JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Anti-Human Herpesvirus 8 antibodies affect both insulin and glucose uptake by virus-infected human endothelial cells

Fabrizio Angius[1], Enrica Piras[1], Stefano Spolitu[1], Luisa Marras[1], Sara Federica Armas[1], Angela Ingianni[1], Pierpaolo Contini[2], Raffaello Pompei[1]

[1] *Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy*
[2] *Metabolic Disease Service, S. Giovanni Hospital, Cagliari, Italy*

Abstract

Introduction: Human Herpesvirus 8 (HHV8) is known to be the cause of the malignant tumour named Kaposi's sarcoma. It is believed to induce an intense modification of cell metabolism in endothelial cells. In this work we analysed the role of anti-HHV8 antibodies in both the insulin and glucose uptake of HHV8-infected primary human endothelial cells (HUVEC).

Methodology: Western blotting, immunofluorescence and radiolabelled glucose were employed to assess the pPI3K expression, insulin binding and glucose-uptake by HUVEC cells, respectively.

Results: We confirmed that HHV8-infection is able to enhance both insulin binding and glucose-uptake in HHV8-infected primary endothelial cells; in addition, we found that anti-HHV8 specific antibodies are able to further increase both insulin and glucose uptake during the late latent phase of HHV8-infection *in vitro*.

Conclusions: These findings suggest that a specific immune response to HHV8-infection may cooperate in boosting the cell metabolism, further enhancing the already increased insulin binding and glucose-uptake in HHV8-infected cells, which is a peculiar property of several oncogenic viruses.

Key words: Human herpesvirus 8; chronic viral infections; viral immunity; anti-viral antibodies; glucose uptake; insulin binding.

J Infect Dev Ctries 2018; 12(6):485-491. doi:10.3855/jidc.10381

(Received 21 March 2018 – Accepted 04 June 2018)

Introduction

The Human Herpesvirus 8 (HHV8) is believed to be the cause of some malignant disorders, namely Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease. HHV8 has a specific tropism to B-lymphocytes and human endothelial cells [1-5]. After a lytic reproductive phase at the first infection, it establishes a latent infection for the entire life span of the host, with occasional reactivation of the acute infection [6,7]. The HHV8 latency nuclear antigen (LANA) is known to be able to immortalise primary endothelial cells, enhance cell survival in critical conditions, and induce the persistency of viral DNA in the cells as an episome [8-10]. HHV8-infection has been reported to induce profound changes in the behaviour of human umbilical vein endothelial cells (HUVEC) [8,11-13], by triggering an over-expression of the insulin receptor, a higher glucose consumption, and production of hypoxia-induced factors [11-15]. Cellular insulin-uptake was detected in the viral lytic phase with a peak during the late-latent phase, whereas glucose-consumption was decreased in the lytic infection, and remarkably increased in the latent phase [16,17]. The increased insulin-uptake induced an increase of glucose accumulation in HUVEC cells. Recently, Piras and colleagues investigated the prevalence of HHV8 DNA and specific antiviral antibodies in diabetes mellitus type 2 (DM2) patients and control subjects [18]. They reported the presence of anti-lytic and -latent antibodies in HHV8-positive DM2 subjects and in non-DM2 controls. A significant prevalence of HHV8 DNA, but no DNA among other Herpesviruses, was found in DM2 patients. Anti-lytic antibodies were more prominent than anti-latent ones in DM2 subjects.

The previous results were obtained in cell cultures *in vitro*. However, *in vivo* the immune system tries to face the viral infection by the production of a specific immune response; therefore, in the present study we aimed to analyse the effect of anti-HHV8 antibodies on some metabolic cell modifications induced by viral infection on human endothelial cells, focusing attention on both insulin and glucose uptake.

