase was kept on cells for 1 h at 37°C, then the cells were washed 3 times and the substrate for peroxidase was added. After 15 min, the reaction was stopped and read in absorbance at a WL of 370 (all the samples were processed in triplicate).

**Insulin-gold assay, microscopy and imaging**

In order to detect and visualize the insulin bound to the cell membrane receptors in situ, 10 nm insulin-gold (I-0391, Sigma Chemical Co., MO, USA) were used. Both controls and HHV8-infected HUVEC cells were diluted from stock cultures and seeded (in triplicate) at a density of 2x10^5 in 35 mm glass-bottomed dishes (MatTek, Ashland, MA, USA) and cultured at 37°C in a 5% CO₂ incubator in growth medium. 3, 14 and 25 days after viral infection, the cells were washed and serum-free M200 medium was added for two hours. After this, cells were thoroughly washed with PBS and fixed with iced methanol for 4 min at 4°C. After washing, the cells at the centre of the dish were covered with 100 µL of insulin-gold, diluted 1:20 according to the manufacturer’s instructions. After one hour of incubation, the cells were washed and silver enhancer (Silver enhancer kit SE100, SIGMA, USA) was added to enlarge colloidal gold labels, as suggested by the manufacturer. Silver enhancer activity was stopped after 5-10 min with 2.5% sodium thiosulfate and the cells were washed with PBS. All observations were performed with an Olympus IX 71 inverted wide-field microscope (Olympus, Tokyo, Japan) using 20x/0.7 and 60x/1.3 plan apochromatic objectives (Olympus UPlanSapo series). Twelve-bit images were captured using a
cooled CCD camera (Sensicam PCO, Kelheim, Germany). Nominal resolutions of images were 0.3 μm/pixel and 0.1 μm/pixel for the 20x and 60x objectives, respectively. In order to simplify image analysis and data reporting, the images were contrast-inverted to obtain positive values (as in fluorescence microscopy). Image analysis was performed with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD, USA). At least 10 microscopic fields and 200 cells were individually selected and measured for each experimental group. Calculations were made with Excel (Microsoft Co., Redmond, WA, USA). Normalized data (reported in Figure 3) represent the average percentage of the density value (intensity per pixel) ± standard error (SE).

Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc. La Jolla, CA, USA) software and Statistica (StatSoft, Tulsa, OK, USA). Comparisons between HHV8 and control data were made by the nonparametric Mann-Whitney U test. Significance was set at p<0.05 and p<0.01.

2-deoxy-D-glucose uptake
HUVEC cells were seeded in 6 multiwell plates at a concentration of 1.5x10⁵/mL in M200 medium with LSGS. After 24 h, the cells were washed and infected for 2 h at 37°C with HHV8 at a multiplicity of 10-20 viruses per cell, as described previously. Then the plates were washed and incubated in complete M200 medium for 1-25 days. Both infected and mock-infected cells were prepared in triplicate for each sample. Glucose uptake was detected by the method described by Nguyen et al. (2005). Briefly, the cells were washed with Hank’s solution and glucose-free medium was added for 1 h at 37°C on the scheduled days. After this, 17 IU of insulin were added.

FIGURE 1 - Lytic and latent HHV8 infection of HUVEC cells. A and B: Morphologic appearance of control and HHV8-infected sub-confluent HUVEC cells at a magnification of 10x10. The cells were always kept in a sub-confluent state and were sub-cultured at least once a week. After 24 h from infection more than 80% of cells showed the typical spindle cell shape of HHV8-infected endothelial cells. C) Detection of ORF26 and ORF73 transcripts during lytic and latent HUVEC infection by HHV 8. M: markers; C+: ORF26 and ORF73 positive controls. The amplification of the housekeeping β-actin gene was used as a control.
to each well for 10 min (recombinant human insulin, Sigma 91077C). Then 0.2 μCi/mL of [3H]2-deoxy-D-glucose (Perkin-Elmer, MA, USA) were added to each well and left for 10 min. The cell monolayers were washed 3 times with cold PBS, dissolved in 1N NaOH and analyzed in a liquid scintillation counter.