Methodology

Cells and viruses

HHV8-positive BC3 cells were grown in RPMI-1640 medium with 10% fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, MA, USA) as previously described [16,17]. A pool stock of HUVEC cells (Thermo Fisher Scientific, Waltham, MA, USA) was grown in a M200 medium (Thermo Fisher Scientific, Waltham, MA, USA) with low serum growth supplement (LSGS, Thermo Fisher Scientific, Waltham, MA, USA). HUVEC cells were always kept in a semi-confluent state and sub-cultured at least once a week. Prior to the experiments, the cells were sub-cultured no more than 3 to 5 times. BC3 cells were used to produce 100x concentrated stocks of HHV8. The cells were infected with HHV8, concentrated at a multiplicity of at least 10-20 genomes per cell in a M200 medium containing 2 μg/mL polybrene for 2 hours at 37°C. After 24-48 hours the infected cells were observed with a light microscope to check the typical spindle cell morphology.

Characterization of HHV8-infection in infected HUVEC cells

Cell infection was assessed by two molecular biology assays as previously reported [16]. In brief, RT-PCR was performed in order to detect the transcripts of the *orf26*, *orf50* and *orf73* genes, and the viral protein expression of K8.1 (mouse monoclonal) and LANA (rat monoclonal) were assessed by western blot in HHV8-infected HUVEC cells. After image acquisition, image processing and densitometric quantification were accomplished by Image J software (NIH Bethesda, MA, USA).

Serum containing anti-HHV8 antibodies

All serum samples were collected in the period between 2008-2016 by the blood donor service at the Cagliari San Giovanni city hospital. Blood samples were collected from voluntary persons after receiving informed consent from all the subjects, and were anonymised before being used. The methods used for experiments involving human subjects were carried out in accordance with the guidelines approved by the Local Ethical Committee (AOU-EC). The samples were tested for the presence of antibodies against both lytic K8.1 and latent LANA HHV8 viral proteins using a validated immunofluorescence kit assay (Scimedx Corp., Denville, NJ, USA), as indicated in the manufacturer's instructions. Only serums with an antibody titre higher or equal to 1:64 were considered positive. Both the HHV8-positive and negative serums from 8-12 persons were matched for age (44-75 years) and gender, then pooled and used for the experiments at a 4% dilution in the cell culture medium.

Western blot for pPI3K detection in HUVEC cells

Cells were lysed at 4°C with lysis buffer in PBS (containing 10% SDS, 50 μg Tris, 1 μM EDTA, pH 7.5, 50 μM DTT and a protease/phosphatase inhibitor cocktail), and homogenised with a UP100H Compact Ultrasonic Laboratory Device (Hielscher Ultrasonic GmbH, Teltow, Germany). The protein content of each sample was determined by the BCA assay (Sigma-Aldrich, Milan, Italy), and processed as previously described [16]. In brief, protein samples (12 μg/lane) were separated by electrophoresis (acrylamide precast gel; Bio-Rad, Laboratories Inc., Milan, Italy) and transferred to a 0.45 μm pore size nitrocellulose (Millipore, Milan, Italy) by standard electro-blotting procedure. The blots were pre-treated with a blocking solution (ChemiBLOCKER, Millipore, Milan, Italy), diluted 1:3 v/v with TBST (50 μM TrisHCl, pH 7.6, 0.15 M NaCl and 0.05% Tween-20) for at least 1 hour at room temperature before the addition of the primary antibodies (dilution 1:200) for pPI3K (mouse polyclonal) and β-actin (goat polyclonal). After overnight incubation at 4°C, the primary antibodies were removed and appropriate horseradish peroxidase-conjugated secondary antibodies were added in a dilution range of 1:5000 for at least 1 hour at RT. All the antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Proteins were detected by enhanced chemiluminescence (Millipore, Milan, Italy) and by exposure to X-ray film (Sigma-Aldrich, Milan, Italy). Densitometric quantification of the protein bands was then accomplished by Image J software (NIH Bethesda, MA, USA) as previously described.

Immunofluorescence assay for insulin binding

The cells were processed for microscopy experiments 3 and 20 days post-infection. Two series of experiments were carried out. Both the controls and HHV8-infected HUVEC cells were diluted from stock cultures and seeded at a density of 2.0×10^5/mL in 35 mm glass-bottomed dishes (MatTek, Ashland, MA, USA) and cultured at 37°C in a 5% CO_2 incubator in growth medium. On the next day, the culture medium in the plates was changed with a serum-free fresh medium for 2 hours at 37°C to clear the residual insulin, then the cells were washed with PBS and incubated with the anti-HHV8 positive serum pools diluted 4%, for 1 hour at 37°C. Subsequently the cells were washed to

remove unbound antibodies, incubated with a 1:2000 solution of insulin-FITC (Santa Cruz Biotech, Dallas, TX, USA) for 1h at room temperature. After that, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min and finally observed using an Olympus IX71 inverted wide-field fluorescence microscope (Olympus, Tokyo, Japan) fitted with a 20×/0.7 plan apochromatic objective. 12-bit-images were captured using a cooled CCD camera (PCO Sensicam, Kelheim, Germany), electronically coupled to a mechanical shutter interposed between the 100 W Hg lamp and the microscope to limit photo-bleaching. Nominal image resolution was 0.3 µm/pixel. Quantitative analysis of images was performed with the Image Pro Plus package (Media Cybernetics, Silver Springs, MD, USA). At least 10 microscopic fields and 100 cells were individually selected and measured for each experimental group. Calculations were made with Excel (Microsoft Co., Redmond, WA, USA). Normalised data represent the percentage of the mean density value (intensity per pixel) ± standard error (SE).

Glucose-uptake by antibody-treated HUVEC cells

HUVEC cells were seeded in 6 multiwell plates at a concentration of $1.5x10^5$/mL in M200 medium with LSGS. After 24 h, the cells were washed and infected for 2 hours at 37°C with HHV8 at a multiplicity of 10-20 viruses per cell, as described previously. The plates were then washed and incubated in complete M200 medium for 3 or 20 days. Both infected and mock-infected cells were prepared in triplicate for each sample. The cells were washed at the scheduled times and serums containing specific anti-HHV8 antibodies diluted 1:100 in M200 medium were added for 1 hour at 37°C. Subsequently glucose-uptake was detected by the method described by Ingianni *et al.* [17]. Briefly, the cells were washed with Hank's solution and glucose-free medium was added for 1 hour at 37°C. Thus, 17 IU of insulin were added to each well for 10 min (recombinant human insulin, Sigma 91077C). Then 0.2 µCi/mL of [^{3}H]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 NaOH solution and analyzed in a liquid scintillation counter.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (La Jolla, CA, USA) and Statistica (StatSoft, Tulsa, OK, USA). All data were expressed as the mean ± SE of experiments in triplicate and analysed by the t-student test or ANOVA, and LSD-Fisher as *post-hoc* test when required. Differences were considered significant when $p < 0.05$.

Results

Characterization of HHV8-infection in HUVEC cells

HHV8-infected HUVEC cells were checked daily under a microscope. The replication rate was normal for up to 6-8 weeks after infection, whereas uninfected control cells lost their mitogenic ability after about 4 weeks, lowering their growth rate. Our experiments were performed from 3 to 20 days post infection (dpi), and viral genes and proteins expression were assessed at each time point as reported in Table 1. On Day 3, lytic genes *orf26* and *orf50* were both clearly expressed, whilst latent gene *orf73* was mostly transcribed 20 dpi. Gene expression data (Table 1A) were confirmed by western blot analysis through the detection of lytic (K8.1) and latent (LANA) viral-antigens (Table 1B and Figure 1).