RESULTS

HHV8 infection of HUVEC cells
After infection with HHV8, within 24 h almost all the HUVEC cells underwent a strong shape modification, with formation of the typical spindle cell aspect (Figure 1A, B). Analysis of the viral genome demonstrated that during the first 7 days, only the orf 26 gene was expressed, which is representative of a lytic infection, and it remained slightly evident until the 14th day (Figure 1C). From day 14 onwards, orf 73 expression (LANA), representative of a latent infection, was clearly detectable and this was the only gene expressed on day 25. By cytofluorometric analysis the number of HHV8 positive cells at day 25 after infection was found to be still higher than 50%.

Production of insulin receptor in HHV8-infected cells
The production of the IR was studied by two different methods. An ELISA assay was used to detect the IRβ-subunit, whilst a RT-PCR at days +3 (lytic phase) and +25 (latent phase) was used to identify and quantify the IRα-subunit. Both tests revealed that IRs were over-expressed after HHV8 infection in HUVEC cells. During lytic infection at day 3, IR production appeared to be unaltered (as well as at days 7-14 in Figure 2A, data not shown) or slightly increased (Figure 2B). A marked increase in the IRβ was evident on day 14 after infection (Figure 2B), whilst a dramatic over-expression of both IRs subunits was detected on the 25th day, which reached an increase of between 90% (IRα, Figure 2A) and 135% (IRβ, Figure 2B) in infected cells compared to the mock-infected ones. The differences observed between infected and control cells on the 25th day were found to be statistically highly significant for both IRα and IRβ (p<0.01). All the tests were repeated at least twice and, when necessary, they were corrected to the number of cells.

FIGURE 2 - Expression of insulin receptor in HHV8-infected HUVEC cells. A: Quantitative RT-PCR of HUVEC cell IRα cDNA. Empty column: mock infected cells; gray column: HHV-8 infected HUVEC cells from 1 to 25 days after infection. B: ELISA test for detection of Insulin Receptor (IRβ) in HUVEC cells infected with HHV8. M: Mock-infected cells (in triplicate); HHV8: Human Herpesvirus 8-infected cells from days 1 to 25 after infection.
FIGURE 3 - Insulin binding and uptake in HHV8-infected HUVEC cells. A: Distinctive microscopic field portions of insulin bound to cell membrane receptors in HHV8 infected and control HUVEC cells after 3, 14 and 25 days from infection; the bar at the bottom left is 30 µm. Images were contrast inverted to analysis and visualization, by this way (as in fluorescence microscopy) cells appear brilliant on dark background. Moreover, to highlight the differences between HHV8-infected and control cells, we used a pseudo-colour algorithm with the ImagePro Plus package (Media Cybernetics, Silver Springs, MD, USA), that clearly identifies and distinguishes the mere silver enhancer single or aggregated particles (blue) bound to the insulin-gold, from the physiological cellular contrast (green) typical in wide-field microscopy. The algorithm sets up a "gate", working as a "watershed", thereby visually depicting the trimmed data in the image. B: Normalized imaging data obtained by insulin gold assay are reported as the average percent of the density value (intensity per pixel) ± standard error (bars); significance was set up at p<0.05 (*) and p<0.01 (**). C: Direct insulin-peroxidase assay of HHV8-infected and mock-infected HUVEC cells from 1 to 25 days after infection. The data indicate the absorbance values at 370 WL. All the tests were done in triplicate and the values were always normalized to the number of cells.
Insulin binding and uptake in HHV8-infected HUVEC cells
To verify if the over-expression of IRs could influence the uptake of insulin, two different methods were used on the HUVEC cells. The insulin-gold assay method was employed for detecting and visualizing the insulin bound to the cell membrane receptors in situ (Figure 3A). Imaging methods were used to highlight the differences in density and concentration of insulin-gold particles, which were additionally enhanced by silver precipitation. In this case, insulin binding started increasing in the lytic phase of infection (+23.6% on day 3, p<0.01), augmented on day 14 (+40.6%, p=0.02) and, finally, reached a maximum during the latent phase on day 25 with an increase of 52.5% (p<0.001) as compared to the control. Insulin binding at day 7 did not show significant differences from day 3 (data not shown). Differences between HHV8-infected and control cells were found to be statistically significant on a daily basis (Figure 3B). No significant difference was detected between uninfected and mock-infected cells. According to these results, the direct insulin-peroxidase assay (Figure 3C) showed an improved insulin uptake by HHV8-infected cells against the control starting from day 3, reaching a maximum value at days 14 and 25. Although a gradual decrease of total insulin binding was observed from day 1 to 25, at day 25 after infection the insulin uptake by HHV8-infected cells was about 71% higher than in control cells.

Uptake of [3H]2-deoxy-D-glucose in HHV8-infected cells
The over-expression of IRs and the rise in insulin uptake by HHV8-infected cells also had a significant influence on glucose uptake (Figure 4). During the lytic infection, glucose uptake appeared to be slightly depressed until day 3, with a decrease of 16-22% compared to the control; from day 7 post infection onwards, the HHV8-infected cells showed a progressively increased uptake of glucose up to 23±7% on day 14, reaching a maximum of 37±9% on day 25. These values are the mean of 3 experiments and in all cases the scored data were normalized for the number of cells. The differences observed between HHV8-infected and control cells on the 25th day post-infection were statistically significant (p=0.026).

DISCUSSION
The results presented in this paper show that HHV8 infection has a profound impact on both insulin and glucose uptake in primary HUVEC cells. It is known that HHV8 is responsible for dramatic changes in the aspect and behaviour of infected endothelial cells. Spindle cell formation is a constant modification of cell morphology. Infected cells are more resistant to stress and toxic substances (Wang and Damania, 2008), present an increase in hypoxia-induced factors (Carroll et al., 2006), and show increases in vascular permeability (Guilluy et al., 2011). Moreover, Rose et al. (2007) demonstrated that HHV8-infected cells undergo an intense modification of cellular insulin receptors. In this paper Rose et al. (2007) data on IRs over-expression were confirmed. In addition, the insulin uptake by infected cells was demonstrated to be highly enhanced, reaching a maximum during the latent phase of HHV8 infection. The enhanced uptake of insulin led to an augmented entry and accumulation of deoxy-glucose inside the infected HUVEC cells. After 25 days of infection, the infected cells presented a glucose uptake that was around 40% higher than the controls. During the lytic infection, a slight decrease of glucose uptake was observed in our
experiments until the 3rd day; this fact could be due to a temporary depression of cell metabolism caused by the viral infection, but after 4-7 days the glucose income into the cells constantly increased as compared to the control, reaching a maximum on the 25th day after infection. There are many demonstrations that HHV8-infected cells acquire new stable properties and are metabolically more efficient, but the biochemical basis of these modifications is still not understood. Wang and Damania (2008) found that HHV8 infection caused the activation of the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin pathway, but which viral product was responsible for these changes has never been clarified. During the latent phase, HHV8 is known to express only a very limited number of genes. LANA (orf 73) is a nuclear antigen, necessary for viral latency (Stuber et al., 2007); during latency, vFLIP (orf 71) and vCyclin are produced in small amounts, while kaposins (A-C, K12) are produced in large amounts and are mainly located in the cell membrane (Wen and Damania, 2010). Work is in progress to ascertain whether one or more of the viral factors produced during latency are involved in the cell membrane modifications and metabolic changes. Whether these findings on increased insulin and glucose uptake in HHV8-infected cells can be referred to general cell metabolism, requires much more investigation. It is known that HHV8, like most other Herpesviruses, can persist in infected persons throughout their life-span (Douglas et al., 2010) and, while clinical Kaposi sarcoma is a rare disease, HHV8 infection shows a high prevalence in some populations (Ingianni et al., 2007, 2009). Recently Guilley et al. (2011) and Gregory et al. (2012) reported that HHV8 infection can activate the PI3K/AKT/mTOR pathway in endothelial cells and that the PI3K/AKT/mTOR signalling pathway is essential for the control of cell proliferation and also regulates anabolic activities within the cell. Since these in vitro growth characteristics mimic early-stage tumorigenesis, Rose et al. (2007) proposed that IRs up-regulation contributes to Kaposi sarcoma tumour development. Also Caselli et al. (2007) suggested that HHV8 infection may play an important role in promoting in vivo inflammation and pathogenic angiogenesis typical of HHV8-associated lesions. In conclusion, over-expression of IRs, enhancement of insulin binding and increase glucose uptake can give HHV8-infected cells a metabolic advantage, that is required for tumour cell proliferation and pathological neo-angiogenesis, as found in clinical Kaposi sarcoma.

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REFERENCES