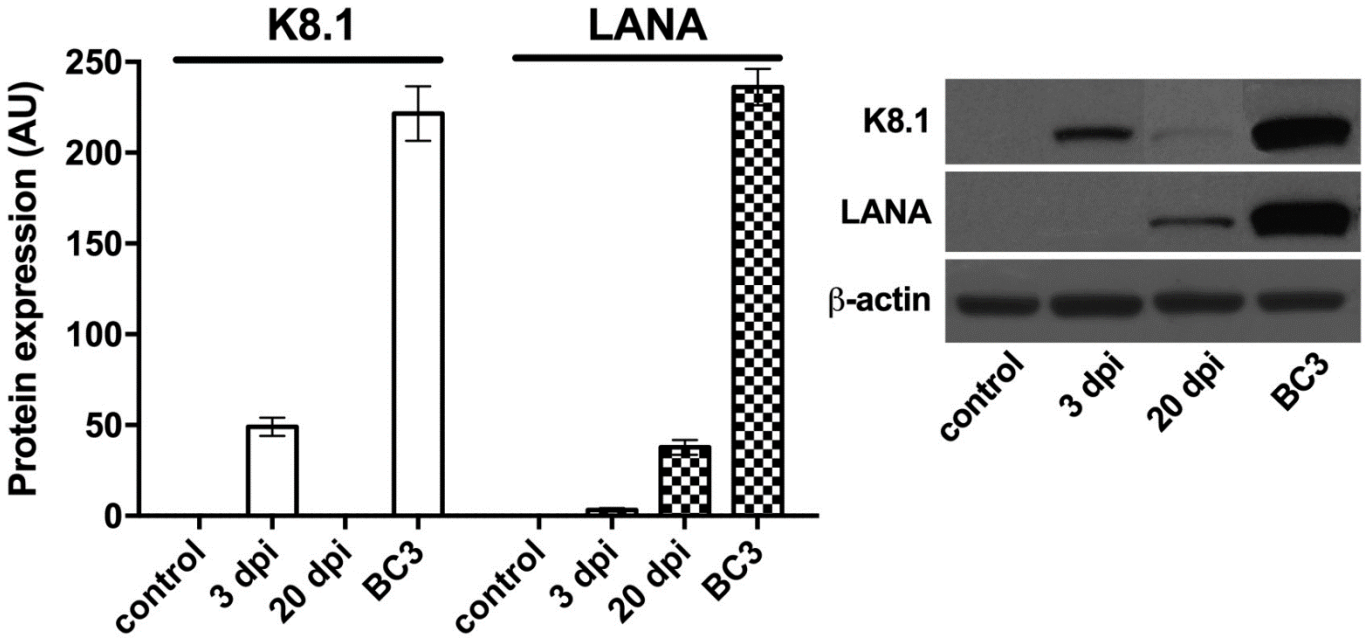
Treatment with serum containing anti-HHV8 antibodies enhances pPI3K expression in latently infected endothelial cells

It is well known that HHV8-infection enhances PI3K activity [10], therefore here we analysed the expression of its phosphorylated active form (pPI3K) during both lytic (Day 3) and latent (Day 20) HHV8-infection, either in the presence or absence of specific anti-HHV8 antibody-positive serum (+). On Day 3, during lytic infection, the pPI3K is clearly expressed in

Table 1. Densitometric data related to genes (A) and proteins (B) expression in HUVEC cells harvested 3 and 20 days post infection (dpi) were normalized by β-actin and reported as arbitrary units (AU). Uninfected HUVEC cells were used as negative control (Control) and HHV8-permanently infected lymphoma cells (BC3) as positive control.

	Control	3 dpi	20 dpi	BC3
(A) Gene expression				
orf50	0	80.26	0	57.28
orf26	0	112.69	15.46	131.14
orf73	0	19.77	89.57	142.71
(B) Protein expression				
K8.1	0	49.06	0	221.64
LANA	0	3.30	37.71	236.15

Figure 1. Lytic and latent phase characterization by K8.1 and LANA protein detection in HHV8-infected HUVEC cells.



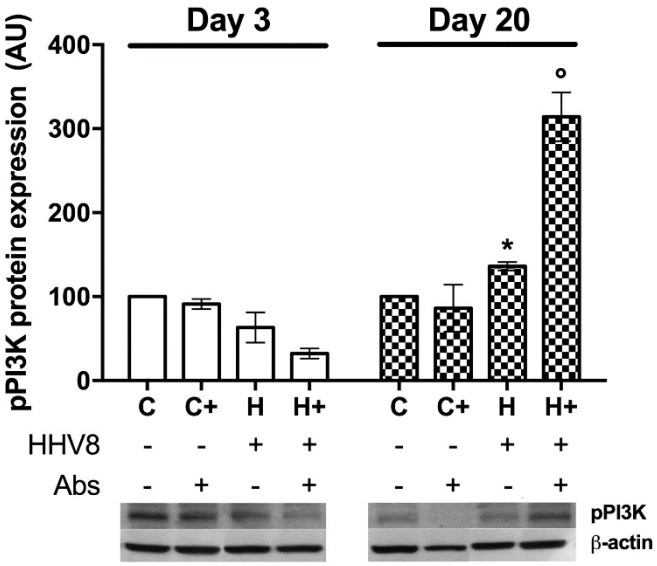
Cells were infected with HHV8, concentrated at a multiplicity of at least 10-20 genomes per cell in a M200 medium containing 2 μg/mL of polybrene for 2 hours at 37°C. Three and twenty days post infection (dpi), sub-confluent cells were harvested and processed for western blotting (for details see Methods). Control: uninfected HUVEC cells; BC3: permanently HHV8-infected lymphoma cells. The densitometric mean values from at least three experiments were normalised for β-actin and data reported as arbitrary units (AU).

the uninfected control cells (C), and the treatment with anti-HHV8 antibody-positive serum (C+) did not induce alteration in the pPI3K expression (Figure 2). Moreover, infected cells that appeared to suffer the viral cytopathic effect on Day 3, showed a decreased pPI3K expression as compared to uninfected controls (C), in cells treated both with HHV8-positive (H+) or negative (H) serum. However, on Day 20 during the latent phase, the control cells expressed lower levels of pPI3K proteins, either in the presence (C+) or absence (C) of anti-HHV8 antibodies, whereas the pPI3K was strongly enhanced in infected HUVEC cells treated with anti-HHV8 antibody-positive serum (H+) compared to HHV8-negative serum infected cells (H). The quantitative difference between infected (H) and infected treated with anti-HHV8 antibody-positive serum (H+) on Day 20 was clearly significant (Figure 2, $p = 0.03$).

Anti-HHV8 antibodies enhance insulin binding in HHV8-infected endothelial cells

Insulin binding was assessed in HUVEC cells treated with specific anti-HHV8 positive or control serum. On Day 3 the infected cells showed a slight increase in insulin binding in anti-HHV8 antibody-treated cells (Figure 3). However, on Day 20 we

Figure 2. Western blot analysis for pPI3K protein expression in HUVEC cells treated with serum containing anti-HHV8 antibodies.



C: uninfected control cells; C+: uninfected control cells treated with serum containing anti-HHV8 antibodies; H: HHV8-infected cells; H+: HHV8-infected cells treated with serum containing anti-HHV8 antibodies; Abs: antibodies. The densitometric mean values normalised for β-actin are reported as arbitrary units (AU). The experiment was repeated at least three times and the statistical significance was set when $p < 0.05$. (*) H vs. C: (°) H+ vs. H.

observed a significant ($p < 0.05$) increase in insulin binding by cells treated with anti-HHV8 antibodies (H+) compared to the relative control (H). As expected, the insulin binding was significantly increased ($p < 0.05$) even between infected (H) and uninfected cells (C). Taken together, these findings demonstrate that specific anti-HHV8 antibodies enhance insulin binding only in HHV8 latently infected HUVEC cells.

Anti-HHV8 antibodies enhance glucose-uptake in latently infected HUVEC cells

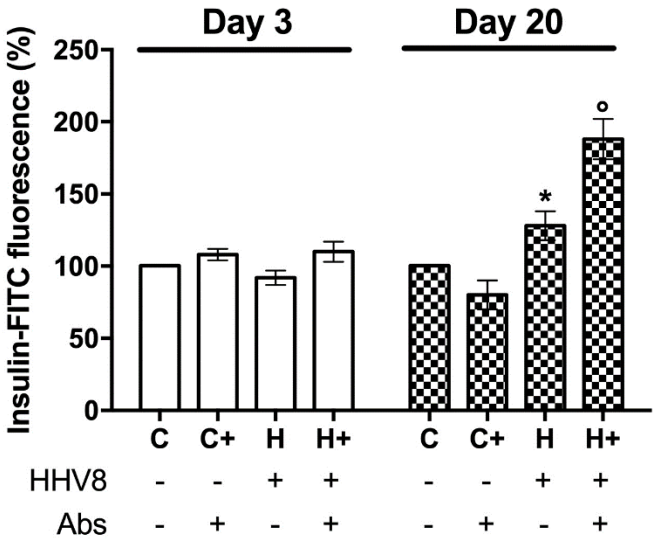
Glucose-uptake was assessed in HUVEC cells 3 and 20 days post-infection (Figure 4). As previously mentioned, on Day 3 the HHV8-infected cells underwent a cytopathic effect due to the acute viral infection, and glucose-uptake dropped in both HHV8-positive (H+) and -negative (H) serum treated cells. However, on Day 20 during the latent phase, the cells recovered, and the glucose-uptake reached the highest level in anti-HHV8 specific antibody-treated HHV8-infected cells (H+) as compared to control serum-treated infected cells (H+ vs. H: $p < 0.05$), and also to uninfected cells (H vs. C, $p < 0.05$).

Discussion

It is well known that HHV8-infection *in vitro* induces important and stable modifications in cell metabolism. However, it is a matter of fact that the immune system tries to face the HHV8-infection *in vivo* with both humoral and cellular responses. The evident issue is whether or not the immune response is able to deal with the metabolic modifications induced by the HHV8-infection. This is the question we have tried to answer in this work, focusing our attention on metabolic modifications that are crucial for cell physiology, namely insulin and glucose-uptake. The present findings indicate that anti-HHV8 antibodies are able to further enhance the already increased uptake of both insulin and glucose previously reported by our group [17] in HHV8-infected primary endothelial cells. Essentially, we found that the pPI3K complex was remarkably promoted by the HHV8-infection particularly during the late latent phase (day 20), whilst it did not show significant changes in the lytic phase (day 3). When the anti-HHV8 antibodies were added to the cell culture during the HHV8 latent phase, we observed a further significant increase in pPI3K expression, and in both insulin binding and glucose-uptake in HHV8-infected cells.

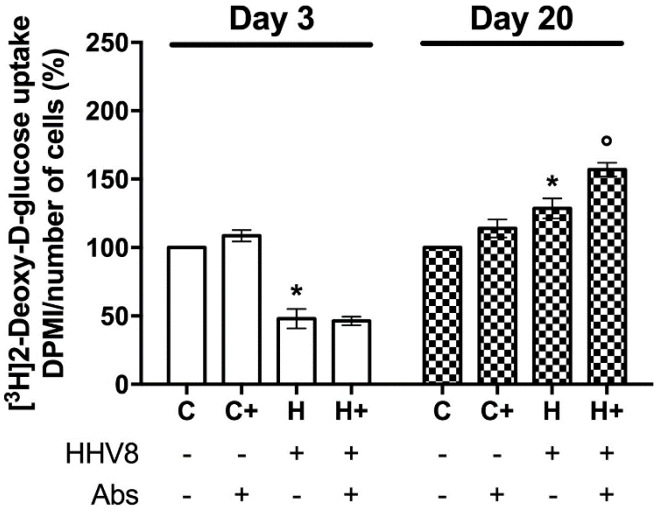
During latent infection, HHV8 is known to produce a small number of proteins, such as the latency factor LANA (*orf73*), vCyclin, which is a cell-cycle regulator, vFlip (*orf71*), that induces a survival advantage for HHV8-infected cells, the K1 and vGPCR proteins that

Figure 3. Insulin-FITC binding in HHV8-infected HUVEC cells treated with serum containing anti-HHV8 antibodies.



The experiment was performed on Day 3, during the viral lytic phase, and on Day 20 during the late latent phase of the virus cycle. The values are reported as % of control (C). C: uninfected control cells; C+: uninfected control cells treated with serum containing anti-HHV8 antibodies; H: HHV8-infected cells; H+: HHV8-infected cells treated with serum containing anti-HHV8 antibodies; Abs: antibodies. The results are the mean of three experiments and the statistical significance was considered when $p < 0.05$. (*) H vs. C: (°) H+ vs. H.

Figure 4. [^{3}H]2-Deoxy-D-Glucose uptake by HHV8 infected HUVEC cells treated with serum containing anti-HHV8 antibodies or with control serum.



The assay was performed on Day 3 (HHV8 lytic phase) and on Day 20 (HHV8 latent phase). The values are reported as % of control. C: uninfected control cells; C+: uninfected control cells treated with serum containing anti-HHV8 antibodies; H: HHV8-infected cells; H+: HHV8-infected cells treated with serum containing anti-HHV8 antibodies; Abs: antibodies. The results are the mean of three experiments and the statistical significance was considered when $p < 0.05$. (*) H vs. C: (°) H+ vs. H.

are also present in the lytic phase, and Kaposin (*orf12*), the most expressed protein in latency. K1 and vGPCR proteins are abundantly present in the cell membrane, while the others are mainly localised either in the nucleus and/or the cytoplasm. Interestingly, the viral K1 and vGPCR oncogenic proteins have been reported to be able to activate the PI3K pathway in BC3 cells conferring a huge advantage on the HHV8 pathogenicity. In addition, PI3K also stimulates the insulin-dependent glucose transporter GLUT4 [10].

Although the mechanism is yet to be clarified and further studies are required, we might speculate that specific anti-HHV8 antibodies binding to the surface K1 and vGPCR viral proteins may activate the PI3K pathway, thus being responsible for the further enhancement of the cell metabolism with an additional increase of insulin and glucose-binding and uptake. This phenomenon would confer an advantage on the HHV8 infected cancer cells in terms of higher growth rate and pathogenicity-related properties.

Conclusion

A possible association of HHV8 infection with type-2 diabetes has recently been proposed [16-19]. Insulin hyper-secretion and consumption is considered a possible initiating event of obesity and diabetes, and discovering the causes that induce insulin hyper-secretion could help to find a solution for diabetes treatment [20]. Therefore, the enhanced cellular binding and uptake of both insulin and glucose induced by HHV8-infection, as demonstrated here, may represent a possible initiating event for those alterations observed in the metabolic syndrome and diabetes. If this alternative and stimulating hypothesis could be demonstrated *in vivo*, this evidence would open a broad and intriguing research path.

Acknowledgements

The authors thank Ms Sally Davies for helping in manuscript preparation and correction. This research was financed by the Department of Biomedical Sciences, University of Cagliari (FIR project 2016-17).

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Corresponding author
Fabrizio Angius, PhD
Dept. of Biomedical Sciences
Via Porcell 4, 09124 Cagliari (Italy)
Phone: 0039-0706758483
Fax: 0039-0706758482
Email: fangius@unica.it

Conflict of interests: No conflict of interests is declared.